Received: 19 May 2021 | Revised: 22 July 2021 | Accepted: 6 September 2021

DOI: 10.1111/ppl.13579

Physiologia Plantarum

1-Aminocyclopropane-1-carboxylic acid stimulates tomato pollen tube growth independently of ethylene receptors

Rasha Althiab-Almasaud[1] | **Huguette Sallanon**[2,3] | **Caren Chang**[4] | **Christian Chervin**[1]

[1]Laboratoire de Recherche en Sciences Végétales, GBF, Université de Toulouse, Toulouse, France

[2]Université d'Avignon, Avignon, France

[3]Qualisud, Université d'Avignon, Université Montpellier, CIRAD, Montpellier SupAgro, Université de La Réunion, Montpellier, France

[4]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland, USA

Correspondence
Christian Chervin, Laboratoire de Recherche en Sciences Végétales, GBF, Université de Toulouse, Toulouse INP, CNRS, UPS, 31320 Auzeville-Tolosane, France.
Email: christian.chervin@ensat.fr

Funding information
French Ministry of Higher Education; U.S. National Science Fundation, Grant/Award Number: MCB-1714993; Toulouse INP; Avignon University

Edited by: T. Greb

Abstract

The plant hormone ethylene plays vital roles in plant development, including pollen tube (PT) growth. Many studies have used the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), as a tool to trigger ethylene signaling. Several studies have suggested that ACC can act as a signal molecule independently of ethylene, inducing responses that are distinct from those induced by ethylene. In this study, we confirmed that ethylene receptor function is essential for promoting PT growth in tomato, but interestingly, we discovered that ACC itself can act as a signal that also promotes PT growth. Exogenous ACC stimulated PT growth even when ethylene perception was inhibited either chemically by treating with 1-methylcyclopropene (1-MCP) or genetically by using the ethylene-insensitive *Never Ripe* (*NR*) mutant. Treatment with aminoethoxyvinylglycine, which reduces endogenous ACC levels, led to a reduction of PT growth, even in the *NR* mutants. Furthermore, GUS activity driven by an EIN3 Binding Site promoter (*EBS*:*GUS* transgene) was triggered by ACC in the presence of 1-MCP. Taken together, these results suggest that ACC signaling can bypass the ethylene receptor step to stimulate PT growth and EBS driven gene expression.

1 | INTRODUCTION

Enhancing crop yields to ensure global food security is becoming a pressing challenge, and in most crop species, flowers, fruits, and seeds are key components for yield control (Dhankher & Foyer, 2018). As such, research efforts on plant reproduction are essential to increase fruit/seed yields.

Ethylene is a phytohormone regulating the reproduction and the development of fruits and seeds, among numerous other processes (An et al., 2020; Binder, 2020). The biosynthesis of ethylene starts from methionine and the immediate precursor of ethylene is 1-aminocyclopropane-1-carboxylic acid (ACC), generated by the ACC synthase (ACS) from *S*-adenosyl methionine. Oxidation of ACC to ethylene is then catalyzed by the ACC oxidase (ACO; Polko & Kieber, 2019). Plant ethylene responses are typically observed upon treatment with exogenous ACC, but several studies have indicated that ACC can act as an ethylene-independent signal (Xu et al., 2008; Tsang et al., 2011; Yin et al., 2019; Vanderstraeten et al., 2019; Mou et al., 2020; Li et al., 2020). Xu et al. (2008) suggested that ACC is involved in the regulation of a cell wall function in Arabidopsis roots even when ethylene perception is blocked by 1-methylcyclopropene (1-MCP). Tsang et al. (2011) demonstrated that the isoxaben-induced inhibition of Arabidopsis primary root elongation depends on ACC biosynthesis, but does not depend on the perception of ethylene. An accumulation of recent studies has uncovered potential signaling roles of ACC in a number of other processes. Vanderstraeten et al. (2019) found that ACC can function as a negative regulator of Arabidopsis early vegetative development, i.e. reducing rosette development, hypocotyl elongation in darkness, and root growth, independently of ethylene signaling. In another study, it has also been shown that ACC itself positively regulates the symmetric division of guard mother cells into two guard cells (Yin et al., 2019). ACC signaling in Arabidopsis ovules was found to be involved in pollen tube (PT) attraction, by stimulating the secretion of the PT attractant LURE1.2

(Mou et al., 2020). Additionally, ethylene-independent ACC signaling has been demonstrated in the liverwort *Marchantia polymorpha* (Li et al., 2020).

Sexual plant reproduction necessitates pollen germination, the elongation of PTs through the style to reach the ovule and the release of sperm cells into the ovular embryo sac for fertilization, among other steps (An et al., 2020). There is evidence that ethylene plays a role in this complex process. For example, increased ethylene production was detected in the pistil after pollination in *Petunia* (Holden et al., 2003). This ethylene burst is correlated with the expression of ACS and ACO genes in the pistil (Llop-Tous et al., 2000). Another study showed that PT growth is stimulated by an ethylene-driven metabolism leading to higher levels of actin filaments (Jia et al., 2018), which are important for the polarized growth of PTs (Mollet et al., 2013). The role of ethylene perception on PT elongation was first observed using 1-MCP (Holden et al., 2003), and later in Arabidopsis *etr1-1* ethylene-insensitive mutants (Jia et al., 2018).

In light of the emerging differences between ethylene- and ACC-induced responses, in this study, we used various strategies to investigate the potential roles of ethylene versus ACC in tomato PT growth.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

This study was carried out with four lines of *Solanum lycopersicum*, cv. MicroTom: wild-type (WT), *Never Ripe* (NR), *EBS:GUS* in WT and *EBS:GUS* in the *NR* mutant. *NR* is a dominant ethylene receptor mutant that displays ethylene insensitivity (Wilkinson et al., 1995). We obtained *NR* and its corresponding WT background from the L.E. Pereira Peres laboratory (Carvalho et al., 2011). The *EBS:GUS* plasmid was obtained from A. Stepanova laboratory (Stepanova et al., 2007) with EBS standing for EIN3 Binding Site. The *EBS:GUS* WT line was generated in our laboratory by transformation with *Agrobacterium tumefaciens* (Jones et al., 2002). The *EBS:GUS NR* line was generated by crossing the homozygous *EBS:GUS* WT line with *NR*. Two homozygous lines were chosen in the T_3 generation. Transgenic plants carrying *EBS:GUS* in WT and *NR* backgrounds were selected for 100% antibiotic resistance to hygromycin B (100 mg l^{-1}). Seeds were sterilized with 5% NaClO for 8 min, then washed three times with sterilized water, and were sown directly on soil and maintained in a culture room, with a 16 h day: 8 h night cycle, associated with a temperature cycle of 22°C:18°C, respectively, under 80% relative humidity and a day light intensity of 250 μmol m^{-2} s^{-1}.

2.2 | Pollen grain germination in vitro assays

Pollen grains were collected from 10 anthers, from approximately five plants, at one and two days post-anthesis, in 1.5 ml centrifuge tubes, by using an electric toothbrush body (Oral-B). For imbibition, pollen grains were placed on the pollen germination medium (300 mM sucrose, 2 mM boric acid, 2 mM calcium nitrate, 2 mM magnesium sulfate, and 1 mM potassium nitrate), as described previously (Firon et al., 2012). The pollen grains were incubated at 25°C, 80% relative humidity for various times, in the dark, in 2 ml vials with a septum and screwed caps (Interchim). For the ACC and aminoethoxyvinylglycine (AVG) treatments, solutions were added in the germination medium at 10 μM final concentration, unless otherwise stated. For the ethylene and 1-MCP treatments, these gases were injected at 100 and 2 ppm final concentration, respectively, based on previously-described dose effects (Althiab-Almasaud et al., 2021). 1-MCP, which inhibits ethylene perception (Binder, 2020), was obtained from AgroFresh and prepared according to their recommendations. All other chemicals were from Sigma-Aldrich.

The germination medium was transferred onto a glass slide prior to microscopic observations. The images were acquired using an inverted microscope (Leitz DMIRB, Leica Microsystems) equipped with a camera CMOS 10 MP (LEÏCA MC 190HD). The objective in place was an NPlan 10×:0.22NA. The length of PTs was measured using the ImageJ software (Abràmoff et al., 2004).

2.3 | ACC content

For PT free ACC content, we optimized the protocol of Bulens et al. (2011) with the following modifications. Then, 2 mg of crushed frozen PTs were placed in 1.5 ml centrifuge tubes, 1 ml of 5% sulfosalicylic acid solution was added to the frozen sample, and incubated and centrifuged as described. The supernatant was collected in a 5 ml glass vial. The ethylene was measured using a gas chromatograph and the following conditions: 2 m × 3 mm 80/100 alumina column, injector at 110°C, N_2 as carrier gas at 30 ml min^{-1} in an isocratic oven temperature at 70°C, and FID detector at 250°C.

2.4 | GUS activity assay and GUS staining

GUS activity was assayed according to Melo et al. (2016) with the following modifications. For each sample, 2 mg of crushed frozen PTs were homogenized with 300 μl extraction buffer composed of 50 mM HEPES-KOH (pH 7.0), 5 mM DTT, and 0.5% PVP (w/v), then the samples were incubated at 4°C for 30 min with gentle shaking and spun at 16 000*g* for 10 min at 4°C. Supernatants (100 μl) were mixed with 100 μl of GUS extraction buffer containing 2 mM 4-methylumbelliferyl-*b*-D-glucuronide and incubated at 37°C for 30 min. Then, 30 μl of this reaction were mixed with 270 μl of stop reagent 0.2 M Na_2CO_3 (pH 9.5).

PTs of *EBS:GUS* transgenic plants were vacuum infiltrated for 5 min in GUS staining buffer (100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% (w/v) Triton X-100, and 1 mg ml^{-1} X-Gluc) and then incubated for 24 h at 37°C. After staining, the PTs were washed in distilled water. GUS-positive samples were examined with an inverted microscope (Leitz DMIRB, Leica Microsystems) equipped with a camera CMOS 10 MP (LEÏCA MC 190HD). The objective was an NPlan 10×:0.22NA.

2.5 | Statistical treatments

All data were processed with the R package (R Core Team, 2021), using ANOVAS and Tukey multiple comparison tests.

3 | RESULTS

3.1 | Ethylene perception stimulates PT growth

The impact of ethylene perception on PT elongation in tomato was studied in the WT and ethylene-insensitive *NR* mutant, 4 h after imbibition. We used exogenous ethylene and 1-MCP treatment as effector or inhibitor of ethylene receptors, respectively. We found that in WT, exogenous ethylene stimulates PT growth, and by contrast, 1-MCP inhibits it (Figure 1A), consistent with the results obtained by Holden et al. (2003) in *Petunia inflata*. Ethylene-stimulated growth was prevented by 1-MCP + ethylene treatment, in which ethylene was applied 30 min after adding 1-MCP, showing that the simulation of PT growth by ethylene goes through ethylene receptors as expected. This also demonstrated that even a small dose of 1-MCP is able to counteract the effect of large concentrations of ethylene. In contrast, *NR* PT growth was not affected by the treatments (Figure 1B), consistent with *NR* being disrupted in ethylene perception (Wilkinson et al., 1995).

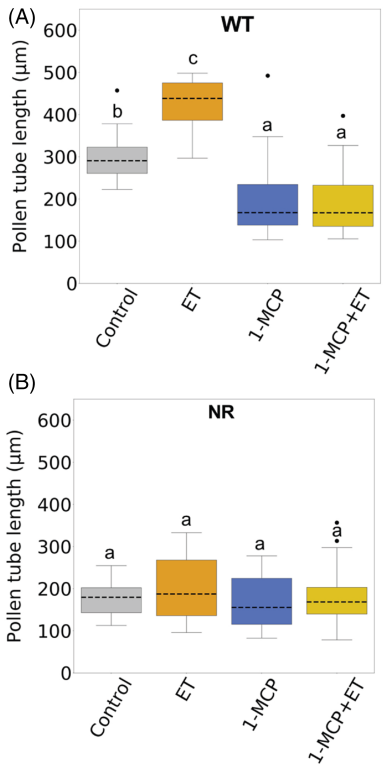

FIGURE 1 Pollen tube (PT) growth is linked to ethylene signaling. The effect of exogenous ethylene (ET, 100 ppm), 1-methylcyclopropene (1-MCP, 2 ppm), on pollen tube (PT) growth in (A) wild-type (WT) and (B) *Never Ripe* (NR), $n = 50$ individual PTs, 4 h after imbibition. In both panels, horizontal dashed lines show medians, and black dots show outliers, and different small letters show significant differences at the 0.05 level (Tukey's HSD)

3.2 | ACC stimulates PT growth, even when ethylene perception is blocked

We next investigated the effect of ACC on PT growth by first performing dose–response experiments. We found that ACC stimulates WT PT growth with an optimal dose of 100 µM (Figure 2A). This biphasic response caused by ACC has already been observed in the liverwort *M. polymorpha* with growth stimulation at 1 µM and growth inhibition at 100 µM (Li et al., 2020). Although ACC treatment likely causes PT growth due to the conversion of ACC to ethylene, we discovered that ACC can also stimulate PT growth in the *NR* mutant (Figure 2B). This result was unexpected given that *NR* cannot perceive ethylene, and it suggested that exogenous ACC can stimulate PT growth independently of ethylene perception.

We then investigated whether endogenous ACC plays a similar role as treatment with exogenous ACC. We began by first measuring the free (nonconjugated) ACC content in PTs (Figure 2C). We did not assay conjugated ACC content, as this has not been shown to play a signaling role in plants. In WT PTs, the endogenous content of free ACC was found to decrease over the imbibition time to 50 nmol g^{-1} (Figure 2C). This corresponds roughly to 50 µM, which fits with a previous determination of ACC in plant tissues (Bulens et al., 2011). Next, we hypothesized that if endogenous ACC induces PT growth, then AVG, an inhibitor of ACS, should exhibit reduced PT growth. By performing an AVG dose response in both WT and *NR*, we found that PT growth linearly decreased with increasing AVG doses up to 10 µM (Figure 2D,E). We next confirmed that the effect of AVG (10 µM) was based on ACC content, rather than a toxic effect. By adding ACC 30 min after the imbibition start in the presence of AVG, we found that ACC restored PT growth in the presence of AVG in both the WT and *NR* (Figures 3A,B). Moreover, by measuring the free ACC content in PTs of both lines, we confirmed that free ACC is indeed reduced by AVG (Figure 3C). Taken together, these results indicated that the stimulation of PT growth by endogenous ACC is partially independent of ethylene perception.

Finally, to confirm that ACC action on PT growth is partially independent of ethylene perception, we treated PTs with exogenous ACC 30 min after imbibition in the presence of 1-MCP, which blocks ethylene perception. We found that we still observed the stimulating effect of ACC on PT growth in the WT and *NR* (Figure 3D). In this case, the relatively shorter PT lengths of all the samples were due to the inhibition of ethylene perception, because1-MCP did not affect the free ACC content (Figure 3E) and likely did not affect ethylene biosynthesis from ACC. These results confirmed that ACC stimulation of PT growth is partially independent of ethylene perception.

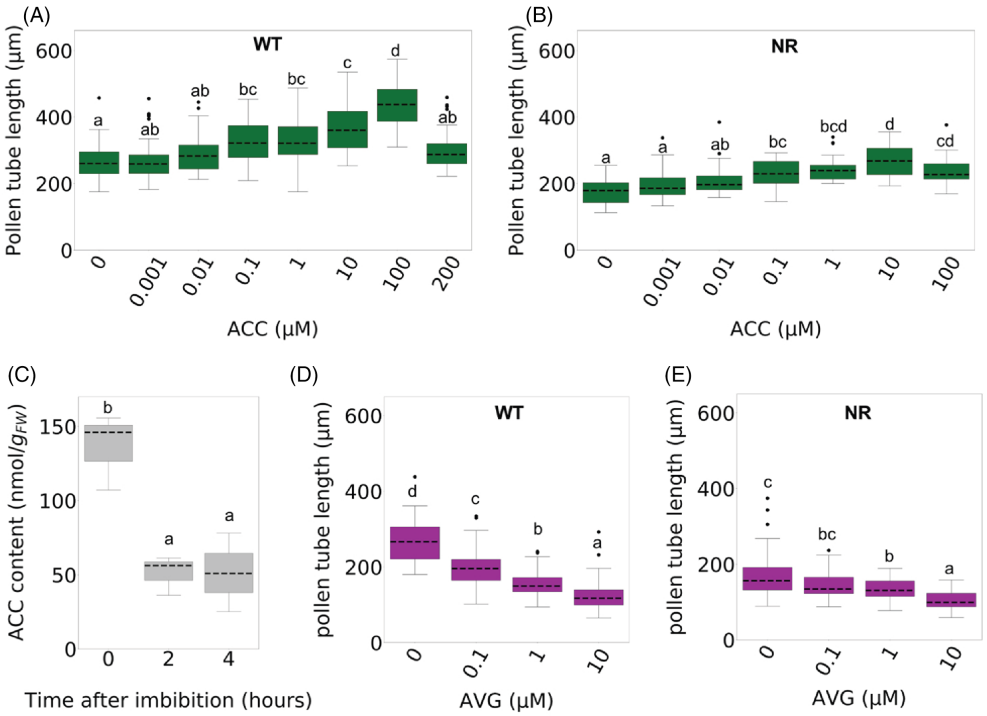

FIGURE 2 Dose effect of exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) on pollen tube (PT) growth and ACC content in PT. Effects of different concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) (A,B) and aminoethoxyvinylglycine (AVG) (D,E) on pollen tube (PT) length in wild-type (WT) and *Never Ripe* (NR), respectively, 4 h after imbibition, $n = 50$ individual PTs. (C) Free ACC content of pollen grains and PTs. In all panels, horizontal dashed lines show medians, and black dots show outliers, different small letters show significant differences at the 0.05 level (Tukey's HSD)

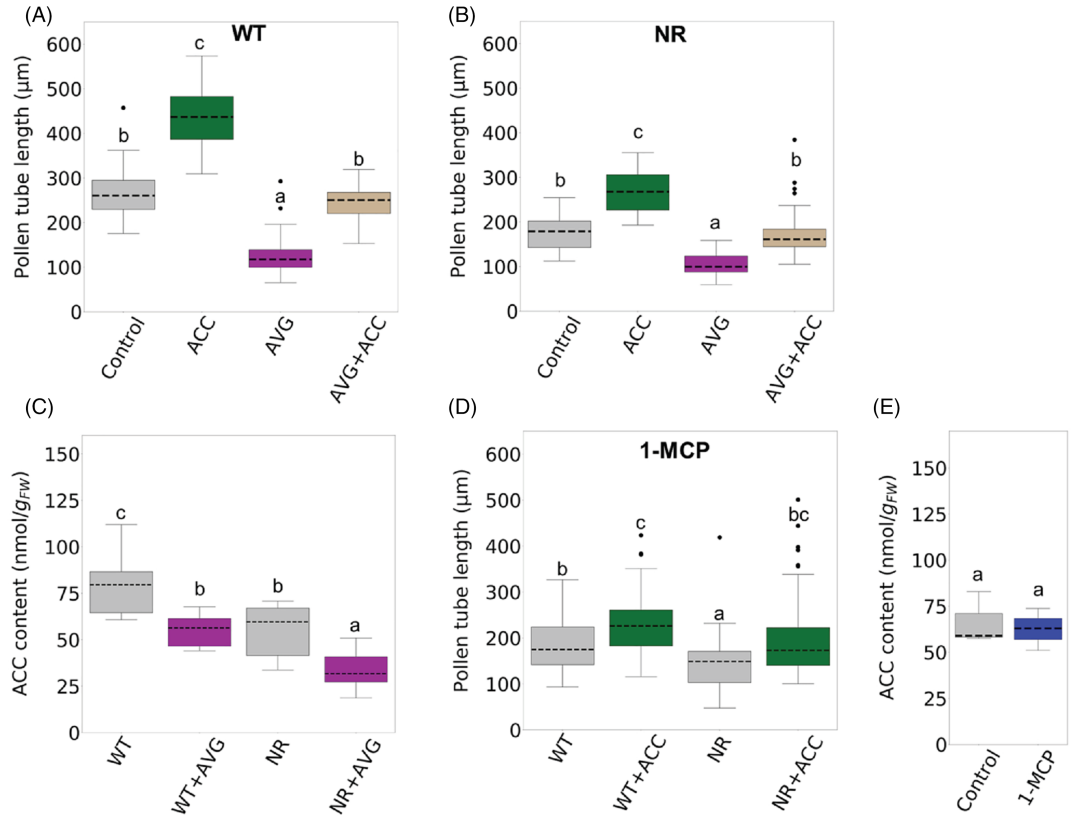

FIGURE 3 The effect of exogenous 1-aminocyclopropane-1-carboxylic acid (ACC, 10 μM) or aminoethoxyvinylglycine (AVG, 10 μM) on PT growth in (A) wild-type (WT) and (B) *Never Ripe* (NR), $n = 150$ individual PTs. (C) Free ACC content in WT and NR PTs with or without AVG, $n = 12$ biological replicates of 0.5 mg of pollen grains each. (D) Effects of ACC on PT growth in presence of 1-methylcyclopropene (1-MCP), $n = 50$ individual PTs. (E) Effect of 1-MCP (2 ppm) on ACC content in PTs, $n = 3$ biological replicates of 0.5 mg pollen grains each. All panel measurements were performed 4 h after imbibition, horizontal dashed lines show medians, and black dots show outliers, different small letters show significant differences at the 0.05 level (Tukey's HSD)

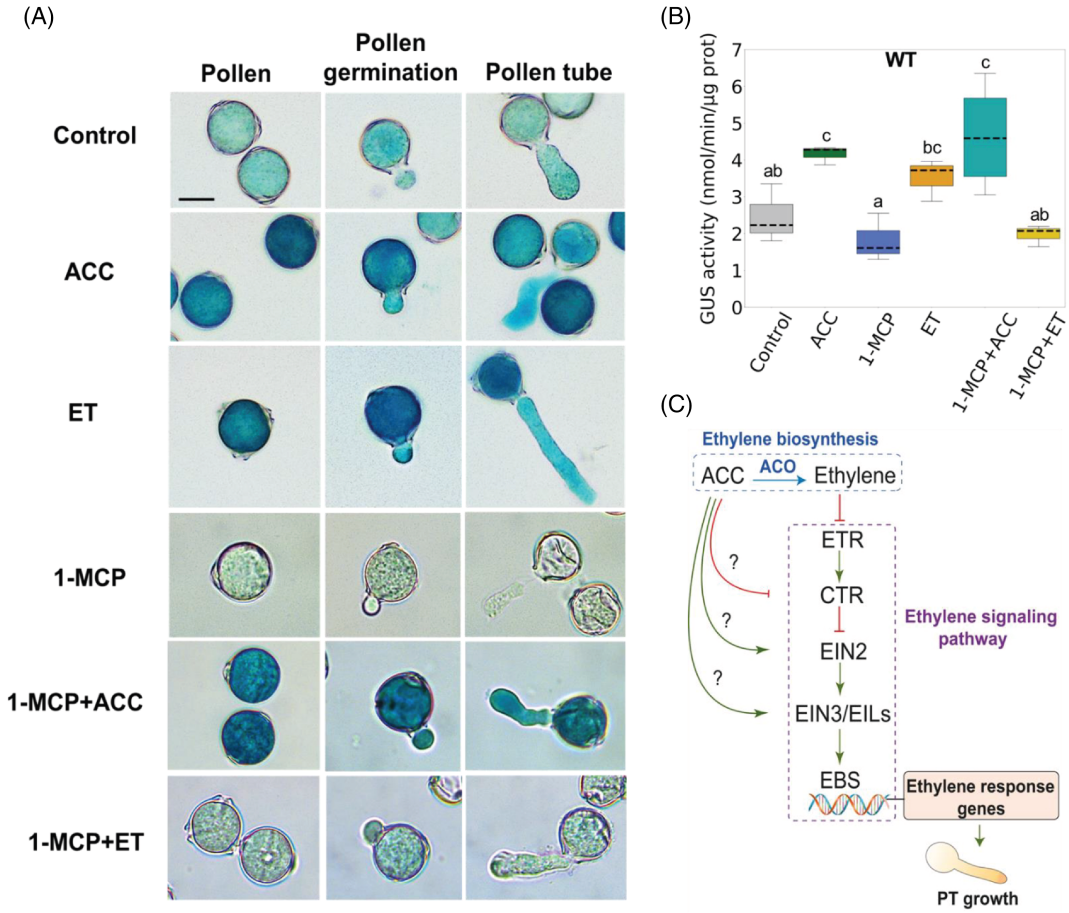

FIGURE 4 1-Aminocyclopropane-1-carboxylic acid (ACC) triggers transcription through EIN3 binding sites independently of ethylene perception. (A) Images of wild-type (WT) tomato pollen grains and tubes expressing the *EBS:GUS* reporter after various treatments. Scale bar = 30 µm. (B) GUS activity assayed 4 h after imbibition in WT pollen tubes (PTs). $n = 3$ biological replicates of 0.5 mg pollen grains each, horizontal dashed lines show medians, different small letters show significant differences at the 0.05 level (Fisher's LSD). (C) Proposed model showing 1-aminocyclopropane-1-carboxylic acid (ACC) signaling bypassing ethylene perception to stimulate PT growth. 1-MCP stands for 1-methylcyclopropene, ET stands for ethylene

3.3 | ACC stimulates GUS driven by EBS, independently of ethylene perception

Given that ethylene and ACC each stimulate PT growth, we asked if ACC is able to trigger a downstream step of the ethylene signaling pathway, i.e. EIN3-LIKE (EIL)-dependent transcription. EILs are primary transcription factors of the ethylene signal cascade (Liu et al., 2015). We observed the effects of various treatments on *EBS:GUS* activity in pools of pollen grains, germinating grains and PTs. This GUS construct was shown to work in tomato fruit tissues (Bianchetti et al., 2017), but had not been tested in pollen cells. First, we observed a degree of GUS activity and staining in the control WT (Figure 4), probably due to endogenous levels of ethylene (Althiab-Almasaud et al., 2021) or to free ACC (Figure 2C). Treatments with exogenous ACC and ethylene were able to stimulate the GUS activity, whereas 1-MCP repressed this activity (Figure 4A,B). Moreover, 30 min after applying 1-MCP, the ACC treatment was able to stimulate GUS activity and staining, despite ethylene perception being blocked (Figure 4A,B). Finally, 30 min after applying 1-MCP, the ethylene treatment was not able to stimulate GUS activity. These results suggest that ACC is able to activate EIL-dependent transcription, independently of the ethylene receptor step. To further confirm this, we conducted experiments in the *NR* mutant background, into which we crossed the *EBS:GUS* transgene, and observed that ACC was able to stimulate GUS activity as in the WT, without or with 1-MCP application (Figure S1). The GUS activity profiles were similar between WT and NR lines (Figures 4B and S1B). This should drive further research to better understand EBS activation in tomato pollen cells.

4 | DISCUSSION

Our results confirmed previous studies showing the essential role of ethylene in PT growth (Holden et al., 2003; Jia et al., 2018). However, we discovered that the ethylene precursor ACC can promote PT growth independently of ethylene perception, adding to the growing evidence that ACC itself can act as a signal. Indeed, when the ethylene receptors were either blocked by 1-MCP or genetically inhibited in the *NR* mutant, the addition of exogenous ACC was able to stimulate PT growth. It is possible that *NR* only partially blocks ethylene

perception as stated in Negi et al. (2010). Furthermore, the use of AVG to inhibit ACC synthesis in the *NR* mutant demonstrated that endogenous ACC plays a role in promoting PT growth independently of ethylene perception. In future experiments to further prove ACC roles independent of ethylene action, pyrazinamide, which blocks ACO (Sun et al., 2017), could be tested.

Ethylene-independent signaling by ACC has already been recently described in various plant organs (Li et al., 2020; Mou et al., 2020; Polko & Kieber, 2019; Vanderstraeten et al., 2019). In contrast to previous findings, where ACC and ethylene induced distinct responses, we found that ACC and ethylene both promote tomato PT growth. Jia et al. (2018) also showed that exogenous ACC was able to promote Arabidopsis PT growth. They observed a reduced PT growth in the *etr1-1* mutant, a gain-of-function ethylene-insensitive mutant similar to *NR*, but they did not observe any stimulation effect by ACC in this mutant. Additionally, they neither tested the effect of ACC in the presence of 1-MCP or other ethylene perception inhibitors, nor measured free ACC content.

In our experiments, we found that there was less free ACC in the *NR* mutant. Thus, the slower PT growth in this mutant compared to WT could be due to a combined effect of impaired ethylene perception and lower free ACC levels.

By testing the downstream activation of the *EBS:GUS* reporter in various conditions impacting the ethylene perception step, we observed results suggesting that ACC can modulate reactions downstream of the ethylene receptor steps. The ACC targets could be signaling steps related to CTR1, EIN2, or EIN3/EILs, which all function upstream of EBS, as outlined in a tentative model (Figure 4C). Previous results by Vanderstraeten et al. (2019) suggest that ACC signaling by-passes the ethylene receptor step and branches to EIN2. Indeed, Vanderstraeten et al. (2019) showed that exogenous ACC reduces the Arabidopsis rosette area in Col-0, even when 1-MCP was applied, but the *ein2-1* ethylene-insensitive mutant was less responsive to the ACC effect. In contrast, Mou et al. (2020) showed that ACC action bypasses the *ein2-5* null mutant in ovules. Additionally, Mou et al. (2020) found that ACC can stimulate an intracellular Ca^{2+} increase in Arabidopsis ovules, and Ca^{2+} is known to enhance PT elongation (Michard et al., 2017). Whether or how Ca^{2+} connects with EBS remains an open question, and examining Ca^{2+} signaling after adding ACC and 1-MCP would be an interesting perspective for a further study.

In conclusion, we observed that both ethylene perception and ACC itself can stimulate tomato PT growth. These findings, particularly the newly uncovered role of ACC that is independent of ethylene perception, open new research perspectives, such as identifying the direct targets of ACC signaling upstream of the EIN3/EIL binding site and elucidating the underlying signaling mechanisms.

ACKNOWLEDGMENTS

Thanks to the French Ministry of Higher Education for the PhD grant to Rasha Althiab-Almasaud. Thanks to Toulouse INP and Avignon University for partial funding of the analyses, and to Mondher Bouzayen for his support. Thanks to Anna Stepanova for the *EBS:GUS* construct, and to Lazaro Pereira Peres for the seeds of *NR* and WT. Caren Chang was partially supported by NSF grant MCB-1714993. Thanks to two anonymous reviewers for helpful comments.

AUTHOR CONTRIBUTIONS

Rasha Althiab-Almasaud: Designed and performed the experiments. Caren Chang and Christian Chervin: Co-designed the experiments. Huguette Sallanon and Christian Chervin: Secured the funding, all authors participated in the manuscript redaction.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Christian Chervin https://orcid.org/0000-0002-0913-6815

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SUPPORTING INFORMATION

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How to cite this article: Althiab-Almasaud, R., Sallanon, H., Chang, C. & Chervin, C. (2021) 1-Aminocyclopropane-1-carboxylic acid stimulates tomato pollen tube growth independently of ethylene receptors. *Physiologia Plantarum*, 173(4), 2291–2297. Available from: https://doi.org/10.1111/ppl.13579