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Effects of rhizobacterial ACC deaminase activity on *Arabidopsis* indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria

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Abstract

1-Amino cyclopropane-1-carboxylic acid deaminase (AcdS) is an enzyme that degrades the precursor of plant hormone ethylene. AcdS activity has been identified in many soil bacteria. It has been proposed to play an important role in plant-growth promotion by rhizobacteria. It would lower ethylene level *via* uncharacterized signaling pathways in the host plant. To further investigate the role of AcdS and the involvement of ethylene signaling pathway in plant development responses to rhizobacteria, we used the model plant *Arabidopsis thaliana*. We compared the changes in root architecture and root hair length induced by four rhizobacteria (*Phyllobacterium brassicacearum* STM196, *Pseudomonas putida* UW4, *Rhizobium leguminosarum* bv. *viciae* 128C53K, *Mesorhizobium loti* MAFF303099) and by their respective *acdS-deficient* mutants. All the mutant strains induced similar changes in lateral root development as their WT counterparts. By contrast, root hairs of seedlings inoculated with the *acdS* mutant strains were significantly longer than those of the plants inoculated with the WT strains. Overall, our results would suggest that rhizobacterial AcdS activity affects local regulatory mechanisms in plant roots, and not lateral root development that is under systemic regulation involving shoot–root dialog.
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Keywords: Ethylene; Root hair; Lateral root; *Arabidopsis thaliana*; Plant growth-promoting rhizobacteria (PGPR); *Phyllobacterium brassicacearum*

1. Introduction

In plants, ethylene regulates several physiological processes including seed germination, root hair development, root and shoot primordia formation, root elongation, leaf and petal abscission, fruit ripening and organ senescence [1]. As evidenced by the phenotype of the loss-of-function *ctr1* mutant of *Arabidopsis*, which has a constitutively activated ethylene response pathway, ethylene is a negative regulator of plant growth [2]. In addition, ethylene has also been shown to act as a messenger of biotic [3] and abiotic stresses [1].

The ethylene biosynthetic pathway was essentially elucidated by Yang and Hoffman [4]. Ethylene is derived from the amino acid methionine that is converted to *S*-adenosyl-methionine (SAM) by SAM synthetase, which metabolite serves as an intermediate in numerous biosynthetic pathways. SAM is usually considered as the earliest precursor of ethylene. It is first converted into 1-amino cyclopropane-1-carboxylic acid (ACC) [5] by the activity of ACC synthase (ACS, EC:4.4.1.14). The ACS is encoded by 11 genes in *Arabidopsis* although *ACS10* encodes a biochemically inactive ACS and *ACS1* is a pseudogene [6]. The ACS activity is generally considered as the rate-limiting step for ethylene biosynthesis [7]. The final step of ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by the enzyme ACC oxidase (ACO or ethylene-forming enzyme; EC:1.14.17.4).

In fields, plant roots interact with a set of soil-borne bacteria known as plant growth-promoting rhizobacteria (PGPR) for their ability to trigger increased biomass production and crop yield [8,9]. The inoculation of plant roots with efficient PGPR strains usually enhances lateral root proliferation and root hairs elongation [10–12]. The ability of many rhizobacteria to

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produce plant hormones or hormone-like substances has often been evoked to explain how PGPR can promote plant growth [13,14]. In addition, it has also been proposed that PGPR could affect hormones level in host plant. For instance, PGPR can lower ethylene level, thus releasing partially the negative regulation exerted on plant growth by this gaseous hormone [15,16]. One mechanism reported to decrease ethylene production consists in the excretion by the bacteria of rhizobitoxine, a toxin that inhibits ACS activity [17–19]. However, the synthesis of rhizobitoxine has been highlighted only in a few bacteria belonging to the *Bradyrhizobium* genus [19–21] and the *Pseudomonas* genus [22].

A second mechanism by which bacteria could lower ethylene level in the host plant involves the enzymatic activity ACC deaminase (AcdS) that catalyzes the degradation of ACC in α-ketobutyrate and ammonia. Since its discovery in 1978 [23], AcdS activity and/or gene has been found in many microorganisms, including the fungus *Penicillium citrinum* [24], the yeast *Hansenula saturnus* [25] and a large range of bacteria, especially soil living bacteria associated with roots including *Achromobacter*, *Agrobacterium*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Rastolnia* and *Rhizobium* [26,27]. It has been postulated that much of the ACC produced by ACS activity in plant roots may be exuded in the rhizosphere [28]. The ACC could then be taken up by the rhizobacteria and subsequently hydrolyzed by AcdS [15]. This can generate a strong sink, which would decrease ACC concentration in plant roots. Such decrease is likely to lower the ethylene biosynthesis rate, and hence to affect plant development. Therefore, AcdS activity aroused great interest from an agronomical point of view. The expression in tomato plant of *Pseudomonas* sp. 6G5 *acds* gene under the control of a 35S promoter led to decreased ethylene synthesis and, as predictable, delayed fruit ripening [29]. Such heterologous expression study provides a functional demonstration of the role of AcdS to lower ethylene level in plants. In addition, these transgenic plants showed higher tolerance to flooding, heavy metal stress and pathogen attack [29–31], as expected from the involvement of ethylene in stress responses. Such observations are not restricted to tomato. For instance, ACC deaminase transgenic *Brassica napus* plants exhibit an improved tolerance to growth inhibition by nickel [32] and salt stress [33].

A positive correlation was found between the *in vitro* AcdS activity of various rhizobacteria isolated from corn rhizosphere and root elongation [34]. Similar results have been obtained by several studies using bacterial mutants deficient in AcdS activity. Thus, *acdS-deficient* mutants of *Pseudomonas putida* GR12-2 [35], *P. putida* UW4 [36] (previously considered as an *Enterobacter cloacae* strain) and *Pseudomonas brassicacearum* Am3 [37] were no longer able to stimulate root growth of *B. napus* and tomato, respectively. Moreover, the expression of the *acdS* gene in strains that do not contain AcdS confers the ability to promote root elongation, as reported when the *E. cloacae* UW4 and CAL2 *acdS* genes have been introduced into *Escherichia coli*, *Pseudomonas* spp., *Azospirillum brasilense* Cd and Cd1843 [38–40].

In this study, we investigated the implication of rhizobacterial ACC deaminase activity in the changes of root development induced during plant–PGPR interaction. For this purpose, we used the model plant *Arabidopsis thaliana* inoculated with *Phyllobacterium brassicacearum* STM196 [41,42]. This strain was isolated from the rhizoplan of field-grown canola roots. *In vivo*, it stimulates *B. napus* and *A. thaliana* growth rates [43]. In gnotobiotic conditions, inoculation with STM196 alters both *B. napus* and *A. thaliana* root development by stimulating lateral root growth and increasing root hair length [43,44]. Preliminary studies have identified an *acdS* gene and a functional AcdS activity (Larcher, personal communication), thus opening the possibility that root responses might be due to the lowering of ethylene production by *P. brassicacearum* STM196. In order to investigate this possibility, we inactivated the *acdS* gene of STM196 and compared the effect of the STM196 *acdS* mutant on *Arabidopsis* root development to the effect of the wild-type strain. We also used *P. putida* UW4, *Rhizobium leguminosarum* bv. *viciae* 128C53K, *Mesorhizobium loti* MAFF303099 and the corresponding *acdS* mutant strains [36,45,46] to check whether the results found for *P. brassicacearum* STM196 are strain-specific.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The beneficial bacteria strains used in this study are listed in Table 1. All rhizobacterial strains were cultivated on E′ medium and inoculated in plant culture medium as described previously for *P. brassicacearum* [43]. *E. coli* DH5α and S17-1 were grown at 37 °C in Luria–Bertani (LB) medium.

Functional characterization of *P. brassicacearum* STM196 *acdS* mutant was carried out using a nitrogen-free broth (NFb) medium (8 ml phosphate buffer (K_2HPO_4 344.47 mM; KH_2PO_4 293.92 mM), 464 ml 40.18 mM malate with or without 15 g l^{-1} agar (Sigma, USA), 20 ml salt solution S1 (20.28 mM $MgSO_4$, 4.42 mM $CaCl_2$), 5 ml salt solution S2 (171.11 mM NaCl, 8.27 mM Na_2MoO_4, 3.44 mM $MnCl_2$), 500 μl iron solution (45.31 mM $FeSO_4$, 31.7 mM EDTA), 500 μl 4.27 mM biotine, supplemented with either 0.8 mM KNO_3 or 3 mM ACC (Sigma, USA). The bacterial strains *P. putida* UW4, *R. leguminosarum* bv. *viciae* 128C53K, *M. loti* MAFF303099 and the corresponding *acdS* mutants were used as controls. All bacteria strains were inoculated to get a final $OD_{595} = 0.01$.

2.2. Plant-growth conditions and inoculation

A. thaliana seeds of wild-type ecotype *Columbia* and *ein2-1* mutant [47,48] were surface-sterilized, sown in 12 cm × 12 cm Petri dishes on a 1% agar (Sigma, USA) mineral medium as described previously [43]. The concentration of KNO_3 in the plant nutrient medium was 5 mM for all experiments presented. The plates were sealed with Micropore^{TM} tape (3 M, USA) and stored at 4 °C during 5 days. Seeds were vertically germinated in a controlled environmental chamber (22 °C/20 °C during the

Table 1
Characteristics of the bacterial strains and plasmids used in this study

Strains, plasmids	Relevant characteristics	Reference or source
Phyllobacterium brassicacearum		
STM196	Erm^R	[41]
STM196 *acdS* mutant	Erm^R, Km^R	This study
Mesorhizobium loti		
MAFF 303099		[45]
MAFF303099 *acdS* mutant	Gtm^R	[45]
Rhizobium leguminosarum bv. *viciae*		
128C53K	Str^R	[46]
128C53K *acdS* mutant	Str^R, Km^R	[46]
Pseudomonas putida		
UW4		[36]
UW4 *acdS* mutant	Tet^R	[36]
Escherichia coli		
DH5α	Cloning strain	[79]
S17-1	Strain used for conjugation and gene disruption	[55]
Plasmids		
pGEM-T Easy	Amp^R, *lacZ*	Promega, USA
pKOK5	Amp^R, *lacZ-Km^R* cassette	[53]
pJQ200-SK	Gtm^R, *sacB*	[54]
pG-*acdS*-pKOK5	pGEMT containing F3-R3 fragment of STM196 *acdS* gene disrupted by pKOK5 cassette	This study
pJQ-*acdS*-pKOK5	pJQ200-SK containing F3-R3 fragment of STM196 *acdS* gene disrupted by pKOK5 cassette	This study

Erm^R, erythromycin resistant; Km^R, kanamycin resistant; Gtm^R, gentamycin resistant; Str^R, streptomycin resistant; Tet^R, tetracyclin resistant; Amp^R, ampicylin resistant; *lacZ*, β-galactosidase; *sacB*, *Bacillus subtilis* invertase/levansucrase.

16 h/8 h light/dark photoperiod). Six days after germination, uniformly looking seedlings were transplanted into new Petri dishes filled with the agar mineral medium supplemented or not with rhizobacteria at a final $OD_{595\ nm} = 0.1$ (four plants per plate, four plates per treatment). Plants were grown vertically another 6 days in the growth chamber.

2.3. Isolation of the acdS gene of Phyllobacterium brassicacearum STM196

A preliminary study identified a 426 pb DNA fragment, which sequence showed homology to a putative *acdS* gene, in the genome of *P. brassicacearum* STM196 (Larcher, personal communication). The complete *acdS* gene was isolated by inverse PCR using this sequence as a template.

Bacterial genomic DNA was isolated according to Chen and Kuo [49]. Aliquots of genomic DNA (10 μg) were digested with 10 U HindIII or HincII (Promega, USA) for 3 h at 37 °C. Ten microliters of digested DNA were circularized with 10 U of T4 DNA ligase (Promega, USA) in a final volume of 200 μl at 4 °C overnight. Five microliters of the different ligation products were used as a template for a first PCR in a final volume of 50 μl using GoTaq DNA Polymerase (Promega, USA). The primers used to amplify DNA fragments from the HindIII ligation were acc-ipcr-F2 (5′-tgcccgccgtacggcagacc-3′) and acc-ipcr-R3 (5′-ggatagggtttgccgcc-3′). To amplify DNA fragments from the HincII ligation, the primers were acc-ipcr-F4 (5′-aatcacacgcgcatggttgcg-3′) and acc-ipcr-R5 (5′-tgaacgccgccaatagacacc-3′). The following PCR program was used: 95 °C, 3 min followed by 35 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min), with a final extension cycle (72 °C, 3 min).

For nested amplification by PCR, 1 μl of the first-round PCR products was used as a template in a final volume of 25 μl, in the same conditions previously described and following the same PCR program. The pairs of primers used were: acc-ipcr-F3 (5′-ccgaggacgacgtggtgc-3′)/acc-ipcrR2 (5′-cccagctttcgcgaatgcc-3′) and acc-ipcr-F5 (5′-caaagatcggctt-caaatgcc-3′)/acc-ipcrR4 (5′-tatcagctcccgacgcgatgg-3′) for DNA containing the HindIII and HincII digested fragments respectively.

The *acdS* and *lrpL* nucleotidic sequences of *P. brassicacearum* STM196 are available from the NCBI database. The GenBank accession numbers are EF452620 and EF452621, respectively.

2.4. Phylogenetic analysis

Sequences included in the phylogenetic analysis were retrieved from GenBank. We included a subset of delta, gamma and beta proteobacteria sequences, including multiple *Pseudomonas* and *Burkholderia* sequences. In order to visualize the phylogenetic relationships of *P. brassicacearum* ACDS sequence with the other alpha-proteobacteria species, we conversely included from one to three sequences of each alpha-proteobacteria species that give a positive result when blasting (using blastp) on GenBank dataset when using the *P. brassicacearum* ACDS sequence.

All amino acid sequences retrieved were aligned using ClustalX [50] and manually corrected using the Genedoc alignment editor programme [51]. Portions of sequences that could not be aligned properly were removed from the data matrix used for the phylogenetic analysis. The phylogenic tree was reconstructed using Protdist and Neighbor software from the Phylip package [52], using the JTT model. The two sequences from the firmicute *Bacillus cereus* and the actinobacteria *Brevibacterium linens* were used as outgroups relatively to the other bacteria included that all belong to the alpha-proteobacteria lineage. Bootstrap values were calculated from 500 replicates.

Blaha et al. [27] and Yao et al. [57] showed that AcdS activities required several specific residues in the protein. In order to assess whether sequences retrieved from the blast process should be considered as true AcdS gene or not, we checked by hand for the presence of such residues in these sequences.

2.5. Disruption of the acdS gene of Phyllobacterium brassicacearum STM196

A portion of the *acdS* gene of *P. brassicacearum* STM196 was amplified by PCR using the forward primer acdSF3 5′-cgcatggttgcggcagttgc-3′ and the reverse primer acdSR3 5′-cggtacggtggactatgtggca-3′ with the previous program (Fig. 1A). The PCR product was purified and subsequently cloned into the pGEM®-T Easy vector (Promega, USA). It was then disrupted by insertion of the pKOK5 (lacZ-KmR) cassette [53] into the unique BamHI site of the fragment (Fig. 1A). The resulting plasmid, pGEM®-T-acdS-pKOK5, was digested with NotI and subcloned into the suicide vector pJQ200-SK [54]. This construct, pJQ-acdS-pKOK5, was introduced by electroporation into *E. coli* S17-1 competent cells [55]. Positive clones were selected for their resistance to kanamycin (50 μg ml^{-1}) and gentamycin (50 μg ml^{-1}). The plasmid of positive clones was then transferred to *P. brassicacearum* STM196 by filter mating. Transformed *P. brassicacearum* STM196 were platted on E′ medium containing erythromycin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Selection for *P. brassicacearum* STM196 containing disrupted *acdS* gene (double recombinant) was performed on E′ solid media supplemented with 7% (w/v) sucrose and 50 μg ml^{-1} kanamycin.

Insertion of the pKOK5 cassette into the chromosomal *acdS* gene and lack of the *sacB* gene was verified by PCR using the following primer pairs: acdSF2 5′-gcaaccagattgcaatcgagga-3′/acdSR2 5′-gcaccacgtcgtcctcggtcag-3′, Kana-F 5′-gctcgaggccgcgattaaattcc-3′/Kana-R 5′-atcctggtatcggtctgctgcgattc-3′, acdSF1 5′-gatgaagcgaatgtttgcgtc-3′/acdSR1 5′-cacggacaaaggattggcgc-3′ (Fig. 1A), SacB-R 5′-ttgattgtttgtctgcgtag-3′/SacB-F 5′-ggttca gccacatttacatc-3′. PCR was performed as described above.

2.6. Plant morphological analysis

Pictures of root sections were captured 4 days after transfer, using an Olympus SZH10 stereo microscope (Olympus, Japan)

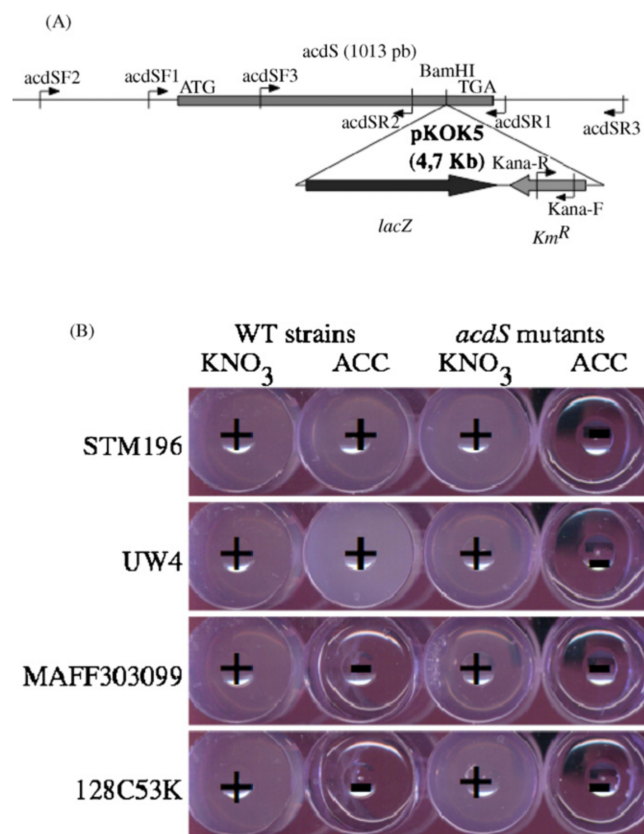

Fig. 1. Construction and functional analysis of *P. brassicarearum* STM196 mutant strain affected in the *acdS* gene. (A) The *acdS* gene was disrupted by double homologous recombination. (B) The growth of the resulting mutant strain was assayed in liquid media. The wild-type strains of *P. brassicacearum* STM196, *P. putida* UW4, *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C23K, and their corresponding *acdS* mutants were grown aerobically in 20 ml of a nitrogen-free broth medium supplemented with 0.79 mM of KNO_3 or 3 mM of ACC, on a rotary shaker (145 rpm) at 25 °C for 48 h. A (+) indicates growth and a (−) no growth.

equipped with a JVC TKC1381 CCD camera (JVC, Japan). Root hair length was measured using Analysis Pro 3.00 software (Soft Imaging System GmbH, Germany). One typical experiment consists of *ca.* 120 measures of root hair length from four different plants per treatment. Root hair experiments have been repeated three times on independent cultures.

For root system architecture analysis, numerical images (TIFF file format) of plates were recorded 6 days after transfer using a flatbed scanner (Epson Perfection 1250; Epson, Japan). The images were analyzed using ImageJ 1.33u (Wayne Rasband, USA) with the plugging NeuronJ 1.01 (Erik Meijering, Switzerland). The lengths of the primary and lateral roots, and the number of visible growing lateral roots were measured. One typical experiment consisted in the analysis of the root system of 13–27 individual plants. Experiments were repeated three times.

For all experiments, the significance of treatment effects was statistically assessed using the Statistica 6.0 software (StatSoft, USA). The analysis of variance using the two-factor ANOVA with a LSD *post hoc* test was used for assessing the differences at $P = 0.05$.

3. Results

3.1. Identification of the acdS gene in Phyllobacterium brassicacearum STM196

We isolated a 2456 pb genomic DNA sequence that putatively contains an AcdS-coding sequence from *P. brassicacearum* STM196. This region contains two ORF of 479 pb and 1013 pb, respectively. The putative protein encoded by the first ORF is 159 amino acid long and exhibited 77% identity with a transcriptional regulator of *Bradyrhizobium japonicum* USDA110 belonging to the leucine-responsive regulatory protein (Lrp) family (AAB49303) [56]. The second and largest ORF is showing high homology to bacterial *acdS* genes. Analyses of the putative amino acid sequence showed high identity with AcdS of Rhizobia, model bacterial strains for symbiotic nitrogen fixation (84% amino acid identity with the *M. loti* AcdS sequence).

The data matrix used for the phylogenetic reconstruction includes 43 sequences. It respectively comprises 19, 7 and 14 alpha, beta and gamma proteobacteria sequences. From 1 to 37 positions at the beginning and from 1 to 10 at the end of the alignment (depending on sequences) were removed due to ambiguous positions in the final alignment. The final data matrix is 344 amino acids long and is available upon request.

The phylogeny obtained clustered all proteobacteria in a single clade (Fig. 2). The basal emerging delta-proteobacteria *Desulfovibrio desulfuricans* branch follows the classical "alpha–beta–gamma" proteobacteria monophyly. Two main clades are strongly supported among the other proteobacteria. A first group only contained gamma proteobacteria, such as *E. coli* or several pseudomonales sequences, and is strongly supported in the phylogeny (100% bootstrap value, BV). The second clade (100% BV) grouped together beta (mainly burkholderiales), gamma (pseudomonales) and alpha proteobacteria.

However, only sequences that clustered in this later alpha–beta–gamma clade showed the specific domains and residues playing a key role in AcdS conformation activities [27,57], thus suggesting that they are truly AcdS-coding gene. Such residues were not observed in sequences that clustered in the first emerging clade.

Within this "true AcdS" clade, all alpha-proteobacteria sequences that could be retrieved from GenBank clustered together (81% BV). It includes Rhizobiales and marine Rhodobacterales. Within this group, *P. brassicacearum* appears as closely related to *Rhizobium sullae*, *Azorhizobium caulinodans* and *M. loti*. The three Rhodobacterales fall within Rhizobiales sequences and do not even form a clade.

3.2. Construction and characterization of an acdS-deficient mutant in Phyllobacterium brassicacearum STM196

In order to investigate the impact of the AcdS activity of the *P. brassicacearum* STM196 PGPR strain on root development in *Arabidopsis*, the *acdS* gene was disrupted to generate a knock-out mutant strain (Fig. 1A). Nine individual clones were recovered on selective media and the disruption of the *acdS* gene by homologous double recombination was confirmed by PCR (data not shown). Because the *acdS* gene on these clones was found disrupted, we postulated that AcdS activity should be absent.

Disrupted and WT *P. brassicacearum* STM196 strains have been grown with either ACC or NO_3^- as a unique nitrogen source to functionally check the absence of AcdS activity in the mutant strain (Fig. 1B). The *P. putida* UW4, *R. leguminosarum* bv. *viciae* 128C53K, *M. loti* MAFF303099 and their corresponding *acdS* mutant strains described earlier [36,45,46] were used as controls. Whereas the *P. brassicacearum* STM196 WT strain grew on both NO_3^- and ACC media, the *acdS*-deficient mutant was unable to grow on ACC medium, suggesting that it did loose AcdS activity. This conclusion is strengthened by the fact that the well-characterized *P. putida* UW4 WT and *acdS* mutant strains [36,38] displayed similar growth patterns. By contrast, both the *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K WT were unable to grow on ACC medium. For these two bacteria, the bioassay did not discriminate the mutant from the WT strains. The inability of these two WT strains to grow in ACC medium suggests that, in our conditions, the enzyme was not operating in pure bacteria culture.

3.3. Effect of bacterial ACC deaminase activity on Arabidopsis root system morphology

In order to determine whether the rhizobacterial AcdS activity has an impact on root development of *Arabidopsis* seedlings, we compared the effects of the *acdS*-deficient mutant strains and those of their WT counterparts on three parameters that characterize the root system morphology, namely the primary root length, the lateral root number and the total lateral root length (Fig. 3). As previously described [43], the main effect of *P. brassicacearum* STM196 was seen on lateral root length (Fig. 3C). By comparison to *P. brassicacearum* STM196, the other bacteria had significant but much weaker effect on lateral root length (Fig. 3C). The primary root of *P. putida* UW4 inoculated *Arabidopsis* plants was shorter than the primary root of control plants, while the other three rhizobacteria had about no effect on the primary root growth (Fig. 3A). *P. brassicacearum* STM196 and *P. putida* UW4 did not affect significantly lateral root number (Fig. 3B). Due to the different impacts of either rhizobacteria on primary root length (Fig. 3A), *P. putida* UW4 induced an increase in lateral root density that was not visible in *P. brassicacearum* STM196 inoculated seedlings. By contrast with these two rhizobacteria, *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K induced a small increase in lateral root number (Fig. 3B).

To investigate the effects of the different WT rhizobacterial strains on lateral root development, we evaluated the distribution of lateral roots among length classes every 0.5 cm. Long lateral roots of seedlings inoculated with *P. brassicacearum* STM196, are in higher proportion compared to

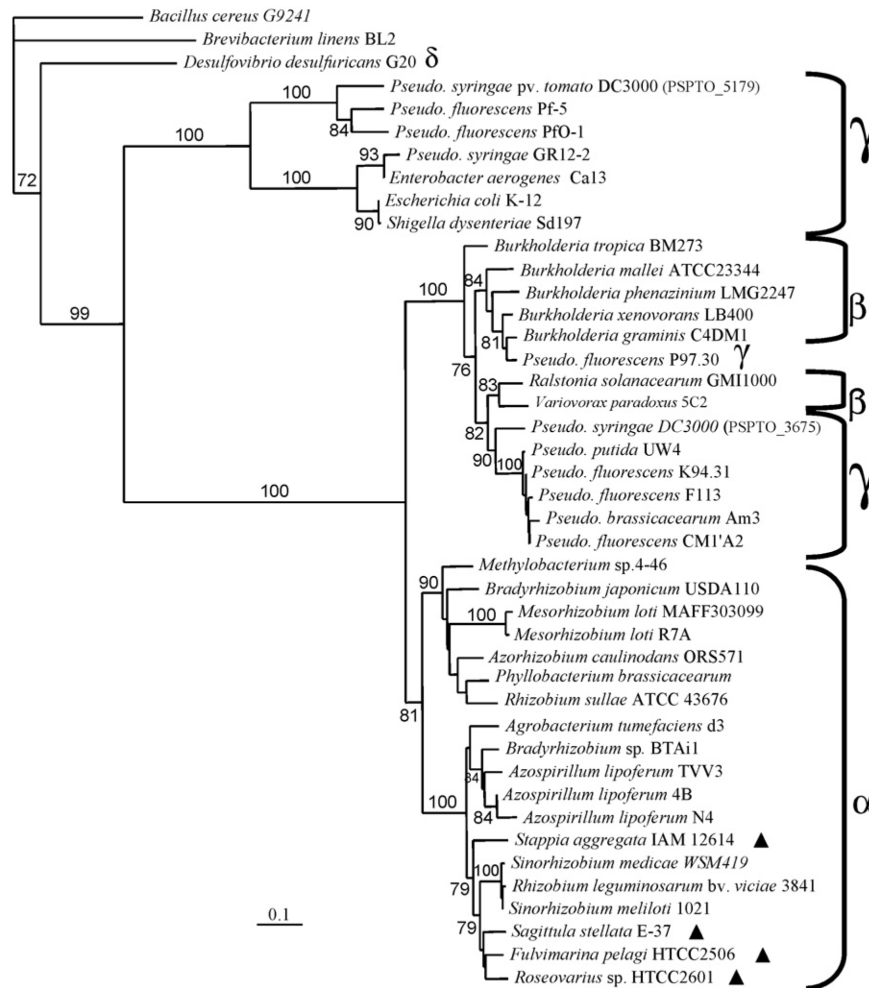

Fig. 2. *P. brassicacearum* STM196 AcdS closely relates to a particular subgroup of proteobacteria AcdS. A distance tree based on alignment of AcdS amino acids sequences has been constructed. Values above each node are bootstrap percentages obtained from 500 replicates. Values below 75% are not indicated. Brackets groups strains of the same proteobacteria phylum(α, β and γ). *Desulfovibrio desulfuricans* is the only delta-proteobacteria strain includes in the tree. Black triangles indicate the four marine strains harboring AcdS sequence (see text). All sequences were retrieved from GenBank with the following accession numbers: *Phyllobacterium brassicacearum* STM196 (ABO31418); *Burkholderia tropica* BM273 (ABE66294); *Burkholderia phenazinium* LMG2247 (ABE66292); *Burkholderia graminis* C4D1M (ABE66289); *Burkholderia xenovorans* LB400 (YP_554094); *Burkholderia mallei* ATCC 23344 (AAU46138); *Azospirillum lipoferum* TVV3 (ABE66297); *Azospirillum lipoferum* N4 (ABE66295); *Azospirillum lipoferum* 4B (ABE66282); *Pseudomonas fluorescens* CM1′A2 (ABE66286); *Pseudomonas fluorescens* F113 (ABE66296); *Pseudomonas fluorescens* K94.31 (ABE66290); *Pseudomonas fluorescens* P97.30 (ABE66288); *Pseudomonas syringae* pv.tomato.DC3000 (AAO57144); *Pseudomonas syringae* pv.tomato.DC3000 (NC_004578.1); *Ralstonia solanacearum* GMI1000 (NP_522207); *Rhizobium sullae* ATCC 43676 (AAT35832); *Rhizobium leguminosarum* bv. *viciae* 3841 (YP_770380); *Sinorhizobium meliloti* (ABA56046); *Agrobacterium tumefaciens* d3 (AAK28496); *Bradyrhizobium* sp.BTAi1 (ZP_00861350); *Bradyrhizobium japonicum* USDA110 (BAC45506); *Mesorhizobium loti* MAFF303099 (BAB52295); *Mesorhizobium loti* R7A (CAD31305); *Variovorax paradoxus* 5C2 (AAT35829); *Pseudomonas syringae* GR12-2 (AAT35840); *Pseudomonas putida* UW4 (AAV73804); *Pseudomonas brassicacearum* Am3 (AAT35827); *Bacillus cereus* G9241 (EAL16846); *Pseudomonas fluorescens* PfO-1 (YP_345978); *Pseudomonas fluorescens* Pf-5 (AAY95661); *Escherichia coli* K-12 (AAC74986); *Azorhizobium caulinodans* ORS 571 (YP_001523183); *Methylobacterium* sp.4-46. (ZP_01848446); *Sagittula stellata* E-37 (ZP_01746018); *Sinorhizobium medicae* WSM419 (YP_001314953); *Stappia aggregata* IAM 12614 (ZP_01546258); *Fulvimarina pelagi* HTCC2506 (ZP_01438810); *Roseovarius* sp. HTCC2601 (ZP_01445585); *Enterobacter aerogenes* (AAT35839); *Brevibacterium linens* BL2 (ZP_00378456); *Desulfovibrio desulfuricans* G20 (YP_389072).

control plants (Fig. 4). This indicates that the rhizobacteria stimulated the growth rate of all lateral roots. *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K led to similar response although their effects were less pronounced as shown by a pattern somewhat intermediary between that of control and *P. brassicacearum* STM196 inoculated seedlings. By contrast, 6 days after inoculation with *P. putida* UW4, the frequency of lateral roots which had lengths in the 0.5–1.5 cm range was higher than in control plants (Fig. 4). On the contrary, the frequency of longer lateral roots (more than 2 cm) dramatically decreased upon inoculation with *P. putida* UW4. This suggests that this rhizobacteria stimulates the growth rate of lateral roots at a developmental stage close to their emergence, but seemingly arrested their growth at a later stage.

The effects of the *acdS* mutants on the root system architecture of *Arabidopsis* seedlings were not significantly different from those of their WT counterparts. The only

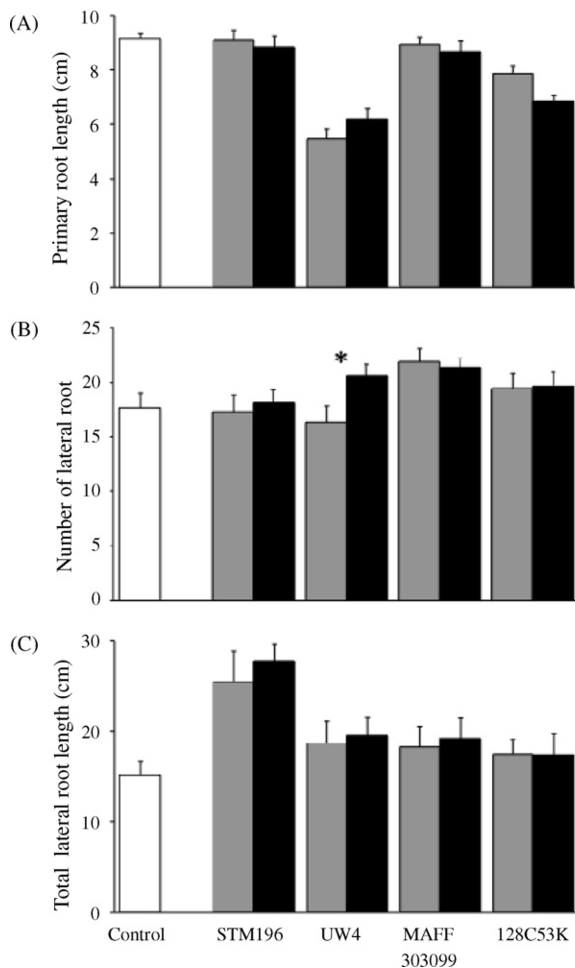

Fig. 3. Bacterial *acdS* mutation does not affect *Arabidopsis* root architecture. Various root architecture parameters of control *Arabidopsis* seedlings (white bars), or inoculated with a WT strain (grey bars) or the respective mutant strain (black bars) were determined. For this study, the following bacterial strain and their respective *acdS* mutant strains were used: STM196, *P. brassicacearum* STM196; UW4, *P. putida* UW4; MAFF303099, *M. loti* MAFF303099; 128C53K, *R. leguminosarum* bv. *viciae* 128C23K. The primary root length (A), the number of lateral roots (B) and the total lateral root length (C) were measured 6 days after inoculation ($n = 13–27$). The experiment was repeated three-fold and representative results of one experiment are shown (average values ± interval of confidence calculated for a P value of 0.05). Asterisk represents the sole significant difference of the means found at $P = 0.05$, according to Fisher's LSD test.

exception found was for the *acdS* mutant of *P. putida* UW4, which triggered an increase in lateral root number while the WT strain did not affect this number (Fig. 3B).

3.4. Effect of rhizobacterial ACC deaminase activity on *Arabidopsis* root hair elongation

Increased ethylene content or a stimulated ethylene signaling pathway results in increased root hair length [58–60]. Therefore, if the hypothesis of an impact of bacterial AcdS activity on ethylene plant production [15] is correct, the roots of plants inoculated with rhizobacteria containing AcdS activity should have shorter root hairs compared to plants inoculated with their AcdS-deficient counterparts.

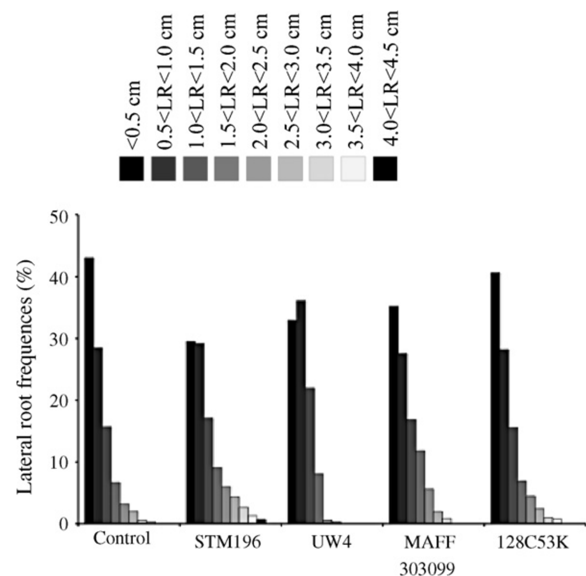

Fig. 4. Distribution of *Arabidopsis* lateral root length is clearly affected by inoculation of *P. brassicacearum* and by *P. putida* but not by *M. loti* and *R. leguminosarum*. Effect of the wild-type strains *P. brassicacearum* STM196, *P. putida* UW4, *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C23K on lateral root distribution among size classes in 14-day-old *Arabidopsis* seedlings. Seedlings were grown in vertically oriented Petri dishes, on mineral solid medium. Six-day-old seedlings were transferred to fresh media inoculated or not with the different strains as indicated. Bars represent the frequencies (percentage) of the lateral roots of 28–36 plants with length falls into each class. Values are mean ($n = 13–27$). The grey code for the different lateral root length classes is indicated in the upper panel.

To test the hypothesis, we calculated the average length of mature root hairs of *Arabidopsis* plants grown in the presence or absence of *P. brassicacearum* STM196, *P. putida* UW4, *M. loti* MAFF303099 or *R. leguminosarum* bv. *viciae* 128C53K, WT or *acdS* mutant strains at 4 days after inoculation (Fig. 5). All the four WT strains tested induced an increase in root hair length. The bacteria-dependent root hair elongation (*i.e.* the difference

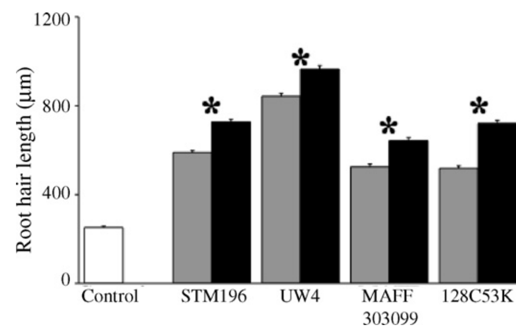

Fig. 5. Wild-type *Arabidopsis* root hair elongation is affected by the *acdS* mutation in response to inoculation by various rhizobacteria mutant strains. Root hair length of control plant (white bar), plant inoculated with WT rhizobacteria (grey bar) and inoculated with the respective *acdS* mutant strain (black bar) were measured in the region between the end of the elongation zone of the primary root and the site of emergence of the lateral roots, 3 days after inoculation ($n = 120$). The experiment was conducted with four different rhizobacteria WT and *acdS* mutant strains (STM196, *P. brassicacearum* STM196; UW4, *P. putida* UW4; MAFF303099, *M. loti* MAFF303099; 128C53K, *R. leguminosarum* 128C53K). The asterisks represent a significant difference of the means at $P = 0.05$, according to Fisher's LSD test.

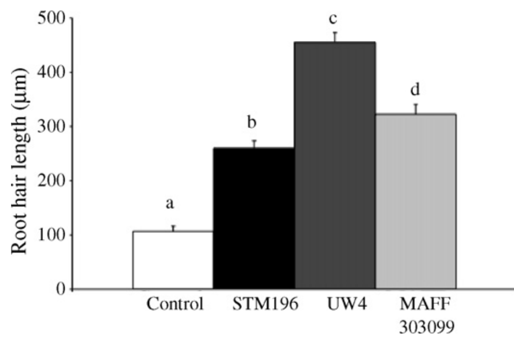

Fig. 6. The root hairs of *ein2-1* ethylene signaling mutant of *Arabidopsis* remains sensitive to inoculation by various rhizobacteria. White bar, control (uninoculated) seedlings; black and grey bars, seedlings inoculated with three WT strains as indicated. The length of between 30 and 40 root hairs growing in the region between the end of the elongation zone of the primary root and the site of emergence of the lateral roots were measured per plant, and at least 12 plants were analyzed for each treatment. The experiment was repeated threefold and representative results of one experiment are shown (average values ± interval of confidence calculated for a P value of 0.05). Letters represent a significant difference of the means at $P = 0.05$, according to Fisher's LSD test.

between the root hair lengths of inoculated and uninoculated plants) represents 110% of the root hair length of control seedlings with *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K, 135% with *P. brassicacearum* STM196 and 235% with *P. putida* UW4. Whatever the strain of bacteria inoculated, the *acdS* mutation led to an even stronger stimulation of root hair elongation. With comparison to the root hair elongation attributable to the WT strains effect, the *acdS* mutant strains induced a 21–75% supplementary increase in root hair length.

Since ethylene is a positive regulator of root hair elongation, we questioned whether the stimulation of root hair elongation by the WT rhizobacteria involved ethylene or if it was controlled by an independent process. For this purpose, we used the *Arabidopsis* ethylene insensitive mutant *ein2-1* that is impaired in the ethylene transduction pathway [47,48]. All the three bacterial strains (*P. brassicacearum* STM196, *P. putida* UW4 and *M. loti* MAFF303099) led to increased root hair length (Fig. 6), indicating that this effect does not involve the ethylene signaling pathway. In fact, root hair length was even increased to a higher extent in the *ein2-1* mutant than it was in the WT *Arabidopsis* plants.

4. Discussion

Colonization of the rhizosphere by PGPR triggers developmental changes in the host plant, especially of the root system [10–12]. It is widely accepted that such responses are the result of pleiotropic effects, involving several signaling pathways simultaneously elicited by the PGPR [9]. These signaling pathways are usually considered to be activated by hormones or hormone-like substances released by the PGPR, or by endogenous plant hormones which level is modified by bacterial activity [14,61,62]. Among these hormones, ethylene is a likely candidate to explain the impact of PGPR on root development. Indeed ethylene has been implicated in many plant processes such as root development and plant microbe interaction [1,3]. Many rhizobacteria do have an AcdS activity that hydrolyses the ethylene precursor ACC into ammonia and α-ketobutyrate. It has thus been proposed that the AcdS activity of many rhizobacterial strains could decrease the level of ethylene in root tissues, which in turn may promote root growth [15].

The PGPR strain *P. brassicacearum* STM196 appears as an interesting model, since the inoculation with this strain affects root architecture and root hair elongation in the model plant *A. thaliana* [43]. Preliminary work showed that *P. brassicacearum* STM196 has both an *acdS* gene and an AcdS activity (Larcher, personal communication). In this context, it is interesting to determine whether the AcdS activity significantly affects plant root architecture. We isolated a complete coding sequence of *P. brassicacearum* STM196 showing homology with previously described AcdS sequences. To better characterize this homology, we performed a phylogenetic analysis of AcdS protein sequence from various bacteria (Fig. 2). After alignment and analyses, two major groups of amino acids sequences were obtained from the blastp process. The first one groups together only sequences from gamma proteobacteria. These sequences do not harbor specific residues involved in AcdS activities. These sequences correspond to D-cysteine desulfhydrase, a PLP-dependent enzyme involved in the catabolism of D-cysteine and sulfur metabolism [63]. The second group is composed of sequences from alpha, beta and gamma proteobacteria that show the specific residues involved in AcdS activity [27,57]. This group should thus been considered as the true AcdS cluster.

The fully sequenced strain *Pseudomonas syringae* pv *tomato* DC3000 has one amino acid sequence that falls within the first clade and another one that falls within the true AcdS cluster. This suggests an ancestral duplication event that gave rise to the two copies, followed by functional divergence.

P. brassicacearum STM196 AcdS is closely related to *Rhizobium* and *Mesorhizobium* AcdS sequences. Previous phylogenic characterization based on 16S rRNA and on *atpD* nucleotidic sequences also indicated that *P. brassicacearum* STM196 was closely related to these two genera [42]. It suggests that AcdS, 16S rRNA and *atpD* followed the same evolutionary path with no recombination among genera, and that *P. brassicacearum* STM196 most certainly diverged only recently from a common ancestor shared with *Mesorhizobium*.

More surprisingly, although 73 complete alpha-proteobacteria genomes are now available from GenBank, AcdS sequences were only obtained from alpha-proteobacteria strains known to interact with plants. It includes Rhizobium (symbiotic bacteria nodulating with Leguminosae) but also rhizospheric strains such as *Azosipirillum* or pathogenic strains such as *Agrobacterium tumefaciens*. It also includes alpha-proteobacteria collected from the seashore (*Sagittula stellata* E-37, *Labrenzia aggregata* IAM 12614), in the Sargasso Sea (*Fulvimarina pelagi* HTCC2506), or in close association with Dinoflagellate microscopic algae (*Roseovarius* sp. HTCC2601). One could tentatively speculate that interaction with a living photosynthesis organism, terrestrial or not, may

impose the presence of the AcdS gene. It finally suggests that *P. brassicacearum* is truly a bacterial strain that is able to interact with plants.

To test the functional importance of AcdS activity during the interaction of *P. brassicacearum* STM196 with plants, an *acdS* knock-out mutant was constructed using the gene disruption method. The inability of the *acdS* mutant to grow in the presence of ACC as sole N source while the WT strain did grow (Fig. 1B) indicates that the product of the *acdS* gene is involved in the metabolism of ACC. This conclusion is reinforced by the fact that the *P. putida* UW4 WT and its *acdS* strain already characterized for AcdS deficiency [36] displayed similar differential capacities of growing on ACC medium (Fig. 1B).

Close to the *acdS* sequence, an ORF presenting homologies with *lrpL* genes was found in a configuration that has already been described in several bacteria (*E. cloacae/P. putida* UW4 [64,65] and *R. leguminosarum* bv. *viciae* 128C53K [46]). Proteins of the Lrp-like family are transcription factors binding on a specific consensus sequence in bacterial gene promoters [66]. Putative Lrp binding sequences have been found in the upstream regions of *E. cloacae/P. putida* UW4 and *R. leguminosarum* bv. *viciae* 128C53K *acdS* genes [46,65]. Moreover, the requirement of a functional Lrp-like protein for the *acdS* transcription has been shown for both strains [46,64]. Nevertheless, we failed to find a putative Lrp binding sequence in the *acdS* promoting region of *P. brassicacearum* STM196. Therefore, whether the product of *lrpL* gene is involved or not in the regulation of *acdS* expression in *P. brassicacearum* STM196 is unclear. It is worthwhile mentioning that the endosymbiotic bacteria *M. loti* MAFF303099, which has a functional *acdS* gene, has no *lrpL* gene. Thus, the regulation of *acdS* expression by Lrp-like proteins is seemingly not universal among alpha-proteobacteria.

Surprisingly, neither the *acdS* mutant nor the WT strains of *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K were able to grow on the ACC medium, while both *P. brassicacearum* STM196 and *P. putida* UW4 grew in this condition (Fig. 1B). For these latter strains, this suggests that AcdS activity is either constitutive or that the presence of ACC in the medium was sufficient to induce it. An earlier report showed that ACC at concentration in the micromolar range was sufficient to induce expression of AcdS in *R. leguminosarum* bv. *viciae* 128C53K [67]. To reconcile our results with this report, one may imagine that *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K would not be devoid of any AcdS activity, but that this activity is not as strong as it is in *P. brassicacearum* STM196 and *P. putida* UW4 in our experimental conditions. In any case, it is interesting to note that the two bacteria that were unable to grow on ACC in our experiment are those which are known to develop N_2 fixing endosymbiosis with legume plants. In *M. loti* MAFF303099, the *acdS* gene was found in the symbiotic island and its expression depends upon the nitrogen fixation regulator NifA2 [45]. If the *acdS* gene is regulated in *R. leguminosarum* bv. *viciae* 128C53K in the same way as it is in *M. loti* MAFF303099, it would explain why these two strains were unable to grow on ACC medium, some plant originating factor being required for its expression. If this were correct, the *P. brassicacearum STM196* and *P. putida* UW4 strains would not need such factor for a functional AcdS activity to operate.

In order to determine the impact of the AcdS activity of *P. brassicacearum* STM196 on root development, we compared the response of *Arabidopsis* seedlings inoculated with the *acdS* mutant or WT strains. The plant response was characterized using two clear phenotypes induced by the *P. brassicacearum* STM196 WT strain, root architecture modifications and root hair elongation. In addition, three other *acdS* rhizobacteria mutants strains and their WT counterparts were used to address the specificity of the impact of AcdS activity.

The root system architecture was described using three parameters, namely the primary root length, lateral root number and total lateral root length. All the rhizobacteria stimulated lateral root growth rate (Fig. 3C), but a closer look reveals slightly different response patterns depending on the strain inoculated (Fig. 4). However, whatever the rhizobacteria used, inoculation of *Arabidopsis* with an *acdS* strain resulted in exactly the same changes in root architecture than inoculation with the corresponding WT strain (Fig. 3). The only exception was found with *P. putida* UW4 for which the *acdS* mutant strains induced an increased lateral root number compared to plants inoculated with the WT strain (Fig. 3B). Since this difference has no impact on the total lateral root length (Fig. 3C), it probably relates specifically to very short lateral roots. The growth rate of lateral roots was slightly stimulated at the emergence stage, so that a greater number became visible in plants inoculated with the *acdS*-deficient strain, but they seemingly did not maintain a high growth rate in later stage. Observing a potential effect of *P. putida* UW4 AcdS activity on lateral root development would thus require a more detailed analysis of lateral root primordia activity.

The inoculation by any of the rhizobacteria tested led to a 2–3-fold increase in root hair length in *Arabidopsis* (Fig. 5). A similar effect has been reported for many other PGPR strains [10,12,68–72]. Inoculating with the *acdS* mutants led to significant, supplementary increase in root hair length (Fig. 5). Moreover, this increase is independent from the bacterial strain indicating that the observed effect on root hair elongation is in fact due to the *acdS* mutation. This differential pattern response is in favor of the hypothesis proposed earlier according to which the AcdS activity of rhizobacteria decreases ethylene production in root by diverting ACC [15]. Indeed, this hypothesis predicts that *acdS* mutants would not decrease ethylene production in root, the longer root hair phenotype being then explained by the positive role of ethylene on root hair elongation [59].

The fact that several mutant strains of different genetic backgrounds caused similar results indicates that all the respective WT strains have an AcdS activity when they are in contact with *Arabidopsis* roots. On the other hand, as discussed above, the AcdS activity of *M. loti* MAFF303099 and *R. leguminosarum* bv. *viciae* 128C53K were not significantly functional in the absence of plant (*in vitro* assay, Fig. 1B). It could indicate that these strains require some plant factor that is present in *A. thaliana* rhizosphere to induce *acdS* expression. In

contrast, *P. brassicacearum* STM196 and *P. putida* UW4 strains would have a functional AcdS activity without such a factor be needed.

Although comparing the effects of four *acdS* mutants and WT rhizobacterial strains showed that PGPR affect root hair elongation in the host plant *via* a modification of plant ethylene metabolism, the main effect of the PGPR tested on root hair elongation appears to be independent of the ethylene pathway. This is demonstrated by the effect of the rhizobacterial WT strains on the *Arabidopsis* ethylene insensitive mutant *ein2-1*. As described earlier [59], the *ein2-1* mutants had shorter root hairs than the WT plants (Fig. 6), indicating that the ethylene transduction pathway is required for complete elongation of root hairs to occur. Surprisingly, all the three PGPR strains tested with *ein2-1* (*P. brassicacearum* STM196, *P. putida* UW4 and *M. loti* MAFF303099) induced an increase in root hair length. The effect of the WT strains, therefore, involves an ethylene-independent regulation. Alternatively, since many PGPR strains have been shown to produce auxin [73–75], one may imagine that auxin is involved in PGPR induced root hair elongation. However, auxin *Arabidopsis* mutants retain the ability to have longer root hairs upon *P. brassicacearum* STM196 inoculation (data not shown) indicating that the PGPR-mediated root hair elongation is unlikely to depend upon auxin either. This new mechanism that regulates root hair elongation is intriguing since genetic screens for abnormal root hair phenotypes in *Arabidopsis* resulted in the isolation of mutants altered in ethylene or auxin response [60,76,77]. Moreover, our results show that the PGPR WT strains were even more efficient in increasing the length of *ein2-1* root hairs than those of the *Arabidopsis* WT plants. This suggests that ethylene has a negative effect on the ethylene-independent mechanism responsible for root hair elongation induced by the rhizobacteria. In summary, PGPR affect root hair elongation through two mechanisms, one ethylene-dependent signaling pathway that relies on rhizobacterial AcdS activity, and one ethylene-independent mechanism that explains the largest part of the response but is negatively affected by ethylene.

In conclusion, our study shows that the AcdS activity of PGPR does affect ethylene metabolism at a level that can be sensed by *Arabidopsis* host plant. However, the impact of AcdS is seemingly modest and probably affects specifically local regulatory mechanisms, such as the ones controlling root hair elongation. By contrast, lateral root development, which process is integrated at the whole plant level and involves systemic regulation by shoot-derived compounds [78], appears not to be affected by the AcdS activity of rhizosphere colonizing bacteria. Consistent with this conclusion, the stimulation of lateral root development in *Arabidopsis* by *P. brassicacearum* STM196 requires normal auxin transport and transduction pathway (Contesto and Touraine, unpublished results).

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