1-Aminocyclopropane-1-carboxylate deaminase producers associated to maize and other Poaceae species

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Abstract

Background: Complex plant-microbe interactions have been established throughout evolutionary time, many of them with beneficial effects on the host in terms of plant growth, nutrition, or health. Some of the corresponding modes of action involve a modulation of plant hormonal balance, such as the deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). Despite its ecological importance, our understanding of ACC deamination is impaired by a lack of direct molecular tools. Here, we developed PCR primers to quantify the ACC deaminase gene acdS and its mRNA in soil communities and assessed acdS+ microorganisms colonizing maize and other Poaceae species.

Results: Effective acdS primers suitable for soil microbial communities were obtained, enabling recovery of bona fida acdS genes and transcripts of diverse genetic backgrounds. High numbers of acdS genes and transcripts were evidenced in the rhizosphere of Poaceae, and numbers fluctuated according to plant genotype. Illumina sequencing revealed taxonomic specificities of acdS+ microorganisms according to plant host. The phylogenetic distance between Poaceae genotypes correlated with acdS transcript numbers, but not with acdS gene numbers or the genetic distance between acdS functional groups.

Conclusion: The development of acdS primers enabled the first direct analysis of ACC deaminase functional group in soil and showed that plant ability to interact with soil-inhabiting acdS+ microorganisms could also involve particular plant traits unrelated to the evolutionary history of Poaceae species.

Keywords: ACC deaminase functional group, Rhizosphere community, Poaceae evolution

Background

Growth, development, and health of macroorganisms are strongly influenced by the interactions they develop with their associated microbial community [1–3]. These interactions often involve nutrient exchanges between partners, the host typically providing organic carbon [4–7] while microorganisms supply amino acids or mineral nutrients, which results from various processes such as nitrogen fixation or phosphorus solubilization [8]. Many of these interactions are complex and entail also the exchange of molecular signals [2]. In the case of plants, microbial partners might influence host hormonal balance by producing molecules mimicking phytohormones (e.g., auxins, gibberellins, or jasmonates) or enzymes that modulate plant hormonal production, notably by degrading the ethylene biosynthetic precursor 1-aminocyclopropane-1-carboxylate (ACC) [2,8,9].

Ethylene is a plant hormone that regulates plant development and stress responses [10,11]. Microorganisms able to produce ACC deaminase transform the ethylene precursor ACC into α-ketobutyrate and ammonia [12,13]. By degrading ACC within roots or in exudates (thereby leading to a sink effect), root-interacting bacteria are indirectly lowering ethylene level in plant roots, thus stimulating root growth and modulating plant stress resistance [9,14–17]. The acdS gene encoding ACC deaminase is highly conserved among microorganisms and has been used to study the phylogeny and diversity of ACC deaminase producers [18–20] in bacteria and micro-eukaryotes (i.e., fungi and stramenopiles). Although horizontal
transfer of acdS between bacteria was suspected due to incongruence between acdS and 16S rRNA gene-based bacterial phylogenies [21, 22], a more exhaustive assessment suggested that acdS was mainly inherited vertically, with only occasional horizontal gene transfers [20]. Thus, acdS is a marker suitable to assess complex ACC deaminase functional communities.

In the root zone, the plant may be colonized by different types of acdS+ microorganisms [23, 24], which are likely to contribute jointly to degradation of ACC produced by roots, and the overall significance of ACC deamination for the plant is expected to result from the combined functioning of its acdS+ microbial partners [2]. However, even though various kinds of acdS+ microorganisms can be readily isolated from the rhizosphere, there is no direct PCR tool available to assess the entire functional group of root-associated acdS+ microorganisms, i.e., including non-cultured taxa and strains.

It is long established that plants shape their rhizosphere bacterial community from the telluric bacterial reservoir [25, 26]. Plant species and plant genotypes within species exhibit specific phenotypic traits, including root properties that are likely to influence rhizobacterial community composition [7]. And indeed, several studies showed the impact of plant genotype on the taxonomic composition of their associated bacterial community [27–30]. This type of effect has also been evidenced when assessing the genetic diversity of functional groups important for plant growth, such as 2,4-diacetylphloroglucinol-producing pseudomonads [31–33], nitrogen fixers [34], or microorganisms involved in other biogeochemical transformations [35], and it might well be that similar effects also take place with acdS+ microorganisms.

Therefore, this work aimed at testing the hypothesis that the functional group of acdS+ microorganisms differed according to plant genotype, which was done in the case of Poaceae. To this end, protocols for quantitative PCR (qPCR) and quantitative reverse-transcription PCR (qRT-PCR) of acdS were developed and validated for analysis of acdS+ microorganisms within the rhizosphere. These tools as well as acdS MiSeq sequencing were implemented on eight Poaceae genotypes previously used in Bouffaud et al. [36] and enabling comparisons at different Poaceae taxonomic levels, i.e., between individual inbred lines within two maize genetic groups (Corn Belt Dent and Northern Flint), between two maize genetic groups, between these maize genetic groups and a teosinte (representing maize’s pre-domestication Zea mays ancestor), and between Z. mays and another member (sorghum) from maize’s Panicoideae subfamily or a member (wheat) from the neighboring Pooideae subfamily.

**Results**

**Validation of qPCR and qRT-PCR tools**

We developed primer sets (Additional file 1: Table S1) to specifically amplify acdS gene sequences in the acdS reference database constructed. Eight primer pairs were discarded as they failed to specifically amplify acdS from pure bacterial genomic samples. Another one was discarded as it could not amplify acdS genes from a soil community, and only pair acdSF5/acdSR8 (Additional file 1: Tables S1, S2 and Additional file 2: Figure S1) was kept.

qPCR conditions were optimized to obtain an amplification efficiency of > 80% and an error below 0.1 with genomic DNA of acdS strains *P. kilonensis* F113 (i.e., 81.2% and 0.06, respectively) and *Burkholderia cenocepacia* J2315 (i.e., 100% and 0.007, respectively). The detection limit of the qPCR method on pure cultures was 6 acdS gene copies (corresponding to 50 fg of J2315 genomic DNA template). When tested on total DNA or cDNA (reverse transcription on total RNA) obtained from rhizosphere of the different Poaceae, amplification efficiencies above 80% and errors below 0.1 were also obtained, for both soils tested. acdS could always be detected by qPCR in the two bulk soils (Additional file 3: Figure S2).

Illumina MiSeq sequencing of acdSF5/acdSR8 amplions from bulk soils and rhizosphere soils gave 3,903,982 reads (44,287 OTUs) from cropped soil and 1,673,758 reads (28,759 OTUs) from meadow soil. Similarities of acdS sequences with known references were assessed with the in-house core-acdS database extracted from the FunGene database. The phylogenetic tree showed that none of the sequences clustered within the D-cystein-sulphhydrase outgroup (Additional file 4: Figure S3). The OTUs obtained clustered in numerous clades encompassing all the known diversity of the acdS gene (*Proteobacteria, Actinobacteria*, and microeukaryotes; Additional file 4: Figure S3).

**Size of acdS group and number of acdS transcripts in Poaceae rhizosphere**

The size of the acdS group amounted to 0.5–6.2 × 10^6 acdS gene copies per g of rhizosphere soil in the cropped soil at 21 days, 0.5–2.0 × 10^5 acdS copies in the meadow soil at 21 days, and 0.2–3.0 × 10^5 acdS copies in the cropped soil at 42 days (Fig. 1A). Compared to bulk soil, acdS group size was higher in the presence of plant for all four maize lines and wheat at 21 days in cropped soil (all five displaying similar levels), for the two maize lines in the meadow soil at 21 days, and for all plants in the cropped soil at 42 days. A significant effect of past soil management was observed in all four treatments studied (two maize lines, tomato, and bulk soil), but an influence of sampling time was evidenced only for maize line FV4.
The number of *acdS* transcripts reached 0.06–2.9 × 10³ *acdS* cDNA copies per g of rhizosphere soil in the cropped soil at 21 days, 0.08–2.1 × 10³ *acdS* copies in the meadow soil at 21 days, and 0.6–2.2 × 10³ *acdS* copies in the cropped soil at 42 days (Fig. 1B). The *acdS* transcripts in the bulk soil samples from cropped soil at 21 and 42 days were below the quantification limit. A strong positive influence of *Poaceae* on the number of *acdS* transcripts was observed in both soils at both sampling times. In addition, *acdS* transcript number in cropped soil was higher for sorghum than maize (except FV4) and teosinte (and tomato) at 21 days, and for FV4 maize than W85 (and tomato) at 42 days, while it was higher for both maize lines studied than for tomato and bulk soil in meadow soil at 21 days. Overall, no significant effect of past soil management or sampling time was found for *acdS* transcript number.

**Diversity of *acdS*⁺ microorganisms colonizing *Poaceae***

Rarefaction analyses resulted in different saturation profiles, suggesting that *acdS* allele diversities differed across the different *Poaceae* rhizospheres (Additional file 5: Figure S4). To estimate *acdS* richness, a subsampling was done with 38,524 sequences per cropped soil sample and with 25,423 sequences per meadow soil sample.

Shannon, Simpson, and Chao diversity indices were calculated for each sample (Tables 1 and 2). In the cropped soil, no significant difference in the diversity of the *acdS* functional group was observed between treatments. In the
Table 1
Diversity indices of acdS functional group. Shannon, Simpson, and Chao indices in bulk soil and rhizosphere were determined at 21 days of plant growth in cropped soil

|               | Bulk soil | NF maize FV4 | NF maize W85 | CBD maize Mo17 | CBD maize FV252 | Teosinte | Sorghum | Wheat |
|---------------|-----------|--------------|--------------|----------------|-----------------|----------|---------|-------|
| Shannon       | 7.12 ± 0.33 | 6.26 ± 1.63 | 7.54 ± 0.19  | 7.18 ± 0.70    | 6.93 ± 0.14     | 7.21 ± 0.41 | 7.23 ± 0.11 | 7.28 ± 0.16 |
| Simpson       | 0.0051 ± 0.0039 | 0.0613 ± 0.1174 | 0.0028 ± 0.0009 | 0.0075 ± 0.0102 | 0.0076 ± 0.0044 | 0.0076 ± 0.0044 | 0.0030 ± 0.0006 | 0.0054 ± 0.0041 |
| Chao          | 14,326 ± 2006 | 12,578 ± 2984 | 15,996 ± 604 | 14,156 ± 3100 | 13,783 ± 579    | 14,030 ± 3095 | 14,049 ± 439 | 15,585 ± 1237 |

NF Northern Flint genetic group of maize, CBD Corn Belt Dent genetic group of maize. Data are means ± standard deviations (n = 5)

Relationship between Poaceae evolution and acdS functional group
The assessment of the influence of past Poaceae evolution on recruitment of acdS microorganisms did not yield any significant correlation, regardless of whether pairwise Poaceae phylogenetic distances were crossed with (i) differences in raw numbers (or log-numbers; Additional file 1: Table S3) of acdS genes copies per g of rhizosphere soil (P = 0.64; Fig. 4b) or g of root (P = 0.24; Fig. 4c), or (ii) differences in pairwise Bray-Curtis dissimilarity indices of acdS communities (P = 0.83; Fig. 4a), at 21 days in the rhizosphere of Poaceae grown in cropped soil. When considering the functioning of the acdS community, however, a significant correlation with pairwise Poaceae phylogenetic distances was found when computing differences in raw numbers (but not log numbers; Additional file 1: Table S3) of acdS transcript copies per g of rhizosphere soil (Spearman rho = 0.65; P = 0.002; Fig. 4d) or g of root (Spearman rho = 0.53; P = 0.02; Fig. 4e).

Relationship between maize/Poaceae evolution and particular rhizobacterial populations
Among the 20 most abundant genera representing over 90% of acdS functional group diversity, a significant positive correlation (P < 0.05) was found for four Actinobacteria, i.e., Agromyces (rho = 0.61; P = 0.006), Kinesosphera (rho = 0.61; P = 0.005), Saccharopolyspora (rho = 0.57; P = 0.009), and Phycicoccus (rho = 0.51; P = 0.022) when comparing read abundance for these genera to the log value of the maximum likelihood phylogenetic distance between Poaceae (Additional file 6: Figure S5).

Discussion
Since the rhizosphere microbiota results mostly from the selection of soil-residing microorganisms [25, 26],

Table 2
Diversity indices of acdS functional group. Shannon, Simpson, and Chao indices in bulk soil and rhizosphere were determined at 21 days of plant growth in meadow soil

|               | Bulk soil | NF maize FV4 | NF maize W85 | Tomato |
|---------------|-----------|--------------|--------------|--------|
| Shannon       | 6.09 ± 1.26 ab | 6.71 ± 0.16 a | 6.68 ± 0.13 ab | 6.19 ± 0.25 b |
| Simpson       | 0.054 ± 0.089 ab | 0.009 ± 0.001 a | 0.010 ± 0.002 ab | 0.020 ± 0.007 b |
| Chao          | 12,870 ± 1882 | 12,353 ± 730 | 12,814 ± 834 | 11,957 ± 1316 |

NF Northern Flint genetic group of maize, CBD Corn Belt Dent genetic group of maize. Data are means ± standard deviations (n = 5). Statistical differences (when any) are indicated using letters a and b (Kruskal-Wallis tests; P < 0.05)
root exudates and other rhizodeposits are expected to play a major role [5], which could explain why this microbial selection can differ according to host genetics [37]. Indeed, plant genetic background was shown to be an important factor shaping the root-associated microbiota, when considering both taxonomic [28, 29, 36, 38] and functional microbial groups, e.g., 2,4-diacetylphloroglucinol-producing pseudomonads [39] or nitrogen-fixing bacteria [34].

In the last two decades, much has been done to document the impact of plant genotype and/or development stage on rhizosphere microbial diversity, with an emphasis on taxonomic assessments using 16S and 18S rRNA genes as well as internal transcribed spacers [40]. However, these taxonomic assessments do not tell much about the functional potential of the microbiota, and this gap limits our capacity to understand the functioning of the plant holobiont [41]. This issue may be targeted at the scale of the entire microbial community, by metagenomics or metatranscriptomics [28, 42], as well as with a focus on particular ecological functions of prime significance, such as nitrogen fixation [34, 43, 44], nitrification [44, 45], or production of antimicrobial metabolites [46].

For certain key functional groups of soil microorganisms, primers and PCR protocols necessary for direct analysis of soil and rhizosphere populations have been used for years (e.g., [33, 46, 47]), but for others, the tools are not available yet. This was the case in particular for...
ACC deaminase-positive microorganisms, despite the research emphasis put on this microbial function in the last 20 years (> 10,000 papers on these microorganisms as listed in Google Scholar). The ecological importance of microbial ACC deaminase activity derives from its effect on plant metabolism of ethylene, a major phytohormone involved in various plant physiological pathways such as root elongation and immune response [16]. ACC itself acts as a phytohormone [17]. Root-colonizing microorganisms with ACC deaminase activity have the potential to indirectly lower endogenous plant levels in ACC and ethylene, and thus to alleviate environmental stress in plant and enhance root growth [16, 48].

Against this background, our first objective was to develop an acdS-based qPCR method targeting all acdS alleles, which was successfully achieved by using a set of acdS strains representative of the highest diversity possible of documented alleles (from Azospirillum lipoferum CRT1 to Ralstonia solanacearum GMI100; Additional file 4: Figure S3). This is first indicated by the ability of the method to minimize acdS quantification bias, as qPCR performance was satisfactory for two contrasted acdS strains based on the criteria of Zhang and Fang [49] (i.e., $R^2 > 0.95$ and slope between $-3.0$ and $-3.9$ corresponding to PCR efficiencies of 80–115%). Second, it is also indicated by (i) primer specificity and (ii) the possibility to target the known diversity of acdS alleles in Proteobacteria, Actinobacteria, Deinococcus/Thermus, and micro-eukaryotes [19, 20, 50], as revealed by MiSeq Illumina sequencing (Additional file 4: Figure S3). Apart from the absence of sequences affiliated to the thermophilic genus Meiothermus of the Deinococcus/Thermus phylum (the unique genus of this phylum shown to harbor acdS [20]), all the other clades were represented, without over-representation of a given type of acdS sequence (Fig. 2). Therefore, this work...
proposes a novel tool that can be used to monitor the size, transcription, and diversity of the acdS functional group in various plant, soil, and environmental conditions.

Our second and main objective was to assess the influence of past Poaceae evolution on root interactions with acdS microorganisms, based on the rationale that bacterial taxa harboring acdS strains colonize roots of different Poaceae [27, 28, 30], acdS microorganisms are readily isolated from these plants [2, 21, 51, 52], and they can stimulate growth of cereals [53–56]. Recruitment of acdS microorganisms by all Poaceae was indeed shown by qPCR (Fig. 1), and it might be an indication of the importance of ACC deamination for the plant. Accordingly, we found a strong increase in the numbers of acdS ARN m (cDNA) when comparing the rhizosphere to bulk soil, showing for the first time the stimulation of acdS transcription, and diversity of the acdS functional group in natural soil and plant systems. We then analyzed the microbial ACC deaminase functional group in the context of Poaceae evolutionary history. Correlations were significant when considering pairwise differences in raw numbers of acdS transcripts versus the genetic distance between acdS groups. This is the first evidence of a link between past Poaceae evolution and the functioning of a root-associated microbial group, such a link having not been observed for instance when considering N-fixing bacteria [34]. It also raises the possibility that microbial modulation of ethylene metabolism has evolved to different extents in various Poaceae lineages, and this issue will deserve further research attention.

Methods
Identification of acdS primers
All available (in February 2016) sequences from the acdS/D-cysteine-sulphydrase FunGene sequence database were retrieved and aligned using MUSCLE. Key amino acid positions known to be important for ACC deaminase activity, namely Lys51, Ser78, Tyr295, Glu296, and Leu322 [59] were searched using Pseudomonas kilonensis F113 as a reference. Sequences presenting different amino acids in the key positions were discarded, as they are likely to correspond to D-cysteine desulfhydrases [59].

The acdS sequences and acdS homologs (also included in the alignment to serve as non-target sequences) from the D-cysteine desulphydrase producer strains Pseudomonas syringae DC3000 (AE16853), Achromobacter xylooxidans BM1 (AY604540), Achromobacter sp. CM1 (AY604541), Rizobium sp. TAL1145 (EU183544), Serratia proteamaculans SUD165 (AY604543), Escherichia coli K12 (CP014348), Pseudomonas marginalis (AY604542), and Enterobacter aerogenes Cal3 (AY604544) were aligned using MUSCLE [60]. This alignment was used to perform a phylogenetic analysis on acdS sequences. The tree was inferred from 1000 nucleotides using the neighbor-joining (NJ) method in MEGA4 [61] with the Kimura two-parameter method for distance calculation [62]. Nodal robustness of the tree was assessed using 1000 bootstrap replicates.

Using the acdS alignment, primers (25–30 bp in length) were visually selected in regions conserved among the acdS sequences and absent from the non-target sequences. The new primers (Additional file 1: Table S1) were then assessed based on the following criteria: (i) a melting temperature (Tm) of 60 to 67 °C, (ii) an absence of predicted hairpin loops and primer-dimer formations [63], (iii) a Tm difference between primers not exceeding 1 °C, (iv) an amplification product not exceeding 300 bp, (v) a maximum of three mismatches between each primer and the 1304 acdS sequences, and (vi) the ability to specifically amplify acdS in genomic DNA samples (using 6 ng of genomic DNA from the acdS+ strains Azospirillum lipoferum TVV3, 4B and RSWT1, Burkholderia cepacia LMG 1222, B.
Development of \( \text{acdS} \) strains

Quantitative PCR assays were conducted using LightCycler 480 SYBR Green I Master mix in a final volume of 20 \( \mu \)L and a LightCycler 480 (Roche Applied Science, Meylan, France). Cycle threshold (Ct) of individual samples was calculated using the second derivative maximum method in the LightCycler Software v.1.5 (Roche Applied Science). The standard curves were obtained by plotting the mean Ct value of the three replicates (per DNA concentration) against the log-transformed DNA concentration. Amplification efficiency (E), calculated as \( E = 10^{(-1/\text{slope})} - 1 \), and the error of the method (mean squared error of the standard curve) were determined using the LightCycler Software v.1.5 (Roche Applied Science). Standard curves were generated using genomic DNA of \( P. \) kilonensis F113 and \( B. \) cenocepacia J2315, two bacterial genomes harboring a single \( \text{acdS} \) copy. \( \text{acdS} \) copy number was computed as \( [\text{DNA} \times \text{Avogadro's number (molecules mol}^{-1})]/[\text{number of DNA base pairs in } \text{acdS} \text{ fragment } \times 660 \text{ (g mol}^{-1})] \), based on the average of 660 g mol\(^{-1}\) per base pair. Amplification specificity was assessed by melting curve analysis of PCR products, done by ramping the temperature to 95 °C for 10 s and back to 65 °C for 15 s, followed by increases of 0.1 °C s\(^{-1}\) up to 95 °C.

qPCR optimization was sought to improve \( \text{acdS} \) amplification efficiency (above 80%) and error (below 0.01) for the \( \text{acdS} \) strains \( P. \) kilonensis F113 and \( B. \) cenocepacia J2315. Three primer concentrations (0.5, 0.75, and 1 \( \mu \)M), four annealing temperatures (66, 67, 68, and 70 °C), two annealing times (30 and 15 s), and three elongation times (30, 15, and 10 s) were tested.

The final qPCR protocol used primers \( \text{acdSF5/acdSR8} \) amplifying a fragment of 133 nt. Reaction mix contained 10 \( \mu \)L of LightCycler 480 SYBR Green I Master (Roche Applied Science), 1 \( \mu \)M of each primer, and 2 \( \mu \)L of DNA extract. The final cycling program included a 10-min incubation at 95 °C, 50 amplification cycles of 30 s at 94 °C, 7 s at 67 °C, and 15 s at 72 °C, and the fusion program for melting curve analysis described above. The generated standard curve from genomic DNA of \( B. \) cenocepacia J2315 was subsequently used as the external standard curve for determination of \( \text{acdS} \) copy number in uncharacterized DNA samples. Two DNA standards from genomic DNA of \( B. \) cenocepacia J2315 were included as reference in each run to detect between-run variations.

Greenhouse experiment

We used samples from a previous greenhouse experiment [36] performed with one wheat (\( Triticum aestivum \) L. cv. Fiorina; Agroscope, Changins, Switzerland), one sorghum (\( Sorghum bicolor \) L. cv. Arprim; Semences de Provence, Fourques, France), one teosinte (\( Zea mays \) ssp. parviglumis; UNAM, Cuernavaca, Mexico), four maize (\( Zea mays \) L.) inbred lines (FV252 and Mo17 from group Corn Belt Dent, and FV4 and W85 from group Northern Flint; INRA, St Martin de Hinx, France), and one tomato (\( Solanum lycopersicum \) L. cv. Marmande; Vilmorin, La Ménitré, France) considered as an arbitrary, external (non-Poaceae) reference.

The plants were cultivated by Bouffaud et al. [36] in one or two sieved (6 mm) soils collected from the top-soil of two neighboring fields (luvisols) at La Côte Saint-André (France). All were grown in soil from a maize-monocropping field (loam, organic matter 2.3%, \( \text{pH}_{120} \) 7.3, N 1.6 g kg\(^{-1}\) and three selected genotypes (see below) also in soil from a permanent meadow (loam, organic matter 5.5%, \( \text{pH}_{120} \) 6.0, N 3.2 g kg\(^{-1}\)). In short, pots of 3 dm\(^3\) holding 2.5 kg soil were sown with surface-disinfected seeds (to get one seedling per pot) or kept as non-planting pots, with five pots per treatment, and they were placed in a greenhouse (randomized blocks). For all treatments, samples were taken at 21 days in cropped soil, whereas maize lines FV4 and W85 and tomato were also studied at 42 days in the same soil and at 21 days in meadow soil. Root systems were individually unearthed and shaken to discard loosely adhering soil. Roots and tightly adhering soil were frozen in liquid nitrogen and lyophilized, and rhizosphere soil was collected from roots (0.5–6 g soil per plant) and placed at –20 °C. Root, rhizosphere soil, and shoot dry weights were measured (Additional file 7: Figure S6). Bulk soil was taken from each non-plant pot (5 g) at 21 (two soils) and 42 days (cropped soil), frozen, lyophilized, and stored at –20 °C.

Extraction of DNA and RNA from rhizosphere and bulk soil

We used total nucleic acids that were extracted by Bouffaud et al. [36], as follows. Briefly, 0.5 g bulk or rhizosphere soil, 0.5 ml extraction buffer (5%
hexadecyltrimethylammonium bromide, 1 mM 1,4-dithio-DL-threitol, in a 0.12 M phosphate buffer at pH 8), and 0.5 g zirconium beads (VWR, Fontenay-sous-Bois, France) were used in a bead beater (TissueLyser II Retsch; Qiagen, Courtaboeuf, France) for 90 s at 30 m s⁻¹. After 10 min centrifugation at 16,000g, supernatants were extracted twice in phenol-chloroform-isooamyl alcohol (24:24:1 v/v/v) and once in chloroform-isooamyl alcohol (24:1 v/v). Nucleic acids were precipitated overnight using absolute ethanol and potassium acetate (3 M, pH 4.8) at -20 °C. After centrifugation for 30 min at 16,000g, pellets were washed in 70% ethanol and dissolved in 100 μL RNase-free DNase-free water (giving 50–100 ng nucleic acids μL⁻¹).

Reverse-transcription synthesis of cDNA
DNA-free RNA was obtained by treating 20 μL of nucleic acid solution with 4 U of DNase I (Invitrogen, Cergy Pontoise, France) in 1× DNase I reaction buffer at room temperature. DNA digestion repeated to remove remaining DNA traces, the reaction was stopped in presence of 1 μL of 25 mM EDTA (10 min at 65 °C), and RNA was purified with RNeasy Mini kit (Qiagen) according to manufacturer’s protocol. DNA contamination after the DNase I treatment of RNA samples was indicated by lack of qPCR amplification (performed as described below), and in the few cases where amplification did take place, an additional DNase I treatment was performed and no qPCR amplification took place then.

Total cDNA synthesis was carried out with 8 μL of resulting purified RNA extract, using random hexanucleotide primers (Invitrogen) and Omniscript reverse transcription kit (Qiagen) following the manufacturer’s instructions. DNA contamination after the DNase I treatment of RNA samples was indicated by lack of qPCR amplification (performed as described below), and in the few cases where amplification did take place, an additional DNase I treatment was performed and no qPCR amplification took place then.

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qPCR and qRT-PCR analyses of acdS
The quantities of acdS genes (qPCR) and mRNA (qRT-PCR) were estimated using 20-μL containing 4 μL of PCR grade water, 1 μL of each primer (final concentration 1 μM), 10 μL of LightCycler-DNA Master SYBR Green I master mix (Roche Applied Science), and 2 μL of sample DNA (10 ng). The cycling program entailed 10 min incubation at 95 °C, followed by 50 cycles of 94 °C for 15 s, 67 °C for 15 s, and 72 °C for 10 s. The fusion program for melting curve analysis is described above. Amplification specificity was assessed by melting curve analysis of PCR and RT-PCR products, done by ramping the temperature to 95 °C for 10 s and back to 65 °C for 15 s, followed by increases of 0.1 °C s⁻¹ up to 95 °C. Melting curve calculations and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software v.1 (Roche Applied Science).

Standard curves were obtained using DNA from B. cenocepacia J2315, whose genome contains one acdS copy, after diluting in triplicate from 5 × 10⁻⁹ to 5 × 10⁻¹⁵ g DNA μL⁻¹. PCR efficiency was derived from standard curves as \( E = 10^{(-1/slope)} \). All five samples for rhizosphere or bulk soil treatment were assessed, and data expressed in g μL⁻¹ were converted into numbers of acdS copies computed as [DNA (g) × Avogadro’s number (molecules mol⁻¹)]/[number of DNA base pairs in PCR template × 660 (g mol⁻¹)], based on an average of 660 g mol⁻¹ per base pair.

acdS sequencing from rhizosphere DNA
Primer specificity was assessed by Illumina MiSeq sequencing of acdSF5/acdSR8 amplicons (size ~ 133 nt), using bulk soils and rhizosphere soils from tomato and six Poa ceae cultivated in cropped or meadow soil. DNA extracts were sent to MR DNA laboratory (www.mrdnalab.com; Shallowater, TX) for acdS sequencing. The qPCR primers acdSF5/acdSR8 were used for the sequencing library, the forward primer carrying a barcode. The 30-cycle PCR (done five times, hence 150 cycles in total) used the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) with 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. Samples were pooled together in same proportions, purified with calibrated Ampure XP beads and the DNA library was obtained with Illumina TruSeq DNA library protocol. Sequencing was carried out on a MiSeq following the manufacturer’s instructions.

Sequence data were treated with MR DNA pipeline. Briefly, sequences were depleted of barcodes, sequences <120 bp or >160 bp or with ambiguous base calls removed, the remaining sequences denoised, operational taxonomic units (OTUs; defined at 3% divergence threshold) generated, and chimeras removed. OTUs were then classified using BLASTn and a curated acdS database (described in acdS database supplemental material). Briefly, the acdS in-house database (see the “Identification of acdS primers” section) developed to define the qPCR primers was adapted to exhibit only the 133 nt corresponding to the amplified PCR fragments. Moreover, when different accessions presented 100% identity in nucleotide sequences and were affiliated to the same bacterial species, only one sequence was kept, reducing the database entries to 1304 different sequences (named core-acdS database; see acdS database supplemental material) representing the phylogenetic diversity of ACC deaminase producers defined based on published data [18–20, 50]. Dataset without singletons was used to generate rarefaction curves and Shannon, Simpson, and Chao diversity indices (calculated using sequencing subsample data for which each sample had the same number of sequences).
Alignment of selected acdS sequences (i.e., ten randomly chosen OTUs per genus) of the core-acdS database and eight related D-cystein desulphhydrase genes (see the “Identification of acdS primers” section) used as outgroup was carried out using MAFFT v7.123b (2013/10/15) [65, 66]. An acdS phylogenetic tree was constructed based on maximum likelihood method using RAxML 8.2.8 software [67]. Trees were annotated using iTOL V3 [68].

Statistical analyses
Experimental treatments were compared based on log-numbers of acdS genes and mRNA, using ANOVA and Fisher’s LSD tests, in each of the soils and at each sampling time. Two-factor ANOVA and Fisher’s LSD tests were also done, to consider sampling time effects in cropped soil (9 treatments × 2 samplings) as well as past soil management at 21 days (4 treatments × 2 past soil managements). Comparisons for bacterial composition data were carried out by between-class analysis (BCA) (ADE4 R and ggplot2 packages) and Kruskal-Wallis rank sum test associated to Tukey’s HSD test.

To evaluate the influence of past Poaceae evolution on root interactions with acdS microorganisms, Spearman correlation analysis was carried out between the phylogenetic distance between Poaceae genera and the corresponding pairwise differences in (i) acdS gene or transcript raw numbers (based on Euclidean distances) or (ii) acdS microbial community composition (based on Bray-Curtis dissimilarity indices). The former was computed by Bouffaud et al. [36] from concatenated chloroplastic sequences of gene rps16 and intergenic regions rps16-trnK and atpI-atpH, using the maximum likelihood method and Kimura two-parameter model.

All analyses were done at P < 0.05, using R 2.10.1 software (https://www.r-project.org).

Nucleotide sequence accession numbers
Reads have been deposited in the European Bioinformatics Institute (EBI) database under accession number PRJEB24637.

Additional files

**Additional file 1:** Table S1. Universal primers designed to target acdS alleles. Table S2. Universal primer pairs tested to amplify specifically acdS alleles, with selected primer pair indicated in bold. Table S3. Spearman correlation analysis of the relation of pairwise plant phylogenetic distance between Poaceae with various Euclidean distances between log-transformed qPCR data describing the corresponding acdS communities. (DOCX 74 kb)

**Additional file 2:** Figure S1. Examples of PCR amplification with the different primer pairs tested. (A) acdS5/acsR7; (B) acdS5/acsR8; (C) acdS5/acsR7; (D) acdS5/acsR8; (E) acdS5/acsR10. The different strains tested were 1. Azospirillum lipoforum 4B; 2. A. lipoforum TVV3; 3. A. lipoforum CRT1 (acdS); 4. A. lipoforum RSWT1; 5. Burkholderia cepacia LMG1222; 6. R. eomycococcum LMG16656; 7. B. stabilis LMG14294; 8. B. dolosa LMG18941; 9. Pseudomonas rhizoalcalis PIR2; 10. P. kilonensis F113; 11. P. protegens CHA1 (acdS); and 12.Ralstonia solanacearum GM1000. (TIFF 13453 kb)

**Additional file 3:** Figure S2. Example of the acdS amplification curves (A), standard curve (B), melting peaks (C) obtained using DNA from Burkholderia eomycococcum J2315, and (D) and (E) obtained using rhizospheric metagenomics DNA serially diluted. (TIF 602 kb)

**Additional file 4:** Figure S3. RAxML bipartition tree of 3322 sequenced acdS alleles from Poaceae rhizosphere. The tree was visualized using iTOL software. Branches colored in violet represent the out-group of D-cystein desulphhydrase genes, whereas acdS alleles affiliated to Betaproteobacteria are shown in khaki, to Gammaproteobacteria in blue, to Actinobacteria in green, to Alphaproteobacteria in red, and to microeukaryotes in orange. Branches are indicated in bold when corresponding to at least two genera (http://itol.embl.de/shared/acdStree). (PDF 892 kb)

**Additional file 5:** Figure S4. Rarefaction curves showing the number of microbial OTUs according to the number of acdS reads, based on observed data obtained from bulk soil or rhizosphere. Data for cropped soil are in full-lines and for meadow soil in dash-lines. (PDF 479 kb)

**Additional file 6:** Figure S5. Pairwise comparison of plant phylogenetic distance between Poaceae (X-axis) with the corresponding Euclidean distance between acdS reads for each of the 20 most abundant microbial genera representing over 90% of acdS functional group diversity (Y-axis). The 18 maize-based comparisons are indicated using black triangles (maize-maize; n = 6), diamonds (maize-teosinte; n = 4), circles (maize-sorghum; n = 4), or squares (maize-wheat; n = 4), and the three other comparisons using a white circle (teosinte-sorghum), white triangle (teosinte-wheat) or white square (sorghum-wheat). Distances were calculated two by two, using Kimura two-parameter model for plant phylogeny and Euclidean distance for taxa. (TIF 155 kb)

**Additional file 7:** Figure S6. Root (A), rhizosphere soil (B), and shoot (C) dry weights. Statistical analyses were performed independently at 21 days in cropped soil, at 21 days in meadow soil, and at 42 days in cropped soil, using ANOVA and Fisher LSD tests (P < 0.05; differences shown with letters a to d). For maize lines FV4, W85, tomato, and bulk soil, two-way ANOVA and Fisher LSD tests (P < 0.05) were also performed to compare treatments according to past soil management or sampling time, and differences with the same genotype at 21 days in cropped soil are indicated by symbols * and #, respectively. (PDF 115 kb)

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Authors’ contributions
MLB, YML, and DM conceived and designed the project. MLB performed the greenhouse experiment and qPCR. SR and AD analyzed the acdS sequences. MLB, SR, YML, and DM analyzed the findings and wrote the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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