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ORIGINAL PAPER

Azospirillum argentinense Modifies Arabidopsis Root Architecture Through Auxin-dependent Pathway and Flagellin

Verónica Mora[1] · Gastón López[1] · Romina Molina[1] · Anahí Coniglio[1] · Sofía Nievas[1] · Nuria De Diego[2] · Sanja Ćavar Zeljković[2,3] · Sara Salcedo Sarmiento[2] · Lukáš Spíchal[2] · Sean Robertson[4] · Olivia Wilkins[4] · Juliana Elías[5] · Raúl Pedraza[5] · José Manuel Estevez[6,7,8] · Mark F. Belmonte[4] · Fabricio Cassán[1]

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Abstract

Toevaluate if root architecture changes observed in *Arabidopsis thaliana* inoculated with *Azospirillum argentinense* Az39 depend exclusively on the bacterial capacity to produce indole-3-acetic acid (IAA) and plant ability to sense IAA levels. *Azospirillum argentinense* Az39, *A. argentinense* Az39 *ipd*C–, flagellin from *A. argentinense* Az39, and pure IAA were applied to *A. thaliana* Col-0 (wild-type) and *tir1.1* (a lateral root deficient mutant) seedlings. Inoculation with heat-inactivated *A. argentinense* Az39 cells and a non-PGPR bacterium (*Escherichia coli* DH5α) was also tested. The primary root (PR) length, lateral roots (LR) number, and root hair (HR) density were assessed, and the root transcriptome was sequenced (Illumina HiSeq), followed by DEGs and GO term enrichment analyses. Inoculation with both *A. argentinense* strains resulted in a shorter PR and an increased number of LR and RH. IAA application (0.1 µM) led to a similar root phenotype than inoculation with Az39 (10^8 CFU mL^{-1}). The addition of 1 µM flagellin, as well as plant exposure to non-lysed *A. argentinense* Az39 or *E. coli* DH5α cells, enhanced RH formation. Genes related to auxin signaling were highly expressed in the roots of Az39-inoculated seedlings; genes related to jasmonate and salicylic acid metabolism were highly expressed in the roots of plants inoculated with i*pdC* − . Root architecture changes in *A. thaliana* inoculated with *A. argentinense* Az39 do not depend exclusively on root IAA levels/IAA plant perception. This PGPR induces root morphological changes through both IAA-dependent and IAA-independent mechanisms. Flagellin may be a key molecule involved in IAA-independent mechanisms.

Keywords *Arabidopsis thaliana* · *Azospirillum argentinense* · Plant growth–promoting rhizobacteria · Flagellin · Root growth · Indole-3-acetic acid

Verónica Mora, Gastón López, and Mark F. Belmonte equally contributed to this manuscript.

✉ Fabricio Cassán
fcassan@exa.unrc.edu.ar

[1] Instituto de Investigaciones Agrobiotecnológicas, Universidad Nacional de Río Cuarto (INIAB, UNRC), CONICET, Río Cuarto, Argentina

[2] Czech Advanced Technology and Research Institute, Palacky University, Šlechtitelů 27, 78371 Olomouc, Czech Republic

[3] Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Genetic Resources for Vegetables, Medicinal and Special Plants, Crop Research Institute, Šlechtitelů 29, 78371 Olomouc, Czech Republic

[4] Department of Biological Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

[5] Facultad de Agronomía, Universidad Nacional de Tucumán, Zootecnia Y Veterinaria, Tucumán, Argentina

[6] Fundación Instituto Leloir and IIBBA-CONICET. Av, Patricias Argentinas 435, C1405BWE Buenos Aires, Argentina

[7] Centro de Biotecnología Vegetal, Facultad de Ciencias de La Vida, Universidad Andres Bello, Santiago, Chile

[8] ANID - Millennium Science Initiative Program - Millennium Institute for Integrative Biology (iBio) and Millennium Nucleus for the Development of Super Adaptable Plants (MN-SAP), Santiago, Chile

1 Introduction

The interaction between plants and beneficial microorganisms usually results in root architecture changes that improve plant capacity to take up water and nutrients from the soil (Grover et al. 2021). In this regard, it has been largely recognized that the well-known plant growth–promoting rhizobacterium (PGPR) *Azospirillum* can improve plant growth due to the production of phytohormones such as auxins, mainly indole-3-acetic acid (IAA), as well as cytokinins, gibberellins, abscisic acid, and free polyamines, among many other mechanisms (Cassán et al. 2020 and references therein). Several studies have demonstrated that *Azospirillum* can alter root architecture by promoting the development and elongation of lateral roots (LR) and root hairs (RH), and inhibiting the elongation of the primary root (PR), thereby presumably increasing the root surface (Cassán et al. 2020; Méndez-Gómez et al. 2020; Rondina et al. 2020; Dubrovsky et al. 1994). These morphological changes at the root level have been mostly related to the bacterial capacity to produce and release IAA (Prinsen et al. 1993; Coniglio et al. 2019); less is known about changes induced by other phytohormones and much less about the role of other bacterial components.

Azospirillum brasilense synthesizes IAA almost exclusively through the indole-3-pyruvic acid-dependent (IPyA) pathway (Costacurta et al. 1994), one of the three tryptophan-dependent pathways described for this genus. The indole-3-pyruvate decarboxylase (IPDC), encoded by the *ipd*C gene, is a key enzyme in this pathway. Silencing this gene reduces IAA biosynthesis (Malhotra and Srivastava 2008); for this reason, *Azospirillum ipd*C – mutants constitute a valuable tool for investigating the role of IAA in plant–microbe interactions. In this regard, inoculation experiments with an *A. brasilense ipd*C – mutant performed by our group suggested that other molecules than IAA might be responsible for some changes in the root system of *Azospirillum*-inoculated plants (Cassán et al. 2020; Puente et al. 2018).

In the present study, we aimed to evaluate more thoroughly root architecture changes in *Arabidopsis thaliana* inoculated with *Azospirillum argentinense* Az39 (formerly *A. brasilense* Az39; dos Santos Ferreira et al. 2022) and try to link these changes to global gene expression in the roots of inoculated plants. To shed light on the involvement of IAA-dependent and IAA-independent routes in root architecture changes, we included in our assays a new *ipd*C – mutant of *A. argentinense* Az39, an experimental *A. thaliana* line impaired in lateral roots formation, as well as other comparators.

2 Materials and Methods

2.1 Bacterial Strains and Culture Conditions

Azospirillum argentinense Az39 was obtained from Instituto de Microbiología y Zoología Agrícola, INTA-Castelar, Argentina, and reactivated in Luria–Bertani (LB) medium with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. Routine *A. argentinense* Az39 multiplication was performed at 30 °C and 120 rpm (orbital shaking) until reaching an optical density at 595 nm (OD_{595}) of 1.2, corresponding to the late exponential growth phase. Aliquots of 100 µl were subsequently transferred to 100-mL flasks containing 25 mL of minimal medium for *A. brasilense* (MMAB) (Vanstockem et al. 1987) supplemented with 10 $mg{\cdot}l^{-1}$ of *L*-tryptophan (Trp) plus 50 $\mu g{\cdot}mL^{-1}$ of kanamycin (Km) when culturing *A. argentinense* Az39 *ipdC* – strain. *Escherichia coli* DH5α (used as a comparator in a subset of experiments) was grown in LB at 37 °C and 120 rpm under orbital shaking until reaching an OD_{595} of 1.8 (late exponential growth phase).

2.2 Construction of the A. argentinense Az39 ipdC – Mutant

A new procedure was developed to obtain an *ipdC* – mutant of *A. argentinense* Az39. This IAA-deficient mutant was obtained by insertional mutagenesis in the *ipdC* gene using the conjugative suicide vector pKNOCK-Km, as described by Alexeyev (1999). An internal fragment of the *ipdC* gene was amplified from the genomic DNA of *A. argentinense* Az39 by PCR with the primer pairs: **Fw_ipdC**, 5′-CG**GAATTC**GCCCGG TCTATCTGGAAATC-3′; **Rv_ipdC** 5′-CG**GAATTC** GTCCATGGCGGTGAACAG -3′ introducing *Eco*RI sites. The PCR product was cloned into pKNOCK-Km and digested with *Eco*RI by T4 DNA ligase (ThermoFisher Scientific®). The resulting vector pKNOCK-Km-*ipdC* was introduced into *E. coli* S17-1/*λpir* (lysogen for strain S17-1) and then transferred to *A. argentinense* Az39 by conjugation (Gullett et al. 2017). The transconjugants were selected using solid Luria–Bertani Congo Red (LBCR) culture medium (Molina et al. 2014) containing kanamycin (50 $\mu g{\cdot}mL^{-1}$) and ampicillin (200 $\mu g{\cdot}mL^{-1}$). The plasmid insertion into an appropriate position was confirmed by PCR with the primers **Fw_ipdC** and **pKnock4_Rev**: 5′-ATGTAAGCC CACTGCAAGCTA-3′ that is homologous to a specific sequence of the pKNOCK-Km. The *ipdC* – mutants of *A. argentinense* Az39 generated by single crossover integration of the plasmids into the genome were named

A. argentinense Az39-*ipdC* −. Further details about the strains and plasmid used are supplied in Table S1.

2.3 Measurement of IAA Production by A. argentinense Strains

An unequivocal methodology combining a one-step solid-phase extraction (SPE) purification method (Torres et al. 2018) with sensitive and selective liquid chromatography–multiple reaction monitoring-mass spectrometry (LC-MRM-MS) (Matsuda et al. 2005) was used to determine IAA production by *A. argentinense* Az39 and its derivative *A. argentinense* Az39-*ipdC* − (onwards referred to as Az39 and *ipdC* −, respectively). The strains were grown in MMAB or LB to late exponential (OD_{595} = 1.2–1.4) and stationary phases (OD_{595} = 1.6–1.8), respectively, centrifuged and filtered to obtain clean supernatants.

UHPLC-MS/MS measurements were performed on Nexera X2 UHPLC (Shimadzu Handels GmbH) coupled with a mass spectrometer MS-8050 (Shimadzu Handels GmbH). Chromatographic separation was performed on an Acquity UPLC BEH C18 (50 × 2.1 mm; 1.7 µm particle size) column (Waters, Milford, MA, USA) with the corresponding pre-column kept at 40 °C. The mobile phase consisted of 0.05% acetic acid in water (component A) and 0.05% acetic acid in methanol (component B). The analytes were separated using a binary gradient starting at 10% of solvent B (0.05% acetic acid in methanol), which increased to 90% of solvent B for 3.5 min, then decreased to 10% of solvent B for the next 0.1 min. The equilibration to the initial conditions took 2.4 min. The flow rate was 0.4 $mL \cdot min^{-1}$, and the injection volume was 2 µL. The identification of IAA was performed via electrospray ionization in positive MRM mode.

2.4 IAA Bacterial Production/IAA Sensing by A. thaliana Seedlings

In a complementary assay, a *DR5::GUS* reporter line of *A. thaliana* was used to assess IAA production by *A. argentinense* strains and IAA sensing by inoculated plants. The seeds of the *DR5::GUS* line kindly provided by prof. Guilfoyle (University of Missouri, Columbia, USA) were placed in 96-well microplates containing half-strength MS medium and stratified at 4 °C in the darkness for 4 days. Subsequently, the microplates were placed into a phytotron under controlled conditions (75 rpm, 21 °C, 16-/8-h light/dark, light intensity 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days, after which suspensions of Az39 or *ipdC* − at titers of 10^8 and 10^9 CFU mL^{-1} (prepared in half-strength MS) were added. Also, 5 µM IAA dissolved in 0.1% acetic acid was prepared and added to a third set of *A. thaliana* seedlings to obtain a positive control. Negative controls were achieved by applying half-strength MS medium with or without 0.1% acetic acid. After 17 h of incubation, the medium was discarded, and the seedlings were directly treated with 150 µl lysis buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100) containing 1 mM of 4-methylumbelliferyl glucuronide (4-MUG), and incubated at 37 °C for 90 min. At the end of the incubation period, 50 µl of 1 M Na_2CO_3 (stop solution) was added to each well, and the fluorescence due to 4-methylumbelliferone (4-MU) formation was measured in a microplate reader (excitation/emission wavelengths 365/460 nm).

2.5 A. argentinense Flagellin Collection

The flagellin from Az39 was obtained according to Elías et al. (2021). Briefly, Az39 was grown in nitrogen-free (NFb) liquid medium at 30 °C and 120 rpm for 24 h, after which cells were collected through centrifugation at 2000 × g for 10 min and washed twice with sterile bi-distilled water. Subsequently, cells were resuspended in 20 mL of 100 mM Tris–HCl buffer pH 7.0, vortexed for 10 min to detach flagella, and centrifuged at 6000 × g at 4 °C for 30 min. The supernatant containing the flagella and other extracellular proteins was recovered and loaded in a 12% (w/v) denaturing polyacrylamide gel for protein electrophoresis, according to standard procedures. The band corresponding to *Azospirillum* flagellin AzFlap (100 kDa) was excised from the gel, electroeluted in Tris–glycine buffer pH 8.3 at 40 V for 12 h, dialyzed, lyophilized, and resuspended in distilled water to a convenient concentration. The identity of the protein was confirmed by Western blot using an *A. brasilense* Sp7 anti-flagellin obtained from rabbit (Viruega-Góngora et al. 2020) as the primary antibody and, as the secondary antibody, an alkaline phosphate-conjugated goat anti-rabbit commercial antibody (Sigma-Aldrich, St Louis, MO, USA).

2.6 Plant Material and Growth Conditions

To check the effects of *A. argentinense* inoculation on *Arabidopsis* root morphology, *Arabidopsis thaliana* (L.) ecotype Columbia 0 (Col-0, wild-type) and the *A. thaliana* mutant *tir1.1*, deficient in various auxin-regulated growth processes, including lateral root formation (Ruegger et al. 1998), were used.

Arabidopsis seeds were surface sterilized with 95% (v/v) ethanol (5 min) followed by aqueous 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Triton X-100 (5 min). After washing four times with sterile distilled water, the seeds were stratified in the darkness at 4 °C in plastic tubes containing sterile distilled water. Two days later, the seeds were sown in Petri dishes containing Murashige and Skoog (MS) medium (Murashige and Skoog

1962) supplemented with 0.8% (w/v) agar and a final pH of 5.7. Subsequently, plates were sealed with micropore tape, and seeds were allowed to germinate and grow vertically for 7 days in a growth chamber at 22 ºC, under 16–8-h light–dark photoperiod (intensity of 60 µmol $m^{-1}·s^{-1}$).

Seven days after sowing, five *Arabidopsis* seedlings per plate were aseptically transferred to new Petri dishes containing MS medium supplemented with 0.8% (w/v) agar and subjected to the following treatments (in different sets of experiments): inoculation with 10^8 CFU mL^{-1} of *A. argentinense* Az39; inoculation with 10^8 CFU·mL^{-1} of *A. argentinense* Az39 *ipdC* −; addition of 0.1 µg·mL^{-1} IAA; addition of *A. argentinense* Az39 cells inactivated by previous exposure to 57 °C for 45 min (Az39 φ); inoculation with 10^8 CFU·mL^{-1} of *E. coli* DH5α (no-PGPR, unable to produce IAA) (Table S2); addition of flagellin from *A. argentinense* Az39 (obtained as described before) at two contrasting concentrations: 200 nM and 1 µM. The *A. argentinense* titer and the IAA concentration were selected based on preliminary experiments (see next item).

For inoculation assays, bacteria from the late exponential growth phase in MMAB medium were obtained through centrifugation (3900x*g*, 12 min, 16 °C), washed with 0.85% (w/v) sterile saline, and resuspended in 0.01 M $MgSO_4$. Subsequently, variable volumes (0.02–2.00 mL) of bacterial suspensions (about 10^9 CFU·mL^{-1}) were mixed with variable volumes (19.98–18.00 mL) of molten MS medium (below 40 °C) to ensure a final titer between 10^3 and $10^{8\ CFU}$·mL^{-1}, homogeneously distributed in the growing medium.

Before the experiments with *A. argentinense* Az39 heat-inactivated cells (Az39 φ), a theoretical titer of 10^9 CFU·mL^{-1} was verified through the microdroplet method (Puente et al. 2018), using as culture medium LB supplemented with 1.5% agar (w/v) and modified by the addition of Congo red dye (LBRC). The same amount of the 0.01 M $MgSO_4$ solution was added and homogenized in the melted medium to obtain uninoculated control seedlings. Flagellin treatment was achieved by adding 5 µL of Az39 flagellin solution 200 nM on the roots of five *A. thaliana* Col-0 seedlings per plate, according to results reported for *A. argentinense* REC3 flagellin by Elías et al. (2021). Also, 1 µM flagellin was evaluated to test dose-dependence effects. All Petri dishes containing *Arabidopsis* seedlings were kept vertically in a growth chamber at 22 °C, and a light/dark photoperiod of 16/8 h (light intensity of 60 µmol·$m^{-1}·s^{-1}$) for 5 days until image analysis.

2.7 Preliminary Experiments

Before conducting the assays designed to evaluate the effects of *A. argentinense* inoculation on the root architecture of *Arabidopsis*, it was necessary to analyze three relevant points: (1) if *A. argentinense* could grow or remain viable in the conditions established for *A. thaliana* growth; (2) which would be the best bacterial concentration to apply; (3) which would be the best IAA concentration to apply (as comparator).

To address point 1, a pure culture of Az39 n (10^9 CFU mL^{-1}) was sown through the microdroplet method in Petri dishes containing Murashige Skoog (MS) medium supplemented with 1.5% (w/v) agar or LB medium supplemented with 1.5% agar (w/v), modified by the addition of Congo red dye (LBRC) (Molina et al. 2014), and incubated at 24 °C and 37 °C. Colonies were counted after 7 days of incubation.

To address points 2 and 3, dose–response tests using incremental concentrations of bacterial cells (10^3 to 10^8 CFU·mL^{-1}) and IAA dissolved in MS medium to reach final concentrations of 0.01; 0.1; 1.0, 5.0; 10.0; 15.0, and 25.0 µg IAA mL^{-1} were conducted.

2.8 Evaluation of Arabidopsis Root Architecture

Arabidopsis thaliana images were acquired with a Canon PowerShot SX510 HS camera 5 days after transplant. The length of the primary root (PR) and the number of lateral roots (LR) ($n = 15$) were analyzed by the use of the RootNav software v1.8.1 (Pound et al. 2013). Lateral root density (LRD) was determined by dividing the number of LR by the length of the PR. Root hairs density (RHD) was calculated by using a 25-mm^2 representative square area (Adu et al. 2017). To obtain more information about root architecture changes, the root system was divided into three sections: basal, middle, and apical root. These sections were observed using a digital stereoscopic magnifying glass (Motic SMZ-171-TLED-Digital). After flagellin treatments, the roots were microphotographed using an Olympus BX-51 microscope. Data were analyzed using Infostat software (Di Rienzo et al. 2012).

2.9 Gene Expression Analysis in A. thaliana Roots Inoculated with A. argentinense

For these studies, *Arabidopsis* Col-0 seedlings were inoculated 4 days after initial planting with 2 µL ($OD_{595} = 1$) of the corresponding *Azospirillum* suspension (Az39 or *ipdC* −) or with 2 µL of saline solution (NaCl 0.9% w/v), as the control.

Root systems were collected 24 h and 7 days post-treatment and flash-frozen in liquid nitrogen. Two biological replicates were collected at each time point, which included at least 50 individual root systems.

Nucleic acids were isolated from root tissue using Invitrogen PureLink Plant RNA Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and DNA contaminants were removed by treating the samples with Ambion TURBO-DNA-free (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. RNA quantity was determined with

a Nanodrop spectrophotometer, and RNA quality was checked with an RNA nanochip on the Agilent 2100 bioanalyzer system. Reverse transcription, fragmentation, end repair, adapter ligation, and PCR enrichment were conducted with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Whitby, ON, Canada), as indicated by the manufacturer's protocols. Library size distribution was determined using a high-sensitivity DNA chip on the Agilent 2100 bioanalyzer system. Library concentration and molarity were determined with the NEBNext Library Quant Kit (New England Biolabs, Whitby, ON, Canada) in conjunction with PerfeCTa qPCR ToughMix (QuantaBio, Beverly, MA, USA) and PicoGreen fluoro-spectrophotometry. Libraries were sent to Genome Québec (Montréal, QC, Canada) for 150 bp single-end sequencing on the Illumina HiSeq 4000 platform.

The raw reads were processed using high-performance computing clusters provided by WestGrid (www.westgrid.ca) and Compute Canada (www.computecanada.ca). The subsequent steps to allow further bioinformatic analyses, including raw reads trimming, alignment to the *Arabidopsis thaliana* TAIR10 reference genome, gene counts, differential gene expression (DEGs) analysis of RNA-seq data and clustering, Gene Ontology (GO) term enrichment analysis, and plots generation were accomplished as described in Robertson et al. (2022). All RNA sequencing data have been deposited at the Gene Expression Omnibus (GEO): GSE192383.

3 Results

3.1 Azospirillum argentinense Az39 Synthesizes IAA if Tryptophan is Available

The IAA production of *A. argentinense* Az39 and its derivative *ipdC* − mutant was analyzed in both LB and MMAB culture media. IAA production by *A. argentinense* Az39 *ipdC* − mutant was not detected in any of the culture media evaluated, while *A. argentinense* Az39 produced 3.45 µg mL^{-1} of IAA in LB, a rich culture medium expected to supply tryptophan (Trp), the precursor necessary to allow this biosynthesis. Conversely, in the MMAB medium (without the addition of the precursor Trp), no IAA production was detected for any *A. argentinense* strain (Table S2). These results confirm that the presence of the precursor Trp is essential for IAA biosynthesis by *A. argentinense* Az39, as demonstrated previously (Puente et al. 2018). Additionally, the inability of the mutant *ipdC* − to produce IAA was confirmed.

3.2 Flagellin from A. argentinense Az39

The identification of the flagellin from *A. argentinense* Az39, after denaturing polyacrylamide gel for protein electrophoresis and electroelution, was assessed by Western immunoblotting using a specific rabbit antibody obtained from the purifed polar fagellin of *A. brasilense* Sp7. A single band of ~ 100 kDa was observed (Fig. S5). This let us infer that the flagellin obtained from the strain Az39 was suitable for the following experiments.

3.3 Corroboration of ipdC – Impairment for IAA Production

The indirect method of GUS activity in the *DR5::GUS Arabidopsis* reporter line was used to confirm that the *ipdC* − mutant obtained through insertional mutagenesis was impaired in IAA production and, therefore, unable to modify endogenous plant IAA levels. The maximum GUS activity was detected under exogenous addition of IAA (Fig. 1). Seedlings inoculated with *A. argentinense* Az39 also showed high fluorescence levels compared to the negative controls, while those inoculated with the *ipdC* − mutant (at the same titers) did not, corroborating the results of the direct IAA quantification. Besides, inoculation with 10^8 and 10^9 CFU·mL^{-1} of *A. argentinense* Az39 or *ipdC* − led to similar responses in terms of GUS activity.

3.4 Selection of Working Variables Based on Preliminary Experiments

Although *A. argentinense* Az39 showed no visible growth in MS at 24 °C, colonies became detectable after 7 days of incubation at 37 °C, demonstrating that *Azospirillum* cells stay viable in the culture medium used for *Arabidopsis* growth (Fig. S1 a, b). As expected, growth in LB was much greater, especially at 37 °C (Fig. S1c, d). The bacterial titers associated with

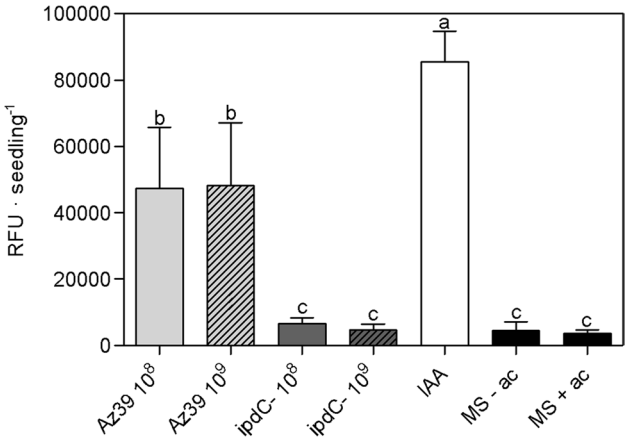

Fig. 1 Expression of *DR5::GUS* in *A. thaliana* Col-0 seedlings inoculated with *A. argentinense* Az39 or *ipdC* −. Seedlings were exposed for 17 h to 10^8 and 10^9 CFU·mL^{-1} of each strain. To obtain a positive control, seedlings were exposed for the same time to 5 µM IAA dissolved in 0.1% acetic acid (ac). Seedlings exposed to Murashige Skoog (MS) medium with or without 0.1% (w/v) ac were used as negative controls

significant changes in the PR length and LR number in *A. thaliana* Col-0 seedlings were 10^7 CFU·mL^{-1} (data not shown) and 10^8 CFU·mL^{-1} (Fig. S2); however, the most significant changes occurred at the latter, for which 10^8 CFU·mL^{-1} was the bacterial titer selected for further assays.

Regarding IAA concentration and its effects on root morphology (Fig. S3), we detected that 0.10 µg·mL^{-1} (equivalent to 0.57 µM IAA) induces a root phenotype similar to that obtained with the inoculation of 10^8 CFU·mL^{-1} of *A. argentinense* Az39. These changes included a reduction in the PR length and a significant increase in LR and RH numbers. IAA concentrations higher than 1.0 µg·mL^{-1} also resulted in a significant PR reduction, but this was accompanied by reductions in LR development. For this reason, 0.10 µg·mL^{-1} of IAA was the concentration selected for further experiments.

3.5 Azospirillum argentinense Az39 and ipdC – Alter Root Architecture in Both A. thaliana Genotypes

Arabidopsis Col-0 plants inoculated with Az39 showed a significant reduction in the primary root (PR) length (Fig. 2a, b) and an increase in the number of LR (Fig. 2c) and LRD (Fig. 2d). Observations with magnifying glasses also revealed an increased number and length of RH (Fig. 2e). In these seedlings, LR proliferation occurred mainly in the middle and apical root zones (Fig. 2e). *Arabidopsis* Col-0 seedlings inoculated with the mutant strain *ipdC* – also showed a significant decrease in the PR length and a significant increase in LR emergence compared to non-inoculated seedlings, but lower than that caused by Az39 (Fig. 2a, b, c). Unlike Az39, the IAA-deficient mutant induced LR development only in the middle zone (Fig. 2e). Additionally, we detected that LR of the seedlings inoculated with the *ipdC* – mutant tended to be longer than those inoculated with Az39. These results show that, despite the impairment for IAA production of the *ipdC* – mutant, both *A. argentinense* strains could change *A. thaliana* root morphology, leading to a shorter PR and increased root density compared to the uninoculated control (Table S3), due to the formation of more LR, with more abundant and large RH, suggesting that the production of IAA by the bacterium is not a strict requirement to change *Arabidopsis* root architecture.

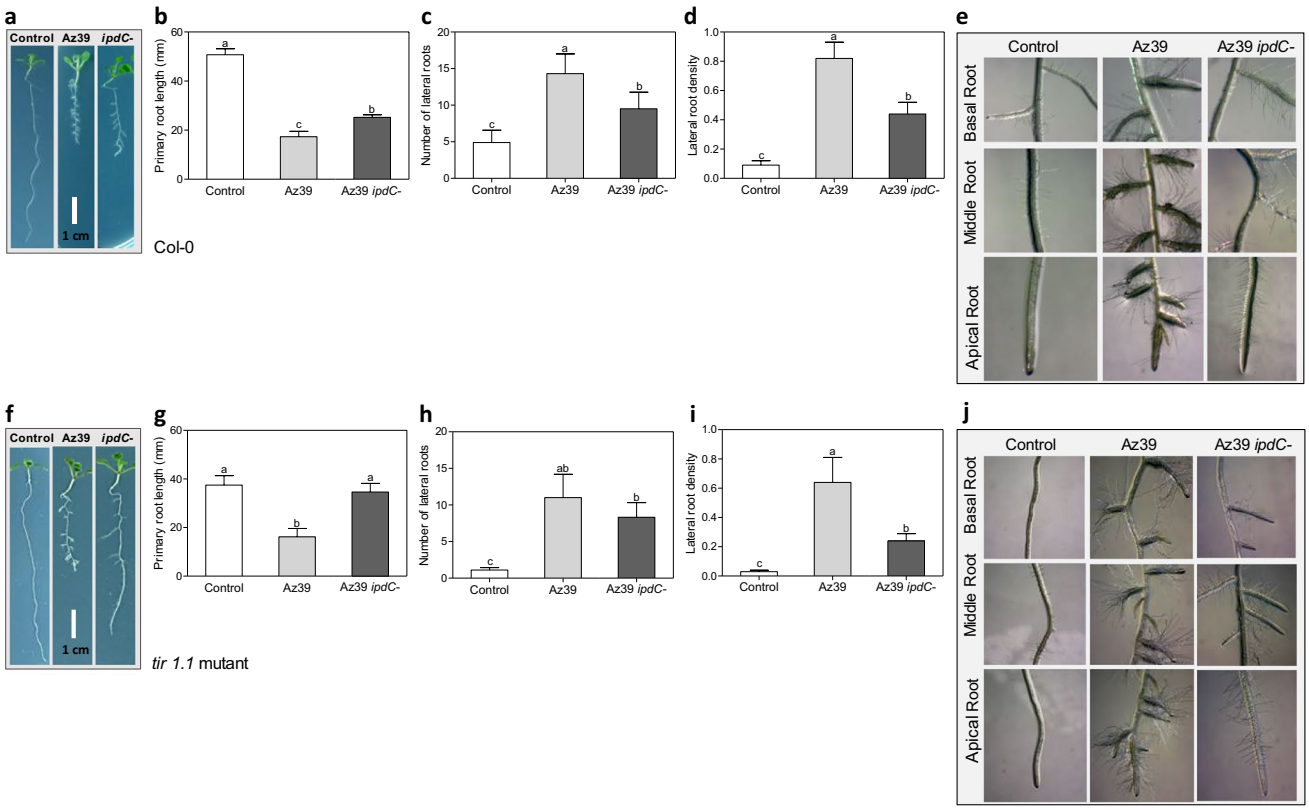

Fig. 2 Root architecture changes in *A. thaliana* inoculated with *A. argentinense* Az39 or *ipdC* – . Upper panel: Col-0; lower panel: *tir1.1* mutant line. **a, f** plant phenotype; **b, g** primary root (PR) length; **c, h** lateral roots (LR) number; **d, i** lateral root density (LRD); **e, j** stereoscopic magnifying glass images showing basal, middle, and apical zones of the primary root. The letters on the bars indicate statistically significant differences according to Tukey's HSD test, $p < 0.05$

The TIR1 protein is a member of a protein family implicated in ubiquitin-mediated processes and is encoded by the *TRANSPORT INHIBITOR RESPONSE 1 (TIR1)* gen. Because *tir1* mutants are deficient in several auxin-regulated growth processes, including lateral root formation, this *Arabidopsis* line would further allow testing to what extent bacterial IAA production and/or IAA sensing by the plant are involved in the root architecture changes described above.

Tir1.1 mutant seedlings inoculated with Az39 showed a similar response as that observed in the WT line, with a significant reduction in the PR development (Fig. 2f, g) and a significant increase in the number of LR (Fig. 2h), also resulting in higher LRD (Fig. 2i). Besides, more RH were formed (Fig. 2j, Table S3), as in the wild-type line. However, *tir1.1* seedlings inoculated with the *ipdC* – mutant presented a PR length similar to that of the uninoculated control (Fig. 2f, g). Despite this, the IAA-deficient strain increased LR formation (Fig. 2h) in the middle zone (Fig. 2j) and stimulated RH proliferation in the apical and middle zones (Fig. 2j, Table S3).

Summing up, at different degrees and with different spatial patterns, inoculation treatments induced LR formation and increased the number of RH in both *A. thaliana* lines, ruling out the strict requirement of bacterial IAA production or IAA sensing by the TIR1 protein to induce these changes. However, the shortening of the PR seems to be a more complex mechanism where the combined effect of IAA level in the roots and IAA sensing by the plant, not necessarily through the TIR1 protein, might be involved. The illustrative images shown in Fig. S4 seem to corroborate this idea, as the exogenous addition of 0.1 $\mu g \cdot mL^{-1}$ IAA resulted in the shortening of the PR in both *A. thaliana* lines.

3.6 Flagellin: a Novel Actor in A. argentinense—Root Interaction?

Because the above-described findings suggested the existence of a mechanism independent of IAA production and sensing behind most morphological changes occurring in *Arabidopsis* roots after *A. argentinense* inoculation, we designed a new set of experiments which included the exposure of *Arabidopsis* Col-0 seedlings to *A. argentinense* Az39, to non-viable Az39 cells (Az39 Ø), to *Escherichia coli* DH5α (a flagellated bacterium, not considered PGPR), or to flagellin obtained from *A. argentinense* Az39.

As shown in Fig. 3a, Az39 Ø did not induce relevant macroscopic changes in the *A. thaliana* root system compared to non-inoculated plants. However, a more detailed analysis using magnifying glasses revealed an increased number of RH in the apical and middle zones (Fig. 3b, Table S3). Moreover, the addition of *E. coli* DH5α cells also resulted in increased numbers of RH in the apical and middle root zones, with no relevant changes in the PR or RH length. Still, no changes in LR numbers were observed under these treatments. On the other hand, the addition of flagellin from Az39 also led to a similar response, with more number and length of RH than control plants (both in the LR and PR), mainly when used at 1 µM (Fig. 3c), suggesting a dose-dependent effect of this compound. In such conditions, the RH density of *Arabidopsis* seedlings exposed to flagellin was similar to that observed in plants inoculated with 10^8 $CFU \cdot mL^{-1}$ of Az39.

We confirmed by viable cell counting on MMAB agar plates ($CFU.ml^{-1}$) and optical density (OD_{595}) that *A. argentinense* Az39 exposed to 57 °C during 45 min (Az39 Ø) was not able to growth and consequently, unable to synthesize IAA. Thus, both the root morphology and root hair proliferation induced by Az39 Ø would be attributed to a mechanism independent of the bacterial capacity to produce IAA (Fig. S6). In this scenario, we assumed that at least part of the changes observed in the root morphology of *Arabidopsis* seedlings could be attributed to the direct physical contact between bacterial cells (even non-viable) and the roots, where flagellin may be a key signaling molecule, contributing to a more developed root system due to increased root hair formation.

3.7 Gene Expression Patterns in the Roots of Arabidopsis Seedlings Inoculated with Az39 or the ipdC – Mutant Show Temporal Differences

The transcriptome of *Arabidopsis* roots was assessed 24 h and 7 days after the inoculation with *A. argentinense* Az39 or *A. argentinense ipdC* –, and a differential expression genes (DEGs) analysis (FDR < 0.05) was conducted (Fig. 4a, Dataset S1). Plants inoculated with the IAA-deficient mutant differentially expressed a large number of genes (1359 genes) after 24 h, and much fewer (281 genes) after 7 days. Conversely, plants inoculated with Az39 differentially expressed only 2 genes after 24 h, whereas 1260 genes were differentially expressed after 7 days. This finding demonstrates that Az39 affects plant gene expression later than the *ipdC* – mutant. At the time points of maximum DEGs, most of these genes were upregulated in plants inoculated with *ipdC* –, whereas in plants inoculated with Az39, they were more balanced.

A Gene Ontology (GO) enrichment analysis revealed that upregulated DEGs in the roots of seedlings exposed to the *ipdC* – mutant at 24 h after inoculation were enriched for many GO terms related to photosynthesis, while 7 days after inoculation were mostly enriched for cell signaling and defense response terms, particularly jasmonic acid (JA) signaling (Fig. 4b). JA signaling-related DEGs common to both treatments included the JA biosynthesis gene *OPC-8:0 COA LIGASE1* (*OPCL1*; AT1G20510) and the

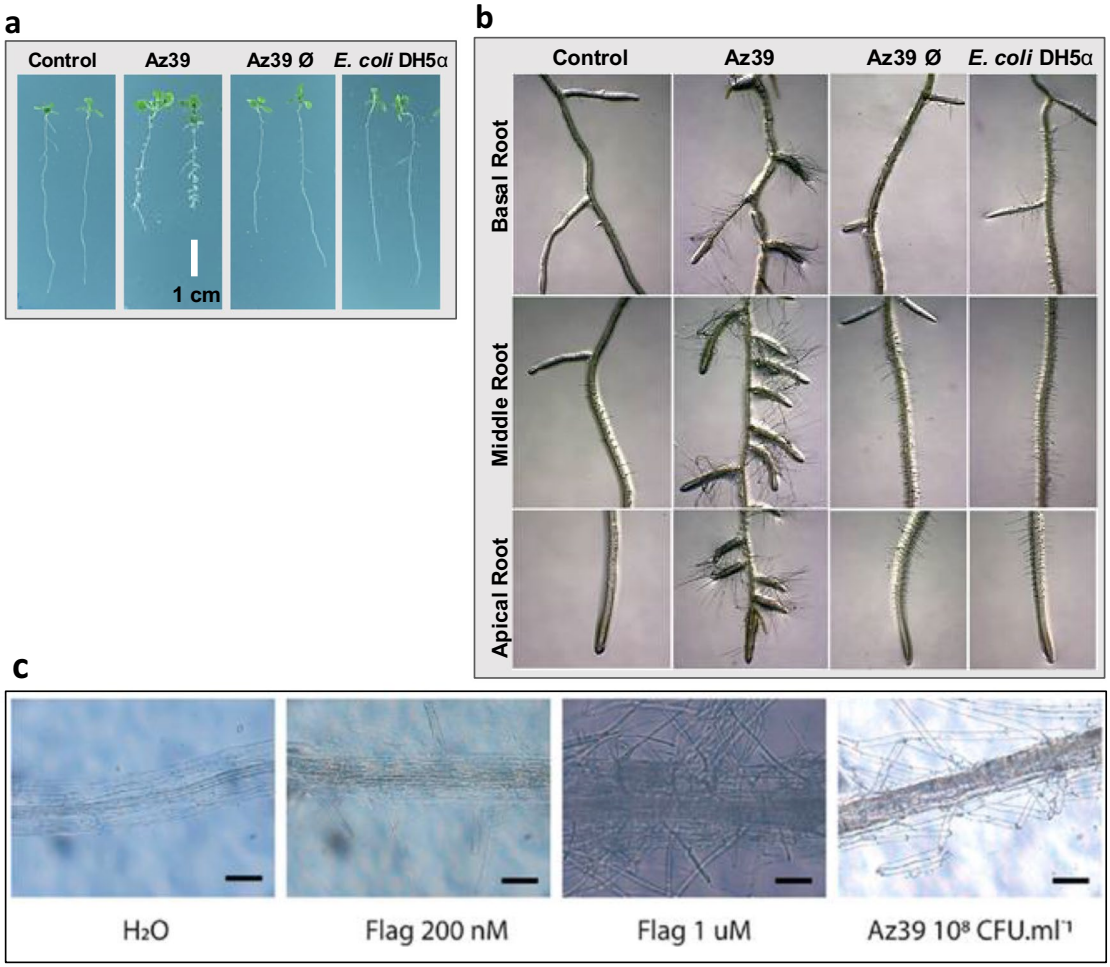

Fig. 3 Comparative effects of different treatments on *A. thaliana* root architecture. *Arabidopsis* wt seedlings were exposed to *A. argentinense* Az39, *A. argentinense* Az39 heat-inactivated (Az39Ø), *E. coli* DH5α (**a, b**), and to 200 nM or 1 µM Az39-flagellin (**c**). Illustrative images obtained by **a** the naked eye, **b** a stereoscopic magnifying glass, or **c** an optical microscope (40×). **c** A segment of a representative lateral root is shown; scale bar: 1 mm at 40×

JASMONATE-ZIM-DOMAIN PROTEIN (*JAZ*) genes *JAZ6* (AT1G72450), *JAZ7* (AT2G34600), *JAZ9* (AT1G70700), and *JAZ10* (AT5G13220).

Az39-upregulated DEGs 7 d after inoculation were additionally enriched for abiotic stress responses (response to cold and to water deprivation), which included genes such as the *LOW TEMPERATURE-INDUCED* (*LTI*) genes *LTI29* (AT1G20450) and *LTI30* (AT3G50970).

In a global analysis considering DEGs at 24 h and 7 days after inoculation, we found that many of them were common to the three treatments (control seedlings, inoculated with Az39, inoculated with *ipdC* –) (971genes) (Fig. 4c). To elucidate differences in gene expression due to developmental stages or bacterial effects, the common DEGs were removed for the subsequent analysis of unique responses. Unique DEGs (i.e., differentially expressed in 1 or 2 of the treatments) were further characterized through a GO term enrichment analysis, which revealed that plants inoculated with *ipdC* – and Az39 activated unique functional programs (Fig. 4d). Thus, control seedlings had GO terms related to housekeeping functions (e.g., water transport, protein ubiquitination, response to nitrate), while bacterial treatments included some overlapping terms related to defense responses (response to wounding, response to fungus, defense response) as well as unique responses. Specifically, plants inoculated with Az39 were enriched for several abiotic stress responses (response to hypoxia, oxidative stress, and water deprivation), and plants inoculated with *ipdC* – were enriched for cell signaling responses (plant-type hypersensitive response, jasmonic acid/salicylic acid signaling, protein phosphorylation, regulation of transcription).

Finally, to better understand how auxin-signaling-related genes were affected by the presence of Az39 or *ipdC* – cells, we investigated the expression of differentially expressed auxin gene families and genes annotated with auxin-related GO terms (Fig. 5). The chosen auxin gene families were the

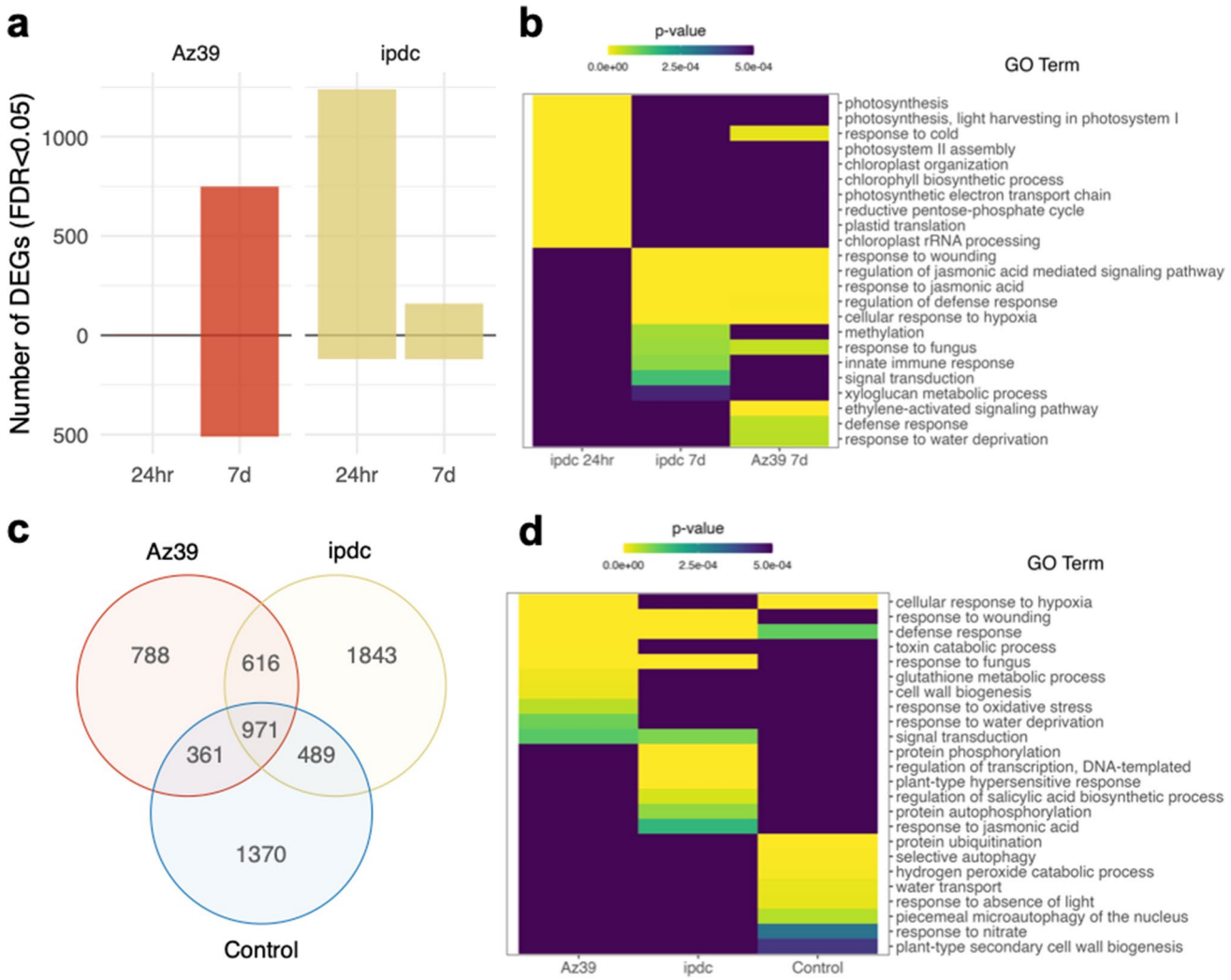

Fig. 4 Differential gene expression analysis of RNA-seq data after inoculation with *A. argentinense* Az39 or *ipdC* – . **a** Number of differentially expressed genes (DEGs; FDR < 0.05) compared to the control at 24 h and 7 days after inoculation. **b** Top 10 enriched upregulated GO terms from comparisons in **(a)**. **c** Venn diagram showing common and non-common DEGs. **d** Top 10 enriched upregulated GO terms from the comparisons in (**c**), after removing the genes common to all 3 treatments. In **b** and **d**, GO terms are colored according to the *p*-value (Fisher's exact test), with yellow being the most significant. All *p*-values > 0.0005 are colored purple. Several GO terms are enriched in more than one sample, as indicated by multiple yellow bars in the row

AUXIN RESPONSE FACTORS (*ARF*), *INDOLE-3-ACETIC ACID* (*IAA*), *YUCCA* (*YUC*), and *PIN-FORMED* (*PIN*), though no *PIN* genes were differentially expressed. Overall, most differentially expressed auxin-signaling genes had the highest expression after 7 days compared to 24 h. The highest overall expression was found at 7 days in the Az39 treatment and the lowest at 24 h in the *ipdC* – treatment. Despite the large number of upregulated DEGs at 24 h in the *ipdC* – treatment, most auxin-related signaling genes were suppressed in the roots of these plants by that time. On the other hand, plants inoculated with Az39 had a similar DEGs pattern to that of the control after 24 h, but, at day 7, auxin signaling was greatly increased in Az39-inoculated plants and not in control plants.

4 Discussion

Several reports have shown that the direct effects of plant growth–promoting rhizobacteria, particularly *Azospirillum*, involve the bacterial production of phytohormones, among which auxins have received great attention (Karimi et al. 2021; Méndez-Gómez et al. 2020), and in many screening works for PGPR selection conducted in the past decades, IAA biosynthetic ability was considered a very important trait.

In this work, changes at the root level induced by *A. argentinense* Az39 and by a mutant strain deficient for IAA biosynthesis were evaluated in *A. thaliana* seedlings to elucidate the mechanisms underlying root growth promotion by

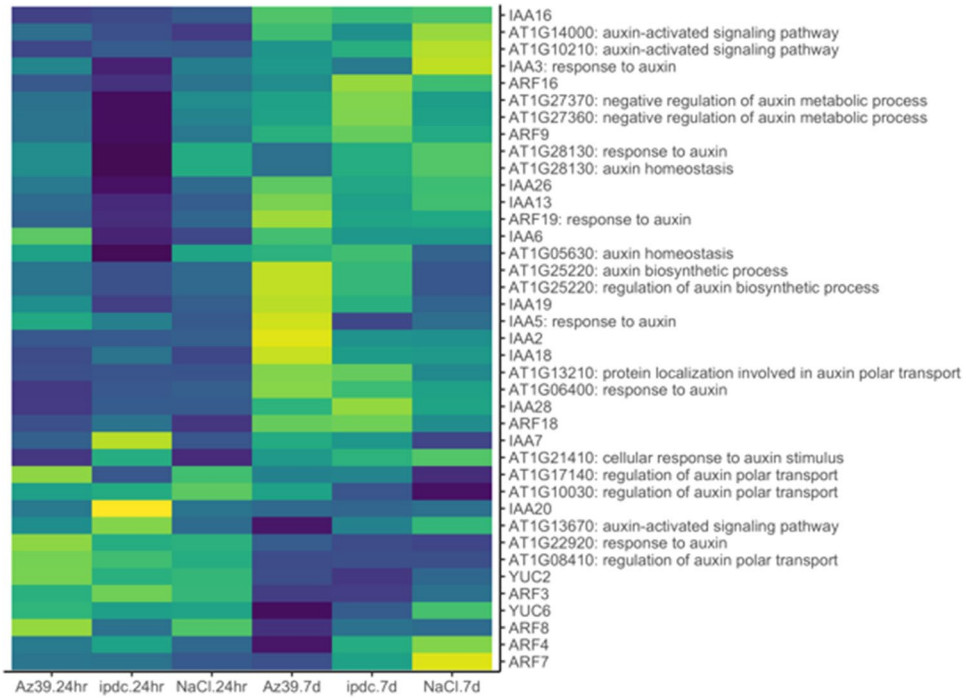

Fig. 5 Differential gene expression (FDR < 0.05) of auxin pathway components. Included are auxin-related gene families (ARF, auxin response factors; IAA, idole-3-acetic acid; YUC, YUCCA) and genes annotated with auxin-related Gene Ontology terms, where applicable. Each gene is colored by a *z*-score, which is the row-wise scaled expression of each gene. Yellow indicates higher expression, while dark blue indicates lower expression, allowing for sample comparison

this PGPR and to understand to what extent bacterial IAA is involved in plant responses.

We found that inoculation of *Arabidopsis* seedlings with both *A. argentinense* strains IAA + (Az39) and IAA − (*ipdC* −) induced a significant reduction in the PR elongation and a significant increase in the number of LR and RH compared to the uninoculated control. Nevertheless, the effects of the IAA + strain were more pronounced.

Based on the results obtained in experiments using *A. brasilense* Sp245 (now *A. baldaniorum* Sp245; dos Santos Ferreira et al. 2020), Spaepen et al. (2014) proposed that the inhibition of the PR growth mainly depends on IAA released by the bacterial partner, while the increase in LR would involve other molecules not related to IAA. Recently, Méndez-Gómez et al. (2020) and Carrillo-Flores et al. (2022) showed that the kinase TOR (target of rapamycin) is involved in the development of LR in *A. thaliana* inoculated with *A. brasilense* Sp245; previous data suggest that PR growth also depends on TOR through the regulation of cell proliferation and elongation (Deng et al. 2016).

Numerous studies have shown that a similar phenotype can be induced by different PGPR species involving different compounds. For example, *Bacillus subtilis* GB03 (Zhang et al. 2007), *Phyllobacterium brassicacearum* STM196 (Contesto et al. 2010), *Pseudomonas aeruginosa* (Ortíz-Castro et al. 2011), *Martelella endophytica* YC6887 (Khan et al. 2016), *Bacillus amyloliquefaciens* UCMB5113 (Asari et al. 2017), and *Achromobacter* sp. 5B1 (Jiménez-Vázquez et al. 2020) induced changes in the root architecture of *A. thaliana* by affecting auxin transport, perception, or signaling. However, more than 10 years ago, Shi et al. (2010) reported that plant inoculation with the rhizobacterium *Serratia marcescens* 90–166 induced a reduction in the PR length and stimulated LR development through both auxin-dependent and auxin-independent pathways, as well as through complex cross-talk between plant hormones including jasmonates, ethylene, and salicylic acid.

Interestingly, we found that genes encoding jasmonic acid synthesis and signaling increased in plants exposed to the IAA + and IAA − strain by day 7 after inoculation. In contrast, genes related to other signaling molecules, such as ethylene or salicylic acid, exhibited differential patterns. These signaling pathways have already been shown to be enriched in the roots of *Arabidopsis* inoculated with other rhizobacteria species (Stringlis et al. 2018). Recent evidence has demonstrated that plants respond similarly to PGPR as they do to pathogens, with past studies noting an induction of both induced systemic resistance (ISR) and systemic acquired resistance (SAR) dependent on the plant–microbe interaction (van Loon et al 2008; Spaepen et al. 2014). Although hormone responses of SAR (namely salicylic acid) and ISR (primarily jasmonic acid and ethylene) were classically thought to be segregated based upon pathogen lifestyle (Kunkel and Brooks 2002), elaborate cross-talk between these two pathways has been more recently described (Tsuda et al. 2009). In the present study, global RNA sequencing revealed upregulation of genes/GO terms involved in SAR and ISR as a result of both *A. argentinese* treatments. Enriched DEGs of the shared interaction between Az39 and *ipdC* − and *A. thaliana* such as the camalexin biosynthesis gene, *PHYTOALEXIN DEFICIENT*

3 (*PAD3*; AT3G26830), and the flagellin responsive kinase *BRI1-ASSOCIATED KINASE 1* (*BAK1*; AT2G13790) have both been noted in other interaction between *A. thaliana* and *A. baldaniorum* (formerly *A. brasilense*) and other PGPR (Spaepen et al. 2014; Pečenková et al. 2017). These reactions stem from the plant sensing PGPR as non-self, due to the presence of microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) (Zamioudis and Pieterse 2012). As a well-studied MAMP involved in plant defense responses, flagellin (specifically the conserved peptide *flg22*) has also been shown to induce inhibition of primary root growth as has been noted in plant-PGPR interactions (Gómez et al. 1999; Millet et al. 2010). Induction of the plant defense response by MAMPs or PAMPs results in complex hormonal cross-talk between salicylic acid, jasmonic acid, and ethylene, which may occur even if the detected molecule originates from non-pathogenic organisms (Tsuda et al. 2009). Although the detection of bacterial flagellin is likely responsible for the global changes in gene activity regarding plant immunity, the full repertoire of effectors in PGPRs, and specifically in Az39 and *ipdC* – have yet to be determined.

Besides, we observed that most genes related to IAA metabolism or transport tended to become more expressed with time, regardless of the strain. Previous reports described gene upregulation in *Arabidopsis* roots under other rhizobacteria interactions (Desrut et al. 2020; Stringlis et al. 2018; Zhao et al. 2018). Global RNA sequencing results also correspond with the observed phenotype of increased lateral root growth. Shared enriched GO terms of Az39 and *ipdC* – included response to jasmonic acid and ethylene, while GO terms specific to Az39 included response to auxin and meristematic regulation. Shared upregulated differentially expressed genes included *AUXIN RESPONSE FACTOR 11* (*ARF11*; AT1G19220). Despite *ipdC* –'s inability to produce IAA, increased ethylene in the plant, possibly occurring as a result of defense response induction, has been shown to stimulate auxin biosynthesis and transport towards the elongation zone of the root, ultimately inhibiting primary root growth (Růžička et al. 2007). Also shared as a result of treatment with the either *A. argentinense* strains was the enrichment for GO terms and differentially expressed genes associated with jasmonic acid, whose increased synthesis been reported to inhibit primary root elongation while promoting lateral root development in *A. thaliana* (Cai et al. 2014; Cheng et al. 2011). Specific only to treatment with Az39 was upregulation for the leucine-rich repeat receptor like kinases, *MUSTACHES* (*MUS*; AT1G75640) and *MUSTACHES-LIKE* (*MUL*; AT4G36180), which hit within the GO terms pertaining to kinase activity and meristematic regulation, respectively. It has been found that *MUS* and *MUL* regulate development of lateral root primordia in *A. thaliana*, with T-DNA insertional *MUS-MUL* knockout mutants exhibiting greatly reduced lateral root formation (Xun et al. 2020). Authors Xun et al. (2020) also found *MUS* transcription to be greatly enhanced as a result of exogenous auxin application. Thus, it is suggested that DEG upregulation as a result of ethylene, jasmonic acid, and auxin, result in the observed phenotype of reduced primary root growth, with enhanced lateral root development after treatment with Az39 and *ipdC* – strains of *A. argentinese*. However, further study characterizing the hormone response/synthesis in developing root tissue upon PGPR-plant interaction is required.

It should be noted that *Arabidopsis tir1.1* inoculation with Az39 resulted in the same root phenotype as the wild-type Col-0, that is, a shorter PR and a higher number of LR. This finding indicates that IAA bacterial production and IAA plant perception, at least with the involvement of the TIR1 receptor, are not mediating the induction of LR and root hairs in *A. argentinense* Az39-inoculated seedlings. Nevertheless, we cannot rule out the existence of redundant functions in proteins belonging to the same family (such as AFB2 or AFB3). In this regard, Jiménez-Vázquez et al. (2020) showed that the rhizobacterium *Achromobacter* sp. 5B1 caused an increase in root branching in *tir1-1* and *axr3-1* mutants. Both auxin-responsive mutants showed some ability to form lateral roots under controlled conditions, strengthening the idea of an auxin-independent mechanism mediating lateral root maturation in *Arabidopsis* seedlings exposed to *Achromobacter* sp. 5B1. However, other auxin-signaling mutants included in that study did not show root growth promotion.

Apart from those involving auxin, other signaling mechanisms or molecules have been suggested to participate in plant growth promotion by rhizospheric microorganisms, particularly in LR and RH formation. For example, cytokinins (López-Bucio et al. 2007), jasmonates, ethylene, salicylic acid (Ribaudo et al. 2006; Shi et al. 2010), and nitric oxide (Creus et al. 2005; Koul et al. 2015) were shown to mediate processes triggered by *A. brasilense*. Likewise, some bacteria affected plant responses by releasing volatile organic compounds (VOCs); this was corroborated for several *Bacillus* spp. (Li et al. 2021), *B. subtilis* GB03, *B. amyloliquefaciens* IN937a (Ryu et al. 2004), *B. megaterium* UMCV1 (López-Bucio et al. 2007), *B. subtilis* SYST2 (Tahir et al. 2017), and *Pseudomonas* PS01 (Chu et al. 2020). Changes in *Arabidopsis* root architecture due to the action of VOCs released by phytopathogens such as *Penicillium aurantiogriseum* have also been documented; root accumulation of auxins and ethylene was detected in this interaction (García-Gómez et al. 2020).

Likewise, the biosynthesis of dimethyl disulfide by *Bacillus* sp. B55 (Meldau et al. 2013) and phenylacetic acid by *Martelella endophytica* YC6887 (Khan et al. 2016) was shown to affect plant growth. In these studies, primary root growth arrest seemed to depend on volatile or diffusible bacterial molecules' sensing, while lateral root development might have been a compensatory mechanism.

Ribaudo et al. (2006) reported that cross-talk between plant ethylene and bacterial auxin is necessary to cause changes in root morphology regarding PR elongation and RH development. These authors suggested that high concentrations of IAA produced by the hyper producer strain *A. brasilense* FT326 could induce the expression of 1-aminocyclopropane-1-carboxylate synthase (ACS; E.C.4.4.1.14), a key enzyme in ethylene synthesis. Tomato inoculation with *A. brasilense* FT326 induced LR production and increased RH number and length. On the other hand, Creus et al. (2005) showed that the induction of LR formation in tomato seedlings is mediated by NO produced by *A. brasilense* Sp245 (now *A. baldaniorum* Sp245).

So far, there is a good body of evidence that plant-microorganism interactions occur through a highly complex communication network involving a variety of molecules, which alter gene expression in plants, in the associated microbe, or both partners. These mechanisms modulate root morphological changes that result in a more developed root system and better plant use of soil resources (nutrients and water), ultimately leading to improved plant growth and health.

Azospirillum brasilense has a polar flagellum, responsible for bacterial movement in liquid media (swimming), and several lateral flagella, synthesized only when this bacterium grows on solid or semi-solid surfaces (Steenhoudt and Vanderleyden 2000); the key role of *A. brasilense* Sp7 polar flagellum in cells attachment on wheat roots was documented many years ago (Croes et al. 1993). The polar flagellum of *A. brasilense* Sp7 consists of flagellin, a glycoprotein of ~ 100 KDa (Moens et al. 1995). In this work, we obtained flagellin from *A. argentinense* Az39 and tested its action on *Arabidopsis* seedlings. More root hairs were formed in the presence of *Azospirillum* flagellin compared to the untreated control, but less if compared with plants inoculated with *A. argentinense* Az39. Interestingly, this phenotype was dose-dependent.

The positive effect of *A. baldaniorum* Sp245 flagellin on root meristem mitotic activity was reported in wheat seedlings (Shirokov et al. 2020). Likewise, Elías et al. (2021) communicated recently that flagellin from *A. argentinense* REC3 can protect strawberry plants against the fungus *Macrophomina phaseolina* by inducing biochemical, histological, and molecular responses. Here, we report for the first time the ability of *Azospirillum argentinense* Az39 flagellin to induce RH formation in *A. thaliana*, thus altering root architecture.

Hernández-Esquivel et al. (2020) proposed that *A. brasilense* Sp245 can alter root morphology through structural molecules such as lipopolysaccharides (LPS), in addition to phytohormone production. These authors refer that LPS may need to contact the root directly to exert a stimulating effect and that plant cells need to distinguish between different LPS components to allow plant response, as these interactions may be species-specific.

There is scarce information on the effects of microbial LPS perception by plants, although plant receptors for typically pathogen-associated molecules (such as flagellin) have been described (Zipfel et al. 2004). Chávez-Herrera et al. (2018) showed that LPS from *A. brasilense* affected some aspects of wheat development, but the molecular mechanism involved is still obscure. More recently, Cassán et al. (2020) suggested that certain cellular components of *A. argentinense*, in direct contact with plant roots, could induce a growth response. In line with these concepts and considering that polar flagellum flagellin from *Azospirillum* also behaves as a microbe-associated molecular pattern (MAMP) (Elías et al. 2021), it is very likely that flagellin from *A. argentinense* Az39 could be involved in the signaling processes resulting in root architecture modification, in addition to phytohormone-mediated mechanisms.

5 Conclusions

IAA-dependent and IAA-independent mechanisms seem to mediate root morphological changes in *A. thaliana* seedlings inoculated with *A. argentinense* Az39—a well-known PGPR strain frequently used in commercial inoculants in Argentina—, opening new horizons regarding selection criteria when screening *Azospirillum* strains intended to become biological products for agriculture. Transcriptome analyses may provide valuable clues to shed light on the complex signaling network resulting in root architecture changes under plant–microbe interactions. The physical presence of *A. argentinense* Az39 cells and the sensing of *A. argentinense* flagellin by roots may be key points in these less-known, IAA-independent routes of root growth promotion.

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Data Availability All RNA sequencing data have been deposited at the Gene Expression Omnibus (GEO): GSE192383. All other relevant data can be found within the manuscript and its supporting materials.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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