Root Responses to Heterogeneous Nitrate Availability are Mediated by trans-Zeatin in Arabidopsis Shoots

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RESEARCH PAPER

Local signalling pathways regulate the Arabidopsis root developmental response to *Mesorhizobium loti* inoculation

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Abstract

Numerous reports have shown that various rhizobia can interact with non-host plant species, improving mineral nutrition and promoting plant growth. To further investigate the effects of such non-host interactions on root development and functions, we inoculated *Arabidopsis thaliana* with the model nitrogen fixing rhizobacterium *Mesorhizobium loti* (strain MAFF303099). *In vitro*, we show that root colonization by *M. loti* remains epiphytic and that *M. loti* cells preferentially grow at sites where primary and secondary roots intersect. Besides resulting in an increase in shoot biomass production, colonization leads to transient inhibition of primary root growth, strong promotion of root hair elongation and increased apoplasmic acidification in periphery cells of a sizeable part of the root system. Using auxin mutants, *axr1-3* and *aux1-100*, we show that a plant auxin pathway plays a major role in inhibiting root growth but not in promoting root hair elongation, indicating that root developmental responses involve several distinct pathways. Finally, using a split root device, we demonstrate that root colonization by *M. loti*, as well as by the *bona fide* plant growth promoting rhizobacteria *Azospirillum brasilense* and *Pseudomonas*, affect root development via local transduction pathways restricted to the colonised regions of the root system.

Key words: Apoplasmic pH, Arabidopsis, colonization pattern, *Mesorhizobium loti*, PGPR, root, symbiosis, split root.

Introduction

In plants the benefits of associative symbiosis can arise from the various effects of rhizobacteria, including changes in root architecture, stimulation of root hair elongation, modulation of plant ethylene synthesis and induction of systemic resistance (ISR) (Vacheron *et al.*, 2013). Directly or indirectly, such effects favour plant growth via the increased capacity of the root system to explore the soil and to take up nutrient ions, or via enhanced protection of the plant against pathogens. Rhizobacteria that can give rise to such beneficial interactions are generically named PGPR for plant growth promoting rhizobacteria. While growth promotion by PGPR can result from a combination of beneficial effects, it never involves the formation of new organs. It thus differs from the mutualistic symbiosis that occurs between nitrogen fixing rhizobacteria and legumes, which is characterized by the formation of a new organ, namely the nitrogen-fixing root nodule (Desbrosses and Stougaard, 2011; Oldroyd *et al.*, 2011).

Abbreviations: ISR, induction of systemic resistance; PGPR, plant growth promoting rhizobacteria; TM, transmembrane; VOC, volatile organic compound.

Associative symbiosis with PGPR has been reported in many plant genera and may be developed by all terrestrial plants. Furthermore, PGPR are found in different orders of bacteria, such as Bacillales, Rhodospirillales and Pseudomonadales, with examples including *Bacillus sp GB03*, *Azospirillum brasilense* and *Pseudomonas fluorescens*. Interestingly, some nitrogen fixing rhizobium species can engage in either mutualistic or associative symbiosis relationships depending on the plant, host or non-host, they are interacting with. For instance, several rhizobium strains have been shown to behave as PGPR on non-host plants such as maize, rice and wheat (Chabot *et al.*, 1996; Webster *et al.*, 1997; Mishra *et al.*, 2006), canola and lettuce (Noel *et al.*, 1996), radish (Antoun *et al.*, 1998), carrots (Flores-Felix *et al.*, 2013) and tomato (Garcia-Fraile *et al.*, 2012). Despite this abundance of reports, there is little information on the mechanisms underlying such interactions that result in growth promotion. It has been suggested that it involves improved phosphorous, nitrogen and iron nutrition (Flores-Felix *et al.*, 2013) and secretion or metabolism of phytohormones, such as auxin and cytokinins, by bacterial cells (Noel *et al.*, 1996).

In this work, we aim to further investigate the beneficial interactions between nitrogen-fixing rhizobacteria and non-host plants. We largely focused on root development and used the model plant *Arabidopsis thaliana* grown *in vitro* and inoculated with the model nitrogen fixing rhizobacteria *Mesorhizobium loti* MAFF303099, which is able to form root nodules with the model legume *Lotus japonicus*. *M. loti* is a close relative of a PGPR strain named *Phyllobacterium brassicacearum* strain STM196 (Mantelin *et al.*, 2006*b*), which has been isolated from field grown canola rhizospheres and shown to promote canola and Arabidopsis growth in soil (Bertrand *et al.*, 2001) and *in vitro* (Mantelin *et al.*, 2006*a*). *In vitro* STM196 inoculation stimulated root hair elongation and increased the total length of lateral roots (Mantelin *et al.*, 2006*a*). While the former response appears to be regulated by the ethylene-signalling pathway (Galland *et al.*, 2012), the latter one relies on the plant auxin pathway (Contesto *et al.*, 2008).

In this report, we describe the colonization pattern of *M. loti* and show that Arabidopsis growth is increased in the presence of the rhizobium. We also show that *M. loti* colonization impacts primary root growth, root hair length and root apoplasm pH. We provide evidence that some root developmental responses rely on the auxin pathway, as in other previously characterised interactions between Arabidopsis and some well-known PGPR strains. A major result, obtained using the split root system device, is that all the root responses are locally triggered by the rhizobacteria in the colonized regions of the root system.

Materials and Methods

Plant material, bacterial strains and growth conditions

All the experiments were conducted with *Arabidopsis thaliana* Col-0. Involvement of the plant auxin pathway in the responses to inoculation was tested with the auxin resistant mutant *axr1-3* (Lincoln *et al.*, 1990; Leyser *et al.*, 1993) and the auxin permease null mutant *aux1-100* (Bennett *et al.*, 1996), while involvement of the ethylene pathway was tested with the ethylene resistant mutant *etr1-1* and the ethylene insensitive mutant *ein2-1* (Guzman and Ecker, 1990). Transgenic Arabidopsis lines expressing either a *pDR5:GFP* reporter construct (Benkova *et al.*, 2003), a *pCYCB1:GUS* construct (Colon-Carmona *et al.*, 1999) or a *pGL2:nls:GFP:GUS* construct (Schnittger *et al.*, 2003) were used to investigate the impact of *M. loti* inoculation on auxin distribution, cell cycle activity in the root tip meristem and root epidermal cell patterning, respectively. Root apoplasm pH was probed using a nanosensor derived from pHluorin (Bagar *et al.*, 2009) (see below).

All plant genotypes were cultivated *in vitro* as previously described, on the same mineral medium (Mantelin *et al.*, 2006*a*) and in the same environmental conditions (Galland *et al.*, 2012).

Bacterial strains and plant inoculation

Plants were inoculated with either *Mesorhizobium loti* MAFF303099 (a gift from Prof. K. Minamisawa, Tohoku University, Japan) or the same bacterial strain but transformed in order to constitutively express a DsRed fluorescent protein (a gift from Dr Y Kawaharada, University of Aarhus, DK). Two PGPR strains were also used: *Azospirillum brasilense* Sp245 (a gift from Dr C. Prigent-Combaret, Ecologie Microbienne, Lyon, France) and *Pseudomonas* sp. WCS417 (a gift from Dr J. Schwachtje, MPIMP, Potsdam-Golm, Germany). The *M. loti* strains were grown on solid TY medium, the Sp245 strain on NAB medium and the WCS417 strain on KingB medium. All the strains were grown at 28 °C.

To prepare an inoculum of *M. loti* or WCS417 cells, an isolated bacterial colony was streaked on a fresh agar plate of the appropriate medium and allowed to grow for 4 days. On the day of inoculation, 8 ml of plant liquid mineral medium was poured onto the plate, which was incubated at room temperature for 20 minutes before the bacterial colonies were stripped from the plate by careful pipetting. The density of the collected bacterial suspension was assessed by spectrophotometry at 595 nm. To prepare the *A. brasilense* Sp245 inoculum, we used a standard protocol (Mantelin *et al.*, 2006*a*) as this strain forms colonies that cannot be easily disrupted in liquid medium when cultivated on solid media.

Inoculation of plant growth medium was achieved as previously described (Mantelin *et al.*, 2006*a*), an aliquot of the bacterial inoculum being introduced into the medium at 42 °C just before filling the agar plates. Plant growth on the inoculated medium ensures bacterial colonization of the roots.

Split root experiments

Square Petri plates with two distinct compartments were obtained by carefully cutting off the two internal walls from square 12 cm x 12 cm Petri dishes with four compartments. The remaining wall split the dish into two separate compartments preventing any diffusion of media or solutes from one compartment to another. Molten plant medium was then poured in one compartment and allowed to solidify before a second molten medium was poured into the remaining empty compartment. The same non-inoculated medium, split root control treatment, was poured into both compartments or one compartment was filled with the non-inoculated medium and the other with the same medium that had been inoculated, split inoculation treatment. In order to get root systems displaying two 'symetrical' parts, the primary root tips of 7-day-old seedlings were removed below the emergence of a second lateral root (Remans *et al.*, 2006). Four days after pruning, seedlings whose root system had developed two distinct halves were transferred onto the split root agar plates, each half of the root system being separately laid onto a different compartment of the plate. All these experimental steps were carried out in a laminar flow hood in order to ensure sterile and controlled conditions.

Root trait analyses

Effects of *M. loti* inoculation on primary root length and root hair elongation were assessed as previously described (Contesto *et al.*, 2010; Galland *et al.*, 2012).

To investigate the bacterial colonization pattern along the root system without physiological disturbance, the Arabidopsis transgenic line that constitutively expresses the fluorescent pHluorin construct (see below) was inoculated with the *M. loti* strain that constitutively expresses the DsRed fluorescent protein. Fluorescence signals were observed with a confocal macroscope (Macroconfocal LSI, Leica, Bensheim, Germany). The same optical equipment was used to observe the fluorescence of Arabidopsis transgenic plants expressing *pDR5:GFP*. A confocal microscope (SP8, Leica, Bensheim, Germany) was used to observe the fluorescence signal from the transgenic line expressing the *pGL2:GFP* construct.

Cell cycle activity at the root tip was evaluated using the Arabidopsis line transformed with the *pCycB1:GUS* reporter construct. Excised root tips were stained as described (Jefferson, 1989) and observed with a macroscope (Z16APO, Leica, Bensheim, Germany) under bright light illumination.

Construction of the pHluorin-TM apoplasmic pH probe

The protein pHluorin was used as a genetically encoded pH nanosensor (Miesenbock *et al.*, 1998; Schulte *et al.*, 2006; Bagar *et al.*, 2009; Geilfus and Muhling, 2011). A pHluorin chimeric construct anchored to the plasma membrane with the pH sensor facing the apoplasm was obtained by creating a modified version of TM23 (Brandizzi *et al.*, 2002) where (i) the GFP sequence was replaced by the pHluorin one and (ii) three amino acids 'VLI' were added to the HsLAMP1 transmembrane (TM) domain. The corresponding construct, named pHluorin-TM, was cloned in the pENTR1a vector between the BamH1 and Sac1 sites and then transferred into the pGWB502 destination vector via recombination (Nakagawa *et al.*, 2007).

Apoplasmic pH measurement in roots expressing the pHluorin-TM construct

Stable transformation of Arabidopsis Col-0 with the pHluorin construct was performed using *Agrobacterium tumefaciens* GV3101 strain according to the floral dip method (Clough and Bent, 1998).

The pattern of apoplasmic pH along transformed roots was probed using the ratiometric pH sensor properties of pHluorin as previously described (Martiniere *et al.*, 2013). In brief, plants expressing the pHluorin-TM construct were grown on agar plates in the presence or absence of *M. loti*. Using a Leica macroconfocal LSI (Leica, Bensheim, Germany), root images were directly acquired *in situ* in the agar plate, without any manipulation of the plant. For each observation, two images were taken upon a sequential illumination at 405 nm and 488 nm, corresponding to the two excitations peaks of pHluorin. In both cases, the emitted fluorescence was collected from 505 nm to 550 nm. Ratio images were obtained by dividing the fluorescence value of each pixel in the image taken at 405 nm by the corresponding value in the image taken at 488 nm. The resulting ratios were converted into pH values using a calibration curve.

This calibration curve was obtained using an extract of pHluorin produced in *Escherichia coli* and diluted in different buffers at various pH values (Martiniere *et al.*, 2013) (see Supplementary Fig. S1 at *JXB* online). The buffered pHluorin solutions were imaged with the Leica macroconfocal LSI at 405 nm and 488 nm. The fluorescence signals were measured and their ratios (Ex^{405}/Ex^{488}) were plotted as a function of pH (Supplementary Fig. S1). The calibration curve was obtained by fitting the experimental points with a sigmoidal function using a Boltzman equation (Gao *et al.*, 2004; Schulte *et al.*, 2006).

Auxin content in bacterial liquid culture supernatant

The amount of auxin produced by *M. loti* was determined as previously described (Contesto *et al.*, 2010). All the experiments were performed in parallel with two reference strains: *A. brasilense* Sp245 and, in the same genetic background, the Δ*IpdC* auxin synthesis mutant strain (Costacurta *et al.*, 1992). For each strain and each condition, nine independent samples were collected and analysed.

Statistics

An independent experiment is defined as an experiment that includes a new plating of seeds and a new rhizobium inoculation. Data were collected and processed using Microsoft Excel. To check whether differences observed between inoculated and non-inoculated plants were statistically significant, we performed Student's T-test. In the figures, a P value below 0.05, 0.01 or 0.001 is highlighted with the symbol *, ** or ***, respectively.

Results

M. loti *interaction with Arabidopsis roots are epiphytic and preferentially localized at secondary root emergence sites*

To check the ability of *M. loti* MAFF303099 to colonize the Arabidopsis root system *in vitro*, plantlets that were either wild type or transformed to constitutively express the GFP-derived fluorescent protein pHluorin-TM, were inoculated with a strain of *M. loti* constitutively expressing a DsRed fluorescent protein. Analysis of the fluorescence signals revealed the presence of *M. loti* colonies or isolated cells at the root surface (Fig. 1A) but not within inner tissues (Supplementary Fig. S2).

To further investigate the colonization pattern of the Arabidopsis root system by the fluorescent *M. loti* strain, we gently removed roots from the agar plate and monitored the intensity of the bacterial fluorescence on the remaining footprint. Strong fluorescent signals were observed in regions corresponding to lateral root emergence sites (Fig. 1B, C). Agar medium was then sampled at different places along the root footprint or far from it, as a control of the inoculated medium. The samples were transferred into sterile water in order to resuspend the bacteria. To count the number of growing fluorescent colonies, dilution series of the bacterial suspensions were plated on solid TY agar medium. All the colonies growing in the plates were fluorescent (data not shown). The agar samples collected at lateral root emergence sites gave rise to the largest number of colonies (Fig. 1D), in agreement with previous data obtained by imaging the fluorescence signals (Fig. 1B, C; Desbrosses *et al.*, 2013).

M. loti *promotes shoot growth, inhibits primary root growth and stimulates root hair elongation*

Since *M. loti* can colonize the Arabidopsis rhizosphere *in vitro*, we checked whether this could affect shoot and root biomass production. One-week-old Arabidopsis plantlets were transferred onto agar plates and either inoculated with *M. loti* or not. After one additional week of growth, they were sampled for shoot and root fresh weight measurements. The presence of *M. loti* significantly increased shoot fresh weight in wild type plants (Fig. 2A, Supplementary Fig. S3) but not in *axr1-3*, *aux1-100*, *etr1-1* and *ein2-1* mutant plants (Supplementary Fig. S3).

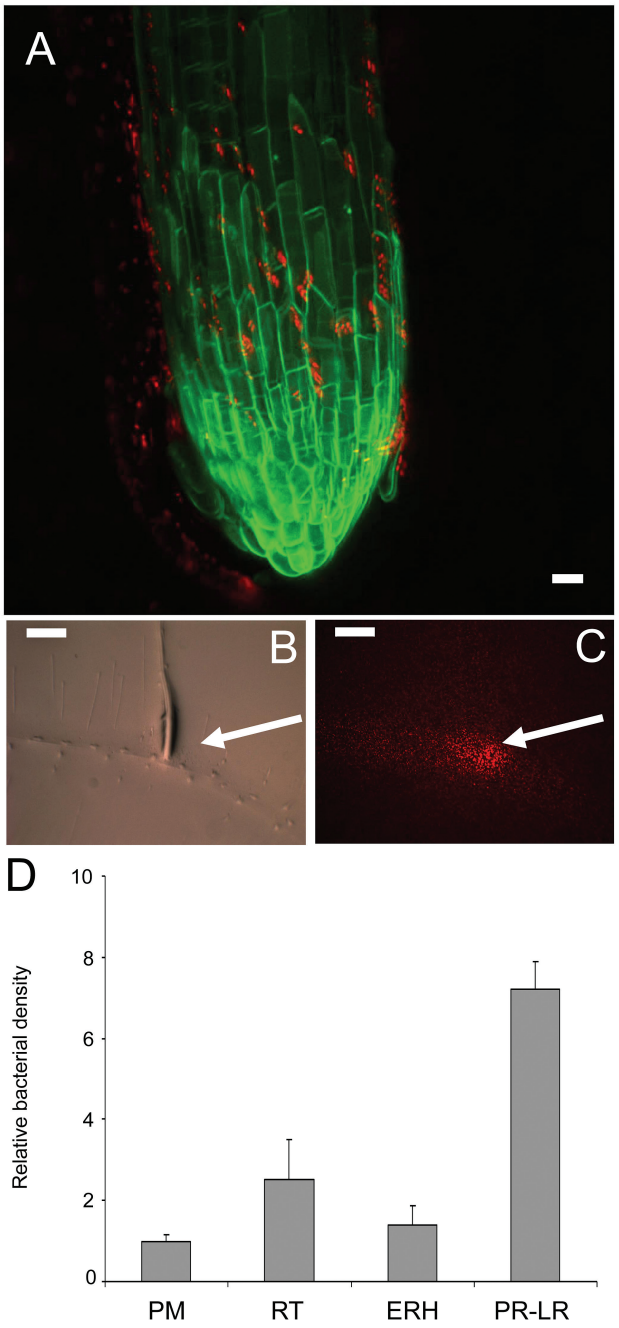

Fig. 1. *M. loti* preferentially grows at the intersection of primary and lateral roots. Transgenic Arabidopsis plants constitutively expressing the fluorescent construct, pHluorin-TM, were inoculated with *M. loti* cells constitutively expressing a DsRed fluorescent protein and grown for a further 5 days. *M. loti* colonization was then observed by confocal macroscopy (A). In the same experiment, the root was removed from the media and the resulting footprint left in the agar was observed under normal light (B) or UV light (C) with a macroscope (Z16APO, Leica, Bensheim, Germany). Large and dense colonies of *M. loti* can be seen in the region (arrow in panel C) corresponding to the intersection of an elongating lateral root and the primary root. Scale bar: 20 μm. Using the same plates, similar agar fragments were carefully sampled with sterile Pasteur pipettes several centimetres away from the root footprint in order to assess bacterial density in the control plant medium (PM), in regions that were previously below the root tip (RT), in the root zone with fully elongated root hairs (ERH) or at the intersection of a primary root with a lateral root (PR-LR). The density of living bacteria was determined in each sample and divided by the density found in the control PM samples (D; 2.76×10^5 CFU/ml). Means±SD of three independent experiments.

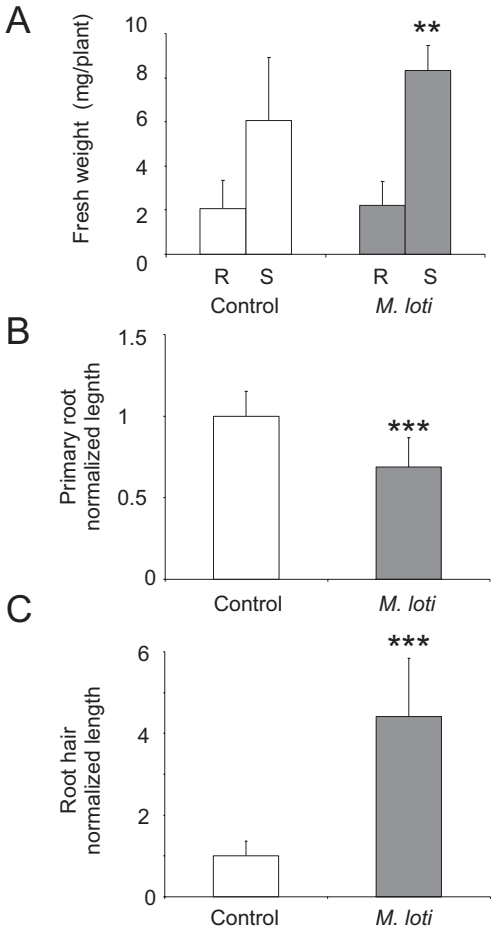

Fig. 2. Inoculation of Arabidopsis roots with *M. loti* promotes plant growth and affects root traits. Seven-day-old *in vitro* grown seedlings of wild type Arabidopsis plantlets were grown for a further 7 days in the presence or absence (histogram black and white bars, respectively) of *M. loti* in the mineral agar-solidified medium. Plants were then sampled for measuring root (R) and shoot (S) fresh weight (A), primary root length (B) and root hair length (C). Means±SD of 26 individuals from seven independent experiments. In each of these seven experiments, the individual values of primary root length and root hair length were divided by the average value of the corresponding length observed in the control non-inoculated plants. For root hair length measurements, an individual value was defined as the mean value of at least 20 root hairs from the same root. In (A), the statistical significance of the difference was tested for each organ.

Thus, *M. loti* behaves as a PGPR on Arabidopsis plantlets growing *in vitro*.

As beneficial rhizobacteria are well known to affect the development of the root system (Vacheron *et al.*, 2013), we checked whether *M. loti* could also modify Arabidopsis root development *in vitro*. Five days after inoculation, we observed that colonization resulted in both shorter primary roots (Fig. 2B) and longer root hairs (Fig. 2C).

Root growth kinetics is affected by the presence of M. loti

The depressive effect of *M. loti* inoculation on primary root growth was further investigated. Time course analyses revealed a very strong inhibition of root growth at 1 day post

inoculation (Fig. 3A). In contrast, measurements performed on days 2 and 3 post inoculation did not reveal any significant inhibition of root growth (Fig. 3A). Thus, the inhibition of root growth triggered by *M. loti* appeared to be transient. Another line of evidence supporting this conclusion was obtained at the molecular level using transgenic Arabidopsis plants transformed with the construct *pCYCB1:GUS*, which encodes a cyclin B1 promoter fused to *GUS* and whose expression reflects the level of cell cycle activity and thus the rate of cell division (Colon-Carmona *et al.*, 1999). Strong GUS staining was displayed by the root tips of control plants, as expected from the cyclin B1 promoter (Fig. 3B, C). In contrast, in roots inoculated with *M. loti*, staining was faint at 24 h post inoculation (Fig. 3B). At 72 h post infection the staining was stronger and similar in intensity to that observed in the non-inoculated roots (Fig. 3C). It remained so for up to 7 days more (Supplementary Fig. S4). Thus, the presence of *M. loti* induced a transient inhibition of cell division in the root meristem.

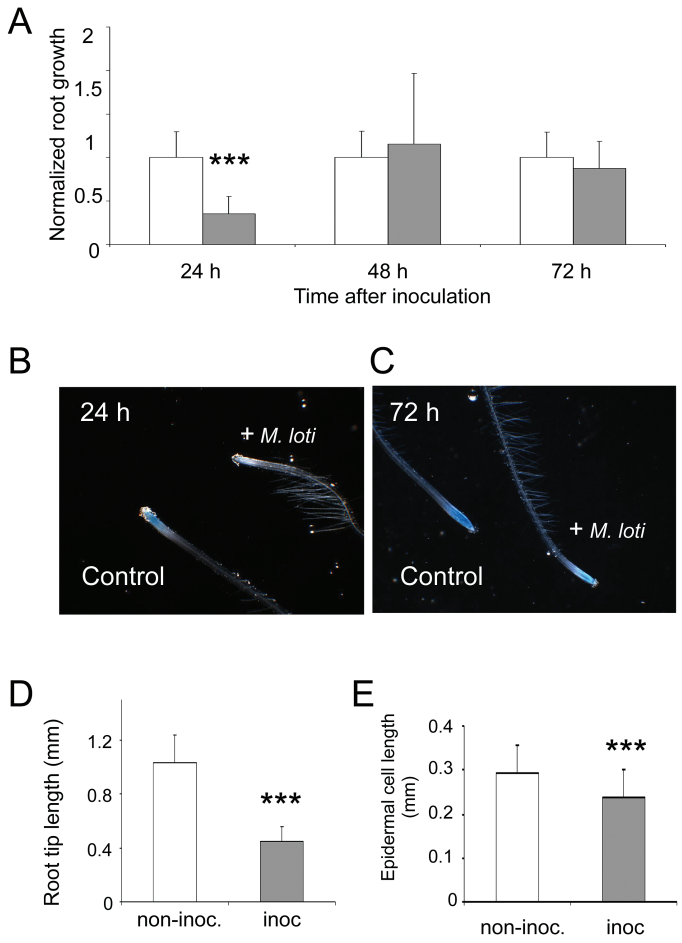

Fig. 3. Analysis of the depressive effect of *M. loti* on Arabidopsis root growth. Arabidopsis seedlings were transferred onto agar plates either inoculated with *M. loti* or not inoculated (control) and grown for a further 3 days. The daily rate of root growth, normalized according to the corresponding mean rate of root growth on the non-inoculated medium, is provided in (A). Black and white bars, inoculated and non-inoculated roots, respectively. Means±SD of five to eight independent experiments, each one comprising 17 to 33 individuals. Arabidopsis seedlings expressing a *pCYCB1:GUS* construct used as a reporter of root meristem mitotic activity were transferred onto inoculated or non-inoculated medium. Roots were sampled 24 h or 72 h after transfer and immediately used for GUS histochemical staining (blue). Representative photographs of inoculated and non-inoculated roots sampled at 24 h (B) or 72 h (C) after the transfer. In parallel experiments, roots were sampled 72 h after the transfer to measure the length of the root tip, operationally defined as the distance between the tip and the first emerging root hairs (D) and the length of the epidermal cells in the fully differentiated zone (E). Means±SD, $n \geq 8$ in (C) and $n \geq 83$ in (D). In (A), the statistical significance of the difference was tested at each time point.

Further analysis of the inhibitory effect of *M. loti* on primary root growth revealed that the presence of bacteria resulted in a significant reduction in size of the region operationally defined as 'meristematic+elongation' zone i.e. the region that extends from the tip of the root to roughly the first emerging root hairs (Fig. 3D). In addition, differentiated root epidermal cells were found to be shorter in *M. loti* inoculated roots (Fig. 3E).

M. loti *inoculation modifies the apoplasmic pH of root periphery cells*

To investigate whether the colonization process affects not only root development but also root physiology, Arabidopsis transgenic plants constitutively expressing a pHluorin-TM construct, which targets the pHluorin pH probe to the outer face of the plasma membrane, were grown on non-inoculated or inoculated agar medium and observed with a confocal macroscope directly *in situ* to prevent any perturbation of pH homeostasis due to plant manipulation. Analysis of the fluorescence of periphery cells from intact roots revealed that the apoplasmic pH pattern along the root axis was different between non-inoculated (Fig. 4A) and inoculated (Fig. 4B) plants. In the absence of *M. loti*, the pH was found to be alkaline at the root tip and rapidly became acidic in the region corresponding to dividing cells (Fig. 4A, C). Along the elongation zone, apoplasmic pH progressively increased from slightly acidic to neutral (Fig. 4C). In the presence of *M. loti*, the apoplasmic pH at the root tip was alkaline, as in the non-inoculated plants (Fig. 4B, C). The meristematic region was acidified, also as in the non-inoculated roots, but the apoplasmic pH was more acidic and remains so in the elongation zone (Fig. 4C). In conclusion, *M. loti* inoculation led to acidification of the root apoplasm.

M. loti *inoculation affects the elongation but not the developmental pattern of root hairs*

M. loti inoculation resulted in longer root hairs (Fig. 2C). To further investigate this stimulating effect, three different types of experiments were performed. First, root hair elongation was observed during the light and the dark periods in plants growing on inoculated or non-inoculated medium. In the absence of *M. loti*, in our experimental conditions, that is growth on a mineral medium devoid of carbon and with a 16 h photoperiod, root hairs elongated during the light period but showed very little elongation during the dark period (Fig. 5A). Interestingly, in presence of *M. loti*, root hairs elongated in a very similar way during both the light and the dark period (Fig. 5A).

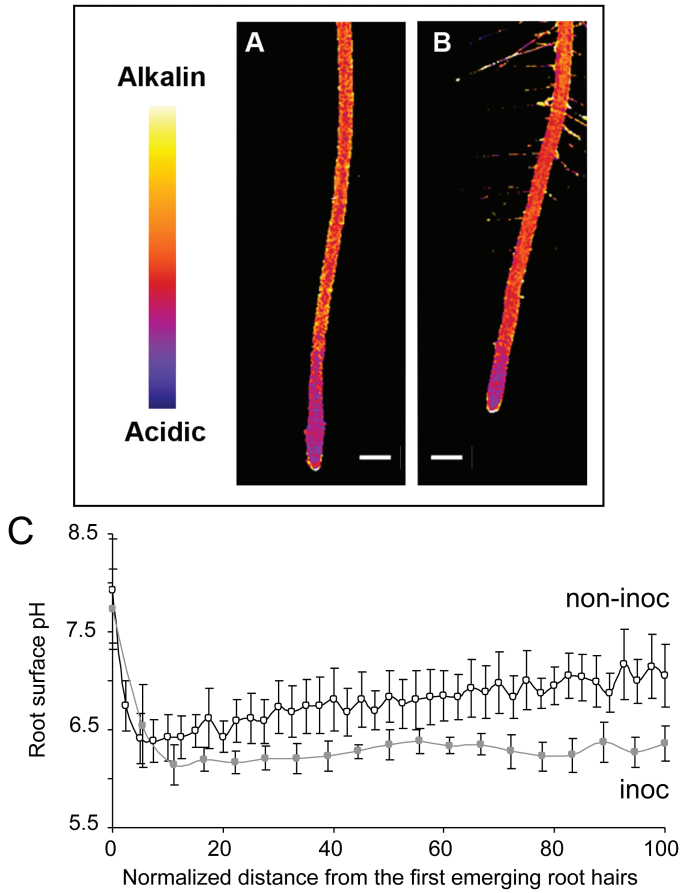

Fig. 4. *M. loti* inoculation affects net excretion of H^+ equivalents into the root apoplasm. Seven-day-old transgenic Arabidopsis lines expressing the pHluorin-TM apoplastic pH probe were transferred onto standard mineral medium supplemented with 0.5 mM MES, the initial pH being adjusted at 5.7. Three days later, roots were illuminated at 405 nm and 488 nm using a confocal microscope. The Ex^{405}/Ex^{488} fluorescence ratio of each pixel was determined, transformed into a pH value and a false colour applied using the calibration curve displayed in Supplementary Fig. S1. Representative images of non-inoculated (A) and inoculated (B) roots. Scale bar, 200 μm. Apoplastic pH values at the root surface were determined using such fluorescence images and plotted as a function of the normalized distance to the root tip, expressed in percent of the distance between the tip and the first emerging root hairs (C). Means±SD, n=10.

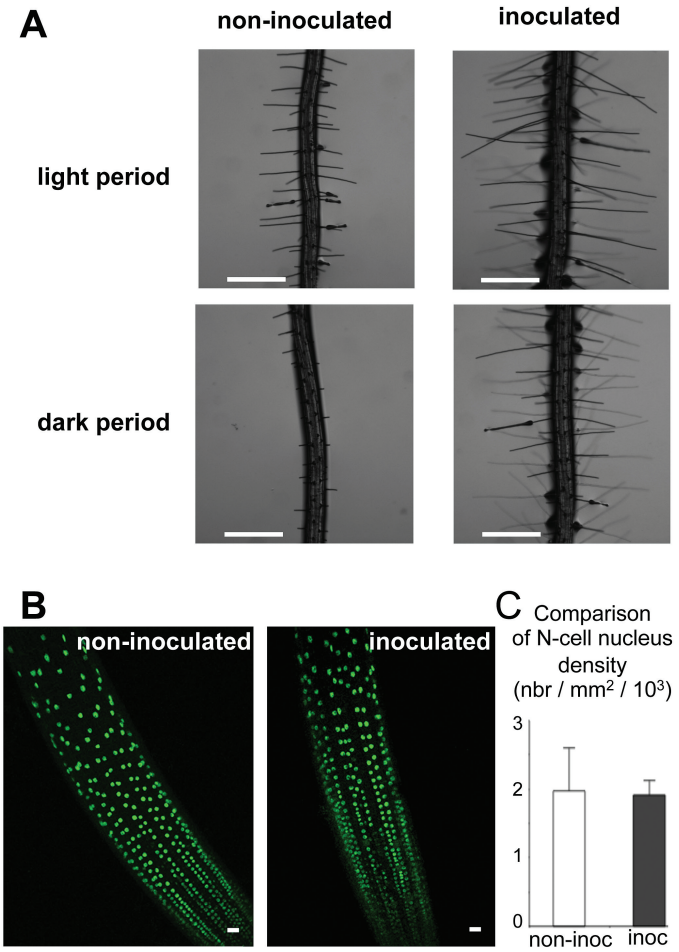

Fig. 5. *M. loti* colonization promotes root hair elongation without significant effects on epidermal cell patterning. Root hair growth in non-inoculated or inoculated Arabidopsis seedlings during the light or dark period (A). Scale bar, 200 μm. Effects of *M. loti* on root epidermal cell patterning were investigated using Arabidopsis transgenic plants expressing a nucleus-localized GFP construct under the control of the promoter of the Arabidopsis gene *GL2*, which encodes a transcription factor that represses root hair elongation in non-hair-forming cells (N-cells). Plants were grown for 7 days after transfer onto inoculated or non-inoculated (control) medium. Root tips were then illuminated by UV light, revealing similar GFP fluorescence patterns, typical of the *pGL2* promoter, in both non-inoculated and inoculated roots (B). Scale bar, 20 μm. The tip of a non-inoculated root was aligned with that of an inoculated root and the number of fluorescent nuclei was counted over the same distance in both roots. These numbers were then divided by the corresponding 2D surface of the roots in order to compare the density of N-cell nuclei in the non-inoculated (non-inoc) and inoculated (inoc) roots (C). Means±SD, n≥7. (This figure is available in colour at *JXB* online.)

Second, we investigated whether *M. loti* inoculation affected the developmental pattern of root hairs by monitoring the expression of a *pGL2:nls:GFP:GUS* reporter construct in transgenic Arabidopsis plants (Schnittger *et al.*, 2003). The GL2 transcription factor represses root hair elongation in non-hair-forming cells (N cells) (Masucci *et al.*, 1996). In the absence of *M. loti*, the GFP signal was localized to the nuclei of N cells (Fig. 5B), as expected from previous analyses (Schnittger *et al.*, 2003), and the plants displayed a succession of cell lines with fluorescent or non-fluorescent nuclei, giving rise to a pattern consistent with *GL2* expression in N cells. In inoculated roots, this overall pattern remained unaffected (Fig. 5B). Furthermore, the density of nuclei expressing *GL2* was similar in the presence or absence of *M. loti* (Fig. 5C).

Third, we checked the effect of *M. loti* on the phenotype of the Arabidopsis *caprice* (*cpc*) mutant, which is characterized by a low root hair density (Wada *et al.*, 1997). Six-day-old *cpc* and wild type Arabidopsis plantlets were transferred onto agar plates inoculated with *M. loti* or non-inoculated plates, where they grew for a further 7 days. For each plantlet, the number of root hairs visible on a photograph of the differentiated root zone was counted and normalized according to the 2D surface of the corresponding root region in order to determine the apparent root hair density. This parameter was lower in *cpc* mutant plants than in control wild type plants by 44% (±10; n=7) on non-inoculated medium, and by 54% (±8; n=8) on inoculated medium, indicating that the presence

of *M. loti* does not rescue the typical low root hair density phenotype of the *cpc* mutant.

Role of auxin in M. loti *effects on root development*

In Arabidopsis, auxin and ethylene are well known positive regulators of root hair elongation [for review see Grierson *et al.* (2014)]. In addition, auxin can stimulate the activity of H^+-ATPase pumps at the plasma membrane, leading to apoplasm acidification (Hager, 2003; Takahashi *et al.*, 2012). Finally, many rhizobacteria strains such as *A. brasilense* Sp245 have been shown to produce significant amounts of auxin, which affects plant root architecture (Spaepen *et al.*, 2014). The effects of *M. loti* on Arabidopsis root system development and physiology might therefore result from auxin production and secretion by the bacteria. To test this hypothesis, we grew *M. loti* and Sp245 bacteria in liquid culture with or without 500 µg.l^{-1} tryptophan, whose supply is required for auxin synthesis by Sp245. Twenty-four hours later, we measured the amount of indolic compounds, including auxin, present in the culture media. The *M. loti* culture medium contained less indolic compounds than the Sp245 culture (Fig. 6A). Furthermore, the amount of indolic compounds was unchanged when the culture medium was not supplemented with tryptophan in the case of *M. loti*, while it was strongly reduced in the case of Sp245 (Fig. 6A). Finally, the medium supplemented with tryptophan in which Sp245 Δ*IpdC* mutant cells, a strain almost completely defective in auxin synthesis (Costacurta *et al.*, 1992), had been grown was found to contain a similar amount of indolic compounds as that of the wild type strain grown in the absence of tryptophan (Fig. 6A). This indicates that the indolic compounds assayed in these experiments were mostly composed of auxin. Thus, when grown in liquid culture and in presence of a large concentration of tryptophan, *M. loti* does not appear to produce much in the way of indolic compounds.

Plant auxin transport and signalling are also involved in responses of the root system to growth promoting rhizobacteria (Contesto *et al.*, 2010; Zamioudis *et al.*, 2013; Spaepen *et al.*, 2014). To check whether *M. loti* affects the root system through an auxin pathway, we inoculated *axr1-3* (Lincoln *et al.*, 1990) and *aux1-100* (Bennett *et al.*, 1996) Arabidopsis mutant plants. Seven days after inoculation, neither the *aux1-100* nor the *axr1-3* mutant lines exhibited root growth inhibition, while they both displayed strong stimulation of root hair elongation (Fig. 6B, C). These results indicate that an auxin transport and signalling pathway is required for primary root growth inhibition by *M. loti*, whereas it is not required for root hair elongation.

M. loti *effects on root development involves local responses*

Reports show that root inoculation with PGPR can affect gene transcription in shoots, suggesting the existence of systemic signalling (Mantelin *et al.*, 2006*a*). We therefore investigated whether Arabidopsis root responses to *M. loti* inoculation were also dependant on a systemic signalling pathway.

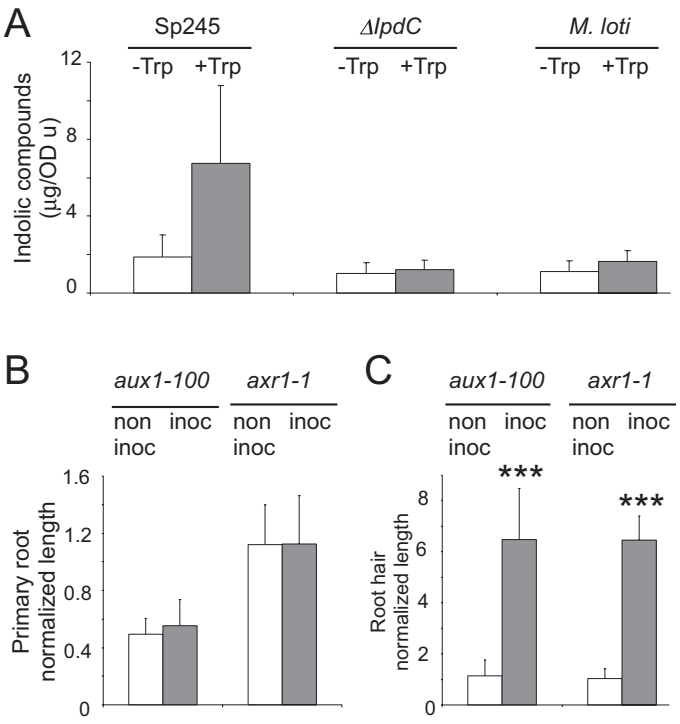

Fig. 6. *M. loti* inhibits primary root growth via a plant auxin pathway. Colonies of the well-known auxin producing strain *Azospirillum brasilense* Sp245, of its auxin synthesis mutant Δ*IpdC* and of *M. loti* were grown in the presence (black bar) or absence (white bars) of 500 µg.l^{-1} of the auxin precursor tryptophan, until the bacterial culture optical density reached an exponential growth phase (OD=0.2 for Sp245 and Δ*IpdC*, OD=0.1 for *M. loti*). The levels of indolic compounds present in the culture media were then determined and standardized by the optical density of the bacterial culture (A) (means±SD, *n*=9). Seven-day-old wild type plants and *aux1-100* and *axr1-3* auxin mutants were transferred onto agar plates inoculated (inoc label) with *M. loti* or non-inoculated (non inoc). Primary root length (B) and root hair length (C) were measured 7 days later and normalized by the average length measured for non-inoculated wild type plants. Means±SD, *n*≥12 (a minimum of 12 individual plants spread over three independent experiments).

To this end, we carried out split root experiments. The primary root tip meristems of 3-day-old plantlets were pruned. The first lateral roots to emerge were split into two new root systems, each of them being grown on an independent 'compartment'. The two compartments were either strictly identical and non-inoculated i.e. control plants, or one compartment was inoculated with *M. loti* and the other left non-inoculated. In the latter split root conditions, the roots grown in contact with rhizobia displayed typical responses to bacterial colonization, namely reduced root growth and longer root hairs, while those grown on the non-inoculated compartment displayed the same phenotype as the roots of the control plants (Fig. 7A, B). Thus, the two effects of *M. loti* on root system development were local, i.e. limited to the roots in contact with the bacteria.

Similar split root experiments were then carried out with *Pseudomonas* sp. WCS417 and *A. brasilense* Sp245 (Supplementary Fig. S5), revealing that the ability of these two rhizobacteria to affect the morphology of the root system by inhibiting root growth and promoting root hair elongation also relies on local responses. Indeed, root growth was inhibited

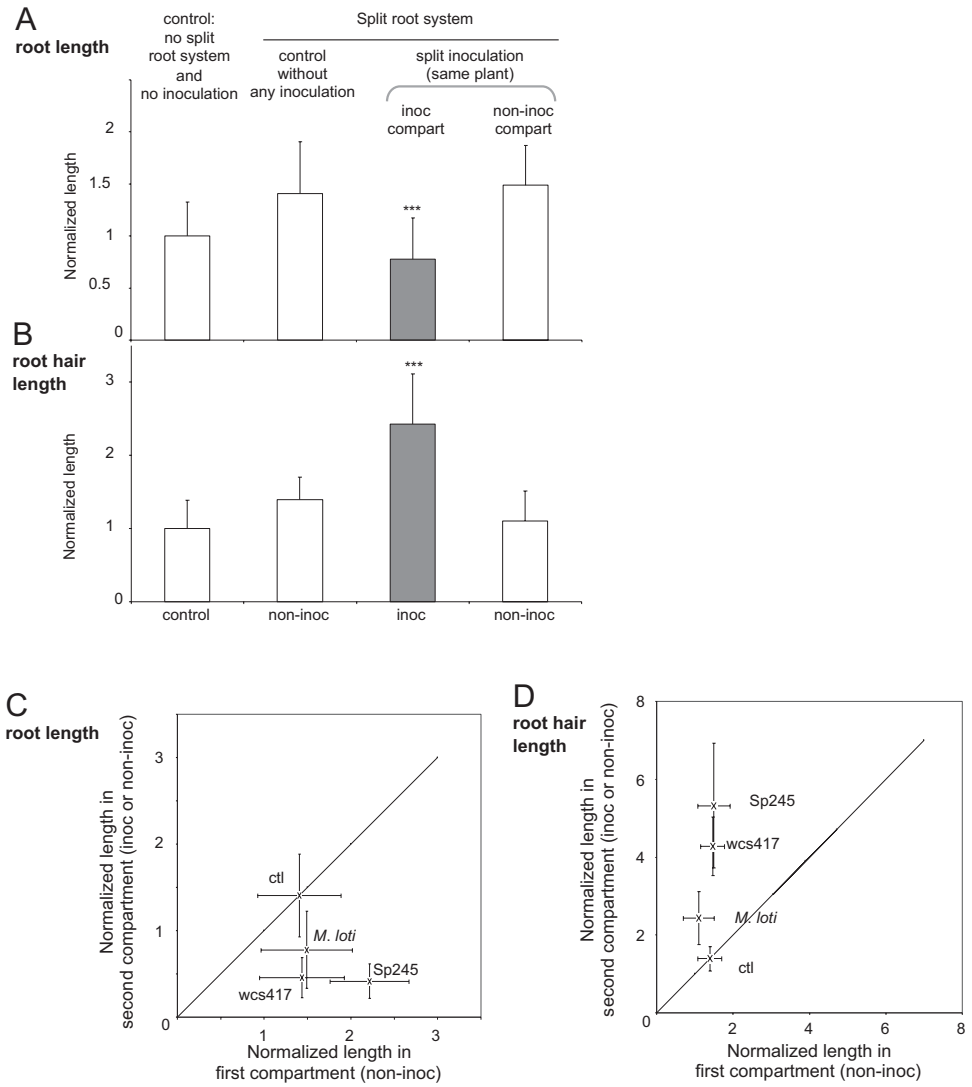

Fig. 7. The inhibition of main root growth and promotion of root hair elongation induced by *M. loti, Azospirilum brasilense* Sp245 and *Pseudomonas* WCS417 are restricted to the colonized parts of the root system. The so-called split root system was used to investigate whether long distance systemic signals are involved in the effects of *M. loti* on root growth and root hair elongation or whether these effects correspond to local responses of the colonized zones of the root system. For split inoculation: one compartment of the device was inoculated with *M. loti* (inoc compart) and the other one left non-inoculated (non-inoc compart). Measurements of main root length (A) and root hair length (B) were taken in both compartments for each plant. Means and standard deviations: for (A) the minimal population size is greater than 11 individuals, for (B) the minimal population size is greater than 100 individuals. For split root system control without any inoculation: both compartments were left non-inoculated. For control i.e. no split root system and no inoculation: intact seedlings whose primary root tip was not excised were grown on non-inoculated medium. The mean values of root length (left bar in panel A; means±SD, *n*=55) and root hair length (left bar in panel B; means±SD, *n*=903) in these control plants were used to normalize the corresponding values obtained for the plants submitted to the split root system treatment. Similar experiments were performed with the PGPR *A. brasilense* Sp245 and *Pseudomonas* WCS417 (see Supplementary Fig. S5). These data have been used to compare the effects of the three bacterial species, in regards to root length (C) and root hair length (D), by plotting the values of these parameters obtained in the inoculated compartment against the corresponding values in the non-inoculated compartment. For control (ctl): both compartments were left non-inoculated. In (A) and (B), the statistical significance of the difference observed in the inoculated compartment was tested against all the other conditions.

in the compartment containing the bacteria but not in the non-inoculated compartment (Supplementary Fig. S5A for WCS417; Supplementary Fig S5C for Sp245). Likewise, root hair elongation was stimulated in the inoculated compartment but not in the non-inoculated compartment (Supplementary Fig. S5B for WCS417; Supplementary Fig. S5D for Sp245).

To compare the capacity of *M. loti*, WCS417 and Sp245 to affect root growth and root hair elongation, we plotted in the same graph (Fig. 7C, D) the data obtained for each individual strain (Fig. 7A, B, Supplementary Fig. S4). The three strains affect root system development in a similar way but with quantitative differences, *M. loti* being less potent (Fig. 7D). Importantly, whatever the bacterial strain, neither root growth nor root hair length in the non-inoculated compartment was significantly different from the corresponding parameters measured in control plants grown on non-inoculated medium without using the split root system (Fig. 7C, D). Thus, the split root system device had no significant effect on root growth and root hair elongation.

Discussion

In this report, *in vitro* colonization of the Arabidopsis root system by the nitrogen fixing rhizobacteria *M. loti* MAFF303099 is investigated using a combination of Arabidopsis mutants and transgenic lines expressing reporter constructs, and the so-called split root system device. We conclude that *M. loti* behaves as a *bona fide* PGPR when interacting *in vitro* with Arabidopsis, and that the different root responses to bacterial colonization probably reflects the involvement of distinct signalling pathways that exert their effects at a local level, in the colonized regions of the root system.

Colonization pattern of M. loti *in Arabidopsis*

In our experimental conditions, *M. loti* bacteria were detected on the root surface, mainly at the root tip (Fig 1A) and at intersections between lateral roots and the primary root (Fig. 1C, D) (Desbrosses *et al.*, 2013). We did not detect *M. loti* colonies inside the root tissues (Supplementary Fig. S2). Furthermore, rapid immersion in water could strip all *M. loti* colonies from the surface of the root (data not shown). Thus, *M. loti* remains epiphytic when interacting with Arabidopsis roots, in contrast to its presence inside root nodule cells when colonizing its regular legume host, *L. japonicus*. This confirms a previous report that bacteria can have different colonization programs depending on the plant species they colonize (Fan *et al.*, 2012).

In our experimental conditions, *M. loti* cells were included in molten agar plant media and consequently evenly distributed throughout the agar and practically immobilized. Yet, 5 days after the plants had been transferred onto the agar plate, larger bacterial colonies were present at the intersection of the primary root with fully emerged lateral roots (Fig. 1C, D), suggesting that the bacteria could divide more rapidly at such places. This colonization pattern is distinct from that reported in the case of other epiphytic rhizobacteria. For example, *Bacillus amyliquefaciens* FZB42 colonize Arabidopsis root tips and root hairs (Fan *et al.*, 2012), *Pseudomonas fluorescens* F133 colonizes all parts of the wheat root system, and *A. brasilense* Cd and Sp245 extensively colonize the root hair zone of the wheat root system (Couillerot *et al.*, 2011). Although the inoculation protocol was not the same, such differences suggest that rhizobia can differ in their colonization patterns. Thus, differences in root colonization patterns can be observed between rhizobacterial species. The underlying mechanism could correspond to successive steps in the development of the biofilm at the root surface, maybe in connection with differences in the quality and quantity of root exudates (Rudrappa *et al.*, 2008).

M. loti *behaves as a PGPR in Arabidopsis*

Inoculation of the plant culture media with *M. loti* resulted in altered root system development and increased shoot biomass (Fig. 2A, B, C, Supplementary Fig. S3). The increase in shoot biomass was on average 34% (Fig. 2A, Supplementary Fig. S3) and therefore close to that induced in Arabidopsis under similar conditions *in vitro* by other PGPR species such as *Bacillus megaterium*, *Variovorax paradoxus* 5C-2 or *P. brassicacearum* STM196 for which growth stimulation by about 30%, 40% and 20% have been reported, respectively (Mantelin and Touraine, 2004; Lopez-Bucio *et al.*, 2007; Chen *et al.*, 2013). The promotion of plant growth observed in the present experiments is unlikely to result from nitrogen fixation by *M. loti* since this activity is transcriptionally controlled by *NifA*, which itself is transcriptionally activated when the oxygen concentration is low (Fischer, 1994; Halbleib and Ludden, 2000). While this condition is met in legume root nodules (Ott *et al.*, 2005), it is unlikely to occur at the surface of Arabidopsis roots, at least when grown under our experimental conditions. A working hypothesis might be that promotion of plant growth by *M. loti* on our mineral medium results from improved nutrient uptake. Indeed, *M. loti* promotes both root hair elongation (Fig. 2C, Fig. 5C, D) and apoplasm acidification on a sizeable portion of the primary root (Fig. 4C). The latter effect could result from larger rates of H^+ excretion by the plasma membrane H^+-ATPase and/or of secretion of organic acids such as malic acid (Ryan *et al.*, 2001). Whatever the underlying mechanisms, acidification of the root cell surface is likely to favour nutrient ion uptake both by steepening the transmembrane electrochemical H^+ gradient that energizes H^+ cotransport systems and by promoting the solubilisation of ions such as iron or even phosphate.

M. loti colonization did not significantly promote shoot and root growth in different auxin and ethylene signalling Arabidopsis mutants (Supplementary Fig. S3), suggesting that auxin and ethylene signalling events play a role in the induction of growth promotion. Recently, a similar hypothesis has been proposed in the case of the interaction between Arabidopsis and *Burkholderia phytofirmans* PsJn (Poupin *et al.*, 2016). Conversely, the interaction between Arabidopsis and *Bacillus megaterium* would involve auxin- and ethylene-independent mechanisms (Lopez-Bucio *et al.*, 2007). The use of different experimental conditions for root inoculation and plant growth makes comparison difficult but such differences suggest that the roles of hormones in PGPR dependent growth promotion in Arabidopsis might strongly depend on the PGPR species.

The transient inhibition of root growth and the stimulation of root hair elongation by M. loti *involve distinct pathways*

Twenty-four hours after inoculation with *M. loti*, root growth and the rate of cell divisions in the root meristem were reduced and both were resumed at 72 hours post inoculation (Fig. 3A, B, C). Thus, *M. loti* transiently inhibited the root tip cell cycle. In contrast, the promotion of root hair elongation as well as the depressive effect on the length of epidermal cells and the size of the 'meristematic+elongation' region were persistent (Fig. 2C, Fig. 3D, E). Such differences in the kinetics of these responses to *M. loti* inoculation are in agreement with the hypothesis that a given rhizobacterium species can activate distinct pathways in the colonized plant (Zamioudis *et al.*, 2013).

In a previous report (Contesto *et al.*, 2008), Arabidopsis inoculation with *M. loti* was not noticed to result in inhibition of primary root growth. This discrepancy with the present report could be due to the fact that the inhibition is transient and that the experimental conditions were different, particularly the inoculation protocol and plant growth conditions.

There is evidence showing that some rhizobacterial species affect plant root architecture via the auxin pathway (Vacheron *et al.*, 2013). The finding that both *aux1-100* and *axr1-1* mutant plants did not display primary root growth inhibition in the presence of *M. loti* (Supplementary Fig. 6B) suggests that activation of the auxin pathway is required for this response. Further supporting this hypothesis, *M. loti* inoculation resulted in an increased GFP fluorescent signal at the root tip of the DR5:GFP transgenic line (Fig. 6B, C, Supplementary Fig. S6), indicating a local accumulation of auxin in root tip cells.

The *aux1-100* and *axr1-3* mutations suppress the reduced root growth phenotype resulting from the presence of *M. loti*, but they were without any significant effect on the increased root hair length phenotype (Fig. 6C). Similarly, evidence that neither auxin transport nor the AXR1 dependent auxin signalling pathway is required for the promotion of root hair elongation was reported in Arabidopsis grown *in vitro* in the presence of *Pseudomonas* sp. WCS417 (Zamioudis *et al.*, 2013). Ethylene might be assumed to play a role in the pathway leading to this promotion since, in absence of rhizobacteria, this hormone is well known to act as a positive regulator of root hair elongation (Pitts *et al.*, 1998). However, previous analyses performed with various ethylene signalling Arabidopsis mutants inoculated with WCS417 or with *P. brassicacearum* STM196, a PGPR that is a close relative of *M. loti* (Mantelin *et al.*, 2006*b*), have revealed that the ethylene pathway is not directly involved in bacterium-induced root hair elongation (Galland *et al.*, 2012; Zamioudis *et al.*, 2013).

M. loti *effects on Arabidopsis root system development involve local responses.*

Several transcriptomic studies have evidenced numerous transcriptional changes in the shoots of inoculated Arabidopsis plants, suggesting the involvement of a systemic signalling pathway (Cartieaux *et al.*, 2003; Mantelin *et al.*, 2006*a*; Cartieaux *et al.*, 2008). In the present report, we used a split root device to test whether a systemic signalling pathway is involved in the root responses to *M. loti* inoculation. For the first time to our knowledge, we show that primary root growth inhibition and root hair elongation are essentially local responses to *M. loti* inoculation (Fig. 7A, B). Furthermore, when we looked at the expression pattern of a *pDR5:GFP* reporter line, we observed that the fluorescent signal was stronger in the inoculated compartment compared with the control compartment (Supplementary Fig. S5). The accumulation of auxin at the root tip can therefore also be assumed to be a local response to *M. loti* inoculation.

Bacterial volatile organic compounds (VOCs) have been shown to promote plant growth (Ryu *et al.*, 2003), to affect auxin homeostasis (Zhang *et al.*, 2007) and to enhance plant defence responses (Ryu *et al.*, 2004). In the present split root system experiments, VOCs could freely diffuse in the Petri plate headspace and affect the root systems growing in each compartment. Thus, the hypothesis that VOC synthesized by *M. loti* are involved in the root response can be ruled out by the fact that altered root development was observed only in the compartment where colonies of rhizobacteria were present (Fig. 7A, B). *M. loti* is therefore unlikely to produce VOCs that could play a major role in the developmental responses of the plant root system, or perhaps they produce too little amounts of VOCs when compared with a VOC producing strain such as *Bacillus subtilis* GB03 (Ryu *et al.*, 2003).

Similarly, the fact that the developmental responses appear to be restricted to the colonized regions of the root system (Fig. 7B) also weakens the hypothesis that ethylene production by root tissues in response to *M. loti* colonization plays a major role in root developmental responses. As a gaseous molecule, ethylene can diffuse freely in the Petri plate headspace and therefore affect the development of the root system similarly in each of the two compartments. This conclusion is in agreement with previous reports showing that the ethylene pathway plays a secondary role in the root hair response to rhizobacterial inoculation (Contesto *et al.*, 2008; Galland *et al.*, 2012; Zamioudis *et al.*, 2013).

The local responses displayed by the root system can be hypothesized to reflect the involvement of a plant defence pathway specific to the root. Indeed, the *NPR1* gene, which encodes a central component of the plant response to pathogens, plays a role in Arabidopsis root responses to inoculation with the nitrogen fixing rhizobacteria *Sinorhizobium loti* (Peleg-Grossman *et al.*, 2009). By the same token, when colonizing inner root tissues in Arabidopsis, *Gluconacetobacter diazotrophicus* transiently inhibits plant growth by activating the plant defence pathway (Rangel de Souza *et al.*, 2016). Furthermore, local responses to biotic stresses are widely documented, especially in the case of the hypersensitive response, which results in programmed cell death around the site of the infection [for review see Coll *et al.* (2011), Mur *et al.* (2008)]. It is important to note that the root-specific defence pathway assumed to be involved in the local responses displayed by Arabidopsis roots upon interaction with *M. loti,* within the framework of the above hypothesis, is essentially different from the one described for the hypersensitive response. This is because it does not involve any obvious cell death and the root goes on to function albeit in a different mode as the root hairs are longer and more abundant and the root tip meristem is smaller.

M. loti *MAFF303099 shares properties with well-known PGPR*

The split root system device has been used to compare, in parallel experiments, the effects of *M. loti* on Arabidopsis root system development to those of two well-known PGPR strains, namely *Pseudomonas* sp. WCS417 and *A. brasilense* Sp245. In the literature, Sp245 is known to synthesise auxin in the presence of tryptophan and to secrete the synthesised hormone into the external medium (Steenhoudt and Vanderleyden,

2000). This auxin of bacterial origin would directly promote plant growth (Steenhoudt and Vanderleyden, 2000). WCS417 has mainly been studied for its capacity to induce systemic resistance in Arabidopsis, a property that can also contribute to the promotion of plant growth in natural conditions (Leon-Kloosterziel *et al.*, 2005; Berendsen *et al.*, 2015). Three main conclusions can be drawn from the present data. First, both Sp245 and WCS417 were found to inhibit root growth and to promote root hair elongation, like *M. loti* (Supplementary Fig. S4A–D). This indicates that the experimental conditions we used allowed all three of these strains to express a qualitatively similar capacity to modify the development of the Arabidopsis root system. Second, as observed with *M. loti*, the effects of Sp245 and WCS417 on Arabidopsis root development remain local, restricted to the colonized parts of the root system (Supplementary Fig. S4A–D). Thus bacterial emission of VOCs or plant emission of ethylene is not likely to play a major role in these effects. Third, although qualitatively similar, the developmental responses of the root system to the three bacterial strains are quantitatively different (Fig. 7C, D). The smaller effects are observed with *M. loti* and the larger ones with Sp245, which is also the strain that secretes the largest amount of auxin into the medium (Fig. 6A). Thus the fact that the three bacterial strains display qualitatively similar effects on root system development in our experimental conditions does not mean that these effects rely on a common mechanism.

In contrast to Sp245 and WCS417, *M. loti* can engage in mutualistic symbiosis, resulting in nitrogen fixation. With respect to this ability, its interaction with the model legume *L. japonicus* has been extensively investigated to decipher the signalling pathways and developmental processes leading to nodule formation (Desbrosses and Stougaard, 2011). *M. loti* has thereby become a model rhizobium. Our work shows that *M. loti* can also engage in associative symbiosis with Arabidopsis, eventually promoting plant growth in gnotobiotic conditions. In other words, this rhizobacterium species possesses the ability to develop two different types of symbiosis. In soil, it would therefore have a higher degree of adaptability to the surroundings roots, depending on its ability to engage in a specific nitrogen fixing-symbiotic relationship. Using such a model rhizobium, which is able to interact with the two model plants *L. japonicus* and Arabidopsis, could open new avenues for deciphering the mechanisms that underly its adaptability, as well as understanding the PGPR-like behaviour of rhizobia species in their interactions with non-host plants. This could also shed new light on rhizobial symbiosis and how it evolved.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Calibration of the pHluorin response to external pH.

Fig. S2. *M. loti* colonization of Arabidopsis roots remains epiphytic.

Fig. S3. The plant auxin and ethylene pathways play a role in plant growth promotion by *M. loti*.

Fig. S4. *M. loti* does not affect root meristem cell division at 7 days post inoculation.

Fig. S5. *M. loti* inoculation results in increased expression of the *pDR5:GFP* auxin reporter construct in Arabidopsis root tips.

Fig. S6. The inhibition of root growth and the promotion of root hair elongation induced by WCS417 and Sp245 PGPR strains are local non-systemic responses of the colonized root.

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