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REGULAR ARTICLE

Development of root system architecture of *Arabidopsis thaliana* in response to colonization by *Martelella endophytica* YC6887 depends on auxin signaling

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Abstract
Background and aims Many rhizobacteria promote plant growth by producing hormones that stimulate the development of plant root system and increase plant biomass. The aim of this study was to investigate the growth promotion activity of the bacterial strain *Martelella endophytica* YC6887 and elucidate the signaling pathways potentially involved in *Arabidopsis* interaction with *M. endophytica* YC6887.
Methods The growth regulation was evaluated by inoculation of strain YC6887 with wild-type *Arabidopsis* Col-0 seedlings and mutants defective in auxin *aux1-7*, *axr4-2*, *eir1-1*, ethylene *ein2-1*, *etr1-3*, jasmonic acid signaling *jar1*, and root hair deficient mutant *rhd6*. The auxin response was further determined by using transgenic line DR5::GUS and a polar auxin transport inhibitor, 1-*N*-naphthylphthalamic acid (NPA).
Results M. endophytica YC6887 increased the number of lateral roots and plant biomass of *Arabidopsis* by producing phenylacetic acid. The growth promotion and improved lateral root development by the bacterium decreased in the auxin related mutants, whereas the ethylene and jasmonic acid mutants had a wild type response. The strain YC6887 increased root hair density in wild type Col-0 and recovered the root hair forming ability in root hair deficient mutant *rhd6*. Moreover, strain YC6887 treatment showed distinct response in DR5::GUS transgenic line compared to the control. Strain YC6887 lost its growth-promoting activity in the presence of NPA, an auxin transport inhibitor. This indicated that strain YC6887 activated the auxin signaling mechanism.
Conclusions Our results showed that *M. endophytica* YC6887 promoted plant growth in terms of plant biomass and root system development. *Arabidopsis* root system development upon *M. endophytica* YC6887 colonization was dependent on auxin signaling, but independent of ethylene and jasmonic acid signaling.

Keywords Lateral root primordia · *Martelella endophytica* · Phenylacetic acid · Root system architecture (RSA)

Abbreviations
IAA (Indole-3-acetic acid)

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LRP (Lateral root primordia)
NPA (1-*N*-Naphthylphthalamic acid)
PAA (Phenylacetic acid)

Introduction

Plant roots interact continuously with their surroundings, in particular with the diverse microbial communities around them that consist of both pathogenic and beneficial microbes. The region affected by these microbes is called the rhizosphere, where the microbial interaction with roots plays an important role in plant development. The development of plant roots is affected by microbial metabolites and secreted exudates from host plants in the rhizosphere, which include carbohydrates, amino acids, and hormones, which in turn affect the microbial community in reverse by activating different signaling in the host plant (Berendsen et al. 2012; Bais et al. 2006). The interaction of roots with microorganisms may be classified as either positive or negative. One of the positive interactions is the promotion of plant growth directly or indirectly by rhizobacteria (Weyen et al. 2009). Often referred to as plant growth-promoting rhizobacteria (PGPR), these microbes promote plant growth by fixing nitrogen, supplying nutrients, suppressing plant pathogens, or producing phytohormones (Compant et al. 2005a; Costacurta and Vanderleyden 1995; Dashti et al. 1998; Chowdhury et al. 2015; O'sullivan and O'Gara 1992; Richardson et al. 2009). Besides phytohormones, other growth factors such as cyclodipeptides (diketopiperazines) and volatile compounds produced by rhizobacteria are also involved in plant growth promotion (Ortiz-Castro et al. 2011; Ryu et al. 2003). Some plant growth promoting rhizobacteria can colonize and enter root tissues, living endophytically in the host plant without causing any harm (Compant et al. 2005b; Timmusk et al. 2005; Reinhold-Hurek and Hurek 2011).

Plant growth and development are complex processes involving the production of phytohormones such as auxin, cytokinin, gibberellins, abscisic acid, ethylene, brassinosteroids, and jasmonic acid. Among these, indole-3-acetic acid (IAA) is an important growth hormone responsible for plant root system architecture development, organogenesis, and several physiological processes (Gray 2004; Ljung 2013; Davies 2004). The naturally active auxins include indole-3-acetic acid (IAA), 4-choloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA), and indole-3-butyric acid (IBA). The architecture of the root system is largely composed of three parts including the primary roots, higher order lateral roots, and the root hairs. The primary root growth depends upon the activity of meristem cells and cell elongation processes, and lateral roots are formed post-embryonically from pericycle cells of the primary root. The root hairs, originating from epidermal cells of primary and lateral roots, absorb water and nutrients from the soil to increase the plant biomass (López-Bucio et al. 2003; Tian et al. 2014). During root development, the plant root system architecture is also affected by certain fungal species such as *Trichoderma virens*, *Trichoderma atroviride*, *Laccaria bicolor*, *Tuber borchii*, and *Tuber melanosporum* which increase the biomass as well as the lateral root and root hair densities of the host plant (Contreras-Cornejo et al. 2009; Felten et al. 2009; Splivallo et al. 2009). Two of these fungal species, namely *T. virens* and *T. atroviride*, have recently been shown to improve the growth of *Arabidopsis* seedlings by enhancing root development and osmolite production through the activation of auxin signaling under salt stress conditions (Contreras-Cornejo et al. 2014).

During root system development, different signaling pathways of plant hormones such as auxin, ethylene, and cytokinin are activated by the rhizobacteria such as *P. fluorescens* WCS417, *Bacillus subtilis*, *Paenibacillus polymyxa*, and *Phyllobacterium brassicacearum* (Arkhipova et al. 2005; Galland et al. 2012; Timmusk et al. 1999; Zamioudis et al. 2013). Cytokinin signaling is involved in the plant growth promotion by *Bacillus megaterium,* which also produces volatile compounds that affect root architecture most likely independently of auxin and ethylene (López-Bucio et al. 2007; Ortiz-Castro et al. 2008). Spermidine, a polyamine produced by *B. subtilis* OKB105, has been found to be involved in the growth promotion of *Arabidopsis* requiring *S*-adenosyl methionine as a precursor; its signaling mechanism, however, has not yet been elucidated (Xie et al. 2014). As macronutrients and micronutrients that include calcium, magnesium, phosphate, potassium, manganese, nitrogen, and sulphur are necessary in plant development associated with hormones, the scarcity of these nutrients may adversely affect root development as well as the growth and activity of rhizobacteria

(Gruber et al. 2013; Kertesz and Mirleau 2004; Svistoonoff et al. 2007).

Biotic and abiotic stresses can also negatively affect plant health and yield (Lacoul and Freedman 2006). Under these stress conditions, endophytic bacteria play important roles in plant growth promotion and stress homeostasis regulation (Sgroy et al. 2009; Cassan et al. 2009). With the view that halophytes could be a source of beneficial bacteria, studies have been conducted to isolate and identify culturable bacteria able to promote plant growth (Bibi et al. 2012, 2013). Strain YC6887 was isolated from the roots of the halophyte *Rosa rugosa* collected from a tidal flat area of Namhae Island, Korea. Phylogenetic analysis as well as physiological, and biochemical characterizations, enabled the assignment of this strain to the species *M. endophytica* with the designation YC6887 (Bibi et al. 2012, 2013).

In this study, we investigated the plant growth-promoting ability of *M. endophytica* YC6887 in pots and in vitro by using *A. thaliana*. We also investigated the effect of *M. endophytica* YC6887 on root hair development and its signaling mechanism for stimulating root system development using *Arabidopsis* mutants.

Materials and methods

Plant/bacterial co-cultivation bioassay

To evaluate the effect of bacterial inoculation on the growth of *A. thaliana*, tests were conducted in pots and in vitro. For preparation of the bacterial inoculum, *M. endophytica* YC6887 was cultivated in liquid medium (10 g yeast extract, 10 g sucrose, 4 g $MgSO_4$, 2 g $MgCl_2$, 1 g K_2HPO_4, and 5 g NaCl per liter of distilled water) at 28 °C for 48 h on a rotary shaker (180 rpm) and then centrifuged at 6000*g* for 15 min to collect the cells (Bibi et al. 2013). The harvested cells were suspended in a buffer solution (10 mM $MgSO_4$) to adjust the density to 2×10^6, 5×10^7, and 5×10^8 CFU mL^{-1} for inoculation. In pot tests, the roots of 2-week-old seedlings of *Arabidopsis* were drenched with 10-ml bacterial suspension at a final concentration of 2×10^6, 5×10^7, and 5×10^8 CFU mL^{-1}, and the same dose of *M. endophytica* YC6887 was reapplied to the roots 2 weeks after the first inoculation (Ramos Solano et al. 2008). Plant height, fresh weight, and the number of siliques per plant were recorded 2 weeks later after harvesting. In an in vitro assay, 4-day-old *Arabidopsis* seedlings cultivated in one fifth strength (0.2×) Murashige and Skoog (MS) media were lined up on one side of the MS plate and co-cultivated with the bacterial suspension (5 μl/seedling) at a concentration of 2×10^6 CFU mL^{-1} or with buffer solution (10 mM $MgSO_4$) as a control and grown for 8 days further in the growth chamber. For auxin transport, a polar auxin transport inhibitor, 1-*N*-naphthylphthalamic acid (NPA, Supelco Analytical, Sigma-Aldrich, St. Louis MO, USA) at a concentration of 1, 5 or 10 μM was supplemented to the MS agar media after autoclaving.

Plant materials and growth conditions

Arabidopsis ecotype Col-0 and its different mutants were used to investigate the plant growth-promoting activity and root hair development by inoculation of *M. endophytica* YC6887. To investigate the relevant signaling mechanism, the transgenic line DR5::GUS and various auxin (*aux1-7*, *axr4-2*, and *eir1-1*), ethylene (*ein2-1*, *etr1-3*), and jasmonic acid (*jar1*) mutants of *Arabidopsis* were used to study the response to *M. endophytica* YC6887. To evaluate root hair development, the mutant *rhd6* was used. For the in vitro assays, the seeds were surface sterilized with 95 % (*v*/*v*) ethanol for 5 min and with 5 % (*v*/*v*) sodium hypochlorite for 5 min, followed by washing with sterile distilled water. The seeds were grown on MS agar media supplemented with 1 % sucrose (*w*/*v*). For stratification, the plates were kept at 4 °C for 48 h and then transferred to a plant growth chamber at a vertical position at an angle of 65° to allow the growth of roots and shoots along the surface of the agar media. The growth chamber was maintained at a long day photoperiod with 16 h of light and 8 h of darkness at 22 °C with a light intensity of 100 μmol m^{-2} s^{-1}.

Gus histochemical staining

To determine whether the growth promotion of *Arabidopsis* by *M. endophytica* YC6887 was due to auxin or auxin-related compounds, an *Arabidopsis* transgenic line expressing auxin inducible DR5::GUS was used to co-cultivate with bacteria. Histochemical detection of the Gus staining was determined in the DR5::GUS transgenic line. *Arabidopsis* seedlings co-inoculated with *M. endophytica* YC6887 for 8 days on 0.2× MS media were incubated at 37 °C in Gus staining

solution containing 50 mM potassium phosphate buffer (PH 7), 0.2 % Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1.0 mM X-Gluc for 10 h. To observe the primary root tips after staining, the treated and control seedlings were cleared by using the method described previously (Malamy and Benfey 1997). After clearing, the representative seedlings were mounted in 50 % glycerol on microscopic slides and examined with a microscope (Olympus Provis AX70, Olympus, Tokyo, Japan).

Purification and structure determination of the auxin-like compound

For purification of the auxin-like metabolite produced by *M. endophytica* YC6887, strain YC6887 was mass cultured in a fermenter containing the liquid culture medium (200 L) at 28 °C under the conditions of 110 rpm agitation, pH 6.8–7.2, and aeration rate of 0.6 volume per volume per minute (vvm). After 60 h of cultivation, the culture broth was centrifuged to remove the cells and the supernatant was extracted twice with an equal volume of ethyl acetate (EtOAc). The EtOAc extract was evaporated under pressure, and the concentrate was loaded on silica gel column (5.0 cm), and then eluted with step wise gradients of solvents (*n*-hexane/EtOAc). A total of 27 fractions were recovered and further separated by semi-preparative reverse-phase HPLC (Alltech C18 column, 10 × 250 mm, Grace Inc., Columbia MD, USA) (flow rate, 2 mL/min; 30 to 100 % CH_3CN in H_2O over 45 min) to obtain the compound with growth promotion activity. To characterize the active compound, 1D and 2D NMR experiments were carried out on a VNS 600 MHz spectrometer, operating at 600 MHz for proton and 150 MHz for carbon. Chemical shifts were expressed in μg/ml and referenced to the residual solvent signals. The mass spectra were recorded on a Micromass LCT mass spectrometer, and lock mass calibration was applied for the determination of accurate masses. Semi-preparative HPLC was carried out on an Agilent system consisting of a vacuum degasser, quaternary pump, diode array detector (DAD) (Agilent, Santa Clara CA, USA) and a Luna 5u C_{18} 100A column (250* 10.00 mm, Phenomenex, Torrance CA, USA). Column chromatography (CC) was performed on silica gel (Merck KGaA, 70–230 mesh) (Merck KGaA, Darmstadt, Germany). TLC was performed on Merck KGaA precoated silica gel 60 F254 plates and spots were visualized under UV light (254 and 365 nm) or by spraying with 20 % H_2SO_4 followed by heating.

Effects of cell free supernatant and phenylacetic acid on the plant growth of *Arabidopsis*

The culture filtrate of *M. endophytica* YC6887 was analyzed for its plant growth-promoting activity on the *Arabidopsis* seedlings. The supernatant of the culture broth collected as described previously for the structure determination of the compound with plant growth-promoting activity was filtered through a 0.2-μm filter (Sartorius Stedim Biotech, Göttingen, Germany) and 10 μl of the culture filtrate was applied to the 4-day-old seedlings of *Arabidopsis*. The growth parameters were evaluated 8 days after treatment. After structure determination of the active compound, the *Arabidopsis* seedlings wild-type Col-0 and auxin mutants were allowed to grow on media supplemented with phenylacteic acid (1 μmol) (Sigma-Aldrich) in DMSO. Solvent only was used as a control. The plant growth-promoting activity was assessed after 8 days of treatment.

Analysis of root development parameters

Root development parameters such as the fresh weight, the number of lateral roots, and the primary root length of the *Arabidopsis* seedlings were measured after 8 days of co-cultivation with *M. endophytica* YC6887. The fresh weight of the seedlings was measured immediately after harvest using an analytical balance, and the primary root length was measured using a ruler. To assess root hair development, seedling segments 1.0 cm above the primary root tip were photographed at a magnification of 40× using a dissection microscope and analyzed with the imageJ software (version 1.38). The number and length of root hairs and the diameter of the primary root were also measured.

Statistical analysis

For comparison of more than two means of the data, statistical analysis was performed with one-way ANOVA followed by Duncan and Tukey's significant difference (HSD) tests. For comparison of two means of the data, Student's *t* test was used (SPSS version 17, SPSS, Chicago IL, USA). Further data was analyzed

with Sigma plot 10. All experiments were repeated three times with three replicates. Bars represent the standard error.

Results

Effect of *M. endophytica* YC6887 treatment on the growth promotion of *Arabidopsis*

The plant growth-promoting activity of *M. endophytica* YC6887 was investigated in pot tests. The seedlings of *Arabidopsis* treated with the suspensions of *M. endophytica* YC6887 at the concentrations of 2×10^6, 5×10^7, and 5×10^8 CFU mL^{-1} showed significantly increased plant height, fresh weight, and the number of siliques compared with the non-treated control plants (Fig. S1 and Table 1). Among the treatments, the bacterial density of 2×10^6 showed significantly higher growth promotion with increased plant height, biomass, and seed production than the other two concentrations (Table 1).

Under in vitro conditions after inoculation of the twelve-day old *Arabidopsis* seedlings with the bacterial suspension at 2×10^6 CFU mL^{-1}, *M. endophytica* YC6887 promoted the growth of the plants by significantly increasing the number of lateral roots and the fresh weight compared with the untreated control seedlings (Fig. 1). The number of lateral roots per seedling in *M. endophytica* YC6887 treated seedlings was 10.2 ± 1.9, almost five times more than the lateral roots of the control seedlings at 2.16 ± 0.5 (Fig. 1a, b). The fresh weight was 5.0 ± 0.5 mg/seedling in *M. endophytica* YC6887 treated seedlings compared with the untreated control at 2.2 ± 0.14 mg/seedling (Fig. 1a, c). However, no significant difference was observed in the primary root length between the treated and untreated control seedlings (Fig. 1a, d). Our results suggest that *M. endophytica* YC6887 has the potential to promote plant growth by altering the plant root system architecture. To further investigate the effect of *M. endophytica* YC6887 on the formation of lateral root primordia across the primary root length, the number of LRP's was determined in *M. endophytica* YC6887 treated seedlings. The number of LRP in *M. endophytica* YC6887 treated seedling was significantly increased compared to that in the control ($p < 0.05$), indicating that strain YC6887 could stimulate lateral root initiation (Fig. 1e).

Analysis of auxin response in *Arabidopsis* by *M. endophytica* YC6887

The response of *Arabidopsis* seedlings to *M. endophytica* YC6887 was observed using the DR5::GUS transgenic line. The primary root tips of 4-day-old seedlings co-cultivated with *M. endophytica* YC6887 for 8 days showed more gus staining (Fig. 2b) compared to the control representing auxin response (Fig. 2a). However, in the presence of NPA (10 μM), the gus staining in *M. endophytica* YC6887 treated seedling was dense at the root tip with increased auxin accumulation and response (Fig. S5d). These results indicated that DR5::GUS responded to inoculation with *M. endophytica* YC6887, which indicated the production of auxin or an auxin-like compound by the bacteria.

To determine whether auxin transport was stimulated by *M. endophytica* YC6887 during development of the root system architecture, the polar auxin transport inhibitor, NPA, was supplemented at different concentrations in the *Arabidopsis* seedling growth media. The addition of NPA at 1 and 5 μM to the *M. endophytica* YC6887 treated seedlings severely inhibited lateral root formation which was almost completely abolished by addition of 10 μM NPA (Fig. 2c). The fresh weight and primary root length also decreased accordingly with increasing concentrations of NPA (Fig. 2d, e). The treatment of

Table 1 *Arabidopsis* (Col-0) plants were treated with the suspension of *Martelella endophytica* YC6887 at different bacterial concentrations and the buffer (10 mM $MgSO_4$) was used as a control in soil. Growth promotion assay measured plant height, fresh weight, and the number of siliques. Means ± standard error within columns are significantly different (Duncan test; $P < 0.05$)

Inoculum conc. (CFU/ml)	Plant height (cm/plant)	Fresh weight (g/plant)	Silique number (/plant)
Control	28.62 ± 0.93c	1.92 ± 0.12c	59.3 ± 4.7c
2×10^6	35.09 ± 0.3a	2.89 ± 0.07a	112.8 ± 5.6a
5×10^7	31.82 ± 1.3b	2.4 ± 0.12b	89.83 ± 6.4b
5×10^8	30.64 ± 0.3ab	2.2 ± 0.14ab	84.23 ± 3.4b

Concentration of bacterial suspension

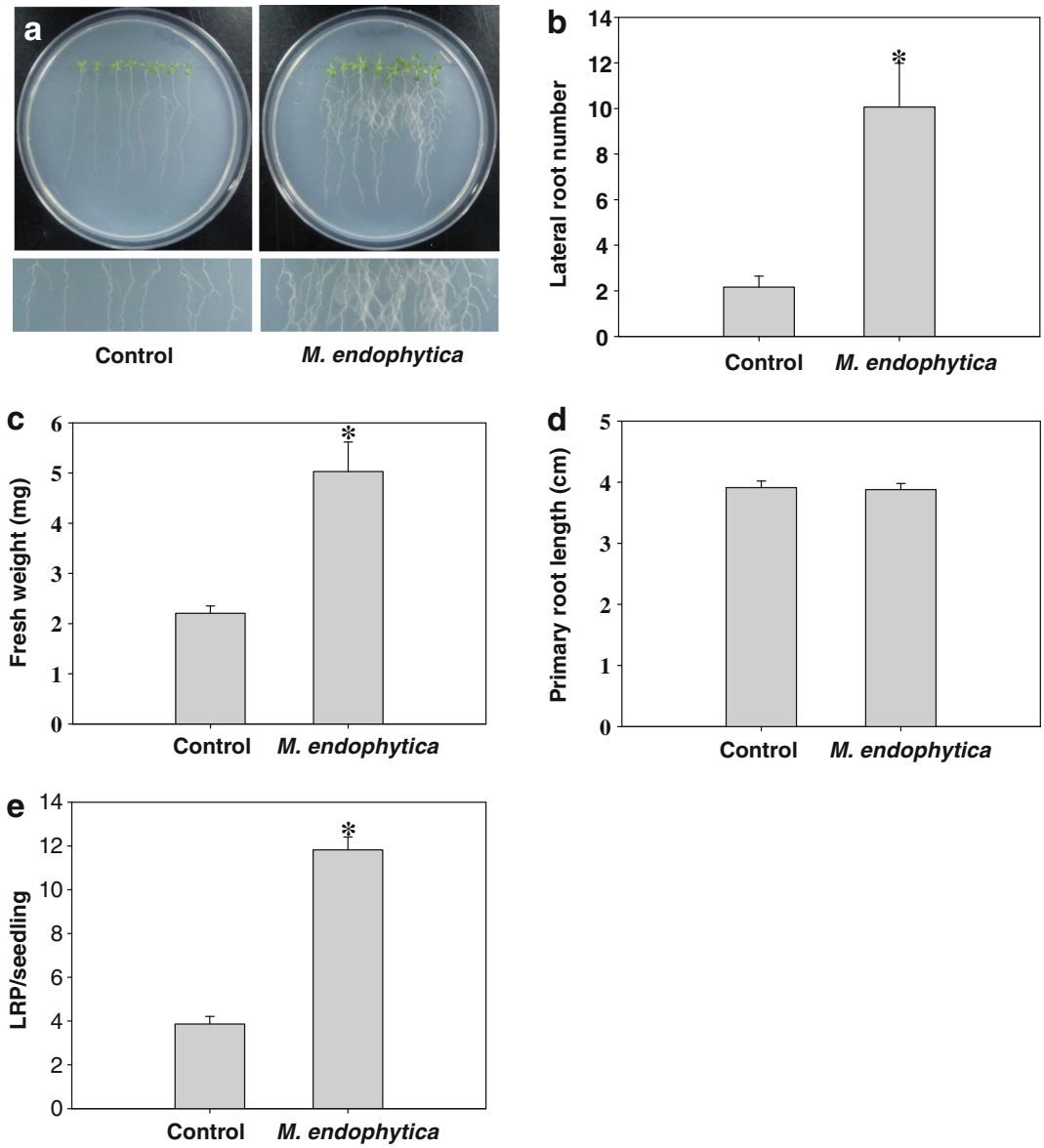

Fig. 1 Effects of *Martelella endophytica* YC6887 treatment on plant growth and root system architecture in *A. thaliana* (Col-0) seedlings. Four-day-old *Arabidopsis* seedlings were inoculated with *M. endophytica* YC6887 on MS media. The bacterial suspension (2×10^6 CFU mL^{-1}) was inoculated with the seedling and grown for 8 days in a growth chamber. The buffer solution (10 mM $MgSO_4$) was used as a control, **a** *Arabidopsis* seedlings co-cultivated with *M. endophytica* YC6887, **b** lateral roots number/seedling, **c** fresh weight/seedling, **d** primary root length/seedling in bacterial treated and non-treated control seedlings, and **e** number of lateral root primordia per seedling. Data shown indicate the mean of 20 seedlings from three replicates (Bars represent the standard error of the mean). The experiment was repeated three times with similar results. Statistically significant differences were designated by *asterisks* ($P < 0.05$)

NPA halted and completely blocked the plant growth-promoting activity of *M. endophytica* YC6887. The loss of the growth stimulating effect of *M. endophytica* YC6887 caused by NPA indicated that the auxin signaling mechanism was required for its plant growth-promoting activity.

Effect of *M. endophytica* YC6887 inoculation on the root system architecture of *Arabidopsis* auxin mutants

To further understand how *M. endophytica* YC6887 inoculation affected the development of the root

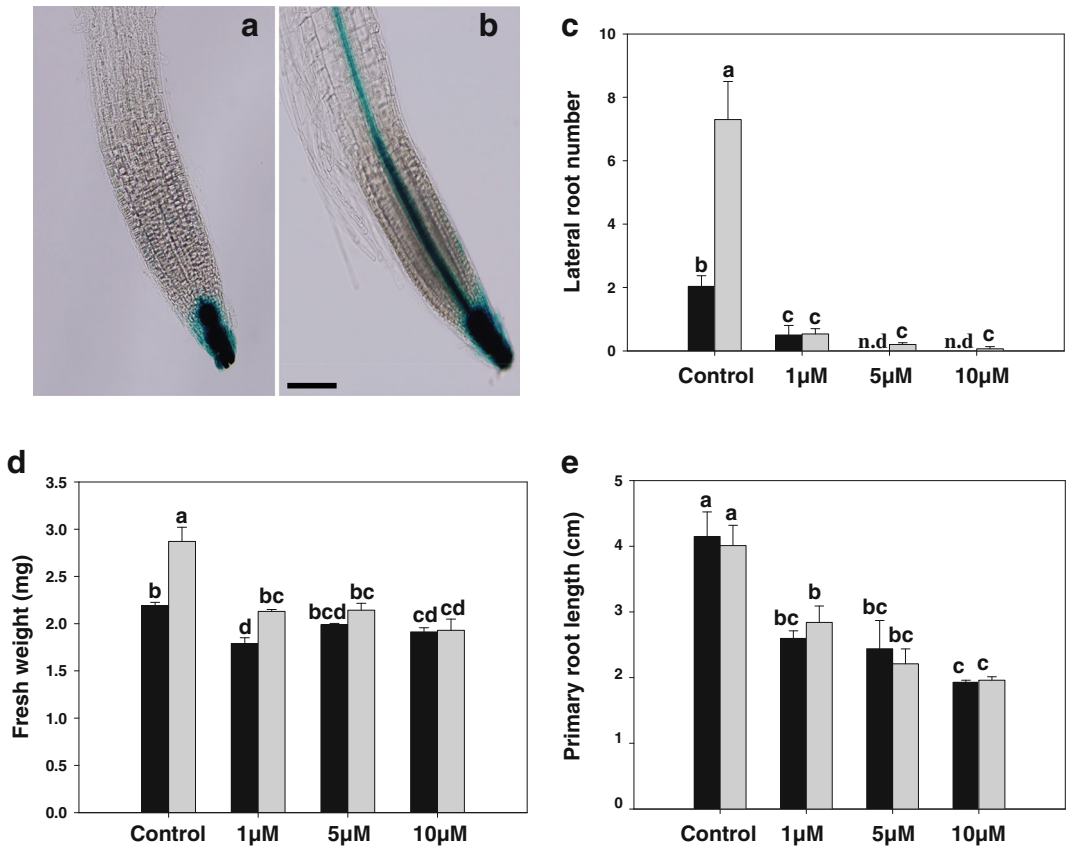

Fig. 2 Effects of *Martelella endophytica* YC6887 inoculation on auxin-regulated gene expression. Primary roots of DR5::GUS transgenic lines after Gus staining were observed under the microscope 8 days after treatment, **a** root tip of control seedlings, **b** root tip of *M. endophytica* YC6887 treated plants. Stained seedling root tips were photographed using an Olympus Provis AX70 microscope with a scale bar 100 μm. Effect of *M. endophytica* YC6887 on the root system architecture of *Arabidopsis* in presence of different concentrations of the polar auxin transport inhibitor, NPA (1 μM, 5 μM and 10 μM), **c** effect of NPA on *M. endophytica* YC6887 induced lateral roots formation, **d** effect of NPA on fresh weight of seedlings, and **e** effect of NPA on primary root length. *Gray bars* represent *M. endophytica* YC6887 inoculated while the *black bars* represent uninoculated *Arabidopsis* seedlings. Different root development parameters indicated show the mean of 10 seedlings ± standard error from three groups. Statistical significant differences are denoted by *different letters* and analysis was repeated three times with similar results (Duncan test; $P < 0.05$)

system architecture, *Arabidopsis* mutants defective in auxin influx (*aux1-7*), auxin efflux (*eir1-1*) carriers, and auxin response (*axr4-2*) were used for analysis of the response to *M. endophytica* YC6887. Roots of *aux1-7* and *axr4-2* mutants failed to express AUX1 and AXR, respectively. They reduced the number of lateral roots and failed to respond to auxin (Marchant et al. 2002; Pickett et al. 1990; Hobbie and Estelle 1995). The mutant *eir1-1* lacking EIR protein was expressed only in roots. EIR is involved in auxin transport and *eir1-1* display resistance to auxin application (Luschnig et al. 1998). In all three tested auxin mutants, a reduced response to *M. endophytica* YC6887 was observed. Analysis of the number of lateral roots had a 4-fold increase in *M. endophytica* YC6887 treated wild-type Col-0 seedlings of *A. thaliana* compared with untreated control seedlings. For auxin related mutants *axr4-2*, *aux1-7*, and *eir1-1,* the number of lateral roots was significantly lower than the treated wild-type seedlings (Fig. 3a). This indicated that *M. endophytica* YC6887 lost its ability to stimulate lateral root formation in the auxin mutants. In *M. endophytica* YC6887 treated wild-type seedlings, an increase in plant fresh weight was observed, but in all auxin mutants, the fresh weight stimulation was significantly lower than the treated wild type seedlings (Fig. 3b). The

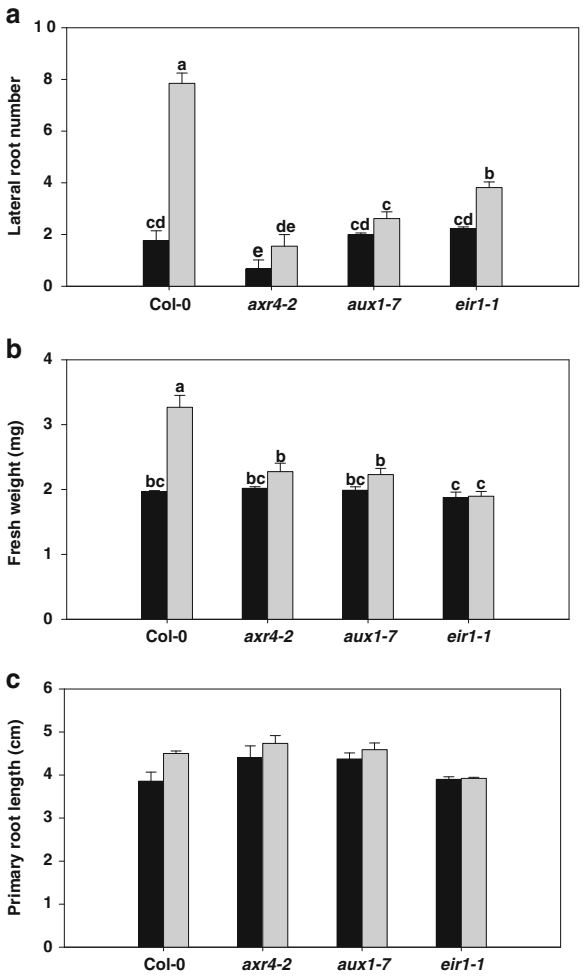

Fig. 3 Effects of *Martelella endophytica* YC6887 co-cultivation on the root system architecture of auxin signaling and response mutants after 8 days of co-cultivation with *M. endophytica* YC6887, **a** lateral roots number/seedling of Col-0 (wild type), auxin influx (*aux1-7*), auxin efflux (*eir1-1*), and auxin response mutants (*axr4-2*) (n = 20), **b** fresh weight/seedling, and **c** primary root length/seedling. *Black bars* indicate the controls, while *gray bars* indicate *M. endophytica* YC6887 treatment. Values for different growth parameters indicate means ± standard error ($n = 20$). *Different letters* indicate statistically significant differences between the control and *M. endophytica* YC6887 treatment (Duncan test; $P < 0.05$). The experiment was repeated three times with the same results

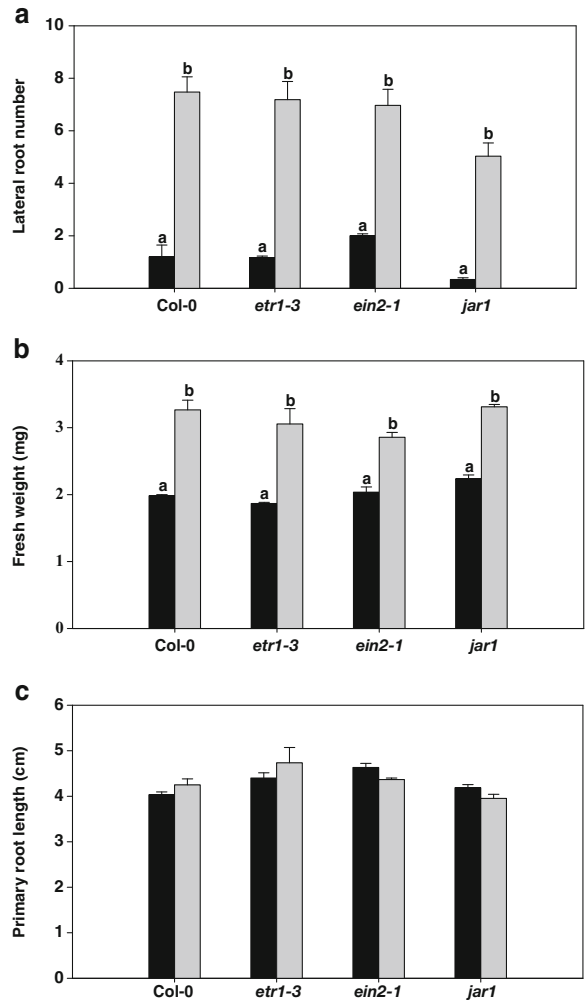

Fig. 4 Response of *Arabidopsis* Col-0 and its ethylene and jasmonic acid mutants to co-cultivation with *Martelella endophytica* YC6887. The bacteria were inoculated with the seedling on MS media and the response was determined after 8 days of co-cultivation in ethylene (*etr1-3, ein2-1*) and jasmonic acid (*jar1*) mutants, **a** lateral root number/seedling, **b** fresh weight/seedling, and **c** primary root length/seedling. *Gray bars* indicate *M. endophytica* YC6887 treatment and *black bars* indicate untreated seedlings. Values for different growth parameters indicate means ± standard error ($n = 20$). *Different letters* indicate statistically significant differences between the control and *M. endophytica* YC6887 treatment (Tukey B; $P < 0.01$). The experiment was repeated three times with the same results

length of the developed primary root, however, was not significantly different between *M. endophytica* YC6887 treated and control seedlings of wild-type and auxin mutants (Fig. 3c). Our results showed that *M. endophytica* YC6887 lost its plant growth-promoting activity in all the auxin mutants.

Development of root system architecture mediated by *M. endophytica* YC6887 is independent of ethylene and jasmonic acid

A phytohormone, ethylene, also plays an important role in the development of root system architecture

by increasing the number and length of root hairs associated with auxin (Pitts et al. 1998; Rahman et al. 2002). The role of ethylene in response to *M. endophytica* YC6887 was analyzed by using the ethylene mutants, *ein2-1* and *etr1-3*. *M. endophytica* YC6887 treated ethylene mutants were found to increase the numbers of lateral roots and fresh weight compared to the non-inoculated seedlings. These values were significantly different between *M. endophytica* treated wild-type Col-0 and the mutants (Fig. 4a, b). It has been reported that jasmonic acid not only affects auxin transport but also activates the jasmonate-dependent auxin biosynthetic gene anthranilite synthase (Sun et al. 2009). In the jasmonic acid mutant, *jar1*, the treatment of *M. endophytica* YC6887 also increased the number of lateral roots and fresh weight (Fig. 4a, b). This showed that *M. endophytica* YC6887 did not utilize the ethylene or jasmonic acid pathways for stimulation of root system development.

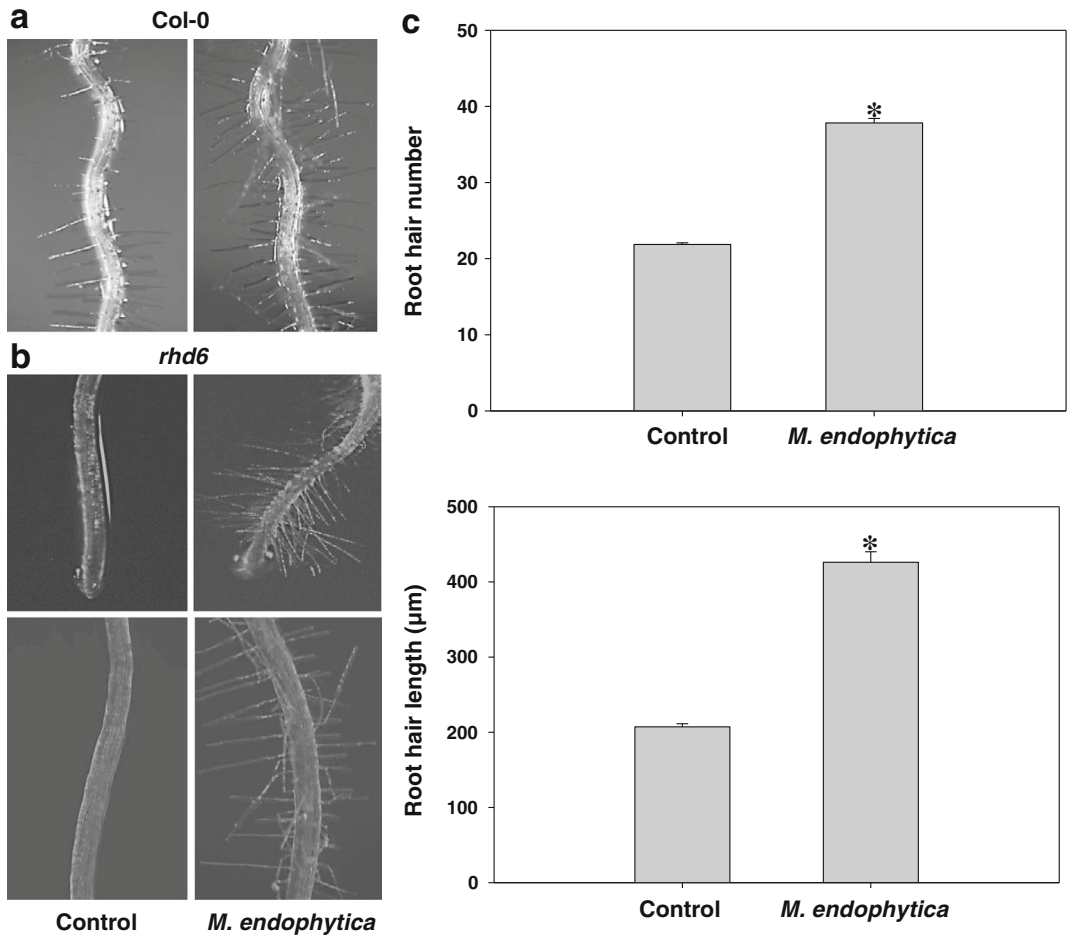

Fig. 5 Effects of *Martelella endophytica* YC6887 inoculation on the root hair density of *Arabidopsis* Col-0 and the root hair mutant *rhd6*, **a** images showing the root hair density after 8 days growth of *Arabidopsis* Col-0. The root segment 1.0 cm above the root tip was treated with buffer solution or co-cultivated with *M. endophytica* YC6887, **b** root hair in *rhd6* mutants treated with buffer solution or *M. endophytica* YC6887 and **c** root hairs number/seedling and root hair length measured in root segments. Values determined were expressed as mean ± standard error measured 1.0 cm above the root tip ($n = 15$). Statistically significant differences between the treatments are denoted by *asterisks* ($P < 0.01$). This experiment was repeated three times with similar results

Stimulation of root hair development by *M. endophytica* YC6887 in different signaling mutants of *Arabidopsis*

To determine whether *M. endophytica* YC6887 affected root hair formation during the development of root system architecture, the bacteria were co-cultivated with wild-type and signaling mutants of *Arabidopsis* seedlings. The inoculation of the bacteria significantly increased the number and length of root hairs compared to the non-inoculated control plants after 8 days of treatment. The number of root hairs in bacterial inoculated seedlings was 37.8 ± 0.59 per seedling as compared to the control seedlings at 21.8 ± 0.21. The length of root hairs in the control was 207 ± 4.2 μm per seedling, while in *M. endophytica* YC6887 treated seedlings, the length increased to 426 ± 14 μm (Fig. 5a, c). The root hair defective mutant *rhd6* of *Arabidopsis* was evaluated to test the stimulation of root hair development by bacterial inoculation. This mutant lost its root hair formation activity. However, with auxin application, it could produce root hair (Masucci and Shieffelbein 1994). The *rhd6* mutant responded to *M. endophytica* YC6887 after 8 days of co-cultivation. It recovered its root hair forming ability, indicating that *M. endophytica* YC6887 with auxin activity was involved in root hair density promotion (Fig. 5b).

Auxin and ethylene can promote root hair initiation and differentiation in *Arabidopsis* (Masucci and

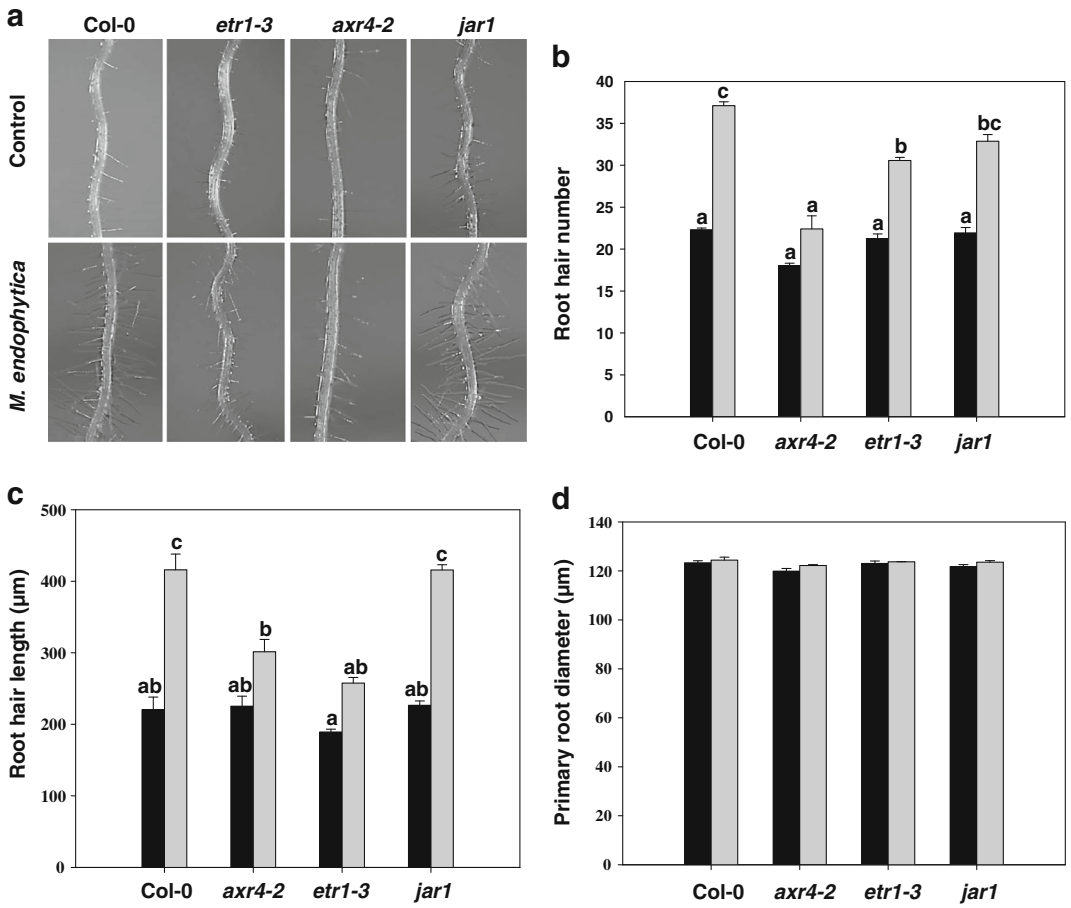

Fig. 6 Effect of *Martelella endophytica* on the root hair density in wild type Col-0, auxin (*axr4-2*), ethylene (*etr1-3*), and jasmonic acid (*jar1*) mutants of *A. thaliana*, **a** root hair formation of *Arabidopsis* Col-0 and mutants at the primary root segment located 1.0 cm above the root tip inoculated with *M. endophytica* YC6887 or treated buffer solution, **b** root hairs number/seedling determined in the 1 mm length of root segment ($n = 15$), **c** root hair length (μm)/seedling measured in 1 mm length of root segment ($n = 15$), and **d** primary root diameter (μm)/seedling ($n = 15$). The *black bars* indicate controls, while *gray bars* indicate *M. endophytica* YC6887 treated seedlings. Values are expressed as mean ± standard error. *Different letters* indicate significant differences between different treatments (Tukey's HSD; $P < 0.01$). Data shown was repeated three times with similar results

Shieffelbein 1994; Tanimoto et al. 1995) while jasmonic acid interacts with ethylene and auxin to promote root hair development (Zhu et al. 2006). The development of root hairs by *M. endophytica* YC6887 was assessed in *Arabidopsis* mutants to determine the underlying pathway. Auxin, ethylene, and jasmonic acid mutants were inoculated with *M. endophytica* YC6887. Inoculation of *M. endophytica* YC6887 in auxin mutants produced fewer root hairs in the mutant *axr4-2*, and the length of root hairs was also significantly shorter compared to *M. endophytica* YC6887 treated wild-type seedlings (Fig. 6a, b). In the ethylene mutant, bacterial treatment increased the number of root hairs significantly, but the length of the root hairs was significantly shorter than that of the treated wild-type Col-0 (Fig. 6a, c). However, in the jasmonic acid mutant, *jar1*, there was no significant difference in the number and length of the root hairs between wild-type and mutants after bacterial inoculation. In the diameter of the primary root, no significant difference was observed in all *M. endophytica* YC6887 inoculated and uninoculated wild-type Col-0 as well as in mutants of *Arabidopsis* (Fig 6a, d). The results showed that *M. endophytica* YC6887 followed a specified signaling pathway dependent on auxin but independent of jasmonic acid pathways to stimulate root hair development in *Arabidopsis*. However, for the ethylene mutant, the response in root hair elongation was compromised (Fig. 6).

Structure determination of auxin-like compound

The compound with plant growth promotion activity was attained as colorless crystals, and its structure was determined by EI mass spectra as well as ^{1}H and ^{13}C NMR. In the EI-MS spectrogram, the molecular ion peak appeared at *m/z* 91 (100 %). NMR data in $CDCl_3$ showed eight hydrogen and eight carbon atoms. The proton NMR (^{1}H-NMR) spectrum of this compound contained a multiplet at δ 7.32–7.20 indicating five protons (5H). These five protons were confirmed to be aromatic benzene protons. Furthermore, a singlet peak at δ 3.58 indicated methylene protons based on the integral. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.32–7.20 (5H, m), 3.58 (2H, s). In addition, the carbon NMR (^{13}C-NMR) spectrum indicated eight carbon atoms in the compound. The peak at δ 177.0 indicated the carbonyl carbon, while four carbon atoms of the benzene ring were distributed at δ 127.3 ~ δ 133.23. ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 177 (C-1), 133.2 (C-3), 129.35 (C-4, C-8), 128.65 (C-5, C-7), 127.35 (C-6), 40.5 (C-2). Based on NMR and EI-MS data, the above compound was identified as phenylacetic acid and the molecular formula was determined to be $C_8H_8O_2$ (Fig. S4a-d).

Effects of culture filtrate and phenylacetic acid on the growth of *Arabidopsis*

In in vitro tests, after treating the 4-day-old *Arabidopsis* seedlings with culture filtrate and phenylacetic acid, the growth parameters related to the number of lateral roots and fresh weight were significantly increased as compared to the untreated control. The number of lateral roots in the culture filtrate and phenylacetic acid treated seedlings was 5.06 ± 0.68 and 7.67 ± 1.1, respectively, as compared to the control seedlings, 0.58 ± 0.12 (media) and 0.67 ± 0.29 (Fig. 7a, b). In the culture filtrate and phenylacetic acid treatments, the fresh weights were 3.3 ± 0.13 and 4.15 ± 0.28 mg, respectively, which were significantly higher than the untreated control (Fig. 7a, c). However, no significant difference was found in the primary root length between the treated and untreated control seedlings (Fig. 7a, d). To examine the effect of PAA on LRP formation, the primary root was observed in PAA-treated seedlings. The PAA treatment significantly increased the number of LRP compared to the control, indicating that PAA could induce the initiation and emergence of lateral roots as IAA (Fig. 7e). The response of *Arabidopsis* to phenylacetic acid was observed using transgenic line DR5::GUS. The primary root and lateral root primordia of phenylacetic acid-treated seedlings were found to have high Gus staining compared to the control, indicating its auxin activity (Fig. 7g, Figs. S5f and S6). The auxin mutants showed no response to phenylacetic acid. They produced lesser number of lateral roots without affecting primary root length compared to wild type treated seedling. Phenylacetic acid at higher concentration inhibited the primary root length. However, its effect was lower than that of IAA (Figs. S2 and S3).

Discussion

Plants inhabiting marine environments associate with diverse microbial communities which are known to have various functions and to interact with host plants, forming a mutualistic relationship to improve plant health. Some of these marine endophytic bacteria are also reported to have antifungal,

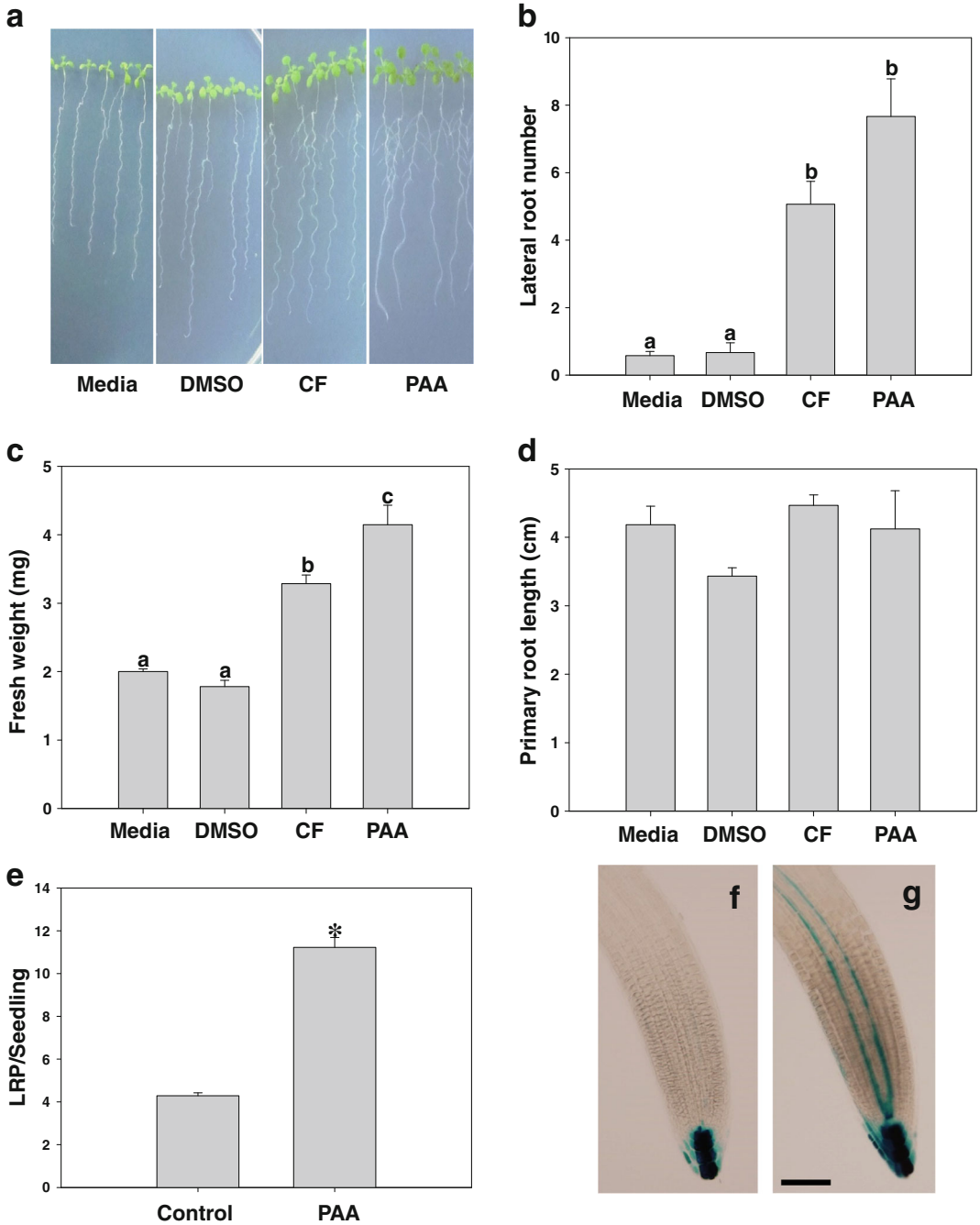

Fig. 7 Effects of culture filtrate (CF) and phenylacetic acid (PAA) on the root system development of *A. thaliana* (Col-0) seedlings. Four-day-old *Arabidopsis* seedlings were treated with CF and PAA (1 μM) and grown for 8 days in a growth chamber. Culture media and DMSO only were used as a control, **a** *Arabidopsis* seedlings co-cultivated with CF and PAA, **b** lateral roots number/seedling, **c** fresh weight/seedling, **d** primary root length/seedling, **e** number of lateral root primordia, **f** and **g** root tip of control and PAA treated DR5::GUS seedlings respectively (scale bar = 100 μm). The experiment was repeated three times with similar results. Statistically significant differences are designated by asterisks (Tukey's HSD; $P < 0.05$)

chitinolytic, and plant growth-promoting activities (Bibi et al. 2012; Shin et al. 2007).

M. endophytica YC6887 isolated from the root of a halophyte was tested for its plant growth-promoting

activities by using *A. thaliana*, a model dicot plant system, to study the interaction with bacteria. *A. thaliana* is attractive as a study model since it has a relatively short life span and its full genome sequence information is available (*Arabidopsis* genome initiative 2000). *M. endophytica* YC6887 was found to affect the development of root system architecture by increasing the number of lateral roots and plant's fresh weight. Also, no significant difference in the length of the primary root of the controls and *M. endophytica* YC6887 treated seedlings were observed. While strain YC6887 originated from the inner root tissues of a *Rosa rugosa*, no evidence of strain YC6887 endophytic colonization in *Arabidopsis* was found in this study (data not shown). The response of *Arabidopsis* DR5::GUS was induced via auxin signaling, which implies the production of auxin or an auxin-like compound by *M. endophytica* YC6887. The inhibition of auxin response by the polar auxin transport inhibitor, NPA, at all concentrations further confirmed the role of auxin stimulus by *M. endophytica* YC6887. Many bacteria such as *P. brassicacearum* STM196, *B. subtilis*, and *Pseudomonas aeruginosa* can promote plant growth through auxin transport, perception, and signaling in *A. thaliana* (Contesto et al. 2010; Ortiz-Castro et al. 2011; Zhang et al. 2007). Other compounds such as dimethyl disulfide and benzoxazinoid produced by *Bacillus* sp. and *Azospirillum,* respectively, are also involved in growth promotion of their associated plants (Meldau et al. 2013; Walker et al. 2011).

In the bacterial interaction with *Arabidopsis*, auxin signaling and transport are very significant and during plant development, the influx and efflux machinery promotes the entry and exit of auxin into the plant body (Grunewald et al. 2009; Mathesius 2008). By comparing the growth of *Arabidopsis* wild type and mutants defective on auxin transport, the response of *Arabidopsis* auxin mutants to *M. endophytica* YC6887 was found to be significantly lower than the wild type Col-0 seedlings. Among the auxin mutants, the *axr4-2* and *aux1-7* responses were very low with respect to lateral root formation in *M. endophytica* YC6887 treated plants compared to the wild-type Col-0, as the auxin influx to the plant body was reduced in the mutants and *M. endophytica* YC6887 failed to promote plant growth in these mutants. The influx carrier AUX protein responsible for the uptake of auxin molecules is actually a member of the permease family of proton driven transporters. Auxin influx can induce lateral root formation and normal gravitotropism. However, the polar localization of AUX1 requires AXR4 gene activity (Dharmasiri et al. 2006; Hobbie 2006; Contreras-Cornejo et al. 2009; Felten et al. 2009). In auxin-dependent pathways, the efflux carrier PIN protein and EIR1 are involved in basipetal auxin transport and transport auxin from cells in the root tip into the elongation zone (Luschnig et al. 1998). In the auxin efflux mutant, *eir1,* treated with *M. endophytica* YC6887, the plant biomass is reduced, indicating that *M. endophytica* YC6887 requires efflux for root system development, as the movement of auxin to the elongation zone is blocked otherwise.

Some bacterial species simultaneously utilize auxin and ethylene pathways to promote root system development (Splivallo et al. 2009). The ethylene and jasmonic acid mutant analysis showed that *M. endophytica* YC6887 maintains its plant growth-promoting activity by increasing the number of lateral roots and fresh weight and is independent of the ethylene or jasmonic acid pathways for its plant growth-promoting activity. Because ethylene and jasmonic acid also interact with auxin, the ethylene in association with auxin causes changes in root system development, while jasmonic acid not only affects auxin transport, but also induces auxin biosynthetic genes (Pitts et al. 1998; Rahman et al. 2002; Sun et al. 2009; Ivanchenko et al. 2008).

During root system development, root hair growth plays an important role in anchorage and nutrient absorption of plants. The development of root hairs involves the auxin and ethylene signaling pathways (Dobbelaere et al. 1999; Ribaudo et al. 2006). *M. endophytica* YC6887 inoculation stimulates root hair development by increasing the length and number of root hairs in wild-type Col-0 seedlings compared to non-inoculated seedlings. However, the root hair defective mutant *rhd6* had no root hair development in the absence of auxin. This indicated that *M. endophytica* YC6887 treatment recovered the root hair deformity in *rhd6* and produced root hair through auxin signaling mechanism. In the *Arabidopsis* auxin mutant *axr4-2*, *M. endophytica* YC6887 lost its root hair formation activity but not in the jasmonic acid mutant, *jar1* as the response to the bacteria was unaffected. In the ethylene related mutant *etr1*, the root hair length was reduced and as such, the role of ethylene appears to be counteractive. However, the number of root hairs was high compared to the number in the auxin mutant, which was significantly lower than in the wild-type Col-0. This confirms that *M. endophytica* YC6887 depends

on auxin signaling as auxin acts upstream of the root hair morphogenic genes and plays an important role in root hair development (Lee and Cho 2013). The active compound showing auxin activity was identified as phenylacetic acid. For further confirmation, the culture filtrate and phenylacetic acid were used to check the growth-promoting activity of *Arabidopsis*. Both the culture filtrate and phenylacetic acid showed growth promotion for lateral roots and fresh weight changes. Our results showed that the phenylacetic acid produced by *M. endophytica* YC6887 promoted the formation of lateral root primordia, resulting in increased number of lateral roots. Lateral roots are originated as a single layered primordia consisting of up to ten small cells from founder cells. They can develop into a dome shaped structure through several developmental stages (Péret et al. 2009). Auxin is one of the important plant hormones responsible for lateral root formation for the different developmental stages (Lavenus et al. 2013). Phenylacetic acid was reported to be a natural auxin produced by many plant species such as wheat, peas, corn, sun flowers, tomato, and barley (Wightman and Lighty 1982). Biological activities of phenylacetic acid in relation to plant growth are lower than that of IAA. However, its endogenous amount is high, and it can respond to the same auxin responsive genes in *Arabidopsis* (Sugawara et al. 2015). Bacterial species isolated from the rhizosphere and the soil such as *Azospirillum* and *Streptomyces* also produce phenylacetic acid, which in turn promotes plant growth and protects the plant from pathogens (Hwang et al. 2001; Somers et al. 2005).

Conclusions

The *M. endophytica* YC6887, isolated from a halophyte, promoted the growth of *A. thaliana* by producing phenylacetic acid. Development of the root system architecture of *Arabidopsis* in terms of lateral roots and root hair density was analyzed by using *Arabidopsis* with hormone-related mutations. The results show that *M. endophytica* YC6887 follows the auxin signaling, albeit independent of other hormone signaling such as ethylene and jasmonic acid. So far, most plant growth-promoting bacteria such as *Pseudomonas* and *Bacillus* species are known to be isolated from terrestrial plants or soils. *M. endophytica* YC6887 is a marine bacterial strain, which may play an important role in its beneficial interactions with the halophyte host.

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