Plant Soil (2017) 419:557–573
DOI 10.1007/s11104-017-3371-1

REGULAR ARTICLE

Arabidopsis thaliana root and root exudate metabolism is altered by the growth-promoting bacterium *Kosakonia radicincitans* DSM 16656^T

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Received: 20 December 2016 / Accepted: 8 August 2017 / Published online: 13 August 2017

Abstract
Aims Plant growth-promoting bacteria (PGPB) affect host physiological processes in various ways. This study aims at elucidating the dependence of bacterial-induced growth promotion on the plant genotype and characterizing plant metabolic adaptations to PGPB.
Methods Eighteen *Arabidopsis thaliana* accessions were inoculated with the PGPB strain *Kosakonia radicincitans* DSM 16656^T. Colonisation pattern was assessed by enhanced green fluorescent protein (eGFP)-tagged *K. radicincitans* in three *A. thaliana* accessions differing in their growth response. Metabolic impact of bacterial colonisation was determined for the best responding accession by profiling distinct classes of plant secondary metabolites and root exudates.
Results Inoculation of 18 *A. thaliana* accessions resulted in a wide range of growth responses, from repression to enhancement. Testing the bacterial colonisation of three accessions did not reveal a differential pattern. Profiling of plant secondary metabolites showed a differential accumulation of glucosinolates, phenylpropanoids and carotenoids in roots. Analysis of root exudates demonstrated that primary and secondary metabolites were predominantly differentially depleted by bacterial inoculation.
Conclusions The plant genotype controls the bacterial growth promoting traits. Levels of lutein and β-carotene were elevated in inoculated roots. Supplementing a bacterial suspension with β-carotene increased bacterial growth, while this was not the case when lutein was applied, indicating that β-carotene could be a positive regulator of plant growth promotion.

Keywords Arabidopsis · Carotenoids · Glucosinolates · Plant growth promoting bacteria · Phenylpropanoids · Root exudates

Abbreviations

AMF	Arbuscular mycorrhizal fungi
CLSM	Confocal laser scanning microscopy

Responsible Editor: Hans Lambers.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-017-3371-1) contains supplementary material, which is available to authorized users.

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eGFP	Enhanced green fluorescent proteinPGBP plant growth promoting bacteria
GC-EI-Q-MS	Gas chromatography-electron ionization-quadrupole mass spectrometry
HPLC-DAD-ESI-MS^n	High-performance liquid chromatography-diode array detection-electrospray ionization-multiple stage mass spectrometry
IAA	Indole-3-acetic acid
UPLC-ESI-QTOF-MS	Ultra-performance liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry

Introduction

Bacteria can colonize the plant rhizosphere, phyllosphere and reproductive organs of plants (Bodenhausen et al. 2013; Compant et al. 2010; Rosenblueth and Martinez-Romero 2006), with some, referred to as plant growth-promoting bacteria (PGPB), capable of stimulating the growth of the host, and others of increasing host fitness. PGPB are able to fix atmospheric nitrogen, enhance nutrient uptake (P, N, Mg, K) and modulate plant development via the modulation of phytohormone production (Berg 2009; Weyens et al. 2009). Biological atmospheric nitrogen fixation by diazotrophic bacteria is of enormous importance to agriculture, as it provides means to optimize nitrogen fertilisation regimes in agriculture and reduce environmental nitrogen pollution (Vessey 2003). Phosphate-solubilizing bacteria are widely distributed in the rhizosphere, operating via the secretion of organic acids and phosphatases in calcareous soils (Richardson and Simpson 2011), as are those which increase the availability of iron and phosphate by the excretion of siderophores or chelators in acidic soils (Matsuoka et al. 2013). A range of bacterial species is known to synthesize (or degrade) the key phytohormones abscisic acid, ethylene, gibberellins and indole-3-acetic acid (IAA) (Dodd et al. 2010). In most cases the presence of PGPB affects the development of primary and lateral roots of inoculated plants as a consequence of bacterial manipulation of host metabolism (El Zemrany et al. 2007; Verbon and Liberman 2016). The overall plant growth benefits from the modified root system architecture either directly by increased uptake of nutrients and water, or indirectly through biocontrol of phytopathogens (Lugtenberg and Kamilova 2009).

While the in vitro characterization of PGPB has provided insights into possible mechanisms underlying plant growth promotion, their mode of action in planta remains unclear. The impact on the *Arabidopsis thaliana* transcriptome following the plant's colonization by *Bacillus subtilis* (Lakshmanan et al. 2013), *Burkholderia phytofirmans* (Poupin et al. 2013), *Pseudomonas thivervalensis* (Cartieaux et al. 2003), *P. fluorescens* strains (van de Mortel et al. 2012; Verhagen et al. 2004; Wang et al. 2005; Weston et al. 2012) and a *Pseudomonas* species (Schwachtje et al. 2011) has been described. While these PGPB induce changes in primary metabolism, defence-related pathways and hormone signalling, the details of the plant's physiological response are strongly dependent on the interaction between the bacterium and its host plant. Recent reports describe the changes to the host metabolome provoked by PGPB (Berger et al. 2017; Vacheron et al. 2013). Especially the composition and concentration of plant secondary metabolites, such as glucosinolates, phenylpropanoids and carotenoids, is affected by the colonisation of bacteria, indicating that these compounds are involved in plant-bacteria interaction, although their function is largely unclear (Chamam et al. 2013; Ruppel et al. 2008; van de Mortel et al. 2012; Walker et al. 2011; Walker et al. 2012). Plant roots secrete constantly a variety of compounds into the rhizosphere as a part of the rhizodeposition process and to influence microbial communities in their immediate vicinity (Bressan et al. 2009). The role of some root secondary compounds in the communication between soil microbes and plants has been resolved. Flavonoids, a major class of phenylpropanoids, are known to induce the expression of nodulation genes in rhizobia for initiation of symbiosis in root exudates (Zhang et al. 2015). Degradation products of carotenoids, mycorradicin and strigolactones, are involved in the establishment of arbuscular mycorrhiza in the host root (Walter 2013). It has been largely recognized that the metabolite constitution of root exudates governs the recognition process between plants and microbes (Bertin et al. 2003; Faure et al. 2009). Hence, the concerted analysis of the metabolite composition of roots and root exudates is of

utmost importance for understanding plant-bacteria interaction (Narula et al. 2009).

Presence of the gram negative bacterium *Kosakonia radicincitans* DSM 16656^T (syn. *Enterobacter radicincitans* (Brady et al. 2013), formerly *Pantoea agglomerans*) in the phyllosphere of winter wheat has been described previously (Kämpfer et al. 2005; Ruppel 1988). It has been shown that the inoculation of this strain stimulates root and shoot growth in a range of plant hosts (Berger et al. 2015; Brock et al. 2013; Höflich and Ruppel 1994; Schreiner et al. 2009). These bacteria, at least in vitro, are capable of both nitrogen fixation and phosphorus solubilization (Ruppel and Merbach 1995; Schilling et al. 1998). A possible involvement in the host's phytohormone status has been inferred by their ability to synthesize IAA and the cytokinins N6-isopentenyl-adenosine and -adenine (Scholz-Seidel and Ruppel 1992). The growth-promoting properties of *K. radicincitans* were demonstrated for a wide range of hosts but the degree of plant beneficial effects is determined by the plant species and genotype (Remus et al. 2000; Schreiner et al. 2009).

The present study focused on characterizing the impact of *K. radicincitans* on plant metabolism when colonizing *A. thaliana* with special emphasis on glucosinolates, phenylpropanoids and carotenoids. We demonstrate that, as a consequence of the bacterial-induced alterations in the root metabolite profile, the root exudate pattern is also greatly affected by the presence of the bacterium. Moreover, we found evidence that the presence of *K. radicincitans*, as visualized by enhanced green fluorescent protein (eGFP)-tagged bacteria, did not correlate with the level of growth promotion when multiple plant accessions were tested.

Materials and methods

Plant material and cultivation

A collection of 18 *A. thaliana* accessions (Bur-0, Can-0, Col-0, Ct-1, Edi-0, Hi-0, Kn-0, Ler-0, Mt.-0, No-0, Oy-0, Po-0, Rsch-4, Sf-2, Tsu-0, Wil-2, Ws-0, Wu-0, kindly provided by L. Westphal, Leibniz Institute of Plant Biochemistry, Germany, Supplemental Fig. S1) was used to assess the growth-promoting effects of *K. radicincitans*. Host plants were raised on non-sterile standard plant growth substrate (Fruhstorfer Erde type P, Germany) under short day conditions (8 h photoperiod) at 22 °C and 40–60% relative humidity. Two week old seedlings were individually potted into sand for inoculation with *K. radicincitans* strain DSM 16656^T. The accession screen was performed once using 20 plants per accession and treatment. Accession Oy-0 grown for plant secondary metabolite profiling and root exudate collection was cultivated as described above. The pots were watered with nutrient solution as described by Gibeaut et al. (1997), and after four weeks of growth, the material was harvested. Roots were washed to remove adhering sand particles and blotted dry on tissue paper. Rosette leaves and roots were separately snap-frozen in liquid nitrogen. These experiments were performed in triplicate using 20–25 plants per treatment that were pooled into one sample.

Bacterial colonisation was quantified in *gtr1gtr2* (At3g47960, At5g62680), *sds1* (At1g78510.1), *ccoaomt1* (At4g34050) and *f6'h1* (At3g13610) knock-out mutants of *A. thaliana*. Here, four-week-old plants were grown as described above and inoculated with *K. radicincitans*. Roots were harvested after 4 days. Three batches of plants were analysed, consisting of 10 plants each.

Accessions Col-0, Ler-0 and Oy-0 grown for in situ localisation studies were raised as in vitro cultures. Seeds were surface-sterilized with a solution containing 5% NaOCl and 0.5% Tween 20 and then incubated at 4 °C for 3 days for stratification. Seeds were sown on sterile plates containing 1/2-strength Murashige-Skoog medium supplemented with 1.5% sucrose and grown as described above under short day conditions. After one week, germinated plants were transferred to square plates with the same medium and kept in a vertical position for further three weeks.

Bacteria cultivation, transformation and plant inoculation

The bacteria were cultured overnight in standard nutrient broth (Merck, Germany) according to Ruppel et al. (2006). The cells were first pelleted by centrifugation, washed twice in sterile 50 mM NaCl, re-suspended in 50 mM NaCl to give an OD_{620} of 0.2 (corresponding to 10^9 cfu mL^{-1}) and finally further diluted to a concentration of 10^7 cfu mL^{-1}. Either a 10 mL aliquot of this cell suspension or 10 mL 50 mM NaCl as control treatment was spread over the surface of each pot.

For in situ localisation studies, electro competent bacterial cells were transformed with plasmid pMP4655 (Bloemberg et al. 2000; Lagendijk et al.

2010). Single colonies of *K. radicincitans* expressing eGFP grown on Luria-Bertani agar plus gentamycin (150 μg/ml) were inoculated in 50 ml standard nutrient broth and preparation of inoculation suspension was as described above. Plants were carefully removed from agar plates, the roots gently soaked in water in a dish and transferred into 12 ml tubes containing 11 ml sterile water. *A. thaliana* plants were left for 24 h to adjust to the changed conditions before 10^5 bacterial cells were added to each plant in 11 ml sterile water. Water levels were adjusted on a daily basis to account for evaporation and plant-based water losses.

Confocal laser scanning microscopy (CLSM)

Bacterial root colonization was monitored after 24 h, 48 h and 6 days. Roots were gently washed in sterile water and fluorescence was recorded with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss Jena GmbH, Germany). Bacterial eGFP fluorescence signals were captured by confocal laser microscopy on a Zeiss LSM 510 META confocal system (excitation/emission 488 nm, samples at 8% argonlaser power, BP505–550 filter, Plan Apo 63/1.4 oil lens) and roots were captured using bright field settings.

Detection of *K. radicincitans* in planta using qPCR

K. radicincitans specific primer design

Comparing the genome of DSM 16656^T (Witzel et al. 2012) to genomes of other Enterobacteriaceae and more distantly related bacteria, a DNA repair protein was found to be specific to *Kosakonia radicincitans* and closely related taxa. NCBI blastn searches confirmed the specificity of this gene in silico (Supplemental Table S1). DNA sequences of DSM 16656^T and close relatives were aligned in order to determine highly variable sites (Supplemental Fig. S2). *K. radicincitans* specific primers were designed using Geneious (http://www.geneious.com/) from Biomatters and Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) from Northwestern University.

Bacterial colonization studies

Root colonization of Col-0 ecotype and *gtr1gtr2*, *sps1*, *ccoaomt1* and *f6'h1* knock-out mutants of *A. thaliana* by *K. radicincitans* was determined by qPCR. Genomic DNA was extracted using DNeasy Plant Kit from Qiagen (Düren, Germany) following the manufacturer's instructions. DNA quantity and quality were assessed with the NanoDrop (Thermo Scientific, Bonn, Germany) system. The qPCRs with strain specific primers were performed using primers fdnaJ_F1 (5`-AAGCCAGCGTTCCG TCGTA-3`) and fdnaJ_R2 (5`-GATCGTTGAACTCG TCGAGCAG-3`) with a product size of 140 bp. Approximately 10 ng of genomic plant DNA was used for each qPCR with SsoAdvanced Universal SYBR Green Supermix (BioRad, Germany). The PCR conditions were as follow: Initially 3 min 95 °C, then 35 cycles of 95 °C for15 sec, followed by 50 s at 72 °C and 5 min of elongation time at 72 °C. Each of the biological replicates was analyzed in three technical replications. Two reference genes (At5g55840, At5g08290) (Witzel et al. 2013) were used for relative quantification (Livak and Schmittgen 2001) of *K. radicincitans* in *A. thaliana* roots. qPCR melting curve analysis was performed to ensure the presence of a single product.

Root secondary metabolite profiling

Glucosinolate analysis

Desulfo-glucosinolate profiles and concentrations were determined as described by Witzel et al. (2013).

Flavonoid determination

Homogenized frozen root (100 mg) material were each extracted in 600 μL 60% methanol, shaken for 60 min at 20 °C and centrifuged (10,000 *g* for 10 min). The pellet was then re-extracted, first in 400 μL 60% methanol for 20 min and then in 200 μL 60% methanol for 10 min. The combined supernatants were filtered through a Spin-X tube (Sigma Aldrich, United States) by centrifugation (1000 *g* for 5 min) and dried by vacuum centrifugation. The residue was dissolved in 200 μL distilled water. Flavonoid glycosides and hydroxycinnamic acid derivatives were detected using HPLC-DAD-ESI-MS^n as described by Neugart et al. (2012), with some modification to the solvent gradient. Specifically, solvent A consisted of 99.5% water, 0.5% acetic acid and solvent B of 100% acetonitrile. The sequence was as follows: 0–12 min: linear increase of B from 5% to 7%; 12–15 min: linear increase of B from 7% to 9%; 15–45 min: linear increase of B from 9% to 12%; 45–100 min: linear increase of B from 12% to 15%; 100–105 min: linear increase of B

from 15% to 75%; 105–115 min: 75% B; 115–120 min: linear decrease of B from 75% to 5%; 120–123 min: 5% B. The flow rate was 0.4 mL min^{-1}, and the detector wavelengths were 320 nm for the hydroxycinnamic acid derivatives and 370 nm for the flavonol glycosides. These were both identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC-DAD-ESI-MS^n using an Agilent series 1100 ion trap mass spectrometer run in negative ionization mode.

Carotenoid determination

Carotenoids were obtained by extracting 100 mg powdered root in chloroform (Baldermann et al. 2010), and detected using an Agilent Technologies 1290 Infinity UPLC device coupled with an Agilent Technologies 6230 TOF LC/MS system. The separation was performed in gradient mode (solvent A was 81:15:4 methanol:methyltert-butyl-ether:water, and solvent B: 6:90:4) on a C_{30} column (YMC Co. Ltd., Japan, YMC C30, 100 × 2.1 mm, 3 μm) at a flow rate of 0.2 mL min^{-1}. To enhance ionization, 20 mM ammonium acetate was added to the mobile phase. Identification was achieved by co-chromatography with reference compounds, and quantification from a dose response curve applying the following standards: neoxanthin $[M\text{-}H_2O + H]^+$ 583.415, lutein $[M\text{-}H_2O + H]^+$ 551.425, zeaxanthin $[M + H]^+$ 569.435 and β-carotene $[M + H]^+$ 537.446.

Primary and secondary metabolite analysis of root exudates

Collection of root exudates was performed as described earlier with some modifications (Xu et al. 2016). In short, roots of plants either inoculated or non-inoculated with *K. radicincitans* were washed to remove any adhering sand and immersed in sterile distilled water for 1 h, and then transferred to a fresh batch of bi-distilled water for a further 4 h. The medium was filtered through a mixed cellulose ester membrane filter (pore size 0.22 μm, Carl Roth, Germany) to remove any cellular debris and external microorganisms, and concentrated tenfold by freeze-drying. The exudate from approximately 25 plants was pooled into a single sample. After collecting the exudate, the roots were weighed. Primary and secondary plant metabolite profiling was performed using GC-MS and LC-MS, respectively, on three replicate samples from three independent biological experiments.

For primary metabolite profiling, freeze-dried root exudates were derivatized and analysed by means of a GC-EI-Q-MS system as described previously (Strehmel et al. 2016). For secondary metabolite profiling, freeze-dried root exudates were reconstituted in 60 μL 30% methanol and analysed by UPLC-ESI-QTOF-MS system (Strehmel et al. 2014). All parameters were maintained as already described.

In vitro bacterial growth assay in the presence of plant secondary metabolites

Overnight cultures of *K. radicincitans* were diluted to 10^5 cfu/mL and grown in the presence or absence of plant secondary metabolites in standard nutrient broth for 15 h at 30 °C and 90 rpm. 2-Propenyl glucosinolate was dissolved in water and diluted to concentrations of 20, 40 and 100 μM, the carotenoids lutein and β–carotene were dissolved in Tween20 (1 g in 10 mL H_20) and diluted to concentrations of 20, 40 and 100 μM, while phenylpronanoids (scopoletin, sinapic acid) and terpenes (squalene, α-humulene, farnesene) were all dissolved in 70% ethanol, each diluted to concentrations of 20 and 40 μM. Control samples of *K. radicincitans* received the same amount of water, Tween20 and 70% ethanol but without the plant metabolites. Each treatment was run in duplicate and the experiment was performed twice.

Statistical analyses

Genotype and treatment effects on rosette biomass were tested using a two-way ANOVA (Holm-Sidak, SigmaPlot v12.3 software, Systat Software, Germany). The scatterplot was generated using SigmaPlot.

Statistical evaluation of rosette biomass accumulation was performed using one-way ANOVA on ranks (Kruskal-Wallis) to detect differences in the mean values among groups of treated and non-treated plants (SigmaPlot). To identify the groups that differ from the other, normality testing (Shapiro-Wilk), equal variance test, followed by a two-tailed t-test was performed (SigmaPlot). Statistical testing of metabolite measurements and in vitro growth experiments was performed using a one-way ANOVA and a two-tailed Student's t-test (SigmaPlot).

Results

Variation in the growth response of *A. thaliana* upon *K. radicincitans* inoculation

In order to survey the natural genetic variation of *A. thaliana* response for growth-promotion provoked by *K. radicincitans*, eighteen accessions were tested for alterations in rosette fresh weight four weeks after bacterial inoculation (Fig. 1a). The variation of biomass accumulation among the accessions ranged from 70% in Ler-0 to 140% in Oy-0, when compared to the non-inoculated plants of the same accession (set at 100%), demonstrating an extensive range of plant responses to the bacterium. Two-way ANOVA testing for genotype-by-treatment interaction revealed a significant effect of the genotype ($p < 0.001$) on rosette weight, while the effect of bacterial inoculation was lower ($p = 0.447$). In turn, there was a significant genotype-by-treatment interaction ($p < 0.001$). Regression analysis revealed a correlation between the fresh weight of control and inoculated plants, while growth promotion was not dependent on the genotype's plant biomass production (Fig. 1b). Additional pairwise statistical analysis (Student's t-tests), subsequent to one-way ANOVA ($p < 0.001$), comparing each accession with and without bacterial inoculation identified accessions with significant alterations in rosette biomass upon treatment (Supplemental Table S2).

K. radicincitans colonizes the root surface of *A. thaliana*

To probe whether the range of growth promotion observed in *A. thaliana* accessions was related to bacterial

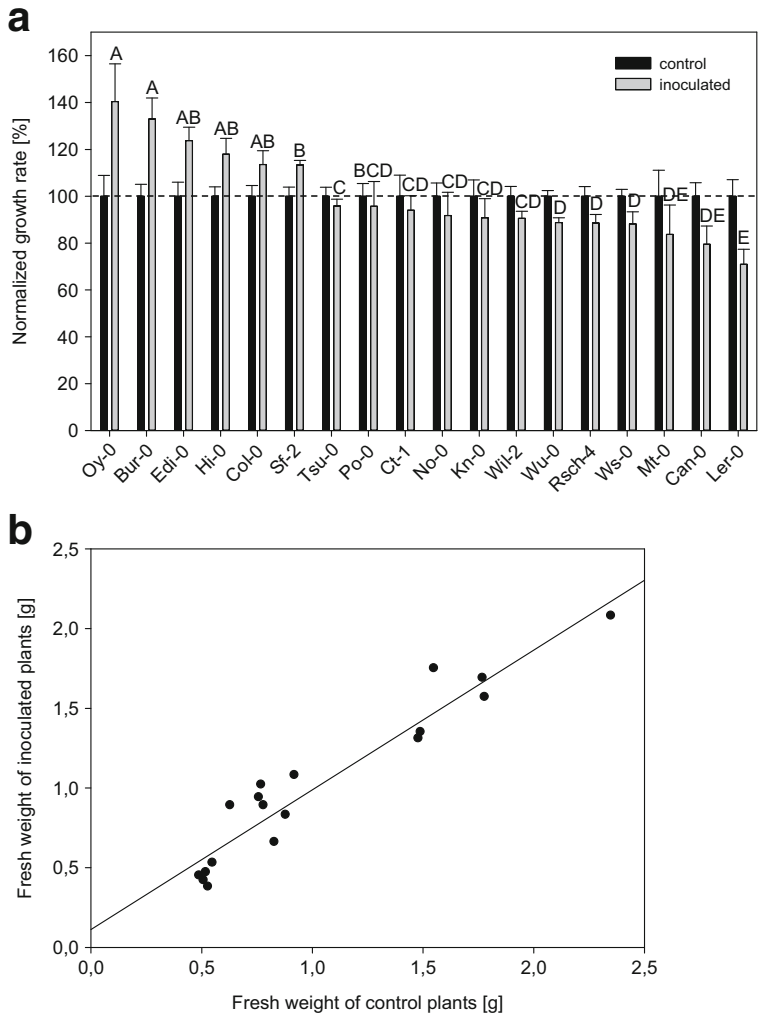

Fig. 1 Relative growth of *A. thaliana* accessions grown in the presence or absence of *K. radicincitans*. The mean rosette fresh weight of inoculated plants ($n = 20$) was normalized to the same of control plants of the respective accession. Error bars indicate the standard error; capital letters denote significant difference between inoculated genotypes ($p < 0.05$, t-test). The dashed line indicates the 1:1 ratio between control and inoculated plants (**a**). Scatterplot comparison of the rosette fresh weight (non-normalized) obtained from inoculated or control plants produced a correlation coefficient of 0.911 (**b**)

colonisation density, pattern of root colonisation were investigated using eGFP-transformed *K. radicincitans* and CLSM. The assay was performed using Oy-0 and Ler-0, representing the most responsive accessions (see Fig. 1), as well as Col-0 where previous testing was done (Brock et al. 2013). Attachment of bacteria onto the root surface and at root hairs was observed after 24 h (Fig. 2a) and adherence was tightly to the plant surface since cells were not washed off during the preparation procedure and were immobile. Bacteria formed a biofilm on the root surface at 48 h post inoculation (hpi) which consisted of only one or two cell layers. Bacterial numbers at individual colonization sites differed from several hundreds to only a few (Fig. 2b, c). The root cap region was not colonized. At 48 hpi bacteria were abundant at emerging lateral roots. At this position bacteria eventually enter the cells close to the junction where lateral root primordia push through the endodermis, the cortex and the epidermis. Colonization of lateral roots was rather sparse and restricted to the lateral root cracks (Fig. 2d, e). Rarely, single plant root cells were densely colonized by bacteria (Fig. 2f). No differences in root colonisation pattern were observed for Oy-0, Ler-0 and Col-0 at 24 hpi, 48 hpi and 6 dpi indicating that growth-promotion may be determined by the plant's genotype specific response to colonisation, rather than actively by the bacterium.

Presence of *K. radicincitans* affects root secondary metabolites

Secondary metabolites present in plant roots are known to govern plant-microbe interactions. Therefore, the composition and concentration of three major classes of secondary metabolites, glucosinolates, phenylpropanoids and carotenoids, were selected for profiling roots of Oy-0 as here the highest beneficial effects of bacterial inoculation were observed (see Fig. 1). Targeted analysis of glucosinolates allowed for the detection and quantification of 11 glucosinolates in roots of Oy-0, confirming earlier findings (Witzel et al. 2013). The amount of total glucosinolate content was lower in roots of inoculated plants and a statistically significant reduction of 8-(methylsulfinyl)octyl glucosinolate was found (Fig. 3a).

The phenylpropanoids present in roots were identified and quantified using a HPLC coupled to an ion-trap mass spectrometer. Analysis allowed for the detection of seven metabolites present in the Oy-0 root. The coumarins, including scopoletin, scopoletin derivates (scopoletin-coniferylalcohol-glucoside, scopoletin-

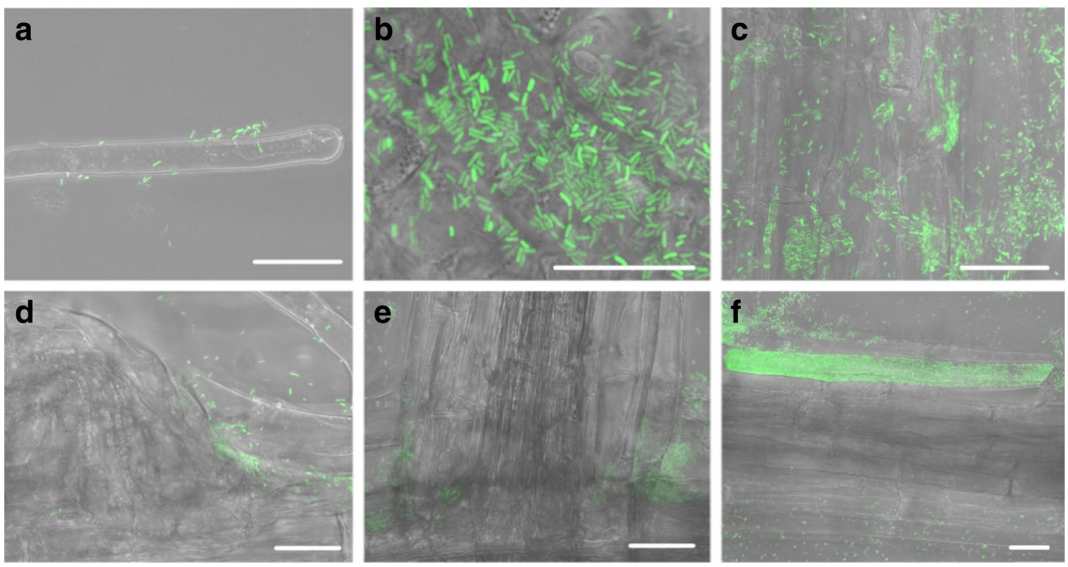

Fig. 2 Confocal laser scanning micrographs of *A. thaliana* Oy-0 colonized by *K. radicincitans* expressing eGFP. (**a**) *K. radicincitans* colonizing a root hair. Image is a 13 μm stack, taken 24 hpi. (**b**) 14 μm stack image of *K. radicincitans* root surface colonization 48 hpi. (**c**) 13 μm stack of root surface 6 dpi. (**d**) Image showing a 36 μm stack taken 6 dpi of *K. radicincitans* colonizing the junctions of a lateral root primordium pushing through the epidermis. (**e**) *K. radicincitans* colonizing the junctions of a lateral root 6 dpi. (**f**) Very infrequently single cells are completely colonized by *K. radicincitans*. The image shows a 20 μm stack at 6 dpi. Scale bar: 20 μm

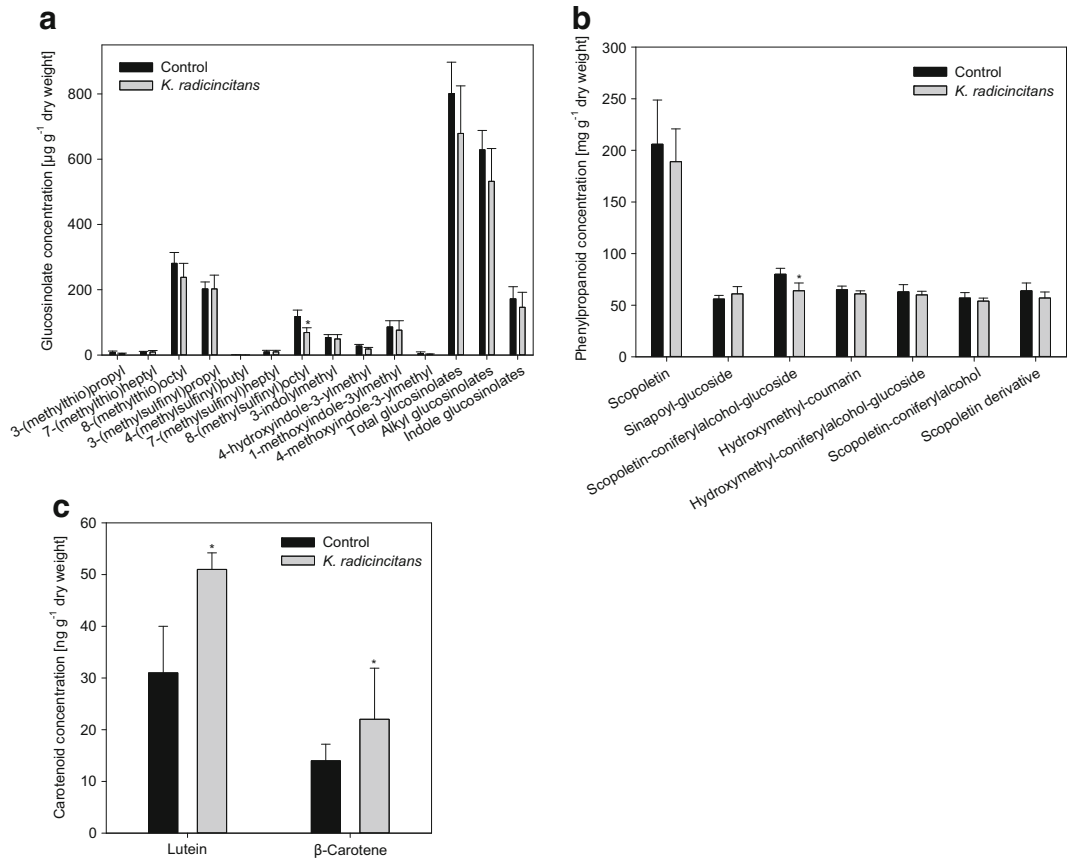

Fig. 3 The decline in glucosinolates (**a**) and phenylpropanoids (**b**) and the induction of carotenoids (**c**) by *K. radicincitans* colonization in *A. thaliana* roots. Values are given as mean ± standard error of three independent experiments, where each mean was derived from two technical replicates. Asterisks indicate statistical differences between inoculated and non-inoculated plants ($p < 0.05$)

coniferylalcohol, a scopoletin derivative) and hydroxymethyl-coumarin, represented the largest quantitative group. In addition to that, cinnamic acids sinapoyl-glucoside and hydroxymethyl-coniferylalcohol-glucoside were detected. The levels of most compounds were lower in the inoculated plants and a statistically significant reduction was found for scopoletin-coniferylalcohol-glucoside (Fig. 3b).

The lowest compound concentration was observed for carotenoids, which were analysed by UPLC-MS. The Oy-0 root contained α-lutein and β-carotene and, in response to the inoculation, both levels increased significantly (Fig. 3c).

K. radicincitans alters the rhizosecretion profile in Arabidopsis

The consequences of the widespread changes to plant secondary metabolites induced by the *K. radicincitans* colonization of roots were next explored with regard to compounds released into the rhizosphere by non-targeted analyses. GC-EI-Q-MS-based metabolite profiling revealed 32 compounds that were significantly affected ($p < 0.05$) by the presence of *K. radicincitans* (Table 1). Of these, 24 compounds were identified based on best mass spectral and retention index match (reverse match >500, retention index deviation <0.5%), and were shown to comprise several nucleobases, amino acids, organic acids, carbohydrates, polyols and phenylpropanoids. Except for lactic acid, all metabolites were reduced by inoculation. A parallel non-targeted LC-MS-based metabolite profiling approach revealed 1398 out of 3915 differentially affected ($p < 0.05$) unique mass-to-charge retention time pairs in positive ionization mode and 670 out of 2955 differential ones in negative ionization mode. The acquisition of collision-induced dissociation mass spectra of quasi-molecular ions, the application of on-column H/D chromatography

Table 1 Differentially accumulated primary plant metabolites in the root exudate following colonization by *K. radicincitans*

RI	*m/z*	Annotation	Substance class	*p*-Value	B/C
1884	264	Adenine (2TMS)	Nucleobase	1.3E-02	0.6
2150	352	Guanine (3TMS)	Nucleobase	2.9E-03	0.3
1825	265	Hypoxanthine (2TMS)	Nucleobase	3.3E-03	0.3
2047	353	Xanthine (3TMS)	Nucleobase	7.9E-03	0.4
2620	217	Inosine (4TMS)	Nucleoside	6.6E-03	0.5
1383	141	3-Cyanoalanine (2TMS)	Amino Acid	7.4E-03	0.7
1537	232	Aspartic acid (3TMS)	Amino Acid	2.1E-03	0.4
1304	158	Isoleucine (2TMS)	Amino Acid	4.5E-02	0.5
1281	158	Leucine (2TMS)	Amino Acid	4.7E-02	0.6
1533	176	Methionine (2TMS)	Amino Acid	2.8E-02	0.6
1841	142	Ornithine (3TMS)	Amino Acid	1.8E-02	0.1
1649	192	Phenylalanine (2TMS)	Amino Acid	2.1E-02	0.5
1849	273	Citric acid (4TMS)	Organic Acid	4.4E-02	0.7
1353	245	Fumaric acid (2TMS)	Organic Acid	1.0E-04	0.3
1345	292	Glyceric acid (3TMS)	Organic Acid	4.3E-03	0.7
1070	**117**	**Lactic acid (2TMS)**	**Organic Acid**	**7.6E-03**	**1.4**
1503	233	Malic acid (3TMS)	Organic Acid	5.7E-03	0.7
2825	361	Cellobiose (1MeOX 8TMS)	Carbohydrate	4.3E-02	0.7
1959	160	Glucose (1MEOX) (5TMS) BP	Carbohydrate	4.1E-02	0.6
2124	205	N-Acetylglucosamine (1MeOX) (4TMS)	Carbohydrate	6.3E-03	0.3
1684	217	Xylose (1MEOX) (4TMS)	Carbohydrate	3.1E-02	0.6
1715	307	Ribose (1MEOX) (4TMS)	Carbohydrate	1.5E-02	0.6
1794	357	Glycerol-3-phosphate (4TMS)	Organophosphate	1.3E-02	0.5
2062	234	Scopoletin (1TMS)	Coumarin	9.4E-03	0.5

All compounds decreased in abundance as a result of colonization, except for lactic acid (2TMS) as highlighted in bold. Numbers shown in parentheses refer to the number of functional groups remaining after derivatization

TMS trimethylsilylated, *MeOX* methoxymated, *B/C* mean ratio between the median intensity of inoculated and non-inoculated plants, calculated from three independent experiments

and a comparison with mass spectral data from literature (Strehmel et al. 2014) successfully resulted in annotating 32 compounds (Table 2). The metabolites were linked to amino acids, dipeptides, aliphatic glucosinolate precursor amino acids, aliphatic glucosinolate degradation products, phenylpropanoids and diverse fatty acid derivatives, among others. The compounds associated to either glucosinolate or phenylpropanoid metabolisms were reduced in concentration by the inoculation, while fatty acid metabolites were enriched.

In vitro effects of secondary metabolites on *K. radicincitans* growth

Several plant secondary metabolites showed an altered accumulation pattern in response to *K. radicincitans* inoculation. To test, whether this is a result of the plant's adaptation to bacterial growth or it is a consequence of bacterial metabolic requirements, pure bacterial cultures were supplemented with pure representative compounds of glucosinolates (2-propenyl), carotenoids (lutein, β-carotene) and phenylpropanoids (scopoletin, sinapic acid). A fourth class of compounds was included to the assay and these were terpenes (squalene, α-humulene, farnesene) that also influence biotic interactions (Tholl 2015). Inhibitory effects on bacterial growth were observed for 2-propenyl, scopoletin, α-humulene and lutein (Fig. 4), while presence of sinapic acid, squalene and farnesene had no influence on *K. radicincitans* in vitro cell density. A beneficial effect was observed for β-carotene in the medium. Here, the highest supplied concentration of 100 μM resulted in an increased bacterial

Table 2 Differentially accumulated secondary plant metabolites in the root exudate following colonization by *K. radicincitans*

Metabolite	RT [s]	Elemental composition	*m/z*	Quantifier ion	# acidic protons	Substance class	B/C	*p*-Value
Phe	76	$C_9H_{11}NO_2$	166.09	[M + H]	3	Aromatic amino acid	0.6	3.6E-04
Tyr	43	$C_9H_{11}NO_3$	182.08	[M + H]	4	Aromatic amino acid	0.8	1.0E-02
Trp	144	$C_{11}H_{12}N_2O_2$	205.1	[M + H]	4	Aromatic amino acid	0.6	1.0E-03
Tetrahomomethionine	221	$C_9H_{19}NO_2S$	206.12	[M + H]	3	Aliphatic GLS precursor amino acid	0.4	4.9E-03
Pentahomomethionine	277	$C_{10}H_{21}NO_2S$	220.14	[M + H]	3	Aliphatic GLS precursor amino acid	0.3	2.0E-03
Methylthiooctanoic acid	489	$C_9H_{18}O_2S$	173.1	[M + H-H_2O]	1	Aliphatic GLS degradation product	0.4	4.1E-04
Methylthiononanoic acid	545	$C_{10}H_{20}O_2S$	203.11	[M-H]	1	Aliphatic GLS degradation product	0.2	3.7E-04
			187.12	[M + H-H_2O]	1		0.3	1.1E-04
Methylthioheptylamine	257	$C_8H_{19}NS$	162.13	[M + H]	2	Aliphatic GLS degradation product	0.3	5.1E-04
Methylthiooctylamine	311	$C_9H_{21}NS$	176.15	[M + H]	2	Aliphatic GLS degradation product	0.3	3.3E-03
Scopoletin	268	$C_{10}H_8O_4$	193.05	[M + H]	1	Coumarin	0.5	5.6E-06
			191.04	[M-H]	1		0.6	2.9E-03
Scopoletin-coniferylalcohol	290	$C_{20}H_{20}O_8$	389.12	[M + H]	3	Dilignol	0.7	7.7E-04
Hydroxymethylcoumarin-coniferylalcohol	275	$C_{20}H_{20}O_7$	355.12	[M + H-H_2O]	3	Dilignol	0.4	3.8E-04
H-Leu-Pro-OH	154	$C_{11}H_{20}N_2O_3$	229.15	[M + H]	3	Dipeptide	0.8	3.8E-03
H-Phe-Gly-OH	102	$C_{11}H_{14}N_2O_3$	223.11	[M + H]	4	Dipeptide	0.7	2.4E-03
H-Leu-Leu-OH	**218**	**$C_{12}H_{24}N_2O_3$**	**245.19**	**[M + H]**	**4**	**Dipeptide**	**2.3**	**1.7E-02**
H-Leu-Phe-OH	**250**	**$C_{15}H_{22}N_2O_3$**	**279.17**	**[M + H]**	**4**	**Dipeptide**	**1.7**	**5.3E-02**
H-Tyr-Ile-OH	175	$C_{15}H_{22}N_2O_4$	295.17	[M + H]	5	Dipeptide	0.7	**3.5E-02**
Pantothenic acid	93	$C_9H_{17}NO_5$	220.12	[M + H]	4	Vitamin	0.7	1.6E-02
Nicotinic acid	41	$C_6H_5NO_2$	124.04	[M + H]	1	Vitamin	0.6	3.3E-042
Uridine	42	$C_9H_{12}N_2O_6$	243.06	**[M-H]**	4	Nucleoside	0.6	**3.6E-03**
Adenosine	46	$C_{10}H_{13}N_5O_4$	268.11	[M + H]	5	Nucleoside	0.5	**8.9E-03**
2′-O-Methyladenosine	68	$C_{11}H_{15}N_5O_4$	282.12	[M + H]	4	Nucleoside	0.3	**3.2E-03**
Undecanedioic acid	**411**	**$C_{11}H_{20}O_4$**	**215.13**	**[M-H]**	**2**	**Fatty Acid Derivative**	**1.7**	**2.0E-03**
Hydroxyundecanoic acid	**424**	**$C_{11}H_{22}O_3$**	**201.15**	**[M-H]**	**2**	**Fatty Acid Derivative**	**2.4**	**6.1E-04**
Oxodecenoic acid	**444**	**$C_{10}H_{16}O_3$**	**207.1**	**[M + Na]**	**1**	**Fatty Acid Derivative**	**3.2**	**5.2E-05**
			183.1	**[M-H]**	**1**		**4.1**	**2.0E-05**
Tetradecadienedioic acid	**496**	**$C_{14}H_{22}O_4$**	**253.15**	**[M-H]**	**2**	**Fatty Acid Derivative**	**1.5**	**4.9E-03**
Hexoside of $C_{13}H_{12}O_4$	**191**	**$C_{19}H_{22}O_9$**	**395.14**	**[M + H]**	**4**	**Hexoside**	**1.2**	**7.7E-03**
Indolic compound	**206**	**$C_{13}H_{17}N_3O_2$**	**248.14**	**[M + H]**	–	**Indolic**	**1.7**	**6.0E-03**
4-MeO-I3CH_2NH_2	194	$C_{10}H_{12}N_2O$	160.08	[M + H-NH3]+	1	Indolic	0.6	1.0E-03
6-(Malonyl-GlcO)-I3CO_2H	**195**	**$C_{18}H_{19}NO_{11}$**	**380.1**	**[M-H-CO2]**	**6**	**Indolic**	**0.5**	**2.4E-03**
Sinapic acid derivative	**223**	**$C_{20}H_{32}O_{10}$**	**431.19**	**[M-H]**	**6**	**Phenylpropanoid**	**2.3**	**6.7E-05**
p-Coumaric acid	260	$C_9H_8O_3$	165.05	[M + H]	2	Phenylpropanoid	0.7	2.3E-4

Those enhanced by the colonization are highlighted in bold. The associated mass spectral data are provided in Supplemental Table S3. B/C: mean ratio between the median intensity of inoculated and non-inoculated plants, calculated from three independent experiments; *GLS* glucosinolate

growth, which was 34% higher as compared to the control treatment, indicating that β-carotene might be metabolized by the bacterium or stimulate growth by other means.

Bacterial colonisation is dependent on presence of specific plant secondary metabolites

To further define the role of secondary plant metabolites in the interaction with *K. radicincitans*, we tested the bacterial colonisation of plants disturbed in root secondary metabolite accumulation or synthesis: *gtr1gtr2*, *sds1*, *ccoamt1*, *f6'h1*. The double knockout of glucosinolate transporters 1 and 2, *gtr1gtr2*, accumulates one third of the wildtype amount of glucosinolates in roots and consequently releases lower amounts into the rhizosphere (Andersen et al. 2013; Xu et al. 2016). Solanesyl diphosphate synthase 1 (sds1) is involved in the synthesis of plastochinone (Liu and Lu 2016), an essential component of phytoene desaturation and

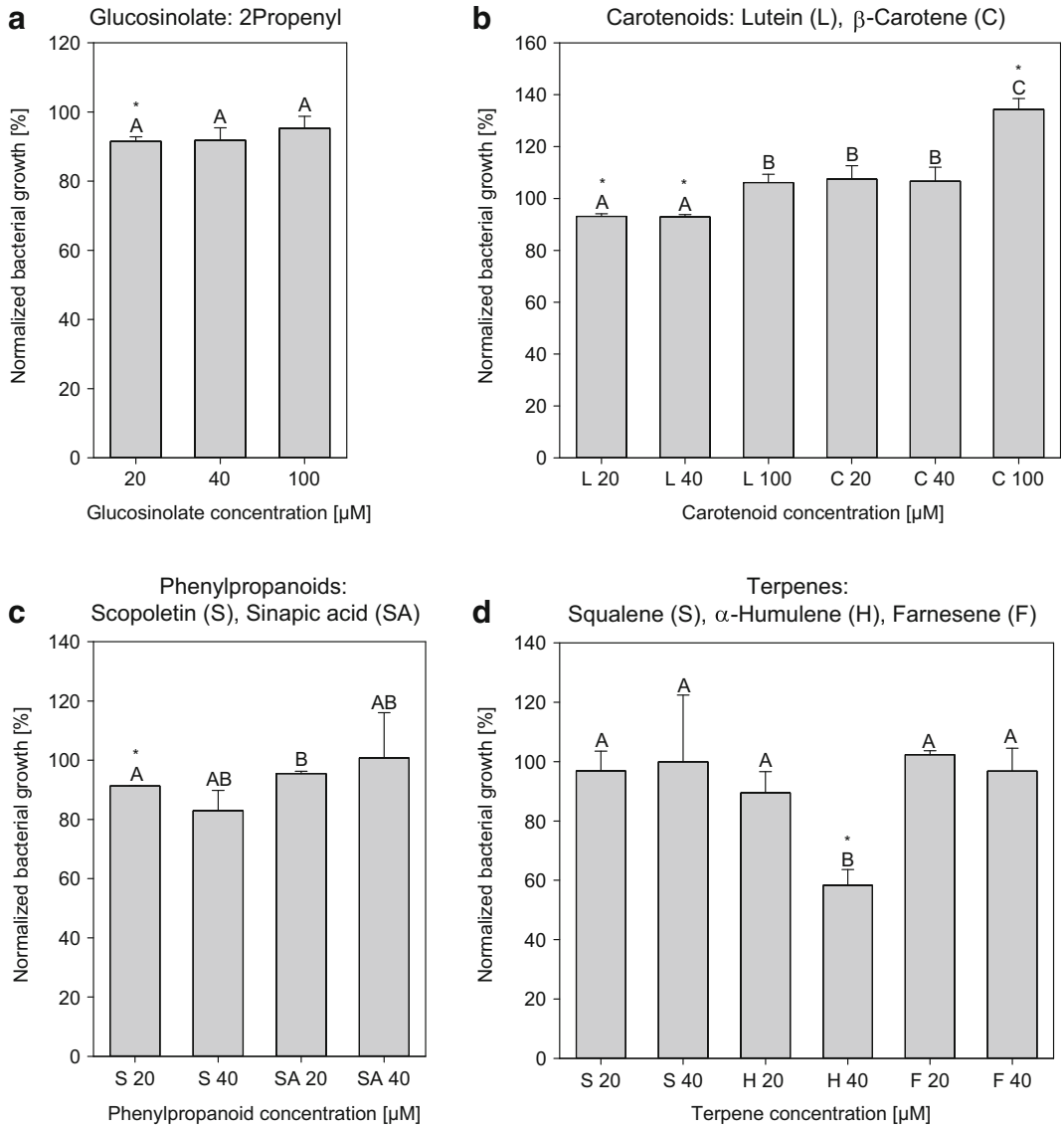

Fig. 4 Response of *K. radicincitans* growth to plant secondary metabolites. Pure liquid cultures were supplemented with standard compounds of glucosinolate (**a**), carotenoids (**b**), phenylpropanoids (**c**) and terpenes (**d**). Bacterial growth was measured after 15 h and presented is the mean of two independent experiments ± standard error, related to the respective control. Capital letters denote significant difference among supplemented compounds and asterisks indicate statistical differences between control samples and supplemented samples ($p < 0.05$)

therefore crucial for carotenoid biosynthesis (Chao et al. 2014). The *sds1* knockout accumulates strongly reduced levels of carotenoids in leaves and roots (Supplemental Fig. S3). Caffeoyl-CoA-O-methyltransferase 1 (CCoAOMT1) is involved in lignin biosynthesis (Vanholme et al. 2012) and feruloyl-CoA-6-hydroxylase 1 (F6'H1) catalyses the synthesis of scopoletin which acts as iron chelator (Fourcroy et al. 2014) or phytoalexin (Sun et al. 2014). The root colonisation was assessed four days after inoculation by qPCR method and revealed considerable lower levels of *K. radicincitans* DNA on roots of *sds1* and *ccoaomt1* (Fig. 5). Levels of bacterial DNA were slightly higher on *gtr1gtr2* and *f6'h1* plants, but these changes were not statistically significant. No qPCR signals for *K. radicincitans* genomic DNA were obtained in samples from non-inoculated plants (not shown).

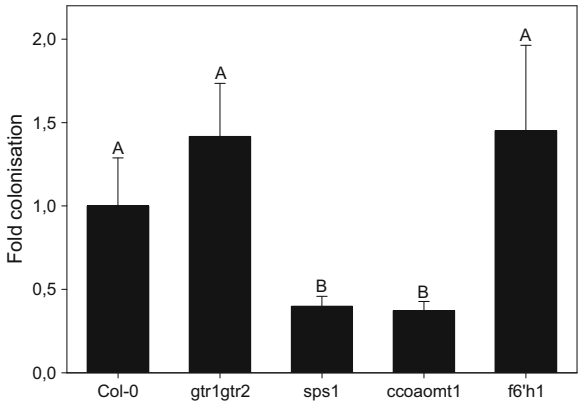

Fig. 5 *K. radicincitans* colonization of the roots of *A. thaliana* knock-out lines four days after inoculation, as detected by qPCR. Values represent the mean ± standard error ($n = 3$) of expression ratios ($2^{-\Delta\Delta CT}$), normalized to two reference genes and to the inoculated Col-0. Capital letters denote significant differences between inoculated genotypes ($p \leq 0.05$)

Discussion

PGPB can contribute significantly to both crop productivity and plant health, but as yet modulation of the host's physiology is poorly understood. Our results demonstrate that the plant genotype determines whether *K. radicincitans* induces a growth promotion in *A. thaliana*. This natural variation within a plant species in the response to PGPB inoculation was also reported earlier for 196 *A. thaliana* accession after *P. fluorescens* inoculation (Haney et al. 2015), a collection of 302 *A. thaliana* accessions tested with *P. simiae* (Wintermans et al. 2016) as well as for 40 *Brachypodium distachyon* accessions colonized by *A. brasilense* or *H. seropedicae* (do Amaral et al. 2016). The host genotypes Oy-0 and Ler-0 were most responsive, but diametrically opposed, to *K. radicincitans* inoculation, whereas the eGFP-based localisation assay revealed that roots of both accessions were well colonized by *K. radicincitans*. This effect has been found before (do Amaral et al. 2016) and demonstrates that growth promotion is a plant genotype-specific response to bacterial inoculation.

The host genotype governs the interaction with rhizobacteria through plant secondary metabolites present in the root and exuded to the rhizosphere (Drogue et al. 2012). Hence, we profiled major plant secondary metabolites in Oy-0 that may be positive regulators of this interaction (summarized in Fig. 6). We found a general decline in the concentration of root glucosinolate and phenylpropanoids in response to bacterial colonisation with significant differences for 8-(methylsulfinyl)octyl glucosinolate and scopoletin-coniferylalcohol-glucoside. Concomitant to this, levels of glucosinolate breakdown products and scopoletin-coniferylalcohol were lower in root exudates of inoculated plants. Glucosinolates are active in counteracting pathogen invasion (Brader et al. 2006), especially in their hydrolysed form (Kliebenstein 2004; Osbourn 1996). A regulatory role in structuring the rhizosphere microbial community was proposed for glucosinolates using transgenic *A. thaliana* (Bressan et al. 2009). The glucosinolate concentration of *Brassica napus* roots determined the colonisation density of the rhizobacterium *Azorhizobium caulinodans* (O'Callaghan et al. 2000). We showed that growth in the presence of different levels of 2-propenyl glucosinolate had no beneficial effects on cell density, while short-term bacterial root colonisation of *gtr1gtr2* was slightly higher as compared to the wildtype, indicating that lower root glucosinolate levels might be favourable for bacterial colonisation. Comparative studies using *A. thaliana* transgenic lines over-accumulating or being devoid of glucosinolates should contribute to our knowledge on the role of this metabolite class in plant-bacteria interactions.

Plant phenylpropanoids are intimately involved in pathogen- and oxidative stress defence. Scopoletin inhibits pathogen growth (Peterson et al. 2003), while scopolin, the glycosylated (inactive) form of scopoletin, promotes the growth of some fungi, and inhibits it in others (Ojala et al. 2000). Our study demonstrated the

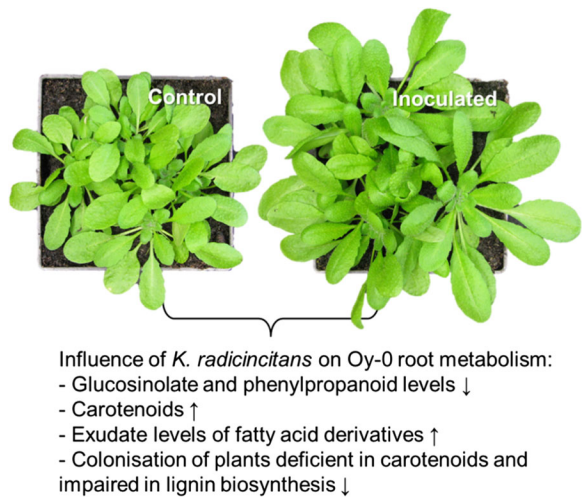

Fig. 6 Schematic representation of metabolic alterations in *A. thaliana* Oy-0 roots provoked by *K. radicincitans*

reduced presence of specific phenylpropanoids in root exudates and in the root itself. In order to test whether this might reflect the growth requirements of *K. radicincitans*, scopoletin and sinnapic acid were supplemented to the bacterial suspension. As for glucosinolates, their moderate inhibitory effects indicate that both substance classes cannot be metabolized by the bacterium. Hence, the decline upon plant inoculation is rather plant-driven than the result of bacterial metabolism and may be linked to the diverting of assimilates to primary metabolism. Short-term bacterial colonisation of *ccoaomt1* was significantly lower as compared to Col-0 and *f6'h1*, indicating that presence of downstream products of *ccoaomt1* are positive regulators for bacterial colonisation. However, no such correlation is established yet and it remains a hypothesis whether structural changes of cell wall or content of flavonol glycosides (Do et al. 2007) account for these observed effects.

Carotenoids are associated with light-harvesting, photoprotection, photosensing and antioxidant protection in the leaf, while their degradation products control biotic interactions in roots (Walter et al. 2010). Reduced apocarotenoid levels in transgenic tomato had negative effects on root colonisation with arbuscular mycorrhizal fungi (AMF) (Kohlen et al. 2012) and C_{13} apocarotenoids act to support the functionality of the AMF symbiosis during later stages of interaction (Walter 2013). The inoculation of *A. thaliana* with *K. radicincitans* raised the carotenoid content of the root, and in vitro testing revealed that β-carotene increases bacterial cell density. Whether β-carotene is metabolized and it's cleavage products act as signalling compounds or if the antioxidant properties play a role in the plant-bacterium interaction, remains to be determined. Our findings, that bacterial colonisation of *sds1* was reduced as compared to the wildtype Col-0, strengthens the assumption that carotenoid-derived compounds play an important role in establishing *K. radicincitans* colonisation in *A. thaliana*.

The root exudates of inoculated plants contained less carbohydrate, amino acid, organic acid and nucleobase, as was also the case in both tobacco and groundnut colonized by *Bacillus cereus* (Dutta et al. 2013), and in tomato colonized by *P. fluorescens* (Kamilova et al. 2006). When supplied with tomato root exudates, *P. fluorescence* WCS365 reduces the amounts of sugars, especially ribose and glucose, and of organic acids, such as citric acid, malic acid and fumaric acid (Kamilova et al. 2006). The PGPB *Enterobacter* sp. strain 638 requires the host to supply glucose, cellobiose or xylose as a source of carbon (Taghavi et al. 2010), and in vitro cultured *K. radicincitans* metabolizes these same carbohydrates (Kämpfer et al. 2005). Therefore, a reduction in the exudate of these metabolites in PGPB colonized roots could reflect their metabolisation by the bacteria. Another explanation for altered root exudation pattern could be a PGPB-mediated reduction in exudation, as shown for potato inoculated with rhizobacteria (Belimov et al. 2015).

Only a limited number of metabolites was enriched in the root exudate of the inoculated plants, e.g. lactic acid. In soybean roots, infection with *Bradyrhizobium japonicum* has been shown to promote the lactic acid content of the root hair (Brechenmacher et al. 2010), while lactic acid is also accumulated in the *Sinorhizobium meliloti* nodules attached to the alfalfa root (Barsch et al. 2006). The function of lactic acid accumulation during symbiotic interactions is vague, and in particular, it is unclear whether the lactic acid is produced by the host and/or by the bacterium. Four derivatives of the fatty acids were prominent in the inoculated *A. thaliana* root exudate: the hydroxylated and oxidized form of the dicarboxylic undecanoic acid, the monounsaturated oxodecenoic acid and the unsaturated long-chain tetradecadienedioic acid. Fatty acids are involved in cell membrane composition, membrane trafficking and signal transduction in the plant cell. However, they may also have a role in extracellular communication with microorganisms, as shown for example by their ability to disrupt the biofilms formed by soil bacteria and fungi (Davies and Marques 2009). Tetradecanoic acid is synthesized by both *P. putida* and *P. aeruginosa*, and is also present in root exudates of maize (Fernandez-Pinar et al. 2012). This fatty acid is known to activate the expression of *ddcA*, a gene required for the colonization of both the seed and root by *P. putida* (Espinosa-Urgel and Ramos 2004). The Brechenmacher et al. (2010) study of the soybean metabolome also demonstrated the increased abundance of six different fatty acids in the root hair, but not in stripped roots, during the early phase of the root's interaction with *B. japonicum*. The data suggest that specific fatty acid derivatives might be involved in the rhizosphere communication, which could also hold true for *K. radicincitans*.

In conclusion, the exploitation of beneficial microbes in the context of devising a more sustainable regime of crop fertilization will require an in-depth understanding

of how PGPBs interact with the host. We have reported here how colonization of *A. thaliana* by *K. radicincitans* altered the root exudation pattern and the profile of secondary metabolites accumulated in a host with a high level of growth promotion. Some of these metabolites are known to function as a carbon source for bacteria, while for others, no role in the plant-bacteria interaction has as yet been established. Furthermore, an increasing number of studies demonstrate the genotype-specific diversity of the host-associated microbiome and its impact on plant health (Haney et al. 2015; Schlaeppi et al. 2014; Zachow et al. 2014). Hence, it can be assumed that also microbes present in the endosphere or rhizosphere affect the plant-growth-promoting ability of *K. radicincitans*. Wheat inoculated with *K. radicincitans* was stronger colonized when plants were grown in sterilized soil as compared to non-sterilized soil and four tested wheat cultivars revealed different intensity levels of colonisation (Remus et al. 2000). Studies on the genotype-specific constitution of the plant microbiome and its influence on PGPB efficiency will contribute to our understanding of the complex plant-microbiome interaction.

Acknowledgements The technical assistance of Sabine Breitkopf, Andrea Jankowsky, Sylvia Krüger, Annett Platalla, Birgit Wernitz and Sieglinde Widiger is gratefully acknowledged. We thank Christoph Böttcher (Leibniz Institute of Plant Biochemistry, Germany) for the gift of leucylproline standard, Ellen Lagendjik (Leiden University, Netherlands) for provision of eGFP plasmid pMP4655, and Stefanie Döll (Leibniz Institute of Plant Biochemistry, Germany), Barbara Halkier and Deyang Xu (University of Copenhagen, Denmark) for transgenic Arabidopsis lines. This work was supported by the German Leibniz association (PAKT project 'Chemical Communication in the Rhizosphere', SAW-2011-IPB-3).

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