Transcriptional regulation of plant sugar transporter genes by beneficial rhizobacteria

Antoine Desrata, Florence Thibaultb, Jesús Mercado-Blancob, Pierre Coutos-Thévenota and Cécile Vrieta,∗

In their natural environment, plants live in close interaction with complex populations of microorganisms, including rhizobacteria species commonly referred to as ‘Plant Growth Promoting Rhizobacteria’ (PGPR). A growing body of evidence demonstrates the importance of sugar transport in plant pathogen resistance and in plant-microorganism mutualistic symbioses. Using an in vitro experimental system, including the model plant species Arabidopsis thaliana, two PGPR strains (Pseudomonas simiae PICF7 and Burkholderia phytofirmans PsJN) and a non-PGPR strain (Escherichia coli), we conducted a comprehensive set of phenotypic and gene expression analyses to explore the role and regulation of sugar transporter genes in plant-PGPR interactions. In physical contact with the seedling roots, or solely via the emission of bacterial volatile compounds, the two PGPR strains tested improved the growth and development of the Arabidopsis seedlings and altered the expression of several plant sugar transporter genes. Our results also revealed both conserved and strain-specific transcriptional regulation mechanisms.

1. Introduction

A growing body of evidence in the literature demonstrates that pathogens are able to manipulate the sugar transport machinery of the host plant to increase the efflux of sugar toward them (Bezrutczyk et al. 2018). Notably, several studies shed light on the capacity of some leaf pathogens to gain sugars from the plant cells by upregulating the expression of genes coding for sugar facilitator proteins of microorganisms, including rhizobacteria species commonly referred to as ‘Plant Growth Promoting Rhizobacteria’ (PGPR). A growing body of evidence demonstrates the importance of sugar transport in plant pathogen resistance and in plant-microorganism mutualistic symbioses. Using an in vitro experimental system, including the model plant species Arabidopsis thaliana, two PGPR strains (Pseudomonas simiae PICF7 and Burkholderia phytofirmans PsJN) and a non-PGPR strain (Escherichia coli), we conducted a comprehensive set of phenotypic and gene expression analyses to explore the role and regulation of sugar transporter genes in plant-PGPR interactions. In physical contact with the seedling roots, or solely via the emission of bacterial volatile compounds, the two PGPR strains tested improved the growth and development of the Arabidopsis seedlings and altered the expression of several plant sugar transporter genes. Our results also revealed both conserved and strain-specific transcriptional regulation mechanisms.

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CONTACT Cécile Vrieta

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nutrients. However, little is known about the underlying molecular mechanisms and volatile compound receptors involved in these biological processes (Finchera and Quiroz 2018; Sharifi and Ryu 2018). Many PGPR species also enhance the host plant tolerance to various abiotic stress and/or display a biocontrol activity against a wide range of soil pathogens via direct antagonism mechanisms involving the production of antimicrobial substances (some of them volatile) or enzymes and/or by competition for nutrients (Vacheron et al. 2013). Lastly, PGPR can also trigger induced systemic resistance (ISR) which stimulates plant defenses against leaf pathogens (Pietere et al. 2014). A large and diverse set of rhizobacteria have been reported for these beneficial effects on plant productivity. Several strains of the genera Pseudomonas and Bacillus, in particular, have been widely investigated as promising PGPR for application in agriculture (Hashem et al. 2019; Hakim et al. 2021). In the light of recent evidence that demonstrates the importance of sugar transport in plant microorganism interactions, changes in the plant carbon fluxes and sugar transporter activities may also play a key role in plant-PGPR interaction and their beneficial effects on plant productivity. A better understanding of the molecular mechanisms involved in these biological processes may help to expand the application of PGPR in sustainable agriculture.

With the aim to identify genes playing an essential role in the efficiency of plant-PGPR interaction, and investigate the importance of plant sugar transport in this biological process, we earlier designed (Desrut et al. 2020) an in vitro experimental system enabling the analysis of PGPR modes of action and molecular mechanisms involved in their beneficial effects on plant growth and development. Using this system, the model plant Arabidopsis thaliana (thereafter named Arabidopsis) was co-cultivated with the well-characterized PGPR strain Pseudomonas simiae WCS417r (PsWCS417r), and a comprehensive set of phenotypic and gene expression analyses (by RNA-sequencing and qRT-PCR) was carried out. Results from this previous study revealed that this strain induces major transcriptional changes of several plant sugar transporter genes. Using a reverse genetic approach, we also demonstrated that two of them, SWEET11 and SWEET12, were functionally involved in the PGPR-triggered plant growth-promoting effects (Desrut et al. 2020).

In order to select additional candidate genes for functional characterization among the sugar transporter genes transcriptionally regulated in Arabidopsis by PsWCS417r (Desrut et al. 2020), and to identify conserved and specific molecular mechanisms of plant growth promotion and sugar transport regulation among PGPR, we aim now to extend this work to two other PGPR strains (Pseudomonas simiae PICF7 and Burkholderia phytofirmans PsJN) and a non-PGPR strain (Escherichia coli DH5α). Overall, our results reveal that the PGPR strains PICF7 and PsJN are able, like PsWCS417r, to alter the expression of several plant sugar genes (essentially genes of the SWEET and ERD6-like families) in both experimental conditions tested: (i) when the seedling roots were inoculated with the PGPR, and (ii) via the emission of volatile compounds only. Noteworthy, the E. coli strain also produced volatile compounds with phytobeneficial effects but did not induce any plant growth-promoting effects in physical contact with the seedling roots. Moreover, our results reveal both conserved and bacterial strain-specific transcriptional regulation of plant sugar transport.

2. Results

2.1. Plant growth-promoting activities of PICF7, PsJN and E. coli DH5α in physical contact with seedling roots

To study the plant growth-promoting effects of the PGPR strains PICF7 and PsJN using our in vitro experimental system, Arabidopsis seedlings were grown axenically on MS medium (0.5X, 0.5% MES, no sucrose) prior their inoculation. Five day old seedlings were inoculated with the bacterial strains and seven days post inoculation their shoot and root fresh weight were measured, and their root architecture parameters were analyzed. Under this experimental condition (PGPR in physical contact with the seedling roots), which included the putative action of diffusible and volatile substances, both strains displayed plant growth-promoting properties, but to a different extent (Figure 2). PICF7 significantly (P < .0001) enhanced the shoot and root biomass of the seedlings (76% and 228% increase, respectively), their lateral root length and number (584% and 254% increase, respectively) and significantly increased (P < .001) their root hair length and density (300% and 373% increase, respectively) in comparison to the control condition (Mock treatment). In contrast, PsJN displayed milder positive, yet significant (P < .01) effects, on the seedling shoot biomass (26%) in comparison to the mock treatment. PsJN also significantly (P < .0001) enhanced the root biomass (147% increase), the primary root length (55% increase), the lateral root length (225%) and the root hair density (126% increase) of the inoculated seedlings, but had non-significant effect on the lateral root number and root hair length parameters (Figure 2 and Table S1). In the same experimental conditions that the one described above, we also evaluated the plant growth-promoting effects of a non-PGPR strain, E. coli DH5α. In physical contact with the seedling roots, E. coli DH5α did not trigger any major plant growth-promoting effects (Figure 2 and Table S1).

2.2. Plant growth-promoting activities of PICF7, PsJN and E. coli DH5α volatile compounds

Volatile compounds produced by PICF7 and PsJN may also be involved in the phytostimulatory effects observed. In order to investigate their effects alone, we set up a second in vitro experimental system in which the PGPR strains were physically separated from the Arabidopsis seedlings (Figure 2). In this condition in which the phenotypic effects observed could only be triggered by the bacterial volatile compounds, those emitted by the PGPR strains PICF7 and PsJN led to marked and significant (P < .0001) beneficial effects on the seedling shoot (322% and 209% increase, respectively) and root fresh weight (587% and 415% increase, respectively). In addition, both PICF7 and PsJN volatile compounds had a significant (P < .0001) influence on the lateral root length (5 and 12.6 fold increase, respectively) and number (486% and 340% increase, respectively), and significantly (P < .01) increased the root hair density of inoculated plants (121% and 64% increase, respectively) in comparison to the control condition (Mock) (Figure 2 and Table S2). Via the production of volatile compounds only, E. coli DH5α also significantly (P < .0001) enhanced the shoot and root biomass (278% and 473% increase, respectively), increased the lateral root length and number (452% and 456% increase,
respectively) of the seedlings, and significantly increased ($P < .05$) their root hair density (34% increase) in comparison to the control (Mock treatment). Lastly, all three strains emitted volatile compounds that significantly ($P < .0001$) enhanced the primary root length in comparison to the mock-treated condition (Figure 2 and Table S2).

2.3. The strains PICF7 and PsJN and E. coli DH5α transcriptionally regulate several plant sugar transporter genes

In a previous study in which genome-wide and targeted gene expression analyses (by RNA-sequencing and qRT-PCR)
were performed, 14 sugar transporter genes (among the 79 ones present in the Arabidopsis genome) were found transcriptionally regulated 7 days post inoculation of five-day-old Arabidopsis seedlings with the PGPR PsWCS417r (Desrut et al. 2020). All these genes, except one (an inositol transporter gene, INT2) belong to the SWEET and ERD6-like gene families of sugar transporters. These genes were differentially expressed in the mock- and PGPR-treated conditions, either in roots (SWEET3, SWEET11, SWEET12, ERD6-like13, ERD6-like15, and ERD6-like18) or in shoots.
SWEET2, SWEET4, SWEET10, SWEET15, ERD6-like7, ERD6-like12, ERD6-like16 and INT2). Among them, only three genes (ERD6-like13, ERD6-like15 and SWEET10) were found up-regulated, and all the others were down-regulated in response to inoculation with PsWCS417r.

In order to identify strain-specific and strain-conserved transcriptional changes, and help prioritize functional characterization studies, we carried out a gene expression analysis by qRT-PCR in both roots and shoots of Arabidopsis seedlings inoculated with PICF7, PsJN, and E. coli DH5α on all the candidate genes identified from a previous study in response to PsWCS417r (Desrut et al. 2020). Data from this analysis are presented in Table S3 and S4, and summarized in Figure 3 as well.

Our results reveal that several of these sugar transporter genes are up- or down-regulated by all three strains in addition to WCS417r, either in physical contact condition with the seedling roots (SWEET4 and INT2 in shoot), or via the emission of volatile compounds only (SWEET3, SWEET11, SWEET12, and ERD6-like18 in root, and SWEET2 in shoot), whereas the others display some strain-specific transcriptional regulation. Furthermore, the transcriptional regulation of the gene SWEET4 in the seedling shoots was specific to the root inoculation condition for all the bacterial strains tested. Lastly, it is interesting to note that the strain PICF7 triggered changes in sugar transporter gene expression very similar to those obtained in response to PsWCS417r in both experimental conditions tested (Figure 3) (Desrut et al. 2020).

3. Discussion

PICF7 and PsJN are well characterized PGPR strains (Prieto and Mercado-Blanco 2008; Poupin et al. 2013; Zuniga et al. 2013; Maldonado-González et al. 2015; Pinedo et al. 2015; Mercado-Blanco et al. 2016; Zhao et al. 2016; Montes-Osuna et al. 2021). In our experimental conditions, PICF7 displayed marked plant growth-promoting activities, both in physical contact with seedling roots and via the production of volatile compounds only (Figures 1 and 2). Note-worthy, PICF7 grew better on LB medium than on MS medium (when placed in physical contact with the seedling.
roots). Hence, a different blend and/or quantity of volatile compounds may be produced between the two experimental conditions. Nevertheless, highly similar PICF7-induced plant growth and development promoting activities were observed in both systems (Figures 1 and 2), suggesting the volatile compounds emitted by this PGPR strain contribute to a large extent to its plant growth-promoting activities. In contrast, the PGPR strain PsJN displayed relatively mild plant growth-promoting effects when the seedling roots were inoculated with this strain. In agreement with previous studies carried out on seed-inoculated Arabidopsis seedlings growing in vitro (Poupin et al. 2013; Zuniga et al. 2013), inoculation of the seedlings with PsJN mostly increased their primary root length in our experimental conditions (Figure 1). However, PsJN triggered strong positive effects on the root and shoot biomasses of the seedlings and their root architecture traits via the production of volatile compounds only (Figure 2). Lastly, the strain E. coli DH5α was used as non-PGPR control in physical contact condition in our study. As expected, only very minor changes in the seedling biomasses and root architecture traits were observed in response to inoculation of the seedling roots with this strain. In contrast, in the second experimental condition in which E. coli DH5α was physically separated from the seedlings so that only its volatile compounds could have an effect, strong plant growth-promoting effects were observed. These unexpected results are in agreement with a previous study demonstrating the phytostimulatory properties of E. coli volatile compounds on the biomass and root architecture system of Arabidopsis seedlings (Bailly et al. 2014). Importantly, the results of this study also revealed the importance of indole in the plant growth-promoting effects triggered by E. coli. Indeed, these effects were abolished in an E. coli mutant devoid of tryptophanase activity (tnaA), and thus unable to produce indole (Bailly et al. 2014). Some evidence also exists in the literature about the volatile compounds potentially implicated in the plant growth-promoting effects observed for PsJN. This PGPR strain was shown to produce the organic compounds 2-undecanone, 7-hexanol and 3-methylbutanol, and its plant growth-promoting effects could be mimicked with exposure to a blend of these three VOCs (Lederger et al. 2016). Overall, our results reveal both PsJN and E. coli DH5α mostly display beneficial effects on the seedling growth and development via the emission of volatile compounds only. Potentially, this might be due to: (i) dose-dependent response (the bacteria growth was better on LB than on MS); (ii) the presence of compounds necessary to produce the volatile substances only or at higher concentration in the LB medium than in the MS medium and/or via the root exudates; and (iii) a mix of beneficial and negative effects when the strain is in physical contact with the seedling roots.

To provide new avenues of investigation by identifying candidate genes transcriptionally regulated by these rhizobacterial strains and their volatile compounds, we also carried out a targeted gene expression analysis on a set of sugar transporter genes (essentially genes of the SWEET and ERD6-like families) earlier identified as being transcriptionally regulated by the PGPR strain PsWCS417r (Sharifi and Ryu 2018). Our results show that several of these genes are regulated by the three strains tested in this study (namely, SWEET4 and INT2 in ‘physical contact’ condition and SWEET2, SWEET3, SWEET11, SWEET12, and ERD6-like18 in ‘volatile compounds’ condition), whereas other genes displayed a strain-specific transcriptional regulation (e.g. SWEET3, SWEET11, SWEET12, ERD6-like13 and ERD6-like18 in response to PICF7 in ‘physical contact’ condition).

It is unlikely that changes in sugar transporter gene expression are solely responsible for all the PGPR-triggered plant growth-promoting effects observed. Notably, regulation of the plant hormonal pathways may also be involved (Dahmani et al. 2020; Desrut et al. 2020). Nevertheless, our results show PICF7, PsJN and DH5α volatile compounds trigger rather similar transcriptional changes for the sugar transporter genes we analyzed (i.e. 5 out of the 14 candidate genes were significantly (P < .05) repressed by all three strains, albeit to a different extent) as well as strong plant growth-promoting effects. Our results also show that the growth-promoting effects of PsJN and DH5α are rather similar and minor, in agreement with the changes they induce in plant sugar transporter gene expression upon physical contact with the roots. Notably, SWEET11 and SWEET12, two genes functionally involved in the efficiency of the interaction between PsWCS417r and Arabidopsis (Desrut et al. 2020) were significantly transcriptionally repressed in response to PICF7 in physical contact condition, and to the volatile compounds of the three strains PICF7, PsJN, and E. coli DH5α (Figure 3). Interestingly, these conditions are those in which the strongest positive effects of the bacterial strains on the seedling growth and development are observed (Figures 1 and 2). Similarly, the bacteria-triggered phytobeneficial effects observed are associated with the transcriptional down-regulation of the genes SWEET3 and ERD6-like18 in root and SWEET2 in shoot (Figures 1–3), which therefore represent good candidate genes for future functional characterization studies in plant-PGPR interactions.

Very little is known regarding the function of ERD6-like genes. In contrast, many studies have focused on the function of SWEET genes in plant-microorganism interactions in the past few years (reviewed in Chandran et al. 2015, Julius et al. 2017). Sugar transporter up-regulation may be part of the microorganism strategy to gain sugar from the host plant, as demonstrated from some SWEET genes in plant-pathogen interactions (Chandran et al. 2015; Julius et al. 2017) and in mutualistic symbioses (Manck-Gotzenberger and Requena 2016). Unexpectedly, almost all the sugar transporter genes analyzed in our study were repressed by the PGPR inoculation or by exposure to their volatile compounds. The biological significance of this finding is unknown, but it is interesting to note that similar results have been obtained in another study exploring the implication of SWEET genes in plant-pathogen interaction (Asai et al. 2016). These authors have studied the expression of all 31 tomato SWEET genes in response to infection with the necrotrophic fungal pathogen Botrytis cinerea. Among them, only one gene (SIWSEE15) was induced, and evidence suggests it may be exploited by the fungus to gain more sugars from the plant cells. Surprisingly, most of the other SWEET genes were repressed. The authors suggested these genes are positively involved in defense reactions and that their downregulation by pathogen effectors may suppress the plant host immunity (Asai et al. 2016). Alternatively, down-regulation of these SWEET genes might be part of the host defense responses aiming at reallocating
and retaining carbohydrates in plant cells to limit pathogen proliferation. Similar regulatory processes may be at play in beneficial plant-microorganism interactions. This hypothesis is supported by evidence in the literature demonstrating the importance of regulating the amount of carbohydrates available to the symbiont in arbuscular mycorrhizal (AM) symbiosis to achieve optimal benefits from the symbiosis for the host plant growth (Bitterlich et al. 2014). Besides, in a previous study profiling the expression of 35 SWEET genes in potatoes in response to inoculation with the AM fungus *Rhizopagus irregularis*, 10 of them (mostly belonging to the clade III SWEETs) were found repressed (Manck-Gotzenberger and Requena 2016). The authors suggested AM fungal colonization may be somewhat perceived as a stress for the plant that could be affecting the partitioning of sugars between roots and shoots via transcriptional regulation of these genes. Further investigations into the function of the *SWEET* genes repressed during plant-microorganism interactions are needed to test these hypotheses and establish whether their down-regulation affects sugar allocation to the symbiotic/pathogenic.

4. Materials and methods

4.1. Inoculation of Arabidopsis thaliana seedlings with the bacteria and in vitro co-cultivation assays

4.1.1. Plant material and growth conditions

*Arabidopsis thaliana* (Arabidopsis) ecotype Columbia (Col-0) was used as model plant species in this study. Arabidopsis seeds were surface sterilized and grown on half strength (0.5X) Murashige and Skoog (MS) medium (M0222, Duchefa Biochemie, Haarlem, The Netherlands), without sucrose, supplemented with 0.5% of MES (Morpholino-Ethane-Sulfonic acid monohydrate; MW=213.2 gmol⁻¹) (ACROS Organics™, 172591000) as previously described (Sharifi and Ryu 2018).

4.1.2. Bacterial strains

*Pseudomonas simiae* (originally designated *P. fluorescens*) PICF7 was isolated from olive roots (Prieto and Mercado-Blanco 2008). This strain is phylogenetically close to *P. simiae* (originally designated *P. fluorescens*) WCS417r (Gómez-Lama Cabánas et al. 2018; Montes-Osuna et al. 2021) and presents the following features: (i) a sequenced genome (Martínez-García et al. 2015); (ii) an endophytic lifestyle in olive roots and cultivated cereals, but it only colonizes the surface of Arabidopsis roots (Maldonado-González et al. 2015; Mercado-Blanco et al. 2016); and (iii) biocontrol activity against *Verticillium dahliae* in olive trees (Montes-Osuna et al. 2021), *Botrytis cinerea* in Arabidopsis (Maldonado-González et al. 2015), and *Fusarium oxysporum* f. sp. *cubense* in banana (Gómez-Lama Cabánas et al. 2021).

*Burkholderia phytofirmans* PsJN (originally designated as *Pseudomonas sp.*, thereafter named PsJN) was isolated from onion roots and is well characterized regarding its plant growth-promoting properties in potatoes, vegetables and grapevines (Sessitsch et al. 2005). In addition, PsJN enhances the seedling primary root length, accelerates the growth rate, flowering time, and enhances salt tolerance in Arabidopsis (seed inoculation) (Poupin et al. 2013; Zuniga et al. 2013; Pinedo et al. 2015; Zhao et al. 2016). Besides, this strain is known to display a biocontrol activity against *B. cinerea*, the agent of gray mould disease in grapevine (Barca et al. 2000) and in Arabidopsis (Miotto-Vilanova et al. 2016). Lastly, its genome has been sequenced (Weilharter et al. 2011; Mitter et al. 2013).

The strain *Escherichia coli* DH5α (thereafter named *E. coli* DH5α) was used as non-PGPR control for our assays with the bacteria in physical contact with the seedlings roots. Unexpectedly, however, *E. coli* has been shown to display phytostimulatory effects on maize seedlings from inoculated seeds growing in non-sterile soil (Walker et al. 2013) as well as on in vitro growing Arabidopsis seedlings exposed to its volatile compounds (Bailly et al. 2014).

4.1.3. Preparation of inoculum and inoculation treatment

For preparation of the inoculum, an aliquot of glycerol stock of the bacteria was streaked on solid KB medium (20 g.L⁻¹ Bacto™ Peptone, 1.5 g.L⁻¹ Dipotassium Phosphate, 1.5 g.L⁻¹ Magnesium Sulfate, 15 g.L⁻¹ Bacteriological agar type E, pH 7) for the strains PICF7 and PsJN, and on solid LB medium (10 g.L⁻¹ Bacto-tryptone, 5 g.L⁻¹ Yeast Extract, 5 g.L⁻¹ NaCl, 15 g.L⁻¹ Agar, pH 7) for the strain *E. coli* DH5α. After 24 h at 28°C, bacterial cells were collected in 10 mM MgSO₄, washed twice with 50 mL of 10 mM MgSO₄ by centrifugation for 5 min at 5000 g, and resuspended in 50 mL of 10 mM MgSO₄. The bacterial titer was adjusted to an OD₆₀₀ nm of 0.002 for PICF7 and *E. coli* DH5α, and 0.01 for PsJN, in order to obtain an inoculum with a bacterial density of 2×10⁸ Colony-Forming Units.mL⁻¹ (CFU.mL⁻¹). For all experiments, this bacterial density was confirmed by counting the number of CFU on LB medium.

Co-cultivation experiments of *Arabidopsis thaliana* with the different bacterial strains either in physical contact with the seedling roots and those involving solely the effects of their volatile compounds were performed as detailed in our previous studies (Desrut et al. 2020; Dahmani et al. 2020).

4.1.4. Phenotypic analyses

Shoot and root fresh weight were measured on an analytical balance. Primary, lateral root, and root hair analyses were carried out using the plugin SmartRoot (Lobet et al. 2011) of the ImageJ software (Schneider et al. 2012).

4.2. Gene expression profiling

4.2.1. Total RNA extraction

Plant samples for gene expression analysis were harvested at mid-day (8 h of light, 16 h photoperiod), 7 days post inoculation. Roots and shoots of Arabidopsis seedlings were harvested separately by sectioning the root-shoot junction, immediately frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from 25 to 100 mg of shoot and root tissues using a phenol/chloroform extraction procedure adapted from (Box et al. 2011) as previously described (Desrut et al. 2020).

4.2.2. Relative gene expression analysis by real-time quantitative RT–PCR (qRT–PCR)

Primers for qRT–PCR were designed using the NCBI Primer-Blast software (Ye et al. 2012), ideally with the following criteria: a primer size comprised between 18 and 25 bp, a GC %
of 45–60%, a melting temperature (Tm) between 58°C and 63°C, and a PCR product size of 50–200 bp. Moreover, preferences were given for primer pairs that were exon-exon shuffling or intron spanning. Sequences of the primers used in this study and efficiency of the primers for the selected candidate genes are listed in Table S5.

Gene expression analyses were performed by qRT-PCR using the GoTaq qPCR MasterMix (Promega) according to the manufacturer instructions (1X GoTaq qPCR Master Mix, 0.33 μM of forward and reverse primer, and 5 μL of 10-fold diluted cDNA per well) and as described in a previous study (Desrut et al. 2020). Target gene expression was normalized using the reference gene At4g26410 (Czechowski et al. 2005; Lemonnier et al. 2014) whose expression remained stable in all conditions evaluated (in the different tissues, time points, and following inoculation with the PGPR strain) according to the results obtained with a second reference gene: AtUPL7 (At3g53090). Results were expressed as relative gene expression values using the 2^(-ΔΔCt) method (Schneider et al. 2012).

4.3. Statistical analysis
Statistical analyses of differences for morphological traits and relative gene expressions were carried out using a non-parametric Mann–Whitney–Wilcoxon test (n < 30) unless otherwise indicated. Tests were performed using the software GraphPad Prism® version 7.0.

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No potential conflict of interest was reported by the author(s).

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Notes on contributors
Dr Antoine Desrut, a PhD student at the University of Poitiers at the time of the study, is currently a teaching and research temporary agent at the University of Tours (France).

Florence Thibault is research technician in the French National Centre for Scientific Research (CNRS). She has expertise in microscopy, microbiology, plant physiology and molecular biology.

Jesús Mercado-Blanco is senior researcher in the Institute for Sustainable Agriculture (Spanish National Research Council, CSIC). His main research interests focus on agricultural microbiology, agro-biotechnology and the development of control tools within integrated disease management strategies, using Verticillium wilt of olive as study model. Specific research topics are the bases underlying plant-microbe interactions by molecular and ‘omic’ approaches and the identification, characterization and use of microbiological control agents, with emphasis on bacterial endophytes.

Prof. Pierre Coutsos-Thèvenot, after a first industrial experience in 1986 in the Francereco Research Laboratories (NESTLE France), joined the Research team of the GIE LVMH Research in 1992. Full Professor since September 1998 at the University of Poitiers (France), he manage a team interested in the role of sugar transporters in defense mechanisms in a plant of agronomic interest, grapevine, and in the model species Arabidopsis thaliana.

Cécile Vriet is associate professor at the University of Poitiers (France). Her current research interests focus on the role and regulation of sugar transport in plant-microorganism interactions, and more particularly those involving Plant Growth Promoting Rhizobacteria (PGPR). From her previous positions, she also gained expertise in the fields of legume-rhizobia symbiosis, plant carbohydrate metabolism, phytohormone biosynthesis and signaling pathways, plant response to oxidative stress, and biotechnology-assisted plant breeding.

ORCID
Cécile Vriet http://orcid.org/0000-0001-6625-268X

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