environmental microbiology reports

Environmental Microbiology Reports (2022) **14**(1), 119–129 doi:10.1111/1758-2229.13039

Brief Report

Two-component system in *Rahnella aquatilis* is impacted by the hyphosphere of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

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Summary

Two-component systems (TCS) are ubiquitous among bacteria, playing key roles in signalling events. However, to what extent the TCS of *Rahnella aquatilis* (a Phosphate solubilizing bacteria) is influenced by the hyphosphere of the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* is totally unknown. Here, the expression of 16 genes encoding the TCS of *R. aquatilis* (i.e. involved in carbon-sensing and nutrient-sensing) and of eight genes regulated by the *PhoR* TCS (i.e. involved in inorganic and organic phosphorus mobilization) were analysed at regular intervals in presence of hyphae of *R. irregularis*. The study was conducted under *in vitro* culture conditions with phytate as the unique source of phosphorus. In presence of the AM fungus, the expression of TCS genes involved in carbon-sensing and nutrient-sensing were stimulated. Only, *BaeS* at 30 and 120 min, and *BaeR* at 60 min were inhibited. In addition, the *PhoR* TCS stimulated the expression of genes encoding phosphatase but inhibited the expression of genes involved in gluconic acid production. As the mechanism of coupling environmental changes with cellular physiological changes, TCS plays a pivotal role in regulating specific gene expression in *R. aquatilis*, recognizing environmental signals. More importantly, TCS genes may regulate bacteria response to hyphal carbon to mobilize phosphorus efficiently in the hyphosphere.

Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous in almost all terrestrial ecosystems forming symbiotic associations with more than two-thirds of plant species (Smith and Read, 2008). They produce 45–100 m of hyphae per gram of soil in prairies and pastures (Miller *et al*., 1995), mobilizing 4%–20% of the total carbon (C) photosynthesized by plants (Hodge, 1996). Part of this C is released by hyphae exudation, modifying the physical, chemical and biological characteristics of the nearby environment (i.e. the so-called hyphosphere). Among the major changes occurring in the hyphosphere are the depletion of minerals, particularly nitrogen (N) and phosphorus (P), because of hyphae uptake (Li *et al*., 1991; Zhang *et al*., 2016a) and reversely, the enrichment in sugars, carboxylates, … due to hyphal exudation (Zhang *et al*., 2018; Luthfiana *et al*., 2021).

The hyphosphere is thus a major marketplace impacting the nutrients and carbohydrates fluxes from soil to plants and vice versa, further providing space and resource niches for soil microorganisms, affecting their population both qualitatively and quantitatively (Wang *et al*., 2016). For instance, Emmett *et al*. (2021) demonstrated that the hyphosphere of *Rhizophagus irregularis* and *Glomus versiforme* were enriched by Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Bacteroidetes and Fibrobacteres, and the relative abundance of Proteobacteria was the highest, up to 50%±11%. Hyphal exudates were shown to increase the number and activities of bacteria by providing labile C sources (Zhang *et al*., 2016b), while the detection and response to specific compounds (i.e. fructose), entailing a regulatory response/cascade (i.e. the induction of expression of phosphatase genes), was reported in the phosphate solubilizing bacteria (PSB) *Rahnella aquatilis* developing in the vicinity of extraradical hyphae of the AM fungus *Rhizophagus irregularis* (Zhang *et al*., 2018). Notwithstanding these

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findings, no study, to the best of our knowledge, addressed how the bacteria respond to the hyphosphere environment using their signal transduction systems.

The two-component signal (TCS) transduction system is a ubiquitous and very conservative regulatory component of bacteria for sensing and responding to external stimuli, and an important metabolic regulatory system as well (Stock *et al*., 2000; Yamamoto *et al*., 2005). This system consists of a sensor histidine protein kinase (HK) and a cognate response regulator (RR) (Hoch, 2000; Yamamoto *et al*., 2005). When bacteria sense external stimuli, the HK typically catalyses an autophosphorylation reaction on a histidine residue, producing a high-energy phosphoryl group, which is then transferred to the RR aspartic acid residue. Phosphorylated RR recognizes and specifically bind to DNA sites located within the regulatory regions of the genes it controls, activating or repressing the transcription of genes, and then causing the specific metabolic response (Bourret, 2010; Santos-Beneit, 2015). As a mechanism of coupling environmental changes with cellular physiological changes, TCS is a major way for bacteria to sense and respond to environmental changes (Capra and Laub, 2012). The expression of TCS gene thus plays an important role in this process. For instance, in *Streptomyces*, the TCS system PhoRP was shown to respond to phosphate starvation. Under this limitation in P, the expression of *PhoR* and *PhoP* increased, and the HK PhoR detected this change. The RR PhoP directly bound to the *atrA* promoter, positively regulating the *atrA* expression and daptomycin production in the bacteria. The expression of *PhoRP* was also increased during fermentation for daptomycin production (Zheng *et al*., 2019). As another example, the BasSR system involved in the regulation of resistance to diverse antibiotics (Baranova and Nikaido, 2002) was shown to activate the transcription of *emrD* by binding directly to its promoter region, decreasing the susceptibility of *Escherichia coli* to various antimicrobial agents. This was supported by mutations of BasSR, increasing the cell susceptibility to antimicrobial agents (i.e. ciprofloxacin and norfloxacin), by downregulating the transcriptional levels of *emrD* (Yu *et al*., 2020).

In the rhizosphere, bacteria use the TCS to sense changes in the environment. A variety of compounds in root exudates (e.g. sugars, amino acids) act as chemoattractants, regulating the expression of a significant number of bacterial genes (Mark *et al*., 2005; Matilla *et al*., 2007) and providing a selective advantage for rhizosphere colonization of specific bacteria (Savka *et al*., 2002). For instance, the VirAG system of *Agrobacterium tumefaciens* is able to recognize some monosaccharides (e.g. D-galactose, D-glucose, D-fucose), and phenolic compounds (e.g. acetosyringone) released by injured plant cells, and activates the expression of *Vir* gene to improve the ability of the bacteria to adapt to environmental changes (Mukhopadhyay *et al*., 2004). However, how this TCS is used by PSB developing in the hyphosphere of AM fungi is totally unknown.

One major difficulty in studying AM fungus–bacteria interactions is to create an environment allowing the production of large amounts of hyphae free of any microorganism other than the target one or soil debris. *In vitro* cultures associating AM fungi to transformed root organs (Fortin *et al*., 2002) in bi-compartmented Petri plates (St-Arnaud *et al*., 1996) is the most reliable system to produce high quantities of hyphae on a gelled medium free of any undesired contaminant. This system has been used in the recent years to study the interaction between AM fungi and PSB or bacterial communities (Zhang *et al*., 2016b, 2018). It is thus adequate to (i) create a reproducible hyphosphere environment and (ii) collect bacterial cells developing close to the hyphal surface for gene expression analysis.

In the present study, we aimed to investigate how the PSB *Rahnella aquatilis* HX2 sense and respond to the hyphosphere environment of *Rhizophagus irregularis* at the transcriptional level. Bi-compartmented *in vitro* culture systems were set up with *R. irregularis* MUCL 43194 associated to carrot roots in a root compartment physically separated from a hyphal compartment (HC) in which only the fungal hyphae were allowed to proliferate in presence of the PSB. Sixteen genes encoding the TCS of *R. aquatilis* and eight genes regulated by the TCS gene *PhoR* were analyzed at regular intervals over a period of 2 h.

Results and discussion

TCS are ubiquitous among bacteria, playing key roles in signalling events, such as cell–cell communication or response to environmental changes. However, to what extent the TCS of the PSB *R. aquatilis* influenced by the hyphosphere of the AM fungus *R. irregularis* is totally unknown, even though these two organisms have been reported to interact intimately (Zhang *et al*., 2018; Jiang *et al*., 2021). Here, both organisms were associated *in vitro* to avoid any interferences with unwanted contaminants. Sixteen genes encoding the TCS (i.e. involved in C-sensing and nutrient-sensing) and eight genes regulated by the *PhoR* TCS (i.e. involved in inorganic and organic P mobilization) were analysed.

In a preliminary experiment, genes expressions were compared between the HC^{+AM} and HC^{-AM} treatments 1, 3 and 6 h after addition of the bacteria to the HC. The expression of most genes decreased greatly from 3 to 6 h (see supplementary materials Figs S1 and S2). It was thus decided to restrict the gene expression analysis

to a period of 2 h (i.e. at 2, 15, 30, 60, 90 and 120 min). A two-way analysis of variance was performed to compare the effects of AM fungus, harvest time and their interaction on expression of the 24 genes (see Table S1).

The expression of the TCS genes was generally modified in presence of the hyphae involved in C-sensing and nutrient-sensing. In addition, the *PhoR* TCS stimulated the expression of genes encoding phosphatase but inhibited the expression of genes involved in gluconic acid production, in an environment with phytate as the unique source of P. Thus, TCS is a key player in regulating specific genes expression in *R. aquatilis*, sensing environmental signals (i.e. hyphae exudation and nutrient changes).

Differential gene expression patterns in TCS involved in C-sensing of R. aquatilis *grown in presence/absence of hyphae*

The exudation of C-rich compounds by the extracellular hyphae of AM fungi has been demonstrated in a growing number of studies conducted in bi-compartmented *in vitro* cultivation systems (e.g. Zhang *et al*., 2018; Luthfiana *et al*., 2021). Sugars, carboxylates, amino acids (Zhang *et al*., 2018) as well as compounds such as deoxyadenosine, deoxycytidine, deoxyguanosine and secreted proteins (Kloppholz *et al*., 2011; Kameoka *et al*., 2019; Luthfiana *et al*., 2021) were detected in the hyphal exudates, some of which stimulating growth and/or modifying the composition of bacterial communities (Zhang *et al*., 2016b, 2018). However, the effects of hyphae exudates on the TCS in the early stages (i.e. within the first 2 h) of AM fungi–bacteria interaction are almost unknown.

Within the first 15 min of interaction, the expression of genes involved in quorum sensing (QS) (i.e. *QseC* and *QseB*) was significantly increased in *R. aquatilis* (Fig. 1A and B). This was also the case at 90 and 120 min for *QseC* and 90 min for *QseB* (Fig. 1A and B). QS is a mechanism by which bacteria sense their population density via a complex cell-to-cell communication system, triggering the expression of specific genes when the density reaches a threshold (Dong *et al*., 2001). Interestingly, plants may impact this mechanism by secreting compounds that may either mimic QS signals in bacteria, thus stimulating QS responses in bacteria (Gao *et al*., 2003), or that may quench pathogen QS, a strategy for preventing and controlling QS-mediated bacterium infection (Dong *et al*., 2001). Our results on the QS genes in *R. aquatilis* suggest that this PSB can sense compounds secreted by the extraradical hyphae of the AM fungus, and may regulate its own behaviour (i.e. control the flagella and motility) through QS. Since AM fungi are unable to directly secrete phosphatase mineralizing soil organic P (Tisserant *et al*., 2012, 2013), we hypothesized that hyphae developing in a nutrient solution containing only organic P may secrete compounds stimulating the QS of the PSB *R. aquatilis*, increasing their beneficial interaction with the hyphae. This was supported by a parallel significant increase, at 15 min, in the expression of *RstB* and *RstA*, two genes involved in biofilms formation and adhesion (Fig. 1C and D). The expression of *RcsC* and *RcsD*, two genes involved in the regulation of biofilms formation and capsule production at 2, 15 and 90 min and *RcsD* at 15 min in the presence of AM fungus, was also significantly greater (Fig. 1E and F). Indeed, biofilms formed by *R. aquatilis* can be detected in a longer time (i.e. at 48 h, see Li, 2016). Moreover, *R. aquatilis* can form biofilms on the roots and hyphae surface to access C sources (Zhang *et al*., 2016b). However, in the present study, we only investigated the gene expression and could not observe the biofilm formation processes, which does not preclude that biofilm was in the early stages of construction. Indeed, in presence of phytates as the unique source of P, *R. aquatilis* may be stimulated by the AM fungus in a process of rewarding with C compounds in exchange of inorganic P (Zhang *et al*., 2018). Thus, TCS genes related to biofilms formation and adhesion could be regulated at the early stages of interaction to induce the formation of bacterial biofilms in the presence of extraradical hyphae. Indeed, it has been reported that in presence of phytates, hyphae exudates (more precisely fructose) stimulated the expression of phosphatase genes in *R. aquatilis* as well as the rate of phosphatase released into the growth medium by regulating its protein secretory system (Zhang *et al*., 2018). Jiang *et al*. (2021) further demonstrated that this flagellated bacterium was able to move along the hyphae towards a localized source of phytate to fulfil its role in P solubilization, being nourished by the exudates of hyphae along the way towards the organic P source. In the present study, the expression of genes involved in P metabolism [i.e. PhoR TCS, acid (*acp*) and alkaline (*alp*) phosphatase] was significantly stimulated at any time of observation (except at 2 min) in the HC^{+AM} treatment, supporting this hypothesis (Figs 2C and 3F, G).

Some bacteria (e.g. various *Streptomyces* strains) colonizing the rhizosphere can produce a whole range of antibiotics against various bacterial and fungal pathogens (Barka *et al*., 2015; Toumatia *et al*., 2015), and these bioactive molecules help to establish *Streptomyces* strains in the rhizosphere to compete with other colonizing bacteria (Rakotoniriana *et al*., 2012; Schlatter and Kinkel, 2015). In *Escherichia coli*, the *BaeR* gene is involved in drug resistance by regulating the expression of genes (i.e. *mdtABC* and *acrD*) encoding multidrug exporter systems, increasing the resistance of this bacterium to novobiocin and deoxycholate (Baranova and

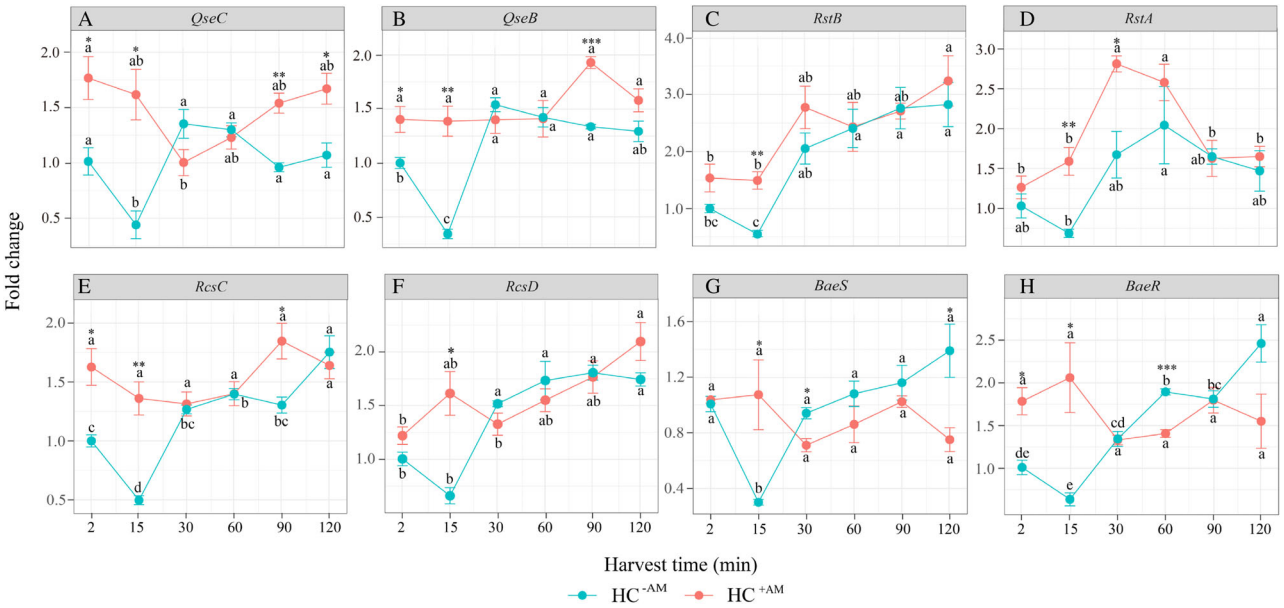

Fig. 1. Dynamics of expression of two-component genes (*QseC*, *QseB*, *RstB*, *RstA*, *RcsC*, *RcsD*, *BaeS* and *BaeR*) involved in C-sensing of *Rahnella aquatilis* in the HC. HC^{-AM} treatment, presence of mycorrhizal roots in the root compartment (RC) but without proliferation of *R. irregularis* in the HC; HC^{+AM} treatment, presence of mycorrhizal roots in the RC and proliferation of *R. irregularis* in the HC. The error bar represents the SE. Differences in lowercase letter mean there is a significant difference between the harvest times within the HC^{+AM} or HC^{-AM} treatment ($P \leq 0.05$ by Tukey's HSD test). The asterisks mean there is a significant difference between the HC^{+AM} and HC^{-AM} treatment at specific time points (*t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

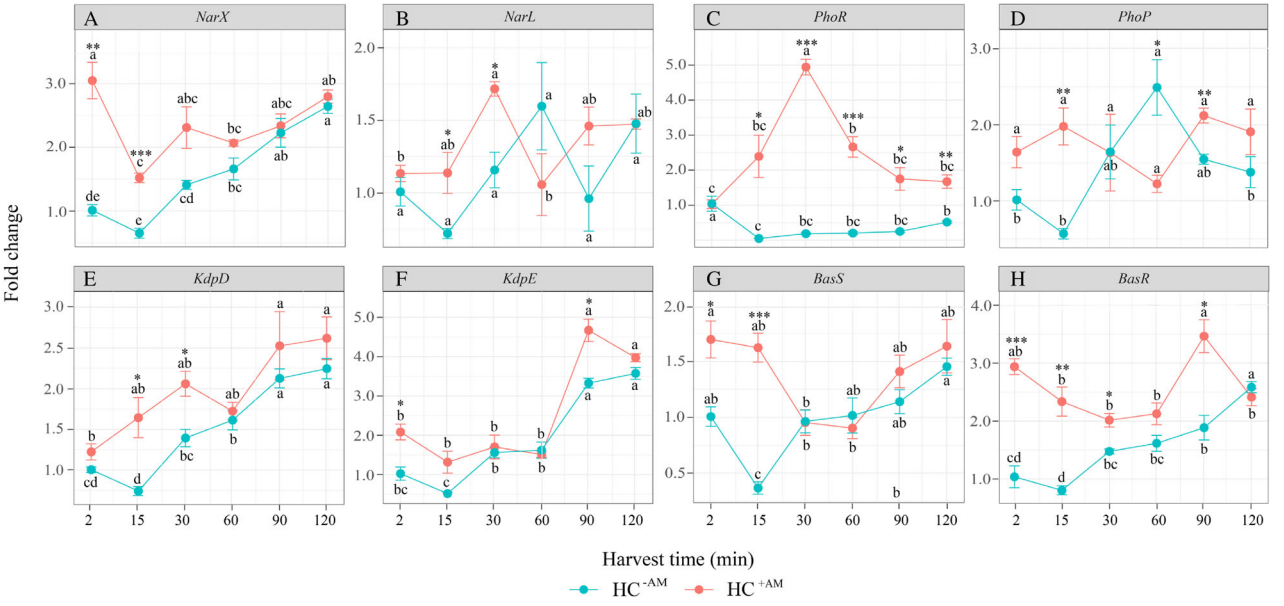

Fig. 2. Dynamics of expression of two-component genes *(NarX*, *NarL*, *PhoR*, *PhoP*, *KdpD*, *KdpE*, *BasS* and *BasR*) involved in nutrient-sensing of *Rahnella aquatilis* in the HC. HC^{-AM} treatment, presence of mycorrhizal roots in the root compartment (RC) but without proliferation of *R. irregularis* in the HC; HC^{+AM} treatment, presence of mycorrhizal roots in the RC and proliferation of *R. irregularis* in the HC. The error bar represents the SE. Differences in lowercase letters mean there is a significant difference between the harvest times within the HC^{+AM} or HC^{-AM} treatment ($P \leq 0.05$ by Tukey's HSD test). The asterisks mean there is a significant difference between the HC^{+AM} and HC^{-AM} treatment at specific time points (*t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

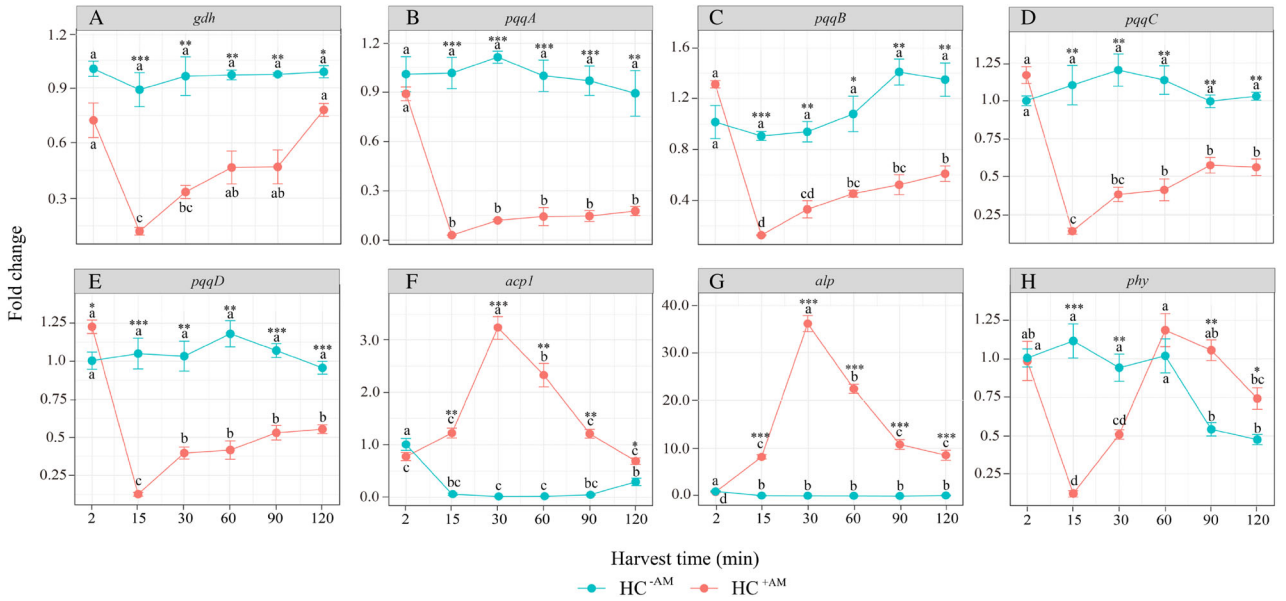

Fig. 3. Dynamics of expression of (A) glucose dehydrogenase (*gdh*), (B–E) cluster of pyrroloquinoline quinoline (*pqqA*, *pqqB*, *pqqC* and *pqqD*) and (F–H) phosphatase genes (*acp1*, *alp*, *phy*) in *Rahnella aquatilis* developing in the HC. HC^{-AM} treatment, presence of mycorrhizal roots in the root compartment (RC) but without proliferation of *R. irregularis* in the HC; HC^{+AM} treatment, presence of mycorrhizal roots in the RC and proliferation of *R. irregularis* in the HC. The error bar represents the SE. Differences in lowercase letters mean there is a significant difference between the harvest times within the HC^{+AM} or HC^{-AM} treatment ($P \leq 0.05$ by Tukey's HSD test). The asterisks mean there is a significant difference between the HC^{+AM} and HC^{-AM} treatment at specific time points (*t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

Nikaido, 2002; Nagakubo *et al*., 2002). Furthermore, the *BaeR* gene increases the resistance of *E. coli* to condensed tannins, which are common secondary metabolites secreted by plants with antimicrobial activities (Schofield *et al*., 2001; Zoetendal *et al*., 2008). In our study, the expression of *BaeS* and *BaeR* was significantly induced in the hyphosphere of the AM fungus at 15 min and 2 and 15 min respectively (Fig. 1G and H), suggesting a mechanism by which the bacterium may prepare itself to compete with other microbes in the hyphosphere.

Differential gene expression patterns in TCS involved in nutrient-sensing of R. aquatilis *grown in presence/absence of hyphae*

Nutrient changes were noticed in the HC of both treatments. The concentrations of N and phytate P were significantly lower in the HC of the HC^{+AM} treatment, while the reverse was noticed for K, the reason of which is still unclear (Table S2). Bacteria use TCS to sense and adapt to environmental changes (Hoch, 2000; Capra and Laub, 2012). Eight genes (i.e. *NarX*, *NarL*, *PhoR*, *PhoP*, *KdpD*, *KdpE*, *BasS* and *BasR*) involved in the response of *R. aquatilis* to nitrogen, phosphorus, potassium, iron and zinc changes in the environment were analyzed in the present study (Fig. 2).

In the HC^{-AM} treatment, the expression of *NarX* decreased from 2 to 15 min and then increased steadily until 120 min and an almost similar trend was noticed for the HC^{+AM} treatment (Fig. 2A). The greatest expression for *NarL* was noticed at 30 min and the lowest at 60 min in the HC^{+AM} treatment (Fig. 2B). The expression of *NarX* at 2 and 15 min and *NarL* at 15 and 30 min was significantly greater in the HC^{+AM} treatment compared to the HC^{-AM} treatment (Fig. 2A and B). In the HC^{+AM} treatment, the expression of *PhoR* significantly increased between 2 and 30 min and then significantly decreased between 30 min and the other sampling times. With the exception of 2 min, the expression of *PhoR* was significantly greater at all time points in the HC^{+AM} treatment compared to the HC^{-AM} treatment (Fig. 2C). The expression of *PhoP* showed reverse tendencies between both treatments. In the HC^{+AM} treatment, the expression of *PhoP* was significantly greater at 15 and 90 min and significantly smaller at 60 min compared to the HC^{-AM} treatment (Fig. 2D). In the HC^{-AM} treatment, the expression of *KdpD*, *KdpE*, *BasS* and *BasR* first decreased between 2 and 15 min and then increased gradually at 30, 60, 90 and 120 min (Fig. 2E–H). In the HC^{+AM} treatment, gene expression trends varied between genes. For *KdpD*, it increased steadily from 2 to 120 min and the expression of *KdpD* was significantly greater in the HC^{+AM} treatment at 15 and 30 min compared to the HC^{-AM} treatment (Fig. 2E). The expression of *KdpE* did

not differ between 2, 15, 30 and 60 min and was significantly greater at 90 and 120 min. The expression of *KdpE* was significantly greater in the HC^{+AM} treatment at 2 and 90 min compared to the HC^{-AM} treatment (Fig. 2F). In the HC^{+AM} treatment, the expression of *BasS* at 2 and 15 min and of *BasR* at 2, 15 30 and 90 min were significantly greater compared to the HC^{-AM} treatment (Fig. 2G and H).

Our results showed that the TCS genes sensing N, P and K (i.e. *NarX*, *PhoR*, *KdpD*) were sensitive to the changes of nutrient concentration. Accordingly, the expressions of these genes were significantly greater at the beginning (2–15 min) in the HC^{+AM} treatment compared to the HC^{-AM} treatment. This suggested that the TCS of *R. aquatilis* responded very quickly to the presence of *R. irregularis*, in order to adapt to the hyphosphere environment.

Differential expression patterns of genes involved in inorganic and organic P mobilization of R. aquatilis *in presence of phytate a unique source of P*

The tradeoff between the supply of C by the hyphae and the mobilization of nutrients (especially P) by bacteria in the hyphosphere is still not clear. Among the hyphal exudates, glucose and fructose are two major compounds released by hyphae (Zhang *et al*., 2018). Their acquisition by the bacterium is feeding the glycolysis and then the tricarboxylic acid cycle to produce ATP. Part of the C is also used to synthesize metabolic compounds capable of solubilizing/mineralizing insoluble soil P. In *R. aquatilis*, the solubilization/mineralization of P follows two main pathways (Jiao *et al*., 2015; Zhang *et al*., 2018). In the first pathway, glucose is used as inducer for gluconic acid production, which is released in the environment for solubilization of inorganic phosphate (Rodriguez *et al*., 2004), further releasing soluble P available to plants. In the second pathway, fructose induces the production of acid and alkaline phosphatase, which are also C-rich compounds, to hydrolyze/mineralize organic P. Therefore, there is a tradeoff that takes advantage of organic C compounds to solubilize/mineralize the insoluble soil P.

In *Escherichia coli* and *Bacillus subtilis* the genes belonging to the pho regulon, including the phosphatase gene and the phosphate-specific transport genes, are regulated by the *PhoR-PhoP* TCS (named *PhoB* in *E. coli*; refs. Kimura *et al*., 1989; Hulett, 1996; Pragai and Harwood, 2002). Analysis of the whole genome of *R. aquatilis* revealed that the glucose dehydrogenase gene and pyrroloquinoline quinoline genes were also regulated by the *PhoR-PhoP* TCS (Guo *et al*., 2012). The *PhoR-PhoP* TCS may play an important role in the tradeoff process as it can regulate different genes expression related to P solubilization/mineralization (Santos-Beneit, 2015). Here, we tested the expression of glucose dehydrogenase gene (*gdh*), pyrroloquinoline quinoline genes (*pqqA*, *pqqB*, *pqqC* and *pqqD*) which are involved in producing gluconic acid for solubilization of inorganic P, and *phy*, *acp1*, *alp* which are involved in encoding phosphatases to hydrolyze/mineralize organic P (Li *et al*., 2014; Zhang *et al*., 2018). An and Moe (2016) found that expression level of the genes (i.e. *gdh*, *pqqA*, *pqqB*, *pqqC* and *pqqD*) involved in solubilization of inorganic P varied significantly with the concentration of C source, showing a trend in expression in the order glucose > glycerol > citrate. However, in our study, the presence of AM fungal hyphae did not stimulate the expressions of those five genes that were rather inhibited at 15, 30, 60, 90 and 120 min (Fig. 3A–E). Conversely, the expression of *acp1* and *alp*, involved in mineralization of organic P, were stimulated by the presence of AM fungal hyphae at the same time points (15, 30, 60, 90 and 120 min) (Fig. 3F and G). The expression of another phosphatase gene (i.e. *phy*) was significantly increased at 90 and 120 min and significantly decreased at 15 and 30 min in the HC^{+AM} treatment compared to the HC^{-AM} treatment (Fig. 3H). Since phytate (an organic P compound) was the unique source of P, we hypothesize that the use by the bacterium of the C released by hyphae was directed towards the production of phosphatases (the second pathway) rather than on the upregulation of genes involved in solubilization of inorganic P (the first pathway). It is not clear whether this mechanism, oriented preferentially towards the production of phosphatases rather than towards the stimulation of genes involved in the solubilization of P, is controlled by the AM fungi or by the metabolism of the bacteria.

Conclusion

The sensor gene of TCS regulates the downstream genes through phosphorylation/dephosphorylation, enhancing the ability of bacteria to adapt to the environment. The expression of TCS will subsequently influence the downstream gene expression involved in bacterial metabolism and nutrient mobilization (Slater *et al*., 2000). In the present study, we demonstrated that the TCS plays a pivotal role in regulating specific gene expression in *R. aquatilis*, recognizing environmental signals (i.e. hyphae exudates and extra-hyphal nutrients – especially P). The TCS provide a conserved mechanism for the coordinated control of gene expression in response to different environmental factors (Alex and Simon, 1994). As such, our results suggested that the TCS genes may regulate bacteria response to hyphal C to mobilize P efficiently in the hyphosphere.

Experimental procedures

The AM fungus used was *R. irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as 'irregulare' MUCL 43194. The strain was provided by the Glomeromycota *in vitro* collection on Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC2 maintained on Petri plates (90 × 15 mm) containing the modified Strullu–Romand (MSR) medium (Declerck *et al*., 1998) solidified with 3 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA). The PSB used was *R. aquatilis* HX2, isolated from a vineyard soil in Beijing, China (Guo *et al*., 2012). This strain was shown to mineralize organic P, solubilize mineral P, fix nitrogen and produce pyrroloquinoline quinine, indole-3-acetic acid and antibacterial substances (Chen *et al*., 2007; Guo *et al*., 2009). It colonizes the hyphal surface of AM fungi (Zhang *et al*., 2016b) and was reported effective in the mineralization and utilization of phytate (Zhang *et al*., 2016b, 2018).

Sixteen genes encoding the TCS of *R. aquatilis* and eight genes regulated by the TCS gene *PhoR* were considered and their primers were designed with the software Primer 5. The sequences of primers and other information related to the genes are described in Table S3.

Experimental set-up

Bi-compartmented Petri plates (90 × 15 mm) were used to grow the roots and AM fungal hyphae (see details in Declerck *et al*., 2003). Briefly, in one compartment (i.e. the root compartment – RC), an excised transformed root of carrot clone DC2 was associated with the AM fungus on 25 ml MSR medium solidified with 3 g L^{-1} Phytagel. After 4 weeks, the extraradical mycelium (ERM) developed profusely in the RC and started to cross the plastic barrier separating the RC from the other compartment (i.e. the HC) in which only the ERM was allowed to develop. In the HC, 4 ml MSR medium solidified with 3 g L^{-1} Phytagel but without sucrose and vitamins was added in a slope from the top of the plastic barrier to the bottom of the HC (Toljander *et al*., 2007). The bi-compartmented Petri plates were incubated in a growth chamber at 27°C in the dark. The cultures were checked on a regular basis, and roots close to the plastic barrier were removed before they started to grow into the HC. One control treatment was included under strictly identical conditions. It comprised carrot roots associated with the AM fungus in the RC without proliferation of ERM in the HC (the MSR medium was cut 1 cm away from the plastic barrier in the RC to prevent the hyphae and roots from crossing the partition wall separating the RC from the HC). Two treatments were thus considered: A treatment with mycorrhizal roots in the RC but without proliferation of *R. irregularis* in the HC (HC^{-AM}) and a treatment with mycorrhizal roots in the RC and proliferation of *R. irregularis* in the HC (HC^{+AM}) (see Fig. 4). Each treatment consisted of three replicates.

After an additional 2 weeks, the ERM of the HC^{+AM} treatment developed extensively on the slope. Ten millilitre of liquid MSR medium without sucrose, vitamins, Ca^{2+}, Fe^{2+} and inorganic P, but containing 450 μM phytate (Na-phytate, Sigma-Aldrich) as the unique source of P, was added to the HC. Phytate is a common source of organic P in the soil. AM fungi cannot utilize phytate-P directly since they are unable to secrete phytases. Part of the inorganic P available to the AM fungi is released from the enzymatic activities of PSB, e.g. *R. aquatilis*. Thus, we select phytate-P as the P source to study the cooperation between AM fungi and bacteria based on our previous studies (see Zhang *et al*., 2016a, 2016b, 2018, 2019).

After another 4 weeks, the HC in the HC^{+AM} treatment contained numerous actively growing hyphae extending from the slope. The remaining liquid MSR medium (approximately 9 ml) in the HC was transferred to a 15 ml tube with a micropipette and adjusted to 10 ml using the same liquid MSR medium as above but without phytate. Then, 2 ml of bacterial supernatant with a concentration of approximately 10^8 CFUs ml^{-1} was added and mixed uniformly. The 12 ml MSR medium containing the bacteria was poured back in the HC of each experimental system with a micropipette.

The *R. aquatilis* inoculum was prepared as follows: bacteria were cultured in liquid Luria–Bertani medium with shaking at 180 rpm at 28°C until OD_{600} reached 0.4–0.6 (logarithmic phase) and then centrifuged at 5878*g* for 6 min. The supernatant was discarded, and the pellet was re-suspended and washed three times with sterilized 0.85% (wt./vol.) NaCl solution. The supernatant was then adjusted to $OD_{600} = 0.8$ with the autoclaved 0.85% NaCl solution and stored for 4 h before use.

Harvest and genes expression analysis

In the three plates of each treatment, the liquid MSR medium containing *R. aquatilis* was added to the HC of each Petri plate. An aliquot of 0.5 ml medium was then sampled with a micropipette 2, 15, 30, 60, 90 and 120 min after addition of the bacteria to the HC, and added to 1 ml RNAprotect® Bacteria Reagent (Qiagen) according to the handbook, to stabilize the bacterial RNA. The samples were then stored at −80°C until RNA extraction.

Total RNA was extracted from the frozen bacteria using the RNAprep Pure Cell/Bacteria Kit (Catalogue number: DP430, Tiangen Biotech, Beijing, China),

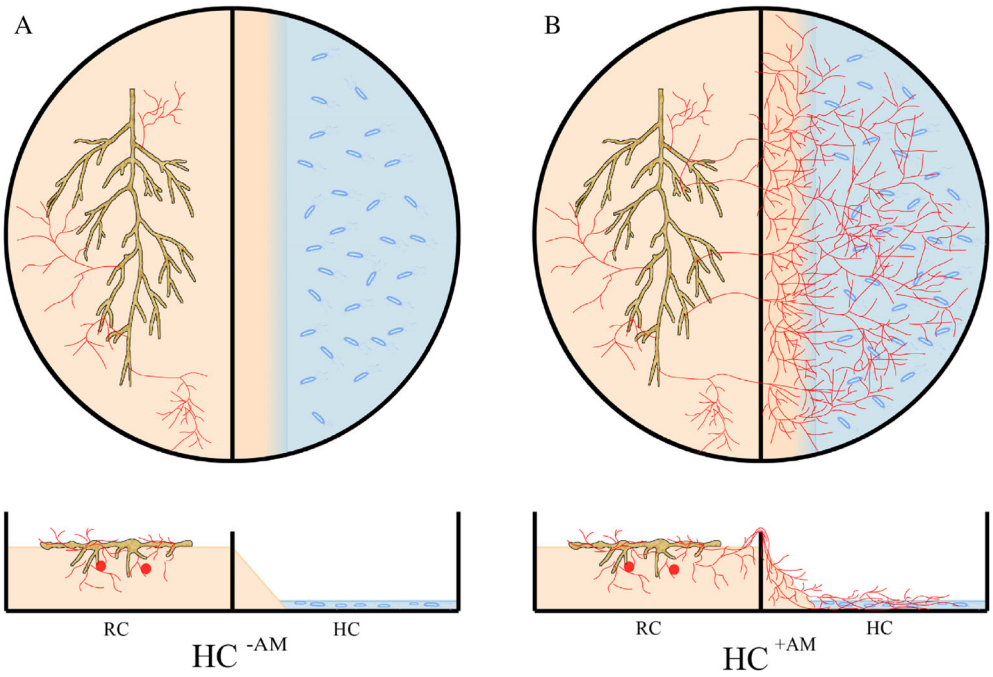

Fig. 4. Schematic representation of the experimental set-up. The system consisted of bi-compartmented Petri plates with a root compartment (RC) containing an excised carrot root clone DC2 associated to *Rhizophagus irregularis* and a HC containing (A) only *Rahnella aquatilis* (the HC^{-AM} treatment) or (B) hyphae of the AM fungus and *R. aquatilis* (the HC^{+AM} treatment). Red lines, extraradical hyphae of *R. irregularis*; red dots, spore of *R. irregularis*; blue ovals, cells of *R. aquatilis*.

according to the manufacturer's instructions and treated with an RNase-Free DNase I (Catalogue number: RT411, Tiangen Biotech) to remove possible DNA contamination. For single-strand cDNA synthesis, 34 ng of total RNA was reverse-transcribed at 42°C for 2 min, 37°C for 15 min and 85°C for 5 s in a final volume of 20 µl using PrimeScript™ RT Reagent Kit with gDNA Eraser (Catalogue number: RR047A, TaKaRa Bio, Japan). The products were then diluted to 200 µl.

Quantitative RT-PCR (qRT-PCR) was performed using a CFX96™ Real-Time System (BIO-RAD, USA). Each PCR reaction was carried out in a total volume of 25 µl containing 2 µl cDNA, 13 µl TB Green® Premix Ex Taq™ II (Catalogue number: RR820A, TaKaRa Bio) and 0.5 µl of each primer (10 µM). The following PCR program was followed: 95°C for 300 s, 40 cycles of 95°C for 10 s, 57°C for 30 s, 72°C for 45 s. A melting curve was recorded at the end of each run to exclude that the primers had generated non-specific PCR products (Ririe *et al*., 1997). All reactions were performed on three technical replicates and their mean used. Baseline range and threshold cycle (Ct) values were automatically calculated using LightCycler® 96 software. The ΔCt was calculated by subtracting the Ct value of a reference gene from the Ct value of each target gene. Relative fold-change of each target gene was normalized by the $2^{-\Delta\Delta Ct}$ method, with reference to the ΔCt value in the control treatment harvested at the 2 min sampling time. The optimal reference gene *atpD* for expression analysis of *R. aquatilis* response to abiotic stresses was used as the housekeeping gene (Li *et al*., 2019).

Determination of mineral element concentrations in the liquid MSR medium of the HC

In parallel to the experiment above, four Petri plates of each treatment were set up for analysis of mineral elements concentrations in the HC. At the end of the experiment, the liquid MSR medium was passed through an Acrodisc® Syringe Filter (0.2 µm Supor® Membrane, Pall Corporation, New York, USA) to remove the bacterial cells, and stored at −80°C for analysis. The concentrations of P, K, Mg, Mn, Zn, Cu and B were evaluated by inductively coupled plasma atomic emission spectroscopy (PerkinElmer, Optima7300DV, USA). The concentration of N was evaluated by the Continuous Flow Analyser (SEAL, AutoAnalyzer3, Germany).

Statistical analysis

Statistical analyses were performed using SPSS v. 16.0 (SPSS, Chicago, IL, USA). Two-way analysis of variance was performed to compare the effects of AM fungus, harvest time and AM fungus × harvest time interaction on

the expression of the 24 genes. Significant differences among the two treatments (i.e. HC^{-AM} and HC^{+AM}) were assessed at each sampling time by *t*-test at $P \leq 0.05$. The concentration of each mineral element in the liquid MSR medium was also evaluated by *t*-test at $P \leq 0.05$. Significant differences among the harvest times in a specific treatment (i.e. HC^{-AM} or HC^{+AM}) were evaluated by a Tukey's honest significant difference (HSD) test at $P \leq 0.05$.

Acknowledgements

We thank associate Professor Yanbin Guo from College of Resources and Environmental Sciences, China Agricultural University, for providing the bacterial strain *R. aquatilis* HX2. This study was financially supported by the Beijing Natural Science Foundation (6212020) and the Program of Advanced Discipline Construction in Beijing (Agriculture Green Development).

Author Contributions

S.L.D., L.Z. and G.F. planned and designed the research. S.L.D. performed experiments. S.L.D., S.D. and L.Z. analyzed data. S.L.D., S.D., L.Z. and G.F. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used for real time q-PCR analysis

Table S2. Comparison of concentration of mineral elements in present/absence of extraradical hyphae of *Rhizophagus irregularis*

Table S3. Functions and information of genes in two component system (TCS) and regulated by the TCS gene *PhoR* of *Rahnella aquatilis*

Fig. S1. Dynamics of expression of two-component genes (*QseC*, *QseB*, *RstB*, *RstA*, *RcsC*, *RcsD*, *BaeS* and *BaeR*) involved in C-sensing of *Rahnella aquatilis* in the hyphal compartment (HC)

Fig. S2. Dynamics of expression of two-component genes (*NarX*, *NarL*, *PhoR*, *PhoP*, *KdpD*, *KdpE*, *BasS* and *BasR*) involved in nutrient-sensing of *Rahnella aquatilis* in the hyphal compartment (HC)