Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H+-pumping pyrophosphatase in pepper plants

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Summary

It has been previously shown that the transgenic overexpression of the plant root vacuolar proton pumps H^+-ATPase (V-ATPase) and H^+-PPase (V-PPase) confer tolerance to drought. Since plant-root endophytic bacteria can also promote drought tolerance, we hypothesize that such promotion can be associated to the enhancement of the host vacuolar proton pumps expression and activity. To test this hypothesis, we selected two endophytic bacteria endowed with an array of *in vitro* plant growth promoting traits. Their genome sequences confirmed the presence of traits previously shown to confer drought resistance to plants, such as the synthesis of nitric oxide and of organic volatile organic compounds. We used the two strains on pepper (*Capsicuum annuum* L.) because of its high sensitivity to drought. Under drought conditions, both strains stimulated a larger root system and enhanced the leaves' photosynthetic activity. By testing the expression and activity of the vacuolar proton pumps, H^+-ATPase (V-ATPase) and H^+-PPase (V-PPase), we found that bacterial colonization enhanced V-PPase only. We conclude that the enhanced expression and activity of V-PPase can be favoured by the colonization of drought-tolerance-inducing bacterial endophytes.

Introduction

Drought is one of the most severe abiotic plant stresses that strongly limits crop productivity (Calanca, 2017). Plants have evolved mechanisms both to cope with drought independently, including escape strategies, avoidance and tolerance (Jarzyniak and Jasiński, 2014), and to cooperate with beneficial plant growth promoting (PGP) microorganisms that provide the plant-host with ecosystem services and activities that mitigate the effects of several abiotic stresses (Lau and Lennon, 2011; 2012; Marasco *et al*., 2013a 2013b; Chen *et al*., 2017; Rolli *et al*., 2017; Vergani *et al*., 2017).

During periods of drought, plants tune their tissue turgor for low water potential by modulating osmotic adjustments, a phenomenon that must be tightly regulated to maintain cell homeostasis (Fang and Xiong, 2015). Perturbation of the osmotic balance involves changes in ion fluxes across the plasma membrane and tonoplast (Gaxiola *et al*., 2007). The plant enzyme vacuolar proton pumps V-ATPase (H^+-adenosine triphosphatase) and V-PPase (H^+-pyrophosphatase) hydrolyse adenosine triphosphate (ATP) and pyrophosphate (PPi), respectively, to increase the ion concentration in the vacuoles by establishing the necessary electrochemical gradient across the tonoplast (Maeshima, 2001; Martinoia *et al*., 2006). The resulting increase in the vacuolar osmotic pressure is coupled with a decrease in the cell water potential, which in turn facilitates water uptake from soil, alleviating the drought stress. The role of V-ATPase and V-PPase in plant tolerance to drought has been demonstrated by their overexpression in several plants, including *Arabidopsis thaliana* (Gaxiola *et al*., 2001), tomato (Park *et al*., 2005; Da-Gang *et al*., 2012), rice (Zhang *et al*., 2011), tobacco (Arif *et al*., 2013), maize (Li *et al*., 2008), barley (Schilling *et al*., 2014), sugarcane (Kumar *et al*., 2014; Raza *et al*., 2016), watermelon rootstock (Park *et al*., 2014), peanuts (Qin *et al*., 2013), alfalfa (Bao *et al*., 2016; Wang *et al*., 2016b), and cotton under both laboratory and field conditions (Pasapula *et al*., 2011).

In arid environments, desert farming favours the selection of drought-protecting microbial assemblages (Marasco *et al*., 2012; TerHorst *et al*., 2014; Soussi *et al*., 2016) that are enriched and rearranged in the plant

rhizosphere and endosphere (Mapelli *et al*., 2013; Marasco *et al*., 2013a; 2016; Cherif *et al*., 2015; Ferjani *et al*., 2015; Santos-Medellín *et al*., 2017). PGP microorganisms use several mechanisms to stimulate drought tolerance in plants (Vurukonda *et al*., 2016; Etesami and Maheshwari, 2018): (i) the microbial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase contributes to control the concentration of the plant stress phytohormone ethylene, by degrading its precursor ACC (Glick, 2014); (ii) microorganisms contribute to modulate plant hormone homeostasis by producing auxin [i.e., indole-3-acetic acid (IAA)], cytokinins and giberellins. These phytohormones are involved in a wide range of adaptive responses and may determine changes in plant root gene expression and root architecture (Egamberdieva *et al*., 2017; Lim and Kim, 2013); (iii) by manipulating the plant antioxidant system microorganisms decrease the reactive oxygen species (ROS) concentration (Wang *et al*., 2012); (iv) the microbial release of osmolytes acts synergistically with those produced by the plant and enhances resistance to water stress (Etesami and Maheshwari, 2018); (v) exopolysaccharides produced by microorganisms recondition the root microenvironment by favouring water retention and protecting plant roots against desiccation (Rossi *et al*., 2012); (vi) microorganisms contribute to enhance the plant induced systemic tolerance to drought by altering the host physiology and the metabolic processes (Cho *et al*., 2013); (vii) an indirect effect of microbial activity is the solubilization of poorly available nutrients such as iron and phosphorus (Pii *et al*., 2015). Interestingly, evidences suggest that the bacteria-mediated protection is a drought-activated mechanism (Rolli *et al*., 2015).

Considering the drought-protecting role of vacuolar proton pumps and the ability of root system-associated bacteria to enhance plant drought-tolerance, we hypothesize that such bacterial-mediated tolerance can be associated with an increment of expression and activity of those pumps. In this study, we selected two endophytic bacteria, denoted by E1 (*Bacillus subtilis*) and E3 (*Paenibacillus illinoinensis*), with plant growth promoting (PGP) phenotypes and genome traits that may influence the plants' response to drought. Hence, to test our hypothesis, we analysed the response to drought conditions of pepper (*Capsicuum annuum* L.), chosen for its high sensitivity to water stress (Jaimez *et al*., 2000), both with and without the endophytic bacteria, and assessed the bacterial effect on the expression of the vacuolar proton pumps, V-ATPase and V-PPase.

Results

Plant growth promoting potential of endophyte strains isolated from drought-resistant plants cultivated under desert farming

Through functional screenings for plant growth promoting (PGP) potential, including *in vitro* assays and a rhizocompetence test (Cherif *et al*., 2015; Marasco *et al*., 2012), we selected two root endophytes of pepper plants (*Capsicuum annuum* L.) cultivated under desert farming conditions (Marasco *et al*., 2012). The two endophytes, E1 and E3, were affiliated to *Bacillus subtilis* and *Paenibacillus illinoinensis* with 16S rRNA gene sequence identity of 100% and 99% respectively (Fig. 1A). Both E1 and E3 showed multiple PGP phenotypes that have the potential to favour plant tolerance to drought (Table 1). However, the two strains showed different tolerances to osmolytes. E1 tolerated higher concentrations of salt (NaCl and KCl), urea and sodium lactate (Table 1 and Supporting Information Fig. S1) than E3, and was also endowed with several traits that encourage plant root colonization, like the formation of a biofilm-like pellicle, swimming and swarming motility, cellulose degradation activity, and the production of biosurfactants involved in the reduction of root surface tension (Table 1 and Supporting Information Table S1). Both strains produced complex mixtures of volatile organic compounds (VOCs), including some capable of promoting plant growth or protecting against phytopathogens, for example, 2,3-butanediol, acetoin and carbon dioxide (among others Cho *et al*., 2008; Cortes-Barco *et al*., 2010; Supporting Information Table S2 and Fig. S2). The complex mixtures of VOCs produced by E1 and E3 promoted the formation of a larger plant biomass in

Arabidopsis thaliana plantlets grown under both normal conditions and osmotic stress induced by 100 mM mannitol (Supporting Information Fig. S3).

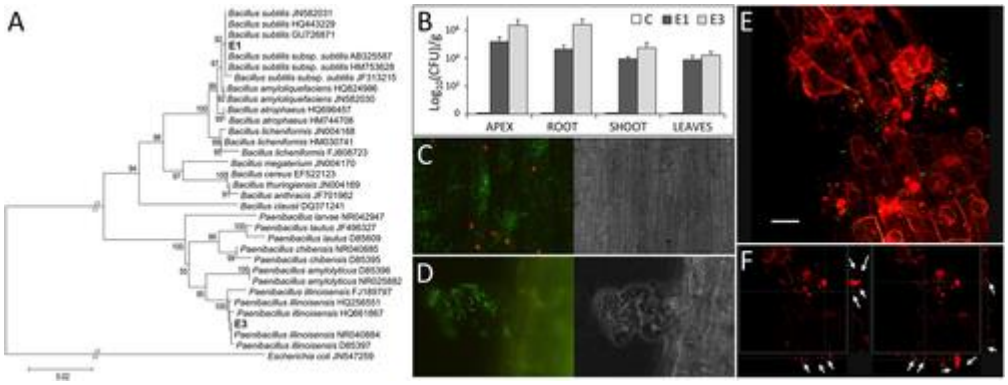

Figure 1

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Phylogenetic affiliation of E1 (*B. subtilis*) and E3 (*P. illinoinensis*), and rhizocompetence/colonization ability.

A. Phylogenetic trees of E1 and E3 using the complete 16S rRNA gene sequence. Neighbour-joining phylogenetic tree-based 16S rRNA gene sequences from E1 and E3, and their closest phylogenetic neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position. B. Enumeration of Rif^R versions of the $E1^{Rif}$ and $E3^{Rif}$ strains after isolation on selective medium. C. Epifluorescence and phase contrast images of pepper roots densely colonized by E1-*gfp*. D. Epifluorescence and phase contrast images of E1-*gfp* cells entrapping root hairs. E. Confocal microscopy analysis of E1-*gfp* colonized roots. F. The boxes indicated by dashed lines show orthogonal views of a three-dimensional confocal image created from a z-stack of x/y-scans. White arrows indicate the potential endophytic colonization of a root cortex by E1-*gfp* cells that were found in the orthogonal views of the inner root tissue.

Caption

Table 1. *In vitro* plant growth promoting activities of bacterial endophytes E1 (*B. subtilis*) and E3 (*P. illinoinensis*).

(A) Plant growth promoting traits		Bacterial strain	
		E1	E3
Biostimulation	ACCd activity	No	No
	Auxin production	No	No
Biofertilization	Phosphate solubilization	Yes	Yes
	Siderophore production	Yes	No
	Exopolysaccharide release	Yes	No

(A) Plant growth promoting traits		Bacterial strain	
		E1	E3
VOCs*	Putative N_2 fixation	Yes	No
	NH_4 production	Yes	No
	VOCs mixture	Yes	Yes
	2,3 butandiol	Yes	No
Plant growth promotion by VOCs*	Normal condition	Yes	Yes
	Stress condition	Yes	Yes
Recolonization ability	Pellicule formation	Yes	Yes
	Cellulase	Yes	No
	Swimming ability	Yes	No
	Swarming ability	Yes	No
	Biosurfactant production*	Yes	No

(B) Resistance to different types of abiotic stresses		Bacterial strain	
		E1	E3
Salt stress	5% NaCl	Yes	No
	8% NaCl	Yes	No
	10% NaCl	Yes	No
Osmotic stress	10% PEG	Yes	Yes
	20% PEG	Yes	Yes

(A) Plant growth promoting traits		Bacterial strain	
		E1	E3
Temperature	4°C	No	No
	42°C	Yes	Yes
	50°C	Yes	No
	NaCl	3%	1%
	Potassium chloride	3%	5%
	Sodium sulfate	5%	5%
	Ethylene glycol	20%	20%
	Sodium formate	1%	1%
Osmolyte (+)*	Urea	2%	5%
	Sodium lactate	3%	4%
	Sodium phosphate	200 mM	200 mM
	Sodium benzoate	20 mM	20 mM
	Ammonium sulfate	100 mM	100 mM
	Sodium nitrate	80 mM	40 mM

- Presence or absence of (**A**) PGP traits and (**B**) abiotic stress tolerance were evaluated following the methods described in the experimental section. Additional details of the results marked with a star (*) are reported in Supporting Information Tables S1 and S2 and Supporting Information Figs. S1–S3. (+) Indicates the highest concentration of osmolytes (% w/v) tolerated by the strains.

The genomes of E1 and E3 have been sequenced (Supporting Information Table S3). A functional survey of the PGP traits in the genomes revealed that E1 and E3 were endowed with multiple metabolic properties that could contribute to the alleviation of drought-induced stress (Table 2). Gene pathways for the synthesis of VOCs were detected, those included the presence of the gene encoding for the butanediol dehydrogenase that

catalyse the reaction to produce the PGP-volatile 2,3-butanediol (i.e., Cho *et al*., 2008). The genome analysis also showed the presence of genes encoding for the acetolactate synthase (*alsS*) and the acetolactate decarboxylase (*alsD*), which catalyse the two-step conversion from pyruvate to acetoin that can be further converted into 2,3-butanediol by the enzyme butanediol dehydrogenase, along with the genes for proline/glycine betaine transporters (Table 2). In E1, the *alsS*, *alsD*, and butanediol dehydrogenase gene showed a 100% similarity to the reference genes of *Bacillus subtilis* 168. In E3, the percentages of similarity to the sequenced *Paenibacillus* spp. genomes were very low for all three of these genes since no reference strain was available (Table 2 and Supporting Information Fig. S4). In both the E1 and E3 genomes, we detected genes encoding for a nitric oxide synthase, ROS detoxifying enzymes, and exopolysaccharide synthesis. Both the E1 and E3 genomes lacked genes known to be involved in the synthetic pathways of auxin derivatives and the ACC deaminase (*acdS*) (Table 2).

Table 2. Genomic screening of E1 (*B. subtilis*) and E3 (*P. illinoinensis*) for PGP metabolic properties and functions possibly involved in plant drought-resistance.

Potential PGP traits involved in plant drought tolerance	**Bacterial strain**	
	E1	**E3**
Glycine betaine pathway	ABC transport enzymes of glycine, betaine/proline (*proX*, *proW* and *proV*)	Absent
Detoxification of ROS	Present	Present
Exoplysaccharide synthesis	*noeJ* and *noeL* genes	*noeJ* and *noeL* genes
ACC deaminase (*acdS*)	Absent	Absent
Butanediol dehydrogenase	Present 100% similarity *B. subtilis* 168	Present 49.7% similarity *B. subtilis* 168
Acetolactate synthase (*alsS*)	Present 99% similarity *B. subtilis* 168	Present 29% similarity *B. subtilis* 168
Acetolactate decarboxylase (*alsD*)	Present 100% similarity *B. subtilis* 168	Absent
Auxin production (*iaaM, iaaH, ipdC*)	Absent	Absent

Potential PGP traits involved in plant drought tolerance	Bacterial strain	
	E1	E3
Nitric oxide synthase	Present	Present

Recolonization ability has been also evaluated as bacterial essential trait to exert PGP functions. Quantification of the bacterial colonization of the plant tissues by the spontaneous rifampicin-resistant mutants $E1^{Rif}$ and $E3^{Rif}$ revealed that they established dense populations ranging from about 10^5 to 10^6 CFU g^{-1} in the root tissue (considering both apex and cortical tissue) and from about 10^3 to 10^4 CFU g^{-1} in the stem and leaf tissues (Fig. 1B). These relatively high bacterial counts were obtained after washing the plant organ surfaces with a physiological buffer several times to eliminate those cells not tightly associated with the plant. Thus, the strains were capable of efficiently colonizing the rhizoplane and the other investigated tissues. Fingerprinting profiles (Internal Transcribed Spacers, ITS-PCR) of randomly picked colonies were identical to those of E1 and E3, confirming that the colonies belonged to the supplemented $E1^{Rif}$ and $E3^{Rif}$ strains (Supporting Information Fig. S5). The *green fluorescent protein (gfp)*-derived E1 strain tightly adhered to the pepper root surface after 24 h (Fig. 1C). Several bacterial cells were found enveloped in the root hairs, suggesting that these plant appendages may represent sites of penetration into the root interior (Fig. 1D). *Gfp*-cells able to colonize the root surface were also detected within the outer cortex (Fig. 1E and F), suggesting that E1 endophytic lifestyle was rapidly established upon inoculation.

Bacterial endophytes promote resistance of pepper plants to drought under hydroponic conditions

The capacity of E1 and E3 to enhance plant drought tolerance was evaluated under hydroponic conditions by adding 20% polyethylene glycol (PEG) to the growth medium in order to modify the osmotic potential and induce a severe drought stress in a relatively controlled manner (Supporting Information Fig. S6). Neither the nutrient solution used to grow the pepper plants nor the PEG added to induce the drought stress supported bacterial growth (Supporting Information Fig. S7), indicating that the bacteria were sustained only by the root exudates of the plants. After 48 h of PEG treatment, the uninoculated plants were strongly affected, while the E1- and E3-inoculated plants did not show any visual symptoms of drought stress (Fig. 2A, top panels). Both the fresh and dry plant biomasses were significantly affected by the PEG treatment ($F_{1,17} = 67.91$, $p < 0.001$ and $F_{1,17} = 41.91$, $p < 0.001$, respectively) and the bacterial treatment ($F_{2,16} = 18.72$, $p < 0.001$ and $F_{2,16} = 8.93$, $p = 0.004$, respectively). Accordingly, the E1 and E3 strains positively affected both the fresh (+40%–50%) and the dry (+20%–30%) weights of the pepper plants, inducing a protection effect independent from the growth condition (–PEG/+PEG; Fig. 2B) compared to the uninoculated plants.

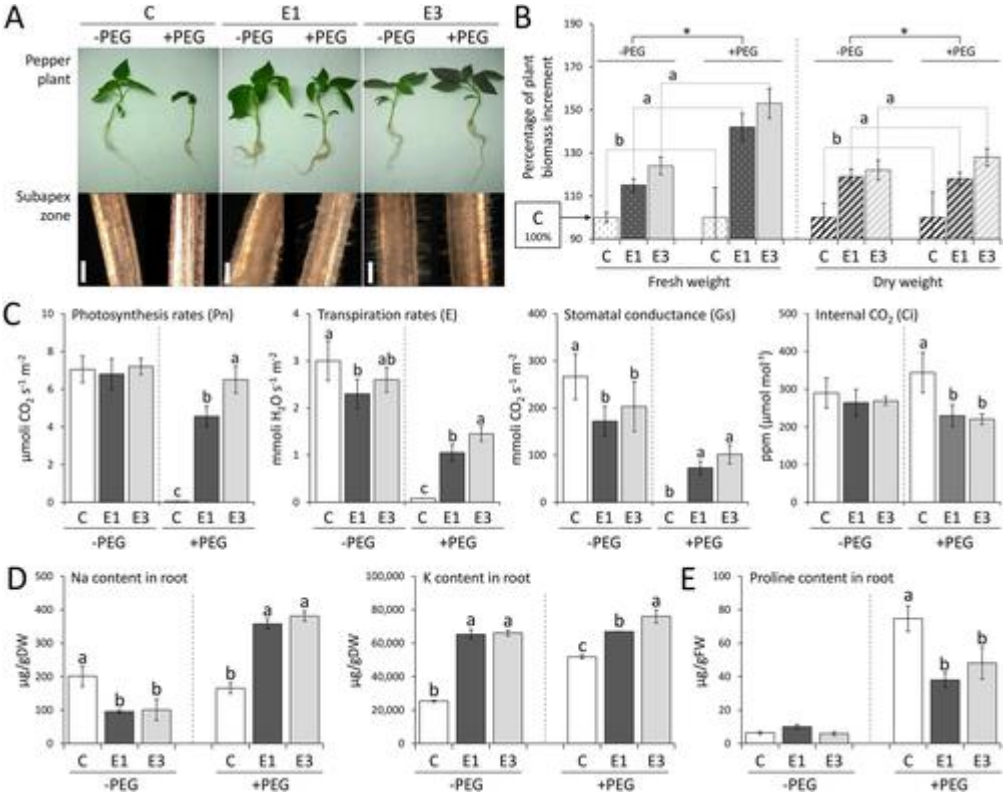

Figure 2

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Bacterial endophytes-induced drought resistance in pepper plants under hydroponic conditions.

A. (Upper panel) Endophyte strains E1 and E3 promoted the growth of pepper plants cultivated under hydroponic conditions and subjected to water stress (20% PEG). (Lower panel) Root sub-apical zone under normal (–PEG) and stress (+PEG) conditions. Bar size, 500 μm. B. Fresh and dry weight of uninoculated and inoculated pepper plants under drought stress expressed as a percentage of increment of inoculated plants compared to the control (i.e., uninoculated) plants (control values set to 100). '*' denotes a significant ($p < 0.05$) difference between +PEG and –PEG roots independent of the bacterial treatment. Different letters denote significantly ($p < 0.05$) different means for uninoculated, E1- and E3-inoculated plants independent of the drought stress. C. Net photosynthesis (Pn), evapotranspiration (E), stomatal conductance (Gs), internal CO_2 (Ci) of inoculated and uninoculated pepper plants measured after 48 h of drought stress in both condition (–PEG and + PEG). Uninoculated control plants are indicated in white and marked with 'C' under the histograms, while inoculated plants are labelled either 'E1' (dark grey) or 'E3' (light grey). Data are relative to one of three independent experiments (four plants per inoculation treatment/stress level; $n = 24$). D. Na, K and (E) proline contents in pepper roots after 48 h of drought stress (three independent experiments; $n = 18$). Different letters indicate significant differences among treatments (C, E1 and E3) and drought stress (–PEG and +PEG).

Caption

A proliferation of the number and changes in the morphology of the root hairs was recorded in the bacteria-inoculated plants in both –PEG and +PEG plants (Fig. 2A). While the E3-inoculated roots developed thin and long root hairs, the E1-inoculated roots showed thick and short hairs (Fig. 2A, bottom panels). PEG, which significantly decreased the root-hair diameter and length in a previous study (Robin *et al*., 2015), may have caused the lower number and shorter length of the hairs in the +PEG (stressed) plant roots.

Leaf-gas exchange measurements confirmed that E1 and E3 alleviated the effect of drought on the plant physiology (Fig. 2C). Under drought stress (+PEG) conditions, significantly higher values were measured for all the physiological parameters in the inoculated plants than in the uninoculated control group (Pn: E1 and

E3, $p < 0.001$; E: E1 and E3, $p < 0.001$; Gs: E1 and E3, $p < 0.001$; Ci: E1, $p = 0.019$ and E3, $p = 0.001$; Fig. 2C). The uninoculated stressed (+PEG) plants showed an impaired photosynthesis system, whereas the inoculated plants had a better physiological status. While both strains, E1 and E3, exerted a protective effect, the E3-inoculated plants maintained the highest net photosynthesis and transpiration rate values under drought stress (Fig. 2C). In the absence of stress (–PEG), no significant differences were found between inoculated and uninoculated plants in the net photosynthesis or the internal CO_2 (Sidak multiple comparisons $p > 0.05$); whereas, depending on the strain, the inoculated plants had significantly lower values of stomatal conductance and transpiration rate than the uninoculated plants (Gs: E1, $p = 0.002$ and E3, $p = 0.044$; E: E1, $p = 0.004$). Thus, the bacterial and PEG treatments caused significant effects that were observed in all the physiological parameters analysed (photosynthesis, Pn: $F_{2,22} = 57.80$, $p < 0.001$; transpiration rate, E: $F_{2,22} = 28.15$, $p < 0.001$; stomatal conductance, Gs: $F_{2,22} = 16.60$, $p < 0.001$; internal CO_2, Ci: $F_{2,22} = 5.41$, $p = 0.014$).

We also evaluated the osmolyte and ion contents in the root and leaf tissues of the plants (Fig. 2D and Supporting Information Fig. S8). The interaction between the bacterial and PEG treatments significantly affected the Na^+ and K^+ contents in particular ($F_{2,16} = 178.16$, $p < 0.001$ and $F_{2,16} = 52.93$, $p < 0.001$, respectively). Under drought stress, treatment with E1 and E3 strongly increased the accumulation of Na^+ (E1 and E3, $p < 0.001$) and K^+ (E1 and E3, $p < 0.001$) ions in the root tissues (Fig. 2D), which improved the cell turgor (Nieves-Cordones *et al*., 2016). Proline content was increased in the roots of plants subjected to water stress compared to the control group, with a significant effect of the interaction between the bacterial and PEG treatments ($F_{2,16} = 21.11$, $p < 0.001$; Fig. 2E). No differences in proline content were observed between the inoculated and uninoculated plants under –PEG conditions (Sidak multiple comparisons $p > 0.05$). However, under +PEG stress conditions, the proline content was higher in the uninoculated plants than in the inoculated ones (E1 and E3, $p < 0.001$), though no significant differences were observed between the E1 and E3 treatments (Fig. 2E).

Bacterial endophytes promote pepper plant resistance to drought in soil

Strains E1 and E3 also conferred drought tolerance to the pepper plants cultivated in soil. The untreated plants were severely affected after 7 days without watering, whereas the treated plants showed turgid tissues and better development (Fig. 3A). Compared to the uninoculated plants, strains E1 and E3 significantly enhanced the pepper plant biomass under drought conditions in terms of both fresh (E1: +34% ± 9%, pair-wise C-,E1: $p = 0.042$; E3: +32% ± 8%, pair-wise C-,E3: $p = 0.028$) and dry weights (E1: +42% ± 9%, pair-wise C-,E1: $p = 0.041$; E3: +37% ± 4%, pair-wise C-,E3: $p = 0.043$; Fig. 3B). The stressed plants inoculated with E1 and E3 showed a similarly fresh and dry biomass not significantly different from that of irrigated plants (Monte Carlo multiple comparisons, $p > 0.05$; Fig. 3B).

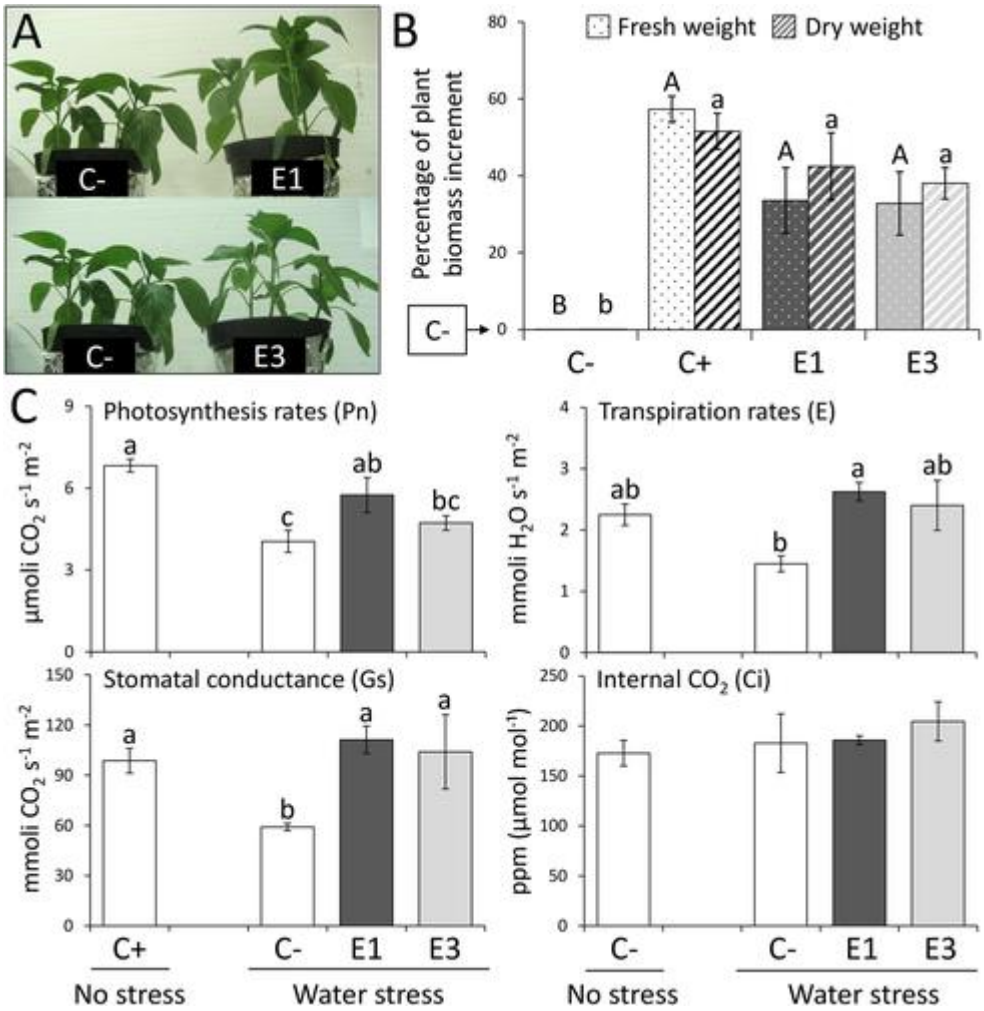

Figure 3

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Endophyte strains show PGP potential to promote drought resistance in pepper plants cultivated in soil.

A. E1- and E3-colonized (pots on the right) and untreated (pot on the left) pepper plants in a non-sterile soil. B. Fresh and dry weights of the plant roots. 'C+' plants properly irrigated. Values are reported as the percentage increase over the negative control, 'C−'. C. Physiological parameters of the inoculated (E1 and E3) and uninoculated (C+: irrigated; C−: drought stressed) pepper plants. Abbreviations are the same as in Fig. 2. Statistical analysis (one-way ANOVA, Tukey's Multiple Comparison Test when a significant *F*-test was obtained) is reported using different letters to indicate the means relative to the treated/untreated and stressed/unstressed plants ($p < 0.05$). The data reported in the graphs are relative to one experiment (three plants per inoculation treatment/stress; $n = 12$), which was representative of the three independent experiments.

Caption

The uninoculated and E3-treated plants under drought stress conditions were characterized by a significantly reduced net photosynthetic rate in the leaves (C−,C+: $p = 0.002$ and E3,C+: $p = 0.005$, respectively), unlike the E1-treated plants (E1,C+: $p = 0.21$). Stomatal conductance was significantly reduced in the uninoculated plants only (C−,C+: $p = 0.009$), and the transpiration rate was not affected at all (Fig. 3C). Comparable internal CO_2 contents were observed in both the inoculated stressed plants and the uninoculated unstressed control group ($p > 0.05$, in both cases), which were significantly higher than those of the uninoculated stressed plants ($p < 0.001$). Taken together, our results confirm that the endophytes E1 and E3 protected pepper plants from drought in soil as well as drought stress induced by PEG in a nutrient solution.

Bacterial endophytes affect pepper root morphology and the expression and activity of root V-PPase

Colonization by the bacterial endophytes also affected the pepper root morphology (Fig. 4A and B). There did not appear to be significant effects of the interaction between bacterial treatment and PEG treatment, thus the two factors have been taken in account separately. The bacterial treatment increased the size of the sub-apical root zone ($F_{2,22}$ = 14.448, p < 0.001), with a large diameter observed in plants treated by either E1 or E3 compared to the untreated plants (E1: +52%, p = 0.02; E3: +90%, p < 0.01; Fig. 4B). Treatment with PEG, on the other hand, did not have any effect on root diameter ($F_{1,23}$ = 0.03, p > 0.05; Fig. 4B).

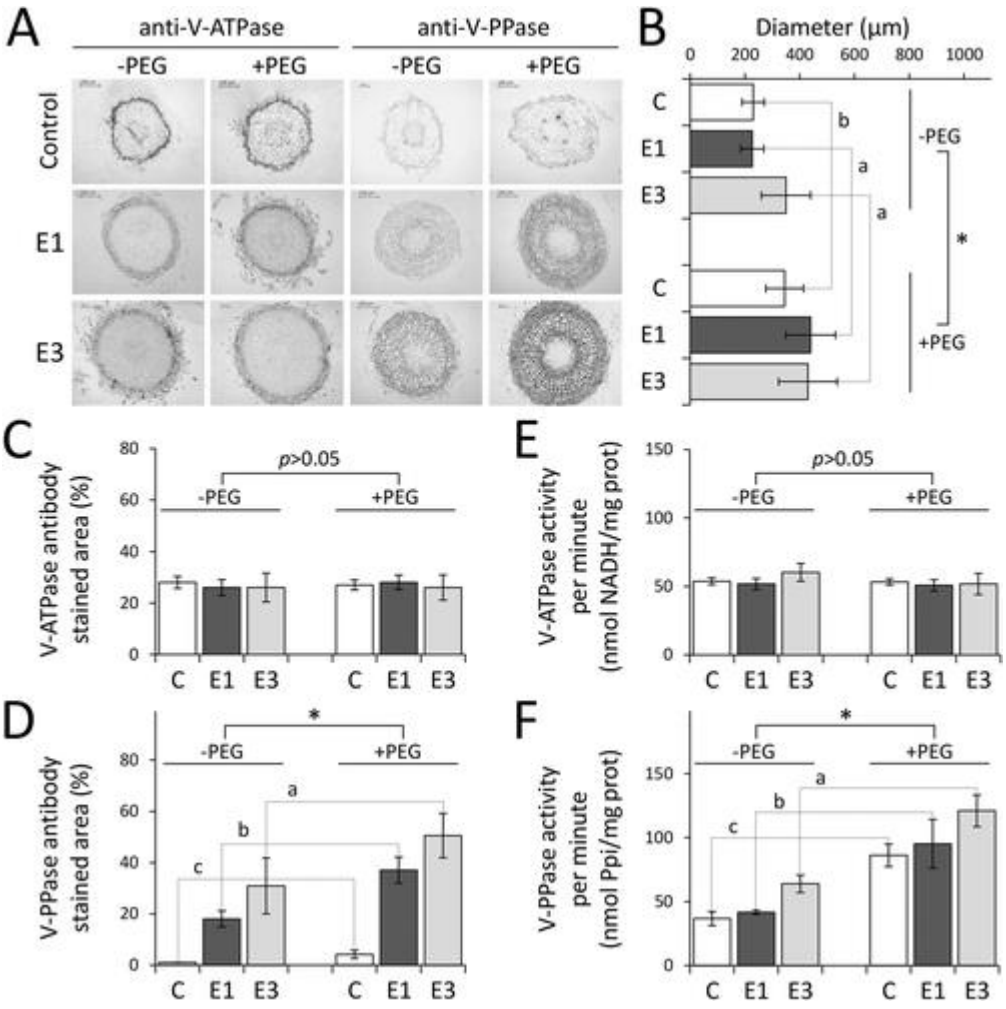

Figure 4

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Treatment with endophyte strains enhances V-PPase expression and activity in pepper roots.

A. Immunolocalization of V-ATPase and V-PPase in root cross sections (900–1000 µm from the tip) of E1- and E3-inoculated and uninoculated pepper plants treated with (+) or without (−) PEG. The images correspond to one experiment representative of three independent experiments. B. Root diameter (µm) measured from root cross sections at a distance of 900–1000 µm from the tip. Results expressed as mean diameter ± standard deviation of all sectioned roots (n = 24). C,D. Quantification of the immunoreactive areas of the vacuolar proton pumps, V-ATPase (C) and V-PPase (D), in the root cross sections. Results expressed as mean ± standard deviation of three independent experiments (n = 18). E,F. Activities of V-ATPase (E) and V-PPase (F) in the tonoplast-enriched fractions of roots of E1- and E3-inoculated and uninoculated pepper plants treated with (+) or without (−) PEG. Results expressed as mean ± standard

deviation of four independent experiments (n = 24). Statistical analysis (two-way ANOVA, REGWQ post hoc test, $p < 0.05$). '*' denotes a significant difference between the drought-stressed (+PEG) and the unstressed roots independent of the bacterial treatment. Different letters denote significantly different means for uninoculated, E1- and E3-inoculated plants independent of the drought stress (REGWQ post hoc test, $p < 0.05$).

Caption
In the untreated plants, the root elongation zone (measured from the tip) was restricted to 350–700 μm, while it extended to 500–1500 μm in the treated plants. Immunostaining experiments performed on thin sections of root tissue from the pepper plants grown under hydroponic conditions revealed that exposure to the bacterial endophytes affected the expression of V-PPase under simulated drought conditions (+PEG; $F_{1,17} = 22.638$, $p < 0.001$; Fig. 4A and D), but not of V-ATPase ($F_{1,17} = 0.042$, $p < 0.05$; Fig. 4A and C). No protein signals were detected in the thin root sections in the absence of the specific primary antibody treatments again V-ATPase and V-PPase, indicating that non-specific reactions attributable to the secondary antibody or to the staining procedure did not occur (Supporting Information Fig. S9). No significant differences were detected in the accumulation or distribution of V-ATPase for bacterial treatment ($F_{2,16} = 0.259$, $p > 0.05$) or PEG treatment ($F_{1,17} = 0.042$, $p > 0.05$; Fig. 4C). The PEG treatment caused the V-PPase accumulation in the external cell layers and in the stele of the root segment corresponding to the elongation zone ($F_{1,17} = 22.638$, $p < 0.001$; Fig. 4A and D). In addition, the bacterial treatments significantly enhanced the accumulation of V-PPase in the cortex root layers ($F_{2,16} = 57.954$, $p < 0.001$) regardless of the PEG treatment, suggesting that colonization by both E1 and E3 is associated with a drought-independent V-PPase protein accumulation (Fig. 4A and D). In order to confirm such results at the enzymatic level, the activity of the two proton pumps, V-ATPase and V-PPase, was assayed on the tonoplast-enriched fraction of the whole root tissues (Fig. 4E and F). The proton pumps activities reflected the respective protein expression observed by immunostaining experiments but with lower increases induced by inoculation with E1 and E3. No significant difference was retrieved in the V-ATPase activity for either factor analysed (bacterial treatment: $F_{2,16} = 2.619$, $p > 0.05$; PEG treatment: $F_{1,17} = 1.728$, $p > 0.05$; Fig. 4E). A significant increase in V-PPase activity was detected in the drought stressed (+PEG) roots compared to the unstressed (–PEG) roots ($F_{1,17} = 16.646$, $p = 0.001$; Fig. 4F). Moreover, the V-PPase activity was significantly affected by the bacterial treatment ($F_{2,16} = 84.383$, $p < 0.001$), with the highest values observed in E3-inoculated plants (Fig. 4F).

Discussion

In this study, we investigated the relationship between the capacity of endophytic bacteria to increase plant drought tolerance and to activate the vacuolar proton pumps, V-ATPase and V-PPase, in the plant host. V-ATPase and V-PPase are key enzymes in plant response to drought, as it was demonstrated that its overexpression induces a strong drought tolerance in many plants (among others Bao *et al*., 2016; Da-Gang *et al*., 2012; Shen *et al*., 2014). V-ATPase and V-PPase establish a proton gradient across the vacuolar membrane that allows the plant tissues to maintain cell turgor at a low soil-water potential (Gaxiola *et al*., 2007, 2016). We found that the pepper plants became resistant to drought and enhanced the expression in the root of V-PPase only, when the plants were colonized by endophytic bacteria (E1 and E3). However, the induction of V-PPase activity in E1 and E3 inoculated plants under drought stress was lower than the protein expression enhancement detected by immunolocalization of the V-PPase under the same conditions. Such discrepancy might be due to a dilution effect. In fact, V-PPase activity was measured from the root tonoplast-enriched fraction obtained from the whole root system, while the V-PPase detection by immunohistochemistry was performed on root sections belonging to the elongation and differentiation zone. The latter is the root portion specifically involved in the water/nutrient uptake process (Barberon and Geldner, 2014), thus any change in expression/activity of proteins engaged in the water/nutrient uptake is expected to be concentrated in this limited root zone. Overall, our finding confirms the association of V-PPase expression with drought resistance and indicates that the endophytic bacteria may activate such expression.

The E1 and E3 strains rapidly adhered to the plant roots and were characterized by many beneficial PGP traits endowed with biopromotion (Patel and Saraf, 2017), biofertilization (Mapelli *et al*., 2012) and

bioprotection against abiotic stresses (Dimkpa *et al*., 2009). A combination of microscopy analysis and strain re-isolation tests showed that both strains had competence in colonizing the endosphere and translocating to the different organs of the pepper plants. High cell counts at the root (between 10^5 and 10^6 CFU g^{-1} of tissue) confirmed that the bacterial cells were tightly attached to the rhizoplane and possibly penetrated the root tissues to multiply in the root endosphere. Emerging lateral roots breaks are considered preferential sites for penetration of bacteria in the inner tissues and from there to the phloem and xylem vessels (Compant *et al*., 2010). Driven by the plant transpiration flux, bacteria can be further translocated to the shoots and leaves (Compant *et al*., 2010). For instance, the PGP strain *Paraburkholderia phytofirmans* PsJN colonizes root rhizodermis cells, internal tissues, internodes and leaves of grapevine (Compant *et al*., 2008). Similarly, *Azoarcus* strain BH72 penetrates the rhizoplane in the elongation and differentiation zone of the root and systemically colonizes the rice plant tissues presumably by longitudinal spreading through vessels (Hurek *et al*., 1994).

Different entry strategies to the root tissue are possible, including a controlled digestion of the root by lytic enzymes or passing through the natural breaks at elongated areas (Hardoim *et al*., 2015). The two strains showed cellulase activity *in vitro*. The ability to produce cell wall-degrading enzymes such as cutinases, pectinases, cellulases, hemicellulases, proteases and lignin-peroxidases is a key strategy adopted by microorganisms to penetrate the cuticle and cell walls and thus to enter into plant tissues (Brader *et al*., 2014). Furthermore, the capacity of E1 and E3 to produce biosurfactants and bioemulsifiers may have played a role in root colonization by changing the root surface wettability, similar to epiphytic bacteria exploiting these molecules to increase their survival times in the phyllosphere (Burch *et al*., 2011). The versatility of bacteria, such as strains E1 and E3, in colonizing different plant tissues should favour the spread of the carried drought tolerance traits in the plant organs, thus potentially enhancing their beneficial effect on the whole plant.

VOC blends produced by strains E1 and E3 promoted the biomass growth of *Arabidopsis* plantlets under both normal and osmotic stress conditions. The characterization of such VOC blends showed that strain E1 produced 2,3-butanediol, a compound that was previously demonstrated to promote plant growth (i.e., Ryu *et al*., 2003). Other bacteria, such as the *B. subtilis* strain GB03 and PGP strains including *Pseudomonas chlororaphis* strain O6, were observed to produce 2,3-butanediol (Ryu *et al*., 2003; Cho *et al*., 2008). The VOCs released by *B. subtilis* GB03 triggered an increased synthesis of auxin in the *Arabidopsis* leaves and translocation of the auxin to the roots at the lateral primordial, facilitating new root formation (Zhang *et al*., 2007). Under drought conditions, *Arabidopsis* plants inoculated with *P. chlororaphis* O6 or exposed to 2,3-butanediol exhibited increased stress tolerance (Cho *et al*., 2008).

Our survey of the two bacterial genomes did confirm the presence of tested PGP traits and did reveal metabolites that can positively affect plant growth. Strains E1 and E3 are endowed with genes for the synthesis of nitric oxide (NO), a key secondary messenger that triggers auxin-mediated rapid cellular and organ responses in plants (Schlicht *et al*., 2013). NO plays a dominant role in the establishment of symbiosis between *Rhizobia*-like bacteria and leguminous plants (Hichri *et al*., 2015), and the ability of *Azospirillum brasilensis* to affect secondary and adventitious root formation in tomato plants was shown to be largely NO-dependent and auxin-independent (Creus *et al*., 2005; Molina-Favero *et al*., 2008). Notably, despite auxin (i.e., IAA) production is a widespread and conserved PGP trait in plant-associated bacterial communities (i.e., Marasco *et al*., 2018), we did not detect such trait in E1 or E3. Both phenotypic assays and our search in their genomes for genes encoding the two major biosynthetic routes of bacterial IAA production, that is, pathways for indole pyruvic acid and indole-3-acetamide (Spaepen *et al*., 2007), were negative. However, regardless of their ability to produce IAA, beneficial bacteria have been shown to manipulate plant auxin homeostasis, thus regulating the postembryonic development of the plant root system (Wang *et al*., 2015).

The *in vivo* potential of the endophyte strains to enhance plant resistance to drought was evaluated in hydroponic conditions and confirmed in a non-sterile soil. Under drought stress conditions, induced either by PEG or by interruption of irrigation, the endophyte-treated plants presented a more robust root system, improved photosynthetic activity, and better physiology than the untreated plants. The plant response was similar to that of the switchgrass *Panicum virgatum* L. treated with the beneficial strain *Paraburkholderia*

phytofirmans strain PsJN, where the positively affected leaf physiology supported higher photosynthetic activity and promoted an increased production of siliques under different levels of drought stress (Wang *et al*., 2016a). The improved drought resistance of plants colonized by E1 and E3 is associated with the enhanced expression and activity of V-PPase, but not with the V-ATPase that did not show significant change when exposed to bacteria/stress. Pepper plants treated with E1 and E3 had increased contents of Na^+ and K^+ in the root tissues compared to untreated plants. Similar effects were observed in *Arabidopsis* and tomato plants subjected to drought and were attributed to cooperation between V-PPase and Na^+/H^+ antiporter activity in vacuole osmotic adjustments (Park *et al*., 2005; Brini *et al*., 2006; Pasapula *et al*., 2011). E1 and E3 colonization did not increase the root content of the osmoprotectant compound proline, suggesting that the drought tolerance was induced through a different mechanism from that observed with other beneficial bacteria (Cohen *et al*., 2015).

We observed that the colonization of the two endophytes was associated with not only the enhanced expression and activity of the V-PPase, but also the formation of a larger root system, the proliferation of root hair, and thicker primary roots. These are all morphological changes previously associated with enhanced plant resistance to drought (Bardgett *et al*., 2014). V-PPase activity has also been associated with the transport of auxin and both the abundance and distribution of PIN1, a major auxin translocator (Li *et al*., 2005), in addition to its effect on vacuolar pH homeostasis. It has been demonstrated that plants overexpressing V-PPase showed an increase in the auxin-mediated cell division at organogenesis, resulting in a larger and more extended root system that may enhance plant performance during drought (Park *et al*., 2005).

In conclusion, from the phenotypic and genomic survey of the two endophytes, we identified PGP traits and genes that may encourage the resistance to drought. Our data show that in drought-stressed pepper plants, endophytic bacteria efficiently colonize the root system, affect plant activity and response to environmental challenges, and support the expression and activity of the V-PPase an enzyme involved in the alleviation of drought stress.

Experimental procedures

Endophyte isolation, identification and in vitro *characterization of the PGP potential*

The two bacterial strains used in this study, E1 and E3, were isolated from the root tissues of pepper plants cultivated under desert farming (Marasco *et al*., 2012). A phylogenetic tree based on an analysis of the entire 16S rRNA sequence was inferred by the neighbour-joining method, with 2000 replicates for a bootstrap test in the molecular evolutionary genetics analysis software, MEGA 4 (Kumar *et al*., 2008).

A functional screening for PGP traits (described in Supporting Information Methods S1) was performed through a series of *in vitro* assays encompassing the (i) biostimulation and (ii) biofertilization activities possibly enhanced by bacteria. Briefly, the indole acetic acid (IAA) production and ACCd activity were measured to evaluate the biostimulation activity. The biofertilization trait was evaluated by measuring the capacity of our strains to solubilize mineral P, release siderophores, regulate atmospheric nitrogen and produce ammonia and exopolysaccharide (EPS).

Abiotic stress tolerance was tested in order to evaluate the ability of selected strains to grow in the presence of salt (NaCl 5%, 8% and 10%), polyethylene glycol (10% and 20%) and at different temperatures (4°C, 42°C and 50°C), as described in Marasco and colleagues (2012).

A phenotype microarray (PM) assay (Biolog) and a PM9 osmolytes microplate containing 96 different conditions (Supporting Information Fig. S1) were used to compare the cellular phenotypes of E1 and E3.

Different inoculating fluid (IF) solutions (proprietary formulation supplied by Biolog) were prepared and used to inoculate the PM plates, following the Biolog PM protocol for gram-positive bacteria (Supporting Information Method S2). The colour change of the redox potential indicator (Dye F, Biolog) due to the metabolic activity of bacterial cells was monitored and measured by PM technology (Biolog) using an OmniLog instrument set at 30°C. Reducing the dye caused the formation of a blue colour, which was recorded by a CCD camera every 15 min (Bochner *et al*., 2001). The kinetic colorimetric data were stored in computer files and analysed using the Biolog software.

The biofilm formation was evaluated as described in Burton and colleagues (2006); the endoglucanase and endopolygalacturonase activity was evaluated according to Compant and colleagues (2008).

To analyse the VOCs produced by strains E1 and E3, 2 ml of MS media were poured in 40 ml airtight-seal glass vials stopped with a silicone PTFA septum and inoculated with 20 µl of 10^8 bacterial cells. A solid-phase microextraction (SPME) manual holder (Supelco, Bellafonte, PA, USA), with a StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco), was inserted through the PTFA cap (Tait *et al*., 2014). The SPME was exposed for 30 min to the bacterial culture headspace and detected using gas chromatography (GC, Agilent Technologies, USA) combined with a mass spectroscopy detector (MS, Agilent Technologies, USA), as described in Supporting Information Method S3. The plant growth promoting effect of bacterial VOCs was tested on *Arabidopsis*. Bi-plate sterile petri dishes (with septum) were used for plant exposure to bacterial VOCs according to previously described methods (Ryu *et al*., 2003). Both sides of the petri dish contained half strength MS, Murashige and Skoog medium (added with 0.8% agar and 1.5% sucrose, adjusted to pH 5.7) for bacterial and plant growth. On the plant side 100 mM mannitol was added to simulate drought stress (Zhang *et al*., 2010). *Arabidopsis* Col0 seeds were surface sterilized, placed on half strength MS plates and vernalized for 2 days at 4°C in the absence of light. The plates were then placed in a growth cabinet with a light and dark cycle of 16 and 8 h, respectively, at 200 µmol photons m^{-2} s^{-1} light, at 22°C temperature and 50% relative humidity, and germinating plantlets were let to grow for 2 days. Bacterial strains were inoculated onto PAF liquid medium and incubated at 28°C under shaking overnight. Bacterial cells were harvested, washed twice with 9 g l^{-1} saline buffer and diluted to 10^9 CFU ml^{-1}, as determined by optical density. Two-day-old *Arabidopsis* seedlings were transferred to new growth media with or without 100 mM mannitol in the first half of sterile bi-plate petri dishes. The other half of the plates was inoculated with 20 µl of bacterial suspension culture or supplemented with the same volume of sterile water. The bi-plates were accurately sealed with parafilm and placed in the growth cabinet for 15 days. At the end of the exposure period, the plants were removed from the agar and fresh weight was determined.

The E1 and E3 biosurfactant productions were evaluated by inoculating cells into a glucose mineral salts medium (GMSM: 10 g l^{-1} glucose; 0.7 g l^{-1} KH_2PO_4; 0.9 g l^{-1} Na_2HPO_4; 2 g l^{-1} $NaNO_3$; 0.4 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$; 2 ml of trace elements [per litre, 2 g $FeSO_4 \cdot 7H_2O$, 1.5 g $MnSO_4 \cdot H_2O$, 0.6 g $(NH_4)6Mo7O_2 \cdot 4H_2O$]; pH = 6.72). The flasks were incubated overnight at 30°C on a rotary shaker (150 rpm). The surface activity of the cell-free supernatants was evaluated by measuring the emulsification index, the drop collapse assays, and the interfacial surface tension (IFT, mn m^{-1}), as reported in the Supporting Information Method S4.

Genome sequencing and assembly, and wide survey of PGP traits

Bacillus subtilis E1 draft genome was recently deposited under the accession number GCA_000724125.1. The functional annotation was performed using the RAST server (Moriya *et al*., 2007). The strain *Paenibacillus illinoinensis* E3 was sequenced using Illumina Hiseq technology; the reads were assembled using velvet. The annotation was then performed by the Indigo Server (Alam *et al*., 2013). The genome was deposited in NCBI under the Bioproject number PRJNA430863.

Genes involved in the PGP mechanisms (i.e., glycine betaine pathway, ROS and auxin production, exopolysaccharide synthesis, nitric oxide synthase, ACC deaminase activity and VOCs production) were selected using several published data (Yan *et al*., 2008; Bertalan *et al*., 2009; Ma *et al*., 2011; Sant'Anna *et al*., 2011; Weilharter *et al*., 2011; Yu *et al*., 2011; Taghavi and van der Lelie, 2013). Using the UniProt database (The UniProt Consortium, 2017) the KEGG orthology (KO) related to the selected PGP genes was extracted. Then the obtained PGP-KO were compared with those present in the two bacterial genomes, which were obtained using the KEGG Automatic Annotation Server (KAAS, Moriya *et al*., 2007). The presence or absence of each PGP-KO in the bacterial genomes was evaluated for both strains.

Bacteria and plant growth conditions

Bacteria were grown in Pseudomonas Agar F (PAF) liquid medium. For plant treatment, a hydroponic solution with 2×10^7 cells ml^{-1}, supplemented with 20% PEG, was prepared from the bacteria after two washes in physiological buffer (9 g l^{-1} NaCl). The ability of bacteria to thrive in the growth medium of hydroponic solution was tested by monitoring the growth curve of endophyte strains inoculated in two solutions, one with PEG and one without (Supporting Information Fig. S3).

Pepper seeds (*Capsicum annuum* L.) were sown in agro-perlite, watered with 0.1 mM $CaSO_4$, and incubated in the dark at 26°C for 6 days to allow plant germination. Plantlets were transferred into a nutrient solution composed of 2 mM $Ca(NO)_3$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, 10 μM H_3BO_3, 1 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.05 μM $(NH_4)Mo_7O_{24}$ and 0.1 mM Fe(III)-EDTA. The final pH was adjusted to 6.0–6.2 with NaOH. The hydroponic cultures were kept in a growth chamber and constantly aerated. Day and night regimes of 16 and 8 h at temperatures of 18°C and 24°C, respectively, and a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} at the plant level were applied.

Evaluation and quantification of bacterial recolonization ability by microscopy analysis and re-isolation procedures

The fluorescent phenotypes of the strains were obtained using a plasmid pGFP-ratiometric carrying the *gfp* gene under the control of P32 promoter. The adopted transformation protocol was described by Tamagnini and colleagues (2008). Only a single transformed colony was retrieved from the E1 transformation, and the E3 transformation was unsuccessful. The resulting strain, E1-*gfp*, was fluorescent under selective conditions and the plasmid-retaining stability was estimated to be 80% after 24 h of growth under non-selective conditions (Cutting and Vander Horn, 1990).

To evaluate the colonization capability of E1-*gfp*, pepper plants grown in hydroponic medium (prepared as described above) were inoculated overnight with 10^7 cells ml^{-1} by agitation. After 20–24 h, the plant roots were washed to remove the weakly bound bacteria and were further observed under an epifluorescent Leica microscope using the GFP filter (excitation at 488 nm). The acquired images were analysed by using the MBF-ImageJ. A confocal analysis was performed using a confocal laser scanning microscope, the Leica TCSNT, equipped with an Argon/Krypton (Ar/Kr) laser. GFP filters (excitation at 488 nm) were used to monitor E1-*gfp*, and dsRED filters (excitation at 558 nm) were used to acquire the root autofluorescence. The experiments were repeated twice on three replicate plants.

To obtain the spontaneous rifampicin mutants of the assayed endophytes, a protocol similar to that described by Dey and colleagues (2004) was adopted. Two isolates were grown overnight in PAF medium at 30°C. Then, the cell cultures were plated onto PAF_{Rif100} plates containing 100 μg ml^{-1} rifampicin and incubated at 30°C for 2 days. Single colonies were purified and streaked at least twice on the PAF_{Rif100} medium before being plated on PAF_{Rif200} plates (containing 200 μg ml^{-1} rifampicin). Colonies demonstrating the ability to grow at this rifampicin concentration were used for further analysis. To evaluate the ability of the spontaneous rifampicin mutants (E1 and E3) to colonize pepper plants, the same protocol used to induce drought stress was applied, unless plants were processed to isolate bacteria. The plant organs were carefully washed in sterile physiological solution to prevent the bacterial cell from actively colonizing the plant surface. One gram of apical (0–3 cm) and subapical (>3 cm) root segments, stems, and leaves were smashed

in a mortar. After 20–30 min, serial dilutions of the samples were plated on PAF plates containing 200 µg ml^{-1} rifampicin. After 2–4 days of incubation at 30°C, the single colonies were counted and at least three colonies per fraction were checked by ITS-PCR to reconfirm bacteria identity (Daffonchio *et al*., 2000). The results are the average of three independent experiments using two replicate plants for each experiment.

Evaluation of the PGP potential of endophyte strains to promote drought resistance of pepper plants in a hydroponic system

We used a hydroponic system to deeply study whether the plant–microbe interaction affected the activity and expression of V-PPase and V-ATPase. Preliminary studies indicated that 20% PEG was necessary to rapidly induce a severe water-stress event (Supporting Information Fig. S2). After germination, the pepper plants were grown in a nutrient solution over 21 days. Then, the plants were inoculated using 10^8 CFU ml^{-1} nutrient solution and were maintained in contact with bacteria for 24 h. Half of the plants were transferred to a complete nutrient solution, while the other half were transferred to a nutrient solution supplemented with 20% PEG6000. The plants were harvested for analysis after 48 h, and the fresh weights of both the shoot and root biomasses were determined. After drying at 65°C in an oven to reach a constant weight, the dry weights of both the shoot and root biomass were also determined. Some freshly collected roots were observed with a LEICA DM R optical microscope, and images were acquired with a Leica EC3 camera and LAS V4.1 software. Statistical analyses were conducted with SSP software. A two-way ANOVA ($p < 0.05$) was used to analyse the independent and interdependent effects of the two factors, 'Bacterial treatment' and 'PEG treatment', and of their interaction. In the presence of a significant *F*-test for interaction, pairwise comparisons were carried out by applying Sidak's correction ($p < 0.05$). In the absence of a significant *F*-test for interaction, the effect of the inoculation treatment was analysed independently on the stress level by comparing the mean to the REGWQ post hoc test ($p < 0.05$).

Evaluation of bacterial effect on physiological parameters of plants under drought stress in a hydroponic system

After 48 h, leaf-gas exchange measurements of the net photosynthesis (Pn), transpiration (E), stomatal conductance (gs) and internal CO_2 (Ci) were performed according to Marasco and colleagues (2012). Analyses of the osmolytes and osmoprotectants were conducted as described in the Supporting Information Methods S5 and S6. For the immunolocalization of the vacuolar proton pumps in the root tissue, the five largest root apical segments were sampled from two plants for each treatment and for three experiments. These samples were fixed in 4% paraformaldehyde (w/v), dehydrated, and then embedded in paraffin as described by (Dell'Orto *et al*., 2002). A microtome was used to cut serial sections of 6 µm and up to 1500 µm from the tip. The sections were mounted on polylysine-treated slides, deparaffinized in xylene and rehydrated through an ethanol series. Immunological detection was performed, as in Dell'Orto and colleagues (2013), by raising polyclonal antibodies against two peptides corresponding to V-ATPase and V-PPase from *Arabidopsis* (Maeshima and Yoshida, 1989; Maeshima, 2001; Kobae *et al*., 2004). A biotinylated secondary antibody, the ExtrAvidin-Peroxidase system (ExtrAvidin Peroxidase Staining Kit, Sigma) catalysing the staining reaction between 3-amino-9-ethylcarbazole (AEC) and H_2O_2 was also used. Root sections were observed with a LEICA DM R optical microscope, and images were acquired with a Leica EC3 camera and LAS V4.1 software. The stained area (%) was quantified using ImageJ software. The cross-section diameters were measured at a distance of 900–1000 µm from the tip.

Isolation of the tonoplast-enriched fraction from roots and determination of V-ATPase and V-PPase activity were performed according to the protocols described in Dell'Orto and colleagues (2013). The immunoreactive bands were quantified as a percentage of the stained area by ImageJ software. Statistical analyses were conducted with SSP software. Two-way ANOVA ($p < 0.05$) was used to analyse the effects of the bacterial treatment, the PEG treatment and their interaction. In the presence of a significant *F*-test for their interaction, pairwise comparisons were carried out by applying Sidak's correction ($p < 0.05$). In the

absence of a significant *F*-test for their interaction, the effect of the inoculation treatment was analysed independently on the stress level by comparing the mean to the REGWQ post hoc test ($p < 0.05$).

Evaluation of the PGP potential of endophyte strains to promote pepper resistance to drought in soil

Pepper seeds (*Capsicum annuum* L.) were spread on wet agro-perlite. After 7 days, three seedlings of the same size were selected and transferred to a plastic pot (14 cm diameter) containing commercial soil. The seedlings were grown in a growth chamber at day and night temperatures of 25°C and 20°C, respectively, with approximately 100 µmol photons m^{-2} s^{-1} of light supplied for 12 h during the daytime. During the second week, the plantlets were inoculated with a bacterial suspension at a concentration of 10^8 cells g^{-1} soil, while the uninoculated plants were watered with tap water. Starting 7 days after inoculation, water was withheld for 12 days. The 'positive control' plants were properly irrigated. After 7 days of withholding water, the physiological parameters were measured as described above. Statistical analysis (one-way ANOVA) was conducted with SPSS software and, when a significant *F*-test was obtained, the means were compared using Tukey's test ($p < 0.05$). After the 12-day drought period, water irrigation was resumed for 3 days before the plants were harvested for biomass measurements. Three independent experiments with three replicate plants per treatments were conducted. Statistical analysis (one-way ANOVA) was used to compare treatments using the Monte Carlo test in Primer 6 software (999 permutations).

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Notes :

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- Presence or absence of (**A**) PGP traits and (**B**) abiotic stress tolerance were evaluated following the methods described in the experimental section. Additional details of the results marked with a star (*) are reported in Supporting Information Tables S1 and S2 and Supporting Information Figs. S1–S3. (+) Indicates the highest concentration of osmolytes (% w/v) tolerated by the strains.