Molecular Insights into the Involvement of a Never Ripe Receptor in the Interaction Between Two Beneficial Soil Bacteria and Tomato Plants Under Well-Watered and Drought Conditions

Pablo Ibor, Sonia Molina, Juan Manuel Ruiz-Lozano, and Ricardo Aroca[†]

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (EEZ-CSIC), Profesor Albareda 1, 18008 Granada, Spain

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Management of plant growth-promoting bacteria (PGPB) can be implemented to deal with sustainable intensification of agriculture. Ethylene is an essential component for plant growth and development and in response to drought. However, little is known about the effects of bacterial inoculation on ethylene transduction pathway. Thus, the present study sought to establish whether ethylene perception is critical for growth induction by two different PGPB strains under drought conditions and the analysis of bacterial effects on ethylene production and gene expression in tomatoes (*Solanum lycopersicum*). The ethylene-insensitive *never ripe* (*nr*) and its isogenic wild-type (*wt*) cv. Pearson line were inoculated with either *Bacillus megaterium* or *Enterobacter* sp. strain C7 and grown until the attainment of maturity under both well-watered and drought conditions. Ethylene perception is crucial for *B. megaterium*. However, it is not of prime importance for *Enterobacter* sp. strain C7 PGPB activity under drought conditions. Both PGPB decreased the expression of ethylene-related genes in *wt* plants, resulting in stress alleviation, while only *B. megaterium* induced their expression in *nr* plants. Furthermore, PGPB inoculation affected transcriptomic profile dependency on strain, genotype, and drought. Ethylene sensitivity determines plant interaction with PGPB strains. *Enterobacter* sp. strain C7 could modulate amino-acid metabolism, while *nr* mutation causes a partially functional interaction with *B. megaterium*, resulting in higher oxidative stress and loss of PGPB activity.

The term plant growth-promoting rhizobacteria was established to provide a designation for the bacterial community of soil that competitively colonized the plant root system and promoted plant growth or diminished incidences of plant

disease or both (Kloepper and Schroth 1978). Later, these were more generally named plant growth-promoting bacteria (PGPB) (Vessey 2003; Weller et al. 2002). PGPB are capable of inducing plant growth either directly or indirectly (Ortiz-Castro et al. 2009), although several mechanisms may simultaneously contribute to the cumulative enhancement of plant growth (Martínez-Viveros et al. 2010). PGPB direct promotion mechanisms include solubilization of one or both iron (Fe) and phosphate (Pi), biological nitrogen (N₂) fixation (Hayat et al. 2010), and modulation of phytohormone levels (production of auxins [Mishra et al. 2010], cytokinins [Ortiz-Castro et al. 2008], and gibberellins [Narula et al. 2006], or ethylene reduction [Glick et al. 2007; Saleem et al. 2007]). Indirect mechanisms, on the other hand, are primarily related to biocontrol processes as a competition for niches within the rhizosphere, chelation of available Fe, interference with toxin production by pathogens (Beneduzi et al. 2012), induction of systemic resistance (Pieterse et al. 2014), and promotion of the establishment of beneficial plant-microbe symbiosis (Lugtenberg and Kamilova 2009; van Loon 2007; Zahir et al. 2003).

Ethylene plays a key role in plant development and has the ability to induce or reduce plant growth, depending on the cell type and plant species (Negi et al. 2010; Pierik et al. 2006; Swarup et al. 2007). Ethylene is biosynthesized from *S*-adenosylmethionine via 1-aminocyclopropane-1-carboxylate (ACC), which is the ethylene immediate precursor. The pathway is catalyzed by ACC synthase (ACS) (Boller et al. 1979) and ACC oxidase (ACO), resulting in ethylene, carbon dioxide, and cyanide (Kende 1993; Yang and Hoffman 1984), although ACC can also be conjugated in several forms (Van de Poel and Van Der Straeten 2014).

Ethylene receptors are predominantly localized into the endoplasmic reticulum, since ethylene is lipophilic and is freely diffused up to endomembrane-located receptors (Grefen et al. 2008). In addition, ethylene signal transduction is triggered when ethylene binds to its receptors via a copper cofactor (Rodríguez 1999; Woeste and Kieber 2000), diminishing the activity of a serine/threonine kinase called constitutive triple response 1 (CTR1), which effectively inhibits further signaling. Ethylene perception results in transcriptional changes denominated as ethylene response, which is mediated by ethylene response factors (ERFs) and ethylene response (*ER*) genes (Ju and Chang 2012; Merchante et al. 2013; Vandenbussche et al. 2012).

Previous analysis established the presence of six ethylene receptors in tomatoes (*SlETR1* to *SlETR6*) and classified them into the following two subfamilies according to their structure:

Transcriptomic data is publicly available in the Gene Expression Omnibus database of the National Center for Biotechnology Information under the accession number GSE10.

[†]Corresponding author: Ricardo Aroca; E-mail: ricardo.aroca@eez.csic.es

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subfamily I (*SIETR1* to *SIETR3*) and II (*SIETR4* to *SIETR6*) (Lashbrook et al. 1998; Tieman and Klee 1999; Wilkinson et al. 1995). *SIETR3* was also named Never Ripe, since a point mutation in the sensor domain, which inhibits further ethylene binding and signal transduction, resulted in an easily recognized phenotype (the inability of fruits to undergo ripening, delayed flowering, leaf senescence, and pedicel abscission), although some residual responsiveness is conserved (Lanahan et al. 1994; Wilkinson et al. 1995). Additionally, four *CTR1*-like genes (*SICTR1* to *SICT4*) were reported to be present in tomatoes (Adams-Phillips et al. 2004; Leclercq et al. 2002) and were called TCTR (tomato CTR). Ethylene receptors and *CTRs* are differentially regulated in response to stimuli and during plant development, possibly regulating varied ethylene responses (Zhong et al. 2008).

Furthermore, ethylene is typically induced in response to environmental stresses (Wang et al. 2013). For instance, drought stress, which causes significant crop losses, induces the production of ethylene (Pierik et al. 2007). Plant mechanisms to cope with water deficiency are mainly mediated by ethylene and abscisic acid (ABA) (Pierik et al. 2007; Sharp et al. 2000), which act as antagonists, since accumulation of ABA could modulate growth response to ethylene and vice versa (Wilkinson and Davies 2010; Wilkinson et al. 2012). The root system is a key player in the regulation of drought stress responses (Chaves et al. 2003; Steudle 2000). Besides primarily diminishing aerial vegetative growth, drought produces physiological effects such as gas exchange inhibition, but it is mainly sensed by roots, thus affecting root-to-shoot signaling (Anjum et al. 2011; Deblonde and Ledent 2001). Additionally, drought is also known to affect the expression of several genes involved in osmotic stress, showing cross-linking with other abiotic stresses (Shinozaki and Yamaguchi-Shinozaki 2007).

ACC is transported from the roots to the shoots via xylem (Else and Jackson 1998), and leaf ethylene evolution is associated with ACC transport in tomatoes under drought stress (Sobeih et al. 2004), thus pointing to ACC as a root-sourced signal and ethylene as a key player in the growth inhibition of leaves (Schachtman and Goodger 2008). Some PGPB containing the enzyme ACC deaminase (ACCD) were defined as stress controllers (Lugtenberg and Kamilova 2009), since these bacterial strains are able to induce plant growth by diminishing ACC content and, thereby, ethylene levels (Belimov et al. 2007; Glick 2014). Nevertheless, other reports suggest ethylene-independent growth promotion (López-Bucio et al. 2007) or the exclusive affection of local regulatory mechanisms in plant roots by ACCD (Contesto et al. 2008). However, little is known about how PGPB inoculation impacts on ethylene production rate in adult plants and on the ethylene transduction pathway and response.

Recently, omics technologies have contributed in the clarification of the complex interaction between bacteria and plants (Su et al. 2016; van de Mortel et al. 2012). Several transcriptomic studies have been carried out in this regard, but many crucial questions remain to be settled. Furthermore, sustainable intensification of agriculture requires an enhancement in the use efficiency of resources (Ghanem et al. 2011; Tilman et al. 2011). PGPB can be used as biofertilizers and phytostimulators as well as biopesticides reducing or replacing chemicals in agriculture (Bhattacharyya and Jha 2012). Moreover, some bacterial strains can increase stress tolerance (Aroca and Ruiz-Lozano 2009). Thus, PGPB management has the potential to become a suitable method for the realization of these goals (Berg 2009; Singh et al. 2011).

A previous study performed concerning tomatoes (*Solanum lycopersicum*) established ethylene sensitivity by *ETR3* as an essential component for plant growth promotion induced by *Bacillus megaterium* but not by *Enterobacter* sp. strain C7, under well-watered (WW) conditions (Ibort et al. 2017). These

PGPB were selected due to their inability to produce ethylene and degrade ACC to avoid the creation of any direct disturbance in plant ethylene metabolism (Ibort et al. 2017), since most studies addressing the involvement of ethylene in the PGPB activity have mainly focused on bacterial strains containing ACCD activity (Glick 2014). The tomato mutant insensitive to ethylene (*nr*) and its isogenic wild-type (wt) parental cv. Pearson line were combined with two PGPB strains, *B. megaterium* (Marulanda-Aguirre et al. 2008) and *Enterobacter* sp. strain C7. The hypothesis was that *B. megaterium* would be unable to promote plant growth in *nr* plants also under drought stress but both PGPB strains could increase the stress tolerance in wt plants. The aim was to shed light on plant-bacteria interaction and PGPB mechanisms with respect to ethylene perception using a transcriptomic approach under both WW and drought conditions. Plant growth was determined at 8 weeks postinoculation, and the expression of ethylene-related genes as well as ethylene production was assayed. Furthermore, the expression levels of tomato genes were simultaneously analyzed by microarray technology to highlight the involvement of other plant pathways in PGPB activity or interaction with plants that are significantly altered.

RESULTS

Biomass production of wt and *nr* plants inoculated with *B. megaterium* and C7.

Dry weight of wt and *nr* plants inoculated with both PGPB (*B. megaterium* and C7) showed similar patterns under WW and drought conditions, but the interaction between the main factors (genotype [G], watering regime [W], and inoculum [I] [G×W×I]) went unnoticed (Fig. 1). In wt plants, PGPB inoculation promoted total dry weight (DW) under WW (20.2 and 20.0% for *B. megaterium* and C7, respectively) and drought conditions (24.7 and 27.4% for *B. megaterium* and C7, respectively). However, in *nr* plants, total DW only showed an increase due to C7 inoculation (25.6 and 21.3% under WW and drought conditions, respectively). Moreover, the drought treatment significantly reduced total DW in both plant genotypes subjected to all inoculation treatments (Fig. 1A).

B. megaterium inoculation only promoted shoot DW in wt plants under drought conditions (26.5%). However, shoot DW was increased by C7 inoculation in both plant genotypes under both watering regimes (22.7 and 24.4% in wt plants under WW and drought conditions, respectively, and 24.8 and 21.7% in *nr* plants under WW and drought conditions, respectively). Furthermore, significant differences due to drought treatments were observed in all cases other than in wt plants under *B. megaterium* inoculation (Fig. 1B). Additionally, significant differences in the case of total and shoot DWs between wt and *nr* plants were only noticed in mock-inoculated plants under WW conditions (Fig. 1A and B).

In addition, root DW depicted an increase as a result of PGPB inoculation in wt plants under both WW (15.7 and 15.9% for *B. megaterium* and C7, respectively) and drought conditions (17.3 and 17.2% for *B. megaterium* and C7, respectively). However, significant differences due to C7 inoculation in *nr* plants were only marked under WW conditions (29.2%). Furthermore, the drought treatment significantly reduced root DW in all cases. However, no differences between plant genotypes were noticed (Fig. 1C).

Modification of ethylene biosynthesis genes expression by PGPB inoculation.

The effects of PGPB inoculation, drought, and ethylene insensitivity on the expression of eight *ACS* and six *ACO* genes were evaluated (Figs. 2 and 3, respectively).

The expression of *ACS2* to *ACS5* and *ACS8* genes in both shoots and roots and *ACS7* gene in roots remained unaffected

by PGPB inoculation, both by plant genotype and drought treatment (data not shown). In the case of roots, *ACS1* expression showed a significant factor interaction (G×W×I). Both bacterial strains showed a decrease *ACS1* expression in comparison with noninoculated wt plants only under drought conditions, depicting lower expression in C7-inoculated plants than *B. megaterium*-inoculated ones. Moreover, drought treatment marked an increase in *ACS1* expression in roots in mock- and *B. megaterium*-inoculated wt plants (Fig. 2A). *ACS6* expression only showed a significant difference between mock-inoculated wt and *nr* plants under drought conditions, showing lower values in the case of *nr* plants than wt ones (Fig. 2B). Furthermore, C7 inoculation exhibited an increase in *ACS7* expression exclusively in *nr* plants under WW conditions, thus producing significant differences between C7-inoculated plants subjected to different watering regimes (Fig. 2C).

In shoots, *ACS1* expression was affected only by plant genotype in mock-inoculated plants under both watering regimes and in *B. megaterium*-inoculated plants only under WW conditions, always showing higher values in wt plants than *nr* ones (Fig. 2D). However, *ACS6* expression was affected by PGPB inoculation in both plant genotypes under both watering regimes. *B. megaterium* inoculation led to a decrease in *ACS6* expression in wt plants exclusively under drought conditions, while its levels were increased by *B. megaterium* in *nr* plants, regardless of the watering regime. C7 inoculation caused a decrease in *ACS6* expression in wt plants under both watering regimes, but no significant effects were noticed as compared with *B. megaterium*-inoculated wt plants. Furthermore, significant effects of drought treatment were only observed in *nr* plants under *B. megaterium* inoculation, causing a repression. In addition, significant, exclusive differences between wt and

Fig. 1. Effects of bacterial inoculation on plant dry weights under well-watered (WW) and drought (D) conditions. **A**, Total **B**, shoot and **C**, root dry weights of wild-type (wt) and never ripe (*nr*) tomato (*Solanum lycopersicum*) plants under WW and D conditions. Treatments are designed as mock-inoculated controls (Mock) (black bars), *Bacillus megaterium*-inoculated plants (Bm) (white bars), and strain C7-inoculated plants (C7) (gray bars). The data comprises means $\pm$ standard error ($n = 7$). The data were analyzed through a three-way analysis of variance (ANOVA) conducted with genotype (G), watering regime (W), and inoculum (I) as sources of variation. The significance of the sources of interaction (G×W×I) was determined through the P values as provided here; ns = not significant, one asterisk (*) indicates $P \leq 0.05$; two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. As no significant interaction between factors was observed, inoculum effects under WW and D conditions were evaluated through separate analyses of wt and *nr* plants with ANOVA. The mean values followed by different letters represent significantly different values ($P < 0.05$) based on the least significant difference test, with values moving higher from the letter a onwards for wt plants and from letter z backward for *nr* plants. The plant genotype effect was measured through an analysis of wt and *nr* plants under the same inoculation treatment (Mock, Bm, or C7) by a Student's t test; the significant difference ($P < 0.05$) is depicted with an asterisk (*) above the *nr* mean values.

Fig. 2. Effects of bacterial inoculation on 1-aminocyclopropane-1-carboxylate synthase (ACS) gene expression under well-watered (WW) and drought (D) conditions. The relative expression of **A**, root ACS 1, **B**, root ACS 6, **C**, root ACS 7, **D**, shoot ACS 1, and **E**, shoot ACS 6 in wild-type (wt) and *never ripe* (nr) tomato (*Solanum lycopersicum*) plants under WW and D conditions. Treatments are designed as mock-inoculated controls (Mock) (black bars), *Bacillus megaterium*-inoculated plants (Bm) (white bars), and strain C7-inoculated plants (C7) (gray bars). The data represents mean values $\pm$ standard error ($n = 3$). The data were analyzed through the three-way analysis of variance (ANOVA) with genotype (G), watering regime (W), and inoculum (I) as sources of variation. The significance of the sources of variation interaction (GxWxl) was evaluated by means of the P value as indicated here; ns = not significant, one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. In case significant interaction between factors was observed, all treatments were compared with each other. The mean values followed by different upper case letters indicate significant difference ($P < 0.05$) based on the least significant difference (LSD) test. In case of not-significant interaction between factors, inoculum effects under WW and D conditions were evaluated by attempting separate analyses of wt and nr plants with ANOVA. The mean values followed by different letters stand for significantly different values ($P < 0.05$) according to the LSD test, with values moving higher from letter a onwards for wt plants and from letter z backward for nr plants. The plant genotype effect was evaluated by the examination of wt and nr plants under the same inoculation treatment (Mock, Bm, or C7), through the Student's t test and analysis of the significant difference ($P < 0.05$), depicted as an asterisk (*) for means values that exceed nr.

nr plants were noticed in mock-inoculated plants independently of watering regime (Fig. 2E).

PGPB inoculation, drought treatment, and plant genotype did not cause any effects in *ACO2* and 5 in shoots, nor in *ACO3* to 5 in roots (data not shown). Furthermore, no significant differences due to PGPB inoculation or drought treatment were noticed in root expression of *ACO1*, 2 and 6. In these genes, only plant genotype differences were noticed; in all cases wt plants showed higher expression values than *nr* ones (Fig. 3A to C). *ACO1* and *ACO2* exclusively showed differences between plant genotypes in mock-inoculated plants under WW and drought

conditions respectively (Fig. 3A and B). Moreover, *ACO6* showed differences between plant genotypes in mock-inoculated and C7-inoculated plants, only under WW conditions (Fig. 3C).

In shoots, both bacterial strains decreased *ACO1* expression in wt plants, while *B. megaterium* inoculation increased its expression only in *nr* plants under WW conditions. Drought treatment decreased *ACO1* expression in mock-inoculated wt plants and in *B. megaterium*-inoculated plants irrespective of the plant genotype. Moreover, significant differences between wt and *nr* plants were only noticed in mock-inoculated plants under both watering regimes (Fig. 3D). Regarding *ACO3* expression,

Fig. 3. The effects of bacterial inoculation on 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene expression under well-watered (WW) and drought (D) conditions. The relative expression of **A**, root ACO 1, **B**, root ACO 2, and **C**, root ACO 6, **D**, shoot ACO 1, **E**, shoot ACO 3, **F**, shoot ACO 4, and **G**, shoot ACO 6 in wild-type cv. Pearson (wt) and *never ripe* (nr) tomato (*Solanum lycopersicum*) plants under WW and D conditions. Treatments are designed as mock-inoculated controls (Mock) (black bars), *Bacillus megaterium* inoculated plants (Bm) (white bars), and strain C7 inoculated plants (C7) (gray bars). The data comprises mean values $\pm$ standard error ($n = 3$). The data were studied by means of a three-way analysis of variance (ANOVA) with genotype (G), watering regime (W), and inoculum (I) as sources of variation. Significance of sources of variation interaction (G $\times$ W $\times$ I)) was evaluated by P value; ns = not significant. one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. As no significant interaction between the factors was observed, inoculum effects under WW and D conditions were evaluated through separate analyses of wt and nr plants with ANOVA. The mean values followed by different letters indicate significant difference ($P < 0.05$) according to the least significant difference test, with values moving higher from the letter a onwards for wt plants and from letter z backward for nr plants. The plant genotype effect was evaluated through separately analyzing wt and nr plants under the same inoculation treatment (Mock, Bm, or C7) by Student's t test, and significant difference ($P < 0.05$) is depicted with an asterisk (*) above nr means.

differences between the plant genotypes were only noticed in mock-inoculated and C7-inoculated plants under WW conditions (Fig. 3E). Only C7 inoculation decreased *ACO4* expression in wt plants, regardless of the watering regime. In addition, significant differences between wt and *nr* plants were exclusively noticed in mock-inoculated plants under WW conditions (Fig. 3F). Regarding the expression of *ACO6*, *B. megaterium* inoculation increased its expression only in *nr* plants, once again discounting the watering regime. However, C7 inoculation decreased *ACO6* expression only in wt plants under WW conditions. Significant effects of drought treatment were observed in mock-inoculated and *B. megaterium*-inoculated wt plants and in *B. megaterium*-inoculated *nr* plants. Moreover, differences between plant genotypes were significantly noticed in mock-inoculated plants, irrespective of the watering regime, and in C7-inoculated plants only under WW conditions (Fig. 3G).

Modification of expression of *Never Ripe* (ETR3) receptor and *TCTRI* genes by PGPB inoculation.

The effects of PGPB inoculation, drought treatment, and plant genotype on expression of *ETR3* and *TCTRI* were evaluated (Fig. 4). In roots, only *B. megaterium* inoculation led to an increase in *ETR3* expression in *nr* plants under drought conditions. Moreover, the differences between plant genotypes were only noticed under C7 inoculation and WW conditions (Fig. 4A). In shoots, PGPB effects were noticed under drought conditions. C7 inoculation decreased *ETR3* expression exclusively in wt plants, while *B. megaterium* caused an increase in its expression in *nr* plants. Moreover, drought treatment only increased the expression of *ETR3* in shoots in mock-inoculated wt plants and in *B. megaterium*-inoculated *nr* plants. Significant differences between plant genotypes were noticed in mock-inoculated plants irrespective of the watering regime and under C7 inoculation in WW conditions, always showing lower values in *nr* plants than those in wt ones (Fig. 4C).

Significant differences in root *TCTRI* expression were only found between plant genotypes under *B. megaterium* inoculation and drought conditions (Fig. 4B). However, *B. megaterium* inoculation increased *TCTRI* in shoots only under drought conditions in *nr* plants. Moreover, mock-inoculated *nr* shoots showed lower *TCTRI* expression than wt ones subjected to drought conditions (Fig. 4D).

Modification of ethylene-responsive genes expression by PGPB inoculation.

Expression of genes involved in ethylene response was also analyzed (Fig. 5). No significant changes were noticed in cases of *ERF1* in root and shoot tissues nor in *ER24* and *ER5* in roots (data not shown). In roots, *ER21* expression interaction between the main factors (G×W×I) was noticed and *B. megaterium* inoculation increased *ER21* transcription in *nr* plants under WW conditions (Fig. 5A).

In shoots, *ER21* expression was affected by bacterial inoculation in both plant genotypes. In wt plants, C7 inoculation reduced *ER21* expression values under drought conditions. On the other hand, C7 induced *ER21* expression in *nr* plants under WW conditions. Moreover, an increase in *ER21* was observed due to *B. megaterium* inoculation in *nr* plants only under drought conditions. Drought treatment led to an increase in *ER21* expression in mock-inoculated wt plants and in *B. megaterium*-inoculated *nr* plants, while *ER21* expression was decreased by drought in *nr* plants under C7 inoculation. Differences between plant genotypes were only noticed in mock-inoculated plants, irrespective of the watering regime, showing lower values in *nr* plants than those in wt ones (Fig. 5B). In addition, shoot *ER24* was affected by *B. megaterium* inoculation in *nr* plants under WW conditions, showing an expression induction (Fig. 5C). Regarding

ER5 expression, mock-inoculated wt plants showed significantly higher values than *nr* plants under drought conditions (Fig. 5D).

Modification of ethylene production by PGPB inoculation.

Ethylene emission by leaflets was determined, but no significant differences due to PGPB inoculation or by drought treatment were observed in either of the plant genotypes. However, notable differences between wt and *nr* plants were noticed in C7-inoculated plants, notwithstanding the watering regime, and in *B. megaterium*-inoculated plants under WW conditions. In mock-inoculated plants, differences were observed exclusively under drought conditions, always showing higher values in *nr* plants than wt ones (Fig. 6A).

In addition, a bioassay for ethylene production was performed to test if ethylene levels emitted by seedlings were affected in response to bacterial inoculation (Fig. 6B). As expected, no ethylene was detected in control vials without seedlings independently of *B. megaterium*, C7, or no inoculation of substrate (data not shown). C7 inoculation exclusively induced the ethylene production rate in wt plants 7 h postinoculation (hpi). However, *B. megaterium* inoculation increased the ethylene production rate 7 hpi. In contrast, no significant bacterial inoculation effects were noticed 26 hpi, but differences between wt and *nr* plants in ethylene production rate were exclusively noticed under *B. megaterium* inoculation 26 hpi.

Transcriptomic analysis of wt and *nr* plants inoculated with PGPB strains.

Microarray data were validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) evaluating correlations between data obtained by means of both methodologies (Supplementary Table S1). The transcriptomic analysis of root samples was performed by comparing different treatments in pairs, to evaluate PGPB inoculation effects on wt and *nr* plants and direct comparison between both bacterial inocula. Moreover, a quantitative evaluation of transcriptomic data was done with the help of Venn diagrams to get a general overview of bacterial inoculation effects (Fig. 7). Additionally, genes that were differentially expressed ($P < 0.05$) and showed changes greater than twofold were evaluated with Plant MetGenMap, to identify significantly altered pathways and perform a functional classification of differentially expressed genes (DEG) with greater than twofold change, using Gene Ontology (GO) terms for biological processes (Supplementary Table S2).

B. megaterium inoculation effects on transcriptomic profiles.

Most of the DEG were specifically affected by *B. megaterium* inoculation in wt and *nr* plants under WW and drought conditions (Fig. 7A; Supplementary Table S3). *B. megaterium* inoculation caused 649 DEG (574 specifically; 88.4%) in wt plants under WW conditions and 26 DEG were shared with DEG affected by *B. megaterium* inoculation in wt plants under drought conditions. Moreover, 11 and 33 DEG were shared with *B. megaterium* DEG in *nr* plants under WW and drought conditions, respectively. In the *B. megaterium* effects in wt plants under drought conditions, 318 DEG (270 specifically; 84.9%) were noticed and three and 16 DEG were shared with *B. megaterium* DEG in *nr* plants under WW and drought conditions, respectively. Moreover, *B. megaterium* inoculation affected 241 DEG (212 specifically; 88.0%) in *nr* plants under WW conditions and 13 DEG were shared with DEG affected by *B. megaterium* in *nr* plants under drought conditions. In the *B. megaterium* effects in *nr* plants under drought conditions, 409 DEG (342 specifically; 83.6%) were noticed. Furthermore, three *B. megaterium* DEG were noticed in common in wt plants under both watering regimes

and in *nr* plants under drought conditions. Two *B. megaterium* DEG were noticed in common in *nr* plants under both watering regimes and wt plants under WW conditions. No DEG affected by *B. megaterium* were common in all cases (Fig. 7A).

In addition, several changed pathways were identified under *B. megaterium* inoculation. In wt plants under WW conditions, an anthocyanin biosynthesis pathway was induced, due to increased transcription of leucoanthocyanidin dioxygenase (LDOX), while lipoxygenase and jasmonic acid biosynthesis pathways were reduced, due to decreased transcription of lipoxygenase A (LOX1.1). In wt plants under drought conditions, *B. megaterium* inoculation caused no significant altered pathways.

In *nr* plants under WW conditions, lipoxygenase and jasmonic acid biosynthesis pathways were reduced by *B. megaterium* inoculation due to decreased transcription of LOX1.1 and two allene oxide synthase (AOS) genes, cytochrome P450 *CYP74C3*

and *CYP74C4*. Flavonoid biosynthesis pathways were also reduced due to a decreased transcription of chalcone synthase B and hyoscamine 6-dioxygenase. Moreover, *B. megaterium* inoculation also caused a reduction in the canavanine degradation pathway, due to a decreased transcription of arginase 2. In *nr* plants under drought conditions, no significant changed pathways were noticed.

Strain C7 inoculation effects on transcriptomic profiles.

As observed for *B. megaterium*, most DEG were specifically affected by C7 inoculation in both plant genotypes and both watering regimes (Fig. 7B). In the case of effects of C7 in wt plants under WW conditions, 582 DEG (457 specifically; 78.5%) were noticed and 68 DEG were shared with DEG affected by C7 inoculation in wt plants under drought conditions. Furthermore, 19 and 27 DEG were shared with C7 DEG in *nr* plants under WW and drought conditions, respectively. C7 inoculation affected 353

Fig. 4. Effects of bacterial inoculation on expression of ethylene receptor (ETR) and signaling (TCTR1) genes under well-watered (WW) and drought (D) conditions. The relative expression of **A**, root ETR 3, **B**, root TCTR 1, **C**, shoot ETR 3, and **D**, shoot TCTR 1 in wild-type (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under WW and D conditions. Treatments were designed as mock-inoculated controls (Mock) (black bars), *Bacillus megaterium*-inoculated plants (Bm) (white bars), and strain C7-inoculated plants (C7) (gray bars). The data consists of mean values $\pm$ standard error ($n = 3$). The data were analyzed by means of a three-way analysis of variance (ANOVA) with genotype (G), watering regime (W), and inoculum (I) as sources of variation. Significance of sources of variation interaction (G $\times$ W $\times$ I) was evaluated by P value; ns = not significant, one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. As no significant interaction between the factors under study was noticed, inoculum effects under WW and D conditions were evaluated through separate analyses of wt and *nr* plants with the application of ANOVA. The means values followed by different letters indicate significant difference ($P < 0.05$) according to the least significant difference test, in which values are employed as higher from letter a onwards for wt plants and from letter z backward for *nr* plants. The plant genotype effect was evaluated through analyses of wt and *nr* plants under the same inoculation treatment (Mock, Bm, or C7), through Student's t test and by examination of the significant difference ($P < 0.05$), depicted as an asterisk (*) beyond *nr* mean values.

DEG (254 specifically; 72.0%) in wt plant under drought conditions and 17 and seven DEG were shared with DEG affected by C7 in *nr* plants under WW and drought conditions, respectively. Regarding C7 inoculation effects on *nr* plants under WW conditions, 504 DEG (443 specifically; 88.0%) were noticed and 16 DEG were shared with C7 DEG in *nr* plants under drought conditions. C7 inoculation affected 242 DEG (185 specifically; 76.5%) in *nr* plants under drought conditions. Moreover, four and two C7 DEG were noticed in common in wt plants under both watering regimes and in *nr* plants under WW and drought conditions, respectively. Four C7 DEG were shared in *nr* plants under both watering regimes and wt plants under WW conditions. Finally, only one DEG affected by C7 was shared in both plant genotypes and both watering regimes (Fig. 7B).

Under C7 inoculation, several modified pathways were also identified. C7 inoculation induced cyanate degradation pathways due to an increased transcription of carbonic anhydrase

under WW conditions. In wt plants under drought conditions, no significant altered pathways were observed.

In C7-inoculated *nr* plants under WW conditions, ethylene biosynthesis from methionine and methionine salvage II pathways were altered due to the induction of one ACS gene. In *nr* plants under drought conditions, tyrosine and phenylalanine degradation pathways were reduced due to a decreased transcription of tyrosine aminotransferase.

Differences between *B. megaterium* and strain C7 inoculation effects on transcriptomic profiles.

The number of DEG experiencing an increase when PGPB were compared with each other, regarding comparisons with mock-inoculated plants, but most of the DEG were also specifically affected in both plant genotypes and both watering regimes (Fig. 7C). PGPB comparison showed 1,579 DEG (1,337 specifically; 84.7%) in wt plants under WW conditions. A total of

Fig. 5. Effects of bacterial inoculation on ethylene-responsive gene expression under well-watered (WW) and drought (D) conditions. Relative expression of **A**, root ER 21, **B**, shoot ER 21, **C**, shoot ER 24, and **D**, shoot ER 5 in wild-type (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under WW and D conditions. Treatments were designed as mock-inoculated controls (Mock) (black bars), *Bacillus megaterium*-inoculated plants (Bm) (white bars), and strain C7-inoculated plants (C7) (gray bars). The data contains mean values $\pm$ standard error ($n = 7$). The data were analyzed by a three-way analysis of variance (ANOVA) with genotype (G), watering regime (W), and inoculum (I) determined as the sources of variation. The significance of sources of variation interaction (GxWxI) were evaluated through P value; ns = not significant, one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. In case of significant interaction between factors, all treatments were compared with others. The mean values followed by different capital letters imply significant difference ($P < 0.05$) according to the least significant difference (LSD) test. In case of mock-significant interaction between factors, inoculum effects under WW and D conditions were evaluated through separate analyses of wt and *nr* plants, using ANOVA. The mean values followed by different letters imply significant difference ($P < 0.05$) according to the LSD test, with higher values from the letter a onwards for wt plants and from the letter z backward for *nr* plants. The plant genotype effect was evaluated through the analysis of wt and *nr* plants under the same inoculation treatment (Mock, Bm, or C7) by Student's t test, and significant difference ($P < 0.05$) is depicted with an asterisk (*) above *nr* means.

110 DEG were shared with those affected between inocula in wt plants under drought conditions. Moreover, 72 and 40 DEG were shared with those in *nr* plants under WW and drought conditions, respectively. A comparison between PGPB affected 904 DEG (624 specifically; 69.0%) in wt plants under drought conditions and 87 and 64 DEG were shared with those in *nr* plants under WW and drought conditions, respectively. In comparison with the effects of PGPB on *nr* plants under WW conditions, 947 DEG (746 specifically; 78.8%) were noticed and 25 DEG were shared with those in *nr* plants under drought conditions. In a comparison of PGPB in *nr* plants under drought conditions, 425 DEG (284 specifically; 66.8%) were noticed. Moreover, 10 and five DEG were shared in wt plants under both watering regimes and in *nr* plants under WW and drought conditions, respectively. Three and two DEG were noticed in common in *nr* plants under both watering regimes and wt plants under WW and drought conditions, respectively. Finally, only two DEG were shared in both plant genotypes and both watering regimes (Fig. 7C). Additionally, a direct comparison between both PGPB strains also showed several changed pathways between *B. megaterium* and C7 inoculations. In wt plants under WW conditions, five pathways were significantly reduced in C7-inoculated plants as compared with *B. megaterium*-inoculated plants. The xanthophyll cycle pathway was decreased due to the transcriptional reduction of violaxanthin de-epoxidase. The dTDP-L-rhamnose biosynthesis II pathway was also reduced by a transcriptional decrease in the NAD-dependent epimerase/dehydratase family protein-like gene. The transcriptional reduction of glycine hydroxymethyltransferase (Cytochrome P450 76A2) decreased glycine betaine degradation, formaldehyde assimilation I, and super pathway of serine and glycine biosynthesis II pathways. In wt plants under drought

conditions, several pathways were significantly induced in C7-inoculated versus *B. megaterium*-inoculated plants. Lip-oxygenase and jasmonic acid biosynthesis pathways were induced due to the transcriptional induction of LOX1.1 and AOS (cytochrome P450 CYP74C3). Furthermore, in comparison with *B. megaterium* inoculation, C7 inoculation led to an increase in the transcription of arginase 2, which significantly affected the canavanine degradation, arginine degradation, citrulline metabolism, and urea cycle pathways.

In *nr* plants under WW conditions, several pathways were significantly induced in C7-inoculated plants as compared with *B. megaterium*-inoculated plants. When compared with *B. megaterium* inoculation, C7 inoculation induced transcription of two arginase 2 genes that had significant effects on canavanine degradation, arginine degradation, citrulline metabolism, and urea cycle pathways. Additionally, ethylene biosynthesis from methionine and methionine salvage II pathways were also induced in C7-inoculated compared with *B. megaterium*-inoculated plants due to the transcriptional induction of two ACS genes.

In *nr* plants under drought conditions, two pathways were significantly reduced in C7-inoculated compared with *B. megaterium*-inoculated plants. The transcriptional reduction of glutamine synthase affected the glutamine biosynthesis pathway and ammonia assimilation cycle.

DISCUSSION

The management of beneficial soil microorganisms can be employed to promote plant growth (Berg 2009; Singh et al. 2011) and reduce the inputs of chemicals in crops (Bhattacharyya and Jha 2012). However, correct and effective implementation in

Fig. 6. Ethylene production by tomato plants. **A.** Relative ethylene production rate in wild-type (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well watered (WW) and drought (D) conditions. The data comprises mean values $\pm$ standard error (SE) ($n = 6$). The data were analyzed by a three-way analysis of variance (ANOVA) with genotype (G), watering regime (W), and inoculum (I) employed as sources of variation. The significance of the sources of variation interaction (G $\times$ W $\times$ I) was assessed through P value; ns = not significant, one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. As no significant interaction between factors, neither plant growth-promoting bacteria inoculation effects were noticed, G was evaluated through the analyses of wt and *nr* plants under the same inoculation treatment (mock, *Bacillus megaterium*–, or strain C7–inoculated [Mock, Bm, or C7]) by Student's t test, and significant difference ($P < 0.05$) is depicted with an asterisk (*) above mean values of *nr*. **B.** Ethylene emission (nanomoles) per hour and grams of dry weight (DW) in wt and *nr* tomato (*Solanum lycopersicum*) plantlets on the day of inoculation (7 h) and 1 day postinoculation (26 h). The data offers mean values $\pm$ SE ($n = 4$). The data were also processed through a three-way ANOVA with G, W, and I as sources of variation. The significance of the sources of variation interaction (G $\times$ W $\times$ I) was evaluated by P value; ns = not significant, one asterisk (*) indicates $P \leq 0.05$, two (**) $P \leq 0.01$, and three (***) $P \leq 0.001$. As no significant interaction between factors was noticed, inoculum effects under WW and D conditions were evaluated, analyzing separately for wt and *nr* plants, using ANOVA. Mean values followed by different letters express significantly different values ($P < 0.05$) according to the least significant difference test, with higher values from the letter a onwards for wt plants and from letter z backward for *nr* plants. The plant genotype effect was evaluated through analyses of wt and *nr* plants under the same inoculation treatment (Mock, Bm, or C7) by Student's t test and significant difference ($P < 0.05$), depicted with an asterisk (*) for above *nr* mean values.

agriculture requires further research to completely comprehend the interaction between plant and soil microorganisms (Berg 2009). Hence, the inoculation effects of two different PGPB strains were evaluated in mature tomato plants for symptoms of ethylene sensitivity and drought stress.

Ethylene sensitivity is also essential for growth promotion by *B. megaterium* but not for C7 under drought conditions.

A previous study using ethylene-sensitive and insensitive tomato plants inoculated with *B. megaterium* and C7 established that both PGPB colonized the plant root system with similar colonization rates, irrespective of the ethylene sensitivity (Ibort et al. 2017). In addition, the plant growth-promotion results noticed in the present study (Fig. 1) were equivalent with those obtained in previous studies (Ibort et al. 2017, 2018), in accordance with PGPB activity dependent on ethylene perception by *SIETR3* for *B. megaterium* but not for C7, once more notwithstanding the watering conditions.

The same growth-promotion effects were noticed under drought stress, which led to a significant reduction in plant growth in all

cases (Fig. 1A). In addition, bacteria-inoculated wt plants under drought conditions reached values of mock-inoculated wt plants under WW conditions, thereby reducing potential yield losses and suggesting that PGPB can be deployed to diminish water inputs in agriculture or to increase resource use efficiency (Dodd and Ruiz-Lozano 2012). Moreover, enhanced promotion effects were noticed in wt plants under drought conditions, suggesting that PGPB increased plant stress tolerance, as previously reported under suboptimal environmental conditions (Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009; Glick 2004). Nevertheless, although C7 was known to be capable of promoting growth in *nr* plants irrespective of the watering regime, the growth promotion was lower under drought conditions. These results suggest that ethylene insensitivity influences C7 effects despite the proposed ethylene-independent activity. In addition, the differences between plant genotypes were observed to be significant only in mock-inoculated plants, pointing to less pronounced ethylene growth-inhibitory effects (Pierik et al. 2006) on *nr* plants in WW conditions due to its mutation (Wilkinson et al. 1995). The differences were not observed under drought

Fig. 7. Venn diagrams of differentially expressed genes by bacterial inoculation in wild-type (wt) and *never ripe* (*nr*) tomato (*Solanum lycopersicum*) plants under well-watered (WW) and drought (D) conditions. **A**, *Bacillus megaterium* effects, **B**, strain C7 effects, and **C**, comparison between *B. megaterium* and strain C7 effect. Each shape represents a comparison between two treatments. The numbers indicate genes that were differentially expressed (P value < 0.05), and those located within overlapping areas represent differentially expressed genes in two comparisons and three or four treatment comparisons.

conditions, probably due to ethylene induction as a result of stress (Pierik et al. 2007).

PGPB inoculation locally affects ethylene biosynthesis and response, resulting in stress alleviation in wt plants, and *B. megaterium* altered recognition in *nr* plants.

In tomatoes, several isoforms of *ACS* and *ACO* genes have been identified (Alexander and Grierson 2002; Blume and Grierson 1997; Jiang and Fu 2000). The rate-limiting enzyme in ethylene biosynthesis is generally *ACS* (Wang et al. 2002), although *ACO* could be limiting under certain conditions (Dorling and McManus 2012). *ACS* expression shows tissue- and cell-specific patterns that are differentially regulated depending on the stage of development and the response to different stimuli, controlling the ethylene production finally (Dorling and McManus 2012; Tsuchisaka et al. 2009). Furthermore, two ethylene regulatory systems were proposed in tomatoes during their vegetative growth (basal ethylene) and fruit ripening (high ethylene), which are, respectively, negatively and positively feedback-regulated (Alba et al. 2005; Alexander and Grierson 2002; Barry et al. 2000).

Altered expression of ethylene biosynthesis genes in tomatoes due to PGPB inoculation was previously reported by Porcel et al. (2014). In the present study, both PGPB modified expression of *ACS* and *ACO* genes (Figs. 2 and 3). *B. megaterium* and C7 showed a decreased expression of ethylene biosynthesis genes in wt plants, although C7 inoculation affected gene expression with higher intensity, in accordance with higher growth promotion. Thereby, both PGPB could diminish ethylene growth inhibitory effects independently of the watering regimes (Pierik et al. 2006; Wang et al. 2013). Nevertheless, PGPB inoculation in *nr* plants induced ethylene biosynthesis genes depicted higher effects by *B. megaterium* inoculation. ACC levels underwent a high increase in response to stresses such as either wounding, pathogen infection, or both (Kende and Boller 1981; van Loon et al. 2006), suggesting that *nr* plants could be recognizing *B. megaterium* as a pathogen-like microorganism.

Additionally, drought effects on ethylene biosynthesis genes pointed to higher stress alleviation by C7 inoculation, in accordance with previously enhanced tolerance against stresses due to PGPB inoculation (Aroca and Ruiz-Lozano 2009). Ethylene emission by aerial tissues was determined to evaluate any alteration of ethylene metabolism, but neither PGPB nor drought modified the ethylene production rate (Fig. 6A). However, previous studies correlated spatiotemporal *ACS* expression with ethylene production (Sobeih et al. 2004; Wang et al. 2002; Zarembinski and Theologis 1994). *ACS* genes present a strong posttranscriptional regulation with high levels of influence on ethylene production (Lyzenga et al. 2012; McClellan and Chang 2008; Xu and Zhang 2014), but *ACS* activity was not assayed and further research is needed to shed more light on the said results. Additionally, drought treatment was observed to generally increase ethylene emission (Pierik et al. 2007; Wang et al. 2013), but harvest was carried out one month after the commencement of the drought treatment and the plants could be adapted (Pan et al. 2012). Moreover, plant homeostasis also has capabilities to counteract bacterial effects, since PGPB bred in roots over two months. Another feasible explanation is that ethylene could be specifically produced by a limited number of tissues or cells, leading to a strong local production while the total amount of ethylene was maintained at low levels in the whole plant or organ.

Furthermore, differences between plant genotypes in ethylene biosynthesis genes were generally observed in mock-inoculated plants, disappearing under bacterial inoculation, probably due to stress alleviation effects (Aroca and Ruiz-Lozano 2009; Dimkpa et al. 2009; Glick 2004). Moreover, ethylene production showed

significant differences between plant genotypes, supporting the independence of ethylene for C7 activity and pointing to ethylene sensitivity as a determinant, in *B. megaterium*–tomato interactions, with interference of drought stress (Pierik et al. 2007).

Additionally, ethylene production after bacterial inoculation of seedlings was assayed (Fig. 6B). At 26 hpi, *B. megaterium*–inoculated *nr* plants showed a higher ethylene production rate than the wt ones. Ethylene emission by plants was previously reported (van Loon et al. 2006) to act as a modulator of interaction between plants with several enemies (Groen et al. 2013), although also with beneficial microorganisms (Zamioudis and Pieterse 2012). Thereby, these results also point to a misinteraction between *B. megaterium* and *nr* plants.

Moreover, the induction of ethylene receptors by PGPB strains was previously reported, pointing to the involvement of the ethylene response pathway in the early stages of the establishment of plant-bacteria association (Ciardi et al. 2000; Vargas et al. 2012). Thus, the expression of *ETR3* was evaluated to determine the ability of PGPB inoculation to modulate ethylene sensitivity by *ETR3* and the involvement (if any) of *ETR3* in the interaction between PGPB and tomato plants (Fig. 4A and C). *B. megaterium* inoculation induced *ETR3* expression in root and shoot tissues exclusively in *nr* plants, pointing to a lower ethylene sensitivity and incompletely functional association between *B. megaterium* and *nr* plants. However, C7 inoculation decreased shoot *ETR3* expression only in wt shoots, suggesting the possibility that aerial tissues of C7-inoculated plants could be less sensitive to ethylene.

In addition, although the presence of four *CTR1*-like genes in tomatoes was established, *TCTR1* expression analysis was selected because it is ethylene-inducible and associated with tissues during the stages of development with high ethylene levels (Adams-Phillips et al. 2004; Leclercq et al. 2002; Zegzouti et al. 1999). The induction of *TCTR1* expression in roots by *B. megaterium* inoculation was noticed in *nr* plants under drought conditions (although it was also significantly, although partially, induced under WW conditions), suggesting local ethylene production in accordance with growth results (Fig. 4B and D). Furthermore, *ETR3* expression on par with *TCTR1* was reported to regulate ethylene responsiveness (Tieman et al. 2000) and *B. megaterium* was able to induce both genes in *nr* plants, suggesting negative feedback regulation in *B. megaterium*–inoculated *nr* plants. That ethylene receptors and *CTRs* are differentially regulated in response to stimuli and during plant development, possibly regulating different ethylene responses, is a confirmed observation (Zhong et al. 2008).

Ethylene-responsive genes were also analyzed to evaluate if the ethylene transduction pathway was activated in PGPB-inoculated plants. *ERF1* was selected as an object of analysis because it acts as an integrator between osmotic stress and ethylene pathways in tomatoes (Huang et al. 2004) and it is induced by drought in several species (Cheng et al. 2013; Müller and Munné-Bosch 2015). Surprisingly, despite the drought treatment, no change in expression was noticed, thus pointing to plant adaptation to drought (Pan et al. 2012). Furthermore, *ERF1* is also known to be involved in defense against pathogens (Cheng et al. 2013; Müller and Munné-Bosch 2015), suggesting that both PGPB did not induce defense mechanisms, although it is possible that *nr* plants could misrecognize *B. megaterium*.

ER5, *ER21*, and *ER24* expressions were analyzed due to their strong ethylene-dependent alteration (Fig. 5). *ER5* and *ER21* are involved in stress response, while *ER24* participates in the regulation of gene expression (Zegzouti et al. 1999). *ER21* encodes a tomato heat-shock protein cognate 70 (hsp70) (Sun et al. 1996). Stress can cause damage to plant cells, thus resulting in osmotic and oxidative stress and induction of heat-shock proteins (Al-Wahaibi 2012; Scarpeci et al. 2008) such as

hsp70, which functions as chaperone (Su and Li 2008; Sung et al. 2001). *B. megaterium* was able to strongly induce *ER21* expression in *nr* roots, suggesting that the presence of *B. megaterium* caused high levels of stress, in accordance with the misrecognition hypothesis.

The *ER21* expression results in shoot tissue points to *B. megaterium*- and C7-produced systemic stress under drought and WW conditions, respectively. However, C7 inoculation could alleviate stress under drought conditions in wt and *nr* plants in accordance with growth results. Furthermore, drought induction of *ER21* in *B. megaterium*-inoculated *nr* shoots and in mock-inoculated wt ones pointed to higher stress levels in these two treatments. Additionally, differences between plant genotypes in shoot *ER21* were only observed in mock-inoculated plants, thereby suggesting that *nr* plants were suffering less stress. The reason behind this could be that ethylene insensitivity prevents further signaling, thus failing to produce some adaptive responses, as previously reported (Feng and Barker 1992; Zhang et al. 2003). In accordance, *ER5* is a lea-like gene (Zegzouti et al. 1999) involved in response to desiccation (van den Dries et al. 2011) and shows lower levels in mock-inoculated *nr* than wt plants under drought conditions. Furthermore, *ER24* is a transcriptional coactivator (Zegzouti et al. 1999), hinting at a higher response by *nr* plants under higher stress triggered by *B. megaterium* misrecognition.

PGPB inoculation affected transcriptomic profiles, showing strain-specific effects mainly dependent on ethylene sensitivity and watering conditions.

Transcriptomic analysis was carried out to evaluate PGPB inoculation effects on the whole set of genes in tomato wt and *nr* plants and to generally point to plant affected pathways. Both PGPB inoculations as well as direct comparison between inocula generally produced specific effects, depending on the bacterial strain, plant genotype, and watering regime (Fig. 7), in accordance with PGPB effects that rely on inoculated bacteria and growth conditions (Long et al. 2008; Ryu et al. 2005). Moreover, both bacterial inoculations modified the expression of a high number of genes under WW conditions, suggesting that drought stress could diminish the effects of bacterial inoculation. Therefore, drought stress indeed has a great impact on transcriptomic profiles in tomatoes (Gong et al. 2010; Iovieno et al. 2016). However, *B. megaterium* altered a lower number of DEG in *nr* plants under WW conditions in accordance with cross-talk between drought stress and *B. megaterium* interaction with *nr* plants.

***B. megaterium* modulates flavonoids enhancing antioxidant status in wt plants but ethylene insensitivity impairs interaction with tomato.**

B. megaterium inoculation significantly altered plant pathways only under WW conditions. *B. megaterium* induced an anthocyanin biosynthesis pathway in wt plants. LDOX produces cyanidin and is essential for biosynthesis of proanthocyanidin (Abrahams et al. 2003; Saito et al. 1999). These molecules belong to the flavonoid family that plays a determinant role in plant interactions with beneficial microorganisms and maintains the redox status within cells (Mierziak et al. 2014; Shirley 1996). Similarly, a previous antioxidant metabolite analysis showed that *B. megaterium* inoculation in wt plants enhanced plant redox status (Ibort et al. 2018). Although flavonoids have been described in symbiotic bacteria and mycorrhiza (Abdel-Lateif et al. 2012), they could also play a role in the interaction with bacteria living in the rhizosphere. Additionally, plant proanthocyanidins can neutralize bacterial lipopolysaccharides (Delehanty et al. 2008), which trigger the immune system of plants (Newman et al. 2013), suggesting that wt plants could avoid an immune response against *B. megaterium*.

Nevertheless, the flavonoid biosynthesis pathway was repressed by *B. megaterium* inoculation in *nr* plants. Chalcone synthase is the first enzyme in the tomato flavonoid biosynthesis pathway (Schijlen et al. 2007), while hyoscyamine 6-dioxygenase catalyzes hydroxylation of hyoscyamine transformation to scopolamine (Matsuda et al. 1991). Chalcone synthase reduction by *B. megaterium* inoculation in *nr* plants suggested that *B. megaterium* was able to reduce a triggered defense response in *nr* plants, since chalcone synthase expression results in the accumulation of flavonoids involved in the defense pathway (Dao et al. 2011). The induction of hyoscyamine and scopolamine accumulation was previously reported using PGPB (Ghorbanpour et al. 2010), but the repression of these genes was in accordance with the diminished defense response against *B. megaterium* by *nr* plants. In addition, among root exudates, canavanine is a nonprotein amino acid that is toxic to many soil bacteria (Cai et al. 2009). The canavanine degradation pathway was also reduced by *B. megaterium* inoculation in *nr* plants, suggesting its accumulation. Indeed, canavanine-mediated inhibition of alfalfa spermosphere colonization by *Bacillus cereus* was previously reported (Emmert et al. 1998), pointing to a partial colonization of *nr* roots by *B. megaterium*.

Furthermore, lipoxygenase and jasmonic acid biosynthesis pathways were repressed by *B. megaterium*, irrespective of plant genotype. However, AOS was exclusively reduced in *nr* plants, probably because of the ability of ethylene to induce AOS expression in tomatoes (Sivasankar et al. 2000). Jasmonic acid functions as a regulator of plant immune responses against pathogens (Browse 2009). Thus, the stated results suggest that *B. megaterium* is not completely recognized as a pathogenic microorganism despite the misinteraction between *B. megaterium* and *nr* plants.

Ethylene sensitivity determines tomato interaction with strain C7, which minimizes the defense response in wt but improves plant fitness in *nr* plants.

C7 inoculation in wt plants under WW conditions induces a cyanate degradation pathway. Carbonic anhydrase participates in the transport of inorganic carbon, thus modulating CO₂ levels during photosynthesis and respiration (Badger 1994; Henry 1996; Price et al. 1994). Carbonic anhydrase is positively correlated with photosynthesis and dry matter accumulation in shoots (Khan 2002; Khan et al. 2004), suggesting an increased respiration and inorganic carbon transport in roots. Surprisingly, C7 inoculation in *nr* plants under WW conditions affected ethylene biosynthesis and methionine salvage II pathways due to induction of an ACS gene. Accordingly, ACS7 expression was induced by C7 inoculation under these conditions. The role of ACS7 in flooding and wounding responses has been reported in previous studies (Shiu et al. 1998). However, ethylene levels remained unaffected by C7 inoculation and the results of this study pointed to its involvement in plant-bacteria interaction. Indeed, several ACS genes participate in response to bacterial elicitors in *Arabidopsis* (Denoux et al. 2008).

Under drought conditions, C7 inoculation in *nr* plants depicted a decrease in tyrosine and phenylalanine degradation pathways. Tyrosine and phenylalanine were used in the biosynthesis of secondary metabolites (Hyun et al. 2011; Tzin and Galili 2010) involved in cell-wall structure (Bonawitz and Chapple 2010) and plant defense or response to stress (Fraser and Chapple 2011; Lee and Facchini 2011), suggesting that a higher amino acid presence in C7-inoculated *nr* plants could counteract stress.

Furthermore, cyanide is a defensive metabolite in plants whose oxidation results in the formation of cyanate (Møller and Seigler 1999). Plants produce cyanide during the process of ethylene biosynthesis (Kende 1993; Peiser et al. 1984). Thus, wt plants could be able to recognize C7, decreasing defensive

metabolites, while *nr* plants respond to the presence of C7 by enhancing the ethylene biosynthesis pathway. Although growth promotion by C7 inoculation was proposed as independent of ethylene perception, these results also suggest that mutation of *ETR3* affects tomato-C7 interaction.

***B. megaterium* could diminish oxidative stress only in wt plants, while C7 could increase it, however, C7 modulates amino-acid metabolism independently of ethylene sensitivity.**

Although PGPB inoculations did not significantly alter pathways in comparison with mock-inoculated plants under drought conditions, a direct comparison between *B. megaterium* and C7 revealed multiple discrepancies. In wt plants under WW conditions, compared with *B. megaterium* inoculation, C7 repressed several pathways. The dTDP-L-rhamnose biosynthesis II pathway produces L-rhamnose, which is a key component of cell wall and secondary metabolites, including flavonoids and anthocyanins (Watt et al. 2004), in accordance with the previous results observed after a comparison of *B. megaterium* and C7 with respect to mock-inoculated plants. The xanthophyll cycle is involved in plant protection against oxidative stress (Latowski et al. 2011), suggesting higher levels of oxidative stress in C7-inoculated than in *B. megaterium*-inoculated wt plants. In fact, increased oxidative stress levels were noticed in C7-inoculated plants under WW conditions (Ibort et al. 2018). Although the metabolites of the xanthophyll cycle could present antioxidant properties in roots, oxidative protection was generally described in photosynthetic tissues (Barickman et al. 2014). However, this pathway also produced substrates for ABA production (Schwartz et al. 1997), suggesting that ABA levels could contribute to PGPB activity of either or both *B. megaterium* and C7. In fact, normal endogenous ABA levels were proposed to be essential for *B. megaterium*-mediated growth promotion (keeping low ethylene production) in tomato plants (Porcel et al. 2014). Additionally, a previous report showed increased ABA levels in *nr* roots inoculated with C7 (Ibort et al. 2017) and ABA was observed to play a key role in the growth of young tissues (Finkelstein 2013). In addition, it has been discovered that glycine hydroxymethyltransferase plays a role in the photorespiratory pathway and contributes to the minimization of reactive oxygen species production and avoidance of cellular damage (Moreno et al. 2005), in accordance with proposed lower oxidative stress in wt plants inoculated with *B. megaterium* than with C7.

In contrast, C7 inoculation in wt plants under drought conditions induced lipoxygenase and jasmonic acid biosynthesis pathways. Furthermore, arginase 2 induction significantly increased canavanine degradation, arginine degradation, citrulline metabolism, and urea cycle pathways. C7 inoculation in *nr* plants under WW conditions also induced arginase 2 genes inducing those pathways, suggesting a common mechanism independent of ethylene sensitivity. In tomato plants, jasmonic acid and wounding are able to induce arginase 2 expression (Chen et al. 2004). Arginase catalyzes the transformation of arginine pool (up to 90% of free nitrogen in vegetative tissues) into urea and ornithine, which is used in the biosynthesis of glutamate, proline, and polyamines. Moreover, nitrogen mobilization has been correlated with arginase expression to meet the metabolic demands (Hwang et al. 2001; Zonia et al. 1995), suggesting that C7 could be able to induce the amino-acid metabolism and/or enhance the nitrogen use efficiency, as previously reported, due to C7 inoculation in ethylene-sensitive and insensitive tomato plants (Ibort et al. 2017). Furthermore, canavanine degradation is induced under C7 inoculation, suggesting lower amount of accumulation of this toxic metabolite (Cai et al. 2009).

In *nr* plants under WW conditions, ethylene biosynthesis and methionine salvage II pathways were also induced by C7 as compared with *B. megaterium* inoculation. As mentioned above, these processes could be affected by *ACS7* expression induction mediated by C7 compared with mock-inoculated plants. In *nr* plants under drought conditions, as compared with *B. megaterium*, C7 showed reduced amounts of glutamine synthase. This enzyme is responsible for primary ammonium assimilation and participates in ammonium detoxification released during metabolic processes as proteolysis or photorespiration (Lea and Mifflin 2011), pointing to higher proteolysis under *B. megaterium* inoculation in accordance with higher stress response.

Conclusions.

In conclusion, both bacterial strains were observed to act as PGPB under WW and drought conditions in ethylene-sensitive tomato plants, which enhanced their water use efficiency. C7 inoculation promoted plant growth in wt and *nr* plants and generally reduced the expression of ethylene biosynthesis genes in wt plants, resulting in alleviation of stress levels. However, *B. megaterium* inoculation induced the expression of ethylene biosynthesis, signaling, and response genes in *nr* plants, thus suggesting local ethylene emission in *B. megaterium*-inoculated *nr* plants. Thus, *ETR3* mutation could impair the interaction between *B. megaterium* and tomato plants, resulting in altered recognition and a lack of PGPB activity. Thus, *B. megaterium* and strain C7 PGPB activity in tomato plants could also be proposed as dependent and independent on ethylene perception by *ETR3*, respectively, under drought conditions, although water stress is known to interfere in plant-bacteria interaction.

Furthermore, PGPB inoculation modified the root transcriptomic profiles in a mode dependent on strain, genotype, and watering regime. Flavonoid biosynthesis was affected by *B. megaterium* inoculation, enhancing antioxidant status in wt plants, while *nr* plants responded to *B. megaterium* as a stressing agent, inhibiting complete functional interaction but avoiding immune response. Incidentally, amino-acid metabolism is modulated by C7 inoculation independently of ethylene sensitivity and *ETR3* mutation also affects the tomato-C7 interaction. However, further research is needed to clarify plant-bacteria interaction, as well as the effects of PGPB inoculation on antioxidant status under drought stress.

MATERIALS AND METHODS

Biological materials.

Tomato (*Solanum lycopersicum*) seeds, a *never ripe* (*nr*) mutant (LA0162) (Wilkinson et al. 1995), and its isogenic wt parental line (cv. Pearson, LA0012) were obtained from the Tomato Genetics Resource Center at the University of California, Davis, CA, U.S.A. PGPB strains were isolated from arid soils in southern Spain. *B. megaterium* was identified in a previous study (Marulanda-Aguirre et al. 2008) and strain C7 was isolated and identified as described by Armada et al. (2014).

Experimental design and growth conditions.

The experiment consisted of a randomized complete block design with two tomato plant lines (wt and *nr*), two watering regimes (WW and D), and three inoculation treatments: i) mock-inoculated control plants, ii) *B. megaterium*-inoculated plants, and iii) strain C7-inoculated plants. Each treatment consisted of 11 replicates. Plants were harvested 8 weeks postinoculation.

The seeds were sterilized, germinated, and inoculated as described by Ibort et al. (2017). Ten-day-old seedlings were transferred to 1-liter plastic pots containing sterile peat moss

and perlite (1:1, vol/vol, autoclaved twice at 120°C for 20 min). Plants were cultivated in a greenhouse under controlled conditions (18 to 24°C, 50 to 60% relative humidity, 16 h of light [$600 \mu\text{mol m}^{-2} \text{s}^{-1}$] and 8 h of dark). Eight 1-liter pots containing the above-mentioned substrate were watered until saturation and were then left to drain for 24 h. Pot weights (water-holding capacity [WHC] of 100%) were measured, resulting in a weight mean of 640.10 g. These pots were dried in a forced draft oven (70°C, 7 days) and pot DWs (WHC of 0%) were determined again, resulting in weight mean of 163.91 g. Thus, watering regime treatments were administered, after an estimation of the grams of water that 1 g of substrate is able to contain, by extrapolating the WHC to substrate weight (SW) in the experimental system and applying the equation $\text{SW} = 4.7568 \times \text{WHC} + 163.91$. All plants were cultivated over a period of 4 weeks under WW conditions, in which plants were watered once every 2 days, in order to maintain constant soil water content close to 100% WHC, which corresponds to 640.10 g. Watering regime treatments were applied thereafter by watering plants up to their WHC of 100% (640.10 g) or 60% (448.10 g) for WW and drought conditions, respectively.

Biomass production.

Plant growth was calculated and analyzed to evaluate PGPB activity. The shoots were separated from root systems, and samples were dried in a forced draft oven (70°C, 3 days). Then, DW was determined, using seven replicates per treatment ($n = 7$). The obtained data were divided by an average of mock-inoculated wt plants under WW conditions to normalize them and evaluate plant growth proportionally in order to control treatment.

Gene expression analysis.

Total RNA was isolated from root tissue from three different plants subjected to each treatment ($n = 3$) using Agilent Plant RNA isolation mini kit, according to the instructions specified by the manufacturer. DNase treatment of the total RNA was carried out on the membrane of column before washing it, using RNase-free DNase (Agilent). RNA integrity and quality were tested by gel electrophoresis as well as measuring 260/230 and 260/280 ratios in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The expression of ethylene-related genes was studied by real-time PCR, using Brilliant III ultra-fast SYBR green qRT-PCR master mix (Agilent) and an iCycler 5 device (Bio-Rad), according to Agilent instructions. Each 10- μl reaction contained 5 μl of 2 $\times$ SYBR green qRT-PCR master mix, 0.2 μl of each primer pair, 0.1 μl of 100 mM dithiothreitol, 0.5 μl of RT/RNase block, 3 μl of RNase-free H_2O , and 1 μl of a dilution of the extracted RNA (100 ng/ μl). The PCR program consisted of a 10-min incubation at 50°C, to perform retro-transcription, and a 3-min incubation at 95°C, followed by 40 cycles of 5 s at 95°C and 10 s at 58°C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked using a heat dissociation protocol ranging 60 to 100°C after the last PCR cycle. Each qRT-PCR was carried out, in duplicate, with three independent biological replicates ($n = 3$). The obtained values were normalized using the threshold cycle (C_t) value for the tomato reference elongation factor gene. Different putative reference genes (actin, tubulin, ubiquitin, and elongation factor) were tested. The elongation factor was selected as reference, using the “Normfinder” algorithm (Andersen et al. 2004), because it was the most stable gene among all the treatments taken into consideration. The relative abundance of transcription was calculated by using the $2^{-\Delta C_t}$ method (Livak and Schmittgen 2001). Negative controls without RNA were used in all PCR reactions. The primers used to amplify each analyzed gene are shown in Supplementary Table S4.

Ethylene measurement.

Ethylene production by leaves was analyzed using gas chromatography as described by Ibor et al. (2017). The production rate of ethylene (nanomoles of ethylene per hour per gram of DW) was evaluated using six replicates per treatment ($n = 6$). Ethylene production by tomato plantlets induced by inoculation of bacterial strains was also analyzed. The seeds were sterilized, as described by Ibor et al. (2017), and were sown directly within 20-ml vials (Supelco Analytical) containing sterile peat moss and perlite (1:1, vol/vol, autoclaved twice at 120°C for 20 min). Plantlets were cultivated over a period of 10 days, without a vial lid, under WW conditions in a growth chamber under controlled conditions (18 to 24°C, 50 to 60% relative humidity, 16 h of light [$600 \mu\text{mol m}^{-2} \text{s}^{-1}$] and 8 h of dark). Bacterial strains were cultured and 10-day-old seedlings were inoculated as described by Ibor et al. (2017). The vials were immediately closed and were incubated inside the growth chamber. Moreover, the vials without seedlings but containing substrate were also inoculated, to assay the ethylene production by either PGPB strains, substrate, or both. The ethylene production rate was evaluated on inoculation day (7 hpi) and on the day after inoculation (26 hpi), using four replicates per treatment ($n = 4$).

Sample preparation for microarray.

The total RNA was isolated from the root tissue of three different plants for each treatment ($n = 3$), using the RNeasy plant mini kit (Qiagen). RNA quality was assessed through the process of gel electrophoresis as well as the measurement of 260/230 and 260/280 ratios in the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and Bioanalyzer RNA kit (Bioanalyzer 2100 de Agilent Technologies). Total RNA was processed to use on the GeneChip tomato genome array (Affymetrix), in accordance with the manufacturer's protocol, by Genomic Analytical service of Cabimer (Consejo Superior de Investigaciones Científicas), Seville, Spain. Total RNA (10 μg) was utilized in a RT reaction for the generation of the first-strand cDNA, using the SuperScript Choice System (Invitrogen) with oligo(dT) 24 fused to the T7 RNA polymerase promoter. Subsequent to the second-strand synthesis, the target complementary RNA (cRNA) labeled with biotin was prepared with the BioArray high-yield RNA transcript labeling kit (Enzo Biochem) in the presence of biotinylated UTP and CTP. After the completion of the purification and fragmentation processes, cRNA (15 μg) was utilized in a hybridization mixture in which the hybridization controls were added. The hybridization mixture (200 μl) was hybridized on arrays for 16 h at 45°C. The standard posthybridization wash and double-stain protocols were applied on an Affymetrix GeneChip fluidics station 450. Finally, the arrays were scanned on an Affymetrix GeneChip scanner 3000 7G. The data were analyzed with the statistical tool LIMMA (Linear Models for Microarrays Analysis) with affyGUI (Wettenhall et al. 2006) in R software (Open Source, v3.2.2), and significant differences were assigned to genes that fell under the threshold ($P < 0.05$) in comparisons between treatments. The obtained transcriptomic data are publicly available in the Gene Expression Omnibus database of the National Center for Biotechnology Information under the accession number GSE106317.

Microarray validation.

The microarray data were validated with the qRT-PCR according to Morey et al. (2006). A set of genes was selected for validation based on their P value ($P < 0.05$) and was assayed with qRT-PCR as described earlier in the gene expression analysis section, using the primers presented in Supplementary Table S5. Relationships between microarrays and the qRT-PCR data were examined through Pearson's correlation. The data

supplied in the correlation analysis comprised the $\log_2$ value of the weighted average for each gene obtained from all replicate plants. Correlation values between the microarray and qRT-PCR results that exceed 0.8 are indicative of strong agreement and renders the study validated.

Microarray data analysis.

To evaluate the effects of bacterial inoculation on wt and *nr* plants, the transcriptomic profiles of *B. megaterium*-inoculated and C7-inoculated plants were compared with those of mock-inoculated ones as well as with each other. Plant MetGenMap software (Joung et al. 2009) was utilized to identify altered pathways from the genomic profile data ($P < 0.05$) and to visualize the profile data in the context of a biochemical pathway, with a threshold of fold change >2 , as well as to perform a functional classification of DEG with more than a twofold change, using GO terms for biological processes. The open source Venn diagram webtool software developed by Ghent University was utilized to quantitatively compare transcriptomic profiles with those obtained under different experimental conditions.

Statistical analysis.

The data were processed through the application of a three-way analysis of variance (ANOVA) with G, W, and I employed as the sources of variation. The significance of the sources of variation interaction (G×W×I) was measured by the P value ($P < 0.05$). In case of significant interaction between factors, all treatments were compared with others in terms of the least significant difference (LSD) test ($P < 0.05$). In case of no interaction between factors, inoculum effects under WW and drought conditions were evaluated, with separate analyses for wt and *nr* plants with ANOVA, followed by an LSD test ($P < 0.05$). The plant genotype effect was evaluated through the analysis of wt and *nr* plants under the same inoculation treatment (no inoculation or inoculated with *B. megaterium* or C7) by a Student *t* test ($P < 0.05$). Ethylene data were also processed through a three-way ANOVA with G, W, and I applied as the sources of variation. Further analysis was performed in the same way as described above, with time after inoculation instead of the application of the watering regime as a source of variation. Plant DW, gene expression, and ethylene production rate were analyzed with the R software (v3.2.2).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Ghent University's Venn diagram webtool software:
<http://bioinformatics.psb.ugent.be/webtools/Venn>
 Open source R software: <http://www.r-project.org>