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Research paper

Proteomic analysis reveals that tomato interaction with plant growth promoting bacteria is highly determined by ethylene perception

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ABSTRACT

Feeding an increasing global population as well as reducing environmental impact of crops is the challenge for the sustainable intensification of agriculture. Plant-growth-promoting bacteria (PGPB) management could represent a suitable method but elucidation of their action mechanisms is essential for a proper and effective utilization. Furthermore, ethylene is involved in growth and response to environmental stimuli but little is known about the implication of ethylene perception in PGPB activity. The ethylene-insensitive tomato *never ripe* and its isogenic wild-type cv. Pearson lines inoculated with *Bacillus megaterium* or *Enterobacter* sp. C7 strains were grown until mature stage to analyze growth promotion, and bacterial inoculation effects on root proteomic profiles. *Enterobacter* C7 promoted growth in both plant genotypes, meanwhile *Bacillus megaterium* PGPB activity was only noticed in wt plants. Moreover, PGPB inoculation affected proteomic profile in a strain- and genotype-dependent manner modifying levels of stress-related and interaction proteins, and showing bacterial inoculation effects on antioxidant content and phosphorus acquisition capacity. Ethylene perception is essential for properly recognition of *Bacillus megaterium* and growth promotion mediated in part by increased levels of reduced glutathione. In contrast, *Enterobacter* C7 inoculation improves phosphorus nutrition keeping plants on growth independently of ethylene sensitivity.

1. Introduction

In soil, a plethora of microorganisms are able to associate with plants (Gray and Smith, 2005). Some of them stimulate plant growth (Adesemoye and Kloepper, 2009; Nadeem et al., 2014). Their management is a cheap, versatile and environmentally-friendly method to improve plant growth (Berg, 2009; Singh et al., 2011). The plant-growth promoting bacteria (PGPB) have been extensively studied (Lugtenberg and Kamilova, 2009; Santoyo et al., 2016) and can stimulate plant growth either by a direct or indirect mechanism (Ortíz-Castro et al., 2009), although diverse mechanisms could be active concurrently (Martínez-Viveros et al., 2010). In consequence, PGPB are pointed as an interesting way to replace chemicals (Bhattacharyya and Jha, 2012), diminishing detrimental environmental impact of crops for a sustainable intensification of agriculture (Tilman et al., 2011).

Ethylene is able to promote or inhibit plant growth depending on plant species and cell type (Pierik et al., 2006), and its production is enhanced under environmental stresses (Wang et al., 2013). Although there are plenty studies about interaction between plant and PGPB, most approaches were only focused on a single biochemical pathway and often miss lots of bacterial effects. Recently, – omics approaches have been carried out to clarify plant-bacteria interaction (Su et al., 2016; Van de Mortel et al., 2012), but many fundamental questions remain to be resolved.

Proteomic information could be interpreted as a photo of bacterial effects on plant physiology (Feussner and Polle, 2015). Proteomic studies regarding plant-bacteria interaction are mainly focused in the nitrogen-fixing rhizobia symbiosis (Mathesius, 2009) and plant-pathogen interaction (Afroz et al., 2013; Mehta et al., 2008). However, little is known about PGPB effects on plant proteomic profiles, despite of their environmental and agricultural importance. Recently, some proteomic analyses have been performed in arabidopsis inoculated with *Paenibacillus polymyxa* E681 (Kwon et al., 2016), in rice inoculated with *Herbaspirillum seropedicae* SmR1 (Alberton et al., 2013) and *Azoarcus* sp.

Abbreviations: PGPB, Plant growth promoting bacteria; 2-DE, Two-dimensional gel electrophoresis; MS, Mass spectrometry; PM, Plasma membrane; MAMPs, Microbe-associated molecular patterns; ER, Endoplasmic reticulum; *nr*, *Never ripe*; wt, Wild type; Bm, *Bacillus megaterium* strai; C7, *Cnterobacter* C7 strain; wpi, Weeks post-inoculation; OD_{600}, Optical density at 600 nm; FW, Fresh weight; DW, Dry weight; PSI, Phosphate solubilizing index

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(Miché et al., 2006), and in maize inoculated with *Azospirillum brasilense* FP2 (Cangahuala-Inocente et al., 2013; Faleiro et al., 2015), but further research is needed because PGPB action mechanisms are often strain-specific (Long et al., 2008; Ryu et al., 2005) and less well characterized (Pühler et al., 2004). Nowadays, the two most commonly used proteomic methods are two-dimensional gel electrophoresis (2-DE) (O'Farrell, 1975) and mass spectrometry (MS) (Mann et al., 2001). However, 2-DE presents some problems as poor resolution for basic, hydrophobic and/or low abundant proteins (Cheng et al., 2010). Nevertheless, shot-gun proteomics (Fournier et al., 2007; Wolters et al., 2001) can be used to perform an integral analysis of proteins extracted from plant cells, subcellular organelles and membranes (Takahashi et al., 2014).

The roots are the bacterial niche for PGPB and the site where a direct plant-bacteria interaction occurs being colonization of root system a fundamental feature for PGPB (Benizri et al., 2001). Furthermore, plant cell membranes are key players in several cellular functions as functional separation as well as transport (Chrispeels, 1999), signalling platforms in response to abiotic (Osakabe et al., 2013) and biotic (Inada and Ueda, 2014) stimuli and molecular trafficking mediated by vesicles (Chen et al., 2011; Murphy et al., 2011). In addition, membrane protein composition mainly define the membrane functionality (Komatsu et al., 2007).

The plasma membrane (PM) acts as a selectively permeable barrier which ensures the interchange of essential metabolites and ions to meet the cell requirements (Chrispeels, 1999). Moreover, the plasma membrane and tonoplast maintain the intracellular homeostasis in the cytoplasm (Sondergaard et al., 2004). A wide range of transport proteins regulate nutrient acquisition in root cells and translocation within the plant. Moreover, nutrient bioavailability determines transporter gene expression at transcriptional and post-transcriptional level (Aibara and Miwa, 2014). Several nutrients are taken up by transporters located in the PM and induced under limited nutritional conditions such as N (Lezhneva et al., 2014) and P (Raghothama and Karthikeyan, 2005). Furthermore, plant development is regulated by phytohormones and there is a close interrelation between nutritional and hormonal homeostasis (Krouk et al., 2011). Ethylene production is induced under several nutrient deficiencies and cross-talking processes have been reported for N (Tari and Szen, 1995) and P (Borch et al., 1999) among others.

In addition, plants interact with a wide variety of microorganisms, and recognition and defence mechanisms have been developed to cope with them (Dodds and Rathjen, 2010). Receptors, which recognize elicitors or microbe-associated molecular patterns (MAMPs), are located in cellular membranes and are able to trigger responses (Boller and Felix, 2009). Furthermore, proteins are processed along the endomembrane system. Firstly, proteins are synthesized in the endoplasmic reticulum (ER) and then transported throughout the secretory pathway to be located in the PM by exocytosis (Murphy et al., 2011). Proteins remain in the PM or are taken up by endocytosis, and stored in endocytic vesicles and recycled back to the PM when needed or targeted for degradation in lytic vacuoles (Chen et al., 2011). Plant cells can respond to microbe interaction by adapting vesicle trafficking (Dörmann et al., 2014; Inada and Ueda, 2014; Ivanov et al., 2010). However, these processes have been observed with intracellular microorganisms (Leborgne-Castel and Bouhidel, 2014) and little is known about PGPB effects on secretory pathways. The microsomal fraction is enriched in membranes such as ER, Golgi, PM, tonoplast and several endosomal vesicles and compartments (Abas and Luschnig, 2010). Thus, proteomic analysis of microsomal fraction is very useful for looking into plant-bacteria interaction regarding signalling and transport processes.

The goal of this study was to shed light on plant-bacteria interaction and PGPB mechanisms regarding to ethylene perception using a proteomic approach. Tomato (*Solanum lycopersiucm*) is the most significant horticultural crop worldwide. The mutant insensitive to ethylene *never ripe* (*nr*) is unable to perceive ethylene due to a mutation in the ethylene receptor ETR3 (Wilkinson et al., 1995), although with some residual responsiveness (Lanahan et al., 1994). Thus, *nr* plants and its isogenic wild-type (wt) parental line were used in combination with two PGPB strains: *Bacillus megaterium* strain (Bm) (Marulanda-Aguirre et al., 2008) and *Enterobacter* spp. (hereafter *Enterobacter* C7 (C7)) since a previous study pointed to ethylene sensitivity by ETR3 as essential for plant growth promotion induced by Bm but not for C7 (Ibort et al., 2017). Most studies addressing the ethylene involvement in the PGPB activity have been mainly focused on bacterial strains which reduce direct ethylene precursor content (Glick, 2014). These PGPB were selected due to their inability to produce ethylene and degrade its precursor to avoid any direct disturbance on plant ethylene metabolism caused by bacteria (Ibort et al., 2017). We aimed to evaluate the bacterial effects on the membranous proteomic profile in mature plants. Plant growth was determined at 8 weeks post-inoculation (wpi) and microsomal proteins analyzed by shot-gun proteomics. Furthermore, antioxidant and phosphorus nutrition statuses were evaluated based on proteomic results.

2. Materials and methods

2.1. Biological materials

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

2.2. Experimental design and growth conditions

The experiment consisted of a randomized complete block design with two tomato plant lines (wt and *nr*) and three inoculation treatments: (1) non-inoculated control plants, (2) *Bacillus megaterium*-inoculated plants and (3) *Enterobacter* C7-inoculated plants. Each treatment consisted in eleven replicates (n = 11). This number of plants has been only chosen because they should confirm the results of a previous paper (Ibort et al., 2017). Plants were harvested at 8 weeks post inoculation (wpi).

Seeds were sterilized (70% ethanol 5 min, 5% sodium hypochlorite 10 min and 3 washing steps with sterile water to remove any trace of chemicals), kept at 4 °C overnight and placed on sterile vermiculite at 25 °C until germination. 10-day-old seedlings were transferred to 1 l plastic pots containing sterile peat moss:perlite (1:1, v:v, autoclaved twice at 120 °C for 20 min). Bacteria were grown in Luria broth (LB) medium with shaking (200 rpm) at 28 °C overnight. The culture optical density was measured at 600 nm (OD_{600}), the cells were centrifuged (2655*g*, 10 min) and the pellet was resuspended in sterile distilled water until OD600 = 1.5 corresponding to a cell density of 10^7 CFU ml^{-1}. One ml of distilled water (control plants), or a bacterial suspension: Bm or C7 (inoculated plants) was sprinkled onto each root seedling at transplantation. Plants were grown for eight weeks in a greenhouse under controlled conditions (18–24 °C, 50–60% relative humidity, 16 h:8 h light (600 μmol m^{-2} s^{-1}):dark). In order to maintain constant soil water content close to water-holding capacity during the whole experiment, water was supplied every two days.

2.3. Biomass production

Plant growth was determined in order to evaluate PGPB activity. Shoots were separated from root systems and fresh weights (FW) were measured. Root samples were immediately frozen in liquid nitrogen