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Transcriptomic analysis reveals the importance of JA-Ile turnover in the response of *Arabidopsis* plants to plant growth promoting rhizobacteria and salinity

Running title: Involvement of JA-Ile in the response to PGPR and salinity

Gorka Erice^{1*}, Juan Manuel Ruíz-Lozano¹, Ángel María Zamarreño², José María García-Mina², Ricardo Aroca¹

¹Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda nº 1, 18008 Granada, Spain.

²Departamento de Biología Ambiental, Universidad de Navarra, Irunlarrea 1, 31008 Pamplona, Spain.

GE*: gorka.erice@eez.csic.es (corresponding author) (+34958181600)

Abstract

The use of plant growth promoting rhizobacteria (PGPR) is a proven management strategy to improve plant growth. The aim was to reveal the genomic and proteomic basis of the plant tolerance to saline soil conditions. Combination of whole transcriptome analysis and proteomic profiling helped further the understanding of the complexity of salt tolerance responses. *Arabidopsis* plants were grown inoculated or not with *Bacillus megaterium* and irrigated or not with salt. Physiological, genomic and proteomic approaches were combined to reveal plant salt tolerance mechanisms. Microarray analyses revealed the up-regulation of the jasmonic acid metabolism (*CYP94B3*, lipoxygenase 4 and allene-oxide cyclase) under saline conditions. Knock-out mutants of the gene of interest *CYP94B3*, responsible of JA-Ile catabolism, were used to confirm the obtained results. Salinity resulted in leaf Na accumulation with decreased chlorophyll content, but PGPR inoculation helped to overcome the stress. Proteomic analysis showed enhanced monodehydroascorbate reductase (MDHAR) content together with ATP synthase. *CYP94B3* knock-out plants confirmed the key role of JA-Ile turnover to overcome moderate saline stress. Subsequent experimentation showed that *CYP94B3* was important for salt tolerance under moderate and severe salt stress. Inoculation with *B. megaterium* was a valuable tool to reveal the importance of JA-Ile turnover and to recover *Arabidopsis* plants from saline stress. A single gene can modify the response of plants to salt stress.

Keywords: *Arabidopsis thaliana*; Salinity; *Bacillus megaterium*; PGPR; *CYP94B3*; Jasmonil isoleucine

1. Introduction

Plant productivity is being constricted by soil salinity. It is estimated that salinity affects 20% of irrigated culture fields, which means over 7% of the land surface worldwide (Gupta and Huang, 2014). Soil salinity decreases plant productivity through osmotic and ion-specific stresses, altering plant capabilities to uptake water and causing ion toxicity. Under future climate change scenarios, salt stress is predicted to increase and more efforts to maintain crop production will be needed (Turrall et al. 2011).

Evolving efficient, low cost, easily adaptable methods for management of abiotic stresses is a major question. It is currently unclear whether the engineering technology will develop fast enough to cope with food demands in the near future (Timmusk and Behers, 2012). In this line, plants usually interact with soil microorganisms that make plants more efficient in coping with environmental stresses including salt (Azcon et al. 2013). These include arbuscular mycorrhizal fungi, yeasts and plant growth promoting rhizobacteria (PGPR) (Zoppellari et al. 2014). As a relatively simple and low-cost alternative strategy, the use of free-living PGPR has been highlighted as a promising broad-spectrum means to improve plant growth (Kim et al. 2013).

The action mechanisms of PGPR include biofertilization, stimulation of root growth, rhizoremediation and induction of plant stress tolerance (Porcel et al. 2014). PGPR can alter plant growth through a wide variety of mechanisms including fixation of atmospheric nitrogen, solubilization and mobilization of phosphorus, sequestration of iron, production of antibiotics, hydrogen cyanide, amino acids, volatile compounds, synthesis of hydrolytic enzymes or the production of phytohormones (Babalola, 2010). In this sense, it is known that abiotic stress response is mediated by phytohormones such as abscisic acid (ABA), salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Ding et al. 2016). Moreover, PGPR may amplify or suppress plant stress signalling to improve water saving in agriculture, i.e. the ability of plants to produce and respond to the main stress hormones previously mentioned (Glick et al. 1998; Arkhipova et al. 2007). Previous studies reported that *Bacillus megaterium* as PGPR could alter leaf symptoms of stress injury in *Lactuca sativa* by increasing the accumulation of proline and reducing the amount of photosynthetic pigments (Marulanda-Aguirre et al. 2008). The same *B. megaterium* strain was then proven

to modify salt response in maize leaves in terms of root growth, necrotic leaf area, leaf relative water content and root hydraulic conductance (Marulanda et al. 2010).

The multiple mechanisms participating in the association of PGPR with plants is consistent with the additive hypothesis of PGPR effects. This hypothesis considers the overall enhancement of plant production and growth as the sum of various effects with small contributions. These components alone do not fully explain the increases in yield, but when combined, this may be a significant contribution (Bashan and de-Bashan, 2010). In this sense the transcriptome analysis and functional characterization of individual genes involved can provide further opportunities for fully understanding these complex interactions. The transcriptome response of *Arabidopsis* plants can be analyzed using whole genome arrays. The use of cDNA microarrays for monitoring gene expression provides an efficient, high-throughput approach to assess the possible functions of large number of genes (Yang et al. 2004, He et al. 2015). Subsequently, candidate genes with potential roles in controlling stress can be selected and their function characterized through the analysis of loss-of-function mutants (Atkinson et al. 2013).

The aim of the present study was to improve the current understanding of the plant-PGPR association, with the goals of revealing the genomic and proteomic basis of the plant tolerance to saline soil conditions. We used the association between *Arabidopsis* and *B. megaterium* PGPR to unveil the mechanisms of plant salt tolerance and how the symbiotic bacteria may improve it. We hypothesize that *Arabidopsis* inoculated with *Bacillus megaterium* could tolerate higher salt stress conditions. In the first experiment, we present data (plant growth and ion accumulation as well as chlorophyll, carotenoid content and TBARS content) of plants grown in an elevated salt treatment or in combination with *B. megaterium*. Combination of whole transcriptome analysis and proteomic profiling, as non-directed techniques, may help us to understand the complexity of plant tolerance to saline soils and discover potential genes and proteins with key roles in mitigating abiotic stresses. The use of knock-out mutants in experiment 2 allows us to confirm the obtained results reported in experiment 1. In Experiment 3, we reinforce the hypothesis developed in the study about the weight of the targeted gene in salt tolerance capacity of *Arabidopsis*. Through the exploitation of plant-PGPR association, we show new aspects of plant abiotic

stress tolerance system and reveal a mechanism used by beneficial microorganisms to improve such plant tolerance.

2. Material and Methods

2.1. Plant growth conditions

Seeds of *Arabidopsis thaliana* (Columbia [WT] or *CYP94B3* -cytochrome P450-like protein [At3g48520]- knockout line obtained from Nottingham Arabidopsis Stock Centre, NASC ID: N518989) were sterilized for 7 min in a solution containing 3.4 g L⁻¹ Bayrochlore and 86% ethanol, followed by 3 rinses with absolute ethanol. The seeds were then kept at 4°C for 24 hours in darkness before sowing. Growth chamber temperature was 24/20 °C (day/night) with supplemental lightening of 200 µE m⁻² s⁻¹ to ensure 16 h photoperiod per day. Plants were grown in sterilized commercial peat moss-based growth medium (Jiffy-7 pellets, Jiffy Products Ltd., Shippagan, New Brunswick, Canada) mixed with vermiculite (1:1 v/v) in plastic trays (53 cm × 27 cm).

Experiment 1 consisted of a randomized complete block design with two factors using *Arabidopsis thaliana* WT: (1) inoculation with *Bacillus megaterium* and (2) salt treatment, with 0 and 50 mM NaCl.

Experiment 2 consisted of a randomized complete block design with three factors: (1) genotype (WT or *CYP94B3*), (2) inoculation with *B. megaterium* and (3) salt treatment with 0 and 50 mM NaCl.

In both experiments, plants were inoculated with 0.1 ml of *B. megaterium* suspension (10⁹ cell ml⁻¹) grown in Luria-Bertani (LB) medium once a week for 5 weeks. Control plants received the same number of applications with the same amount of growth medium without bacteria.

One week after the last inoculation the salt treatment was imposed. Plants submitted to salt treatment were irrigated with 10 mL of 50 mM NaCl for 7 consecutive days. After one week of salt treatments, plants were harvested. Rosette dry mass was obtained after drying plant tissues at 70 °C for 48 h.

Experiment 3 consisted of a randomized complete block design with two factors: (1) genotype (WT or *CYP94B3* mutant), (2) increasing salt concentration treatment of 0, 50, 100, 150 and 200 mM NaCl. Seedlings were germinated in Petri dishes containing MS medium with increasing NaCl concentration and grown for 3 weeks. Plant dry mass was expressed as the percentage of the corresponding genotype (WT or *CYP94B3*) at 0 mM NaCl.

2.2. Sodium and potassium content

Rosette sodium and potassium content was determined by mass spectrometry by the Ionomic Service at Estación Experimental del Zaidín, CSIC (Granada, Spain). Sodium and potassium analysis was analyzed by inductively coupled plasma-optical emission spectrometry following the ICP-OES technique (ICP-OES 720-ES Agilent Technologies, Santa Clara, CA, USA).

2.3. Oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of leaves with an ice-cold mortar and 6 ml of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at $15,000 \times g$ for 20 min. The chromogen was formed by mixing 200 μ l of supernatants with 1 ml of a reaction mixture containing 15% (w/v), trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25 N HCl and then incubating the mixture at 100 °C for 30 min (Minotti and Adust, 1987). After cooling at room temperature, tubes were centrifuged at $800 \times g$ for 5 min, and the supernatant was used for spectrophotometric reading at 532 nm. Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The calibration curve was made using MDA in the range of 0.1-10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artefactual formation of TBARS during the acid-heating step of the assay.

2.4. Pigment concentrations in leaves

Photosynthetic pigments of five leaves per treatment were extracted using 0.1 g of fresh material in 5 ml 80% acetone. After filtering, 1 ml of the suspension was diluted with a further 2 ml acetone, and chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Cx + c) content were determined with a Hitachi U-2001 spectrophotometer (Hitachi Ltd., Japan), at 663.2, 646.8 and 470.0 nm. The concentrations of pigments were calculated according to the formula provided by Lichtenthaler (1987).

2.5. MDHAR activity

Extraction was carried out at 4°C. A 250 mg aliquot of leaves was homogenized with 1 ml of 50 mM MES/KOH buffer (pH 6.0), containing 40 mM KCl, 2 mM CaCl₂, and 1 mM L-ascorbic acid (AsA) (Torres and Andrews, 2006). MDHAR assay was performed in a final volume of 0.2 ml in the UV-microplate well at 25 °C using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). The reaction buffer consisted of 50 mM Hepes buffer (pH 7.6), 2.5 mM L-ascorbic acid (AsA), 0.25 mM NADH. MDHAR activity was measured according to the method from Miyake and Asada (1992) adapted to microplate quantification described by Murshed et al. (2008). Activity of monodehydroascorbate reductase (MDHAR) was assayed by monitoring the decrease in absorbance at 340 nm because of the oxidation of NADH (nicotinamide adenine dinucleotide, reduced) (extinction coefficient: 6.22 mM⁻¹ cm⁻¹).

2.6. Anthocyanin content

Anthocyanin content was assessed spectrophotometrically using the pH differential method described by Rapisarda et al. (2000) and adapted by Zhang et al. (2012). One hundred milligram of ground leaf samples were simultaneously treated with pH 4.5 buffer solution (240 mM HCl and 400 mM sodium acetate) and pH 1 buffer solution (150 mM HCl and 50 mM KCl). Absorbance was measured at 510 nm, and anthocyanin content was calculated as:

$$\text{Anthocyanin content (mg g}^{-1}\text{ DW)} = \Delta A \times 484.82 \times 1000 / 24825 \times \text{FW/DW}$$

where ΔA is the difference of absorbances, $A_{pH1} - A_{pH4.5}$, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24825 is its molar absorptivity (ϵ) at 510 nm in the pH 1 solution, and FW/DW is the ratio between fresh and dry weight rosette biomass.

2.7. RNA isolation synthesis of cDNA and hybridization

RNA was extracted from leaf samples using the RNeasy Plant Mini Kit (Qiagen, Alameda, CA, USA) following the instructions from the manufacturer. The RNA was subjected to DNase treatment and reverse-transcription using the QuantiTect Reverse Transcription Kit (Qiagen, Alameda, CA, USA). To rule out the possibility of a genomic DNA contamination, all the cDNA sets were checked by running control PCR reactions with aliquots of the same RNA that had been subjected to the DNase treatment, but not to the reverse transcription step. cDNA were hybridized to an Affimetrix Gene Chip ATH1 (Affimetrix Inc., Santa Clara, USA), and the measurement of signal intensity was conducted using GCOS version 1.4 (Affimetrix Inc.) in the Genomic Analytical service of CABIMER (Centro Andaluz de Biología Molecular y Medicina Regenerativa, Seville, Spain) facilities.

2.8. Analysis of microarray data

Total RNA (10 μ g) was used in a RT reaction to generate first-strand cDNA using the SuperScript choice system (Invitrogen) with oligo(dT) 24 fused to 7T RNA polymerase promoter. After second-strand synthesis, target complementary RNA (cRNA) labelled with biotin was prepared using the BioArray high yield RNA transcript labelling kit (Enzo Biochem, NY) in the presence of biotinylated UTP and CTP. After purification and fragmentation, cRNA (15 μ g) was used in a hybridization mixture where hybridization controls were added. The hybridization mixture (200 μ l) was hybridized on arrays for 16 h at 45°C. Standard post-hybridization wash and double-stain protocols were used on an Affimetrix GeneChip fluidics station 450. Finally, arrays were scanned on an Affimetrix GeneChip scanner 3000 7G.

Raw microarray data were analyzed in the R environment with Biconductor software. Microarray raw data were first normalized according to the gcRMA method (Wu et al. 2005). After normalization, data were fitted to a moderated linear model using the Limma package (Smyth, 2004). We used the AffyilmGUI package to apply the linear model (Wettenhall et al. 2006). AffyilmGUI is a Graphical User Interface for analysis of Affimetrix microarray data using Gordon Smyth's limma package (Linear Models for MicroArray data). A false discovery rate (FDR) for multiple testing collections (Benjamini and Hochberg, 1995) was applied to extract genes whose transcript levels differed significantly ($P \leq 0.01$). Fold-change was calculated using GeneSpring GX version 7.3.1 (Agilent Technologies, Santa Clara, USA) and set up at 4 as cut value.

Genes from microarray analysis were functionally classified using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system with default settings (Thomas et al. 2003). The Plant MetGenMap Software (Joung et al., 2009) was used to identify changed pathways from genomic profile data and to visualize data in a biochemical pathway context using a threshold of fold change over 4 using false discovery rate (FDR) as correction method.

2.9. Leaf proteomic characterization, analysis of 2-DE gels and protein identification

Frozen leaf tissue (200 mg fresh weight) was processed and Two-dimensional electrophoresis (2-DE) was conducted according to the method described in Aranjuelo et al. (2011). Functional classification of identified proteins was determined according to the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system with default settings (Thomas et al. 2003).

2.10. Phytohormones analysis

Endogenous SA, JA, JA-Ile and OPDA in plants were analyzed using high performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). The extraction and purification of these hormones were carried out using the method described by Ibort et al. (2017) on 0.25 g of frozen plant tissue.

2.11. Statistical analysis

Statistical analyses were performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA). Data were subjected to analysis of variance (ANOVA). The sources of variations were: PGPR inoculation and salt treatment including their interactions (experiment 1); plant genotype, PGPR inoculation and salt treatment including their interactions (experiment 2); and plant genotype and salt concentration including their interactions (experiment 3). Post hoc comparison with the LSD test was used to find out differences between groups with $P < 0.05$ as the significance cut-off.

3. Results

3.1. Experiment 1. Effect of salt stress and *B. megaterium* on *Arabidopsis* plants.

Inoculation with *B. megaterium* increased *Arabidopsis* dry weight under both control and salt conditions (Table 1). Neither the salt treatment nor the inoculation with *B. megaterium* modified the leaf K content, but plants grown under saline conditions exhibited increased leaf Na content (Table 1).

	Rosette dry weight (g plant ⁻¹)	K (mg Kg ⁻¹ DW)	Na (mg Kg ⁻¹ DW)
Control	0.31 ± 0.04 b	11918 ± 2682 a	4425 ± 324 b
Bm	0.48 ± 0.05 a	13037 ± 1132 a	2788 ± 188 b
Salt	0.37 ± 0.03 b	16794 ± 2146 a	13209 ± 1881 a
Salt + Bm	0.55 ± 0.02 a	12785 ± 1730 a	12572 ± 2948 a

Table 1. Effect of salt irrigation (50mM) on plant rosette dry weight, K content and Na content of 7 weeks old *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values ± S.E. Different letters indicate significant differences between treatments ($p < 0.05$) based on LSD test.

Irrigation with salt significantly dropped the content of chlorophylls measured as chlorophyll a, b and total chlorophyll, but inoculation with *B. megaterium* made plants return to control levels (Figure 1). Identical behaviour was seen for carotenoids, which

decreased in the salt treatment; however, the inoculation of *B. megaterium* helped plants maintain the control concentration (Figure 1).

Figure 1. Effect of salt (50mM) irrigation on chlorophyll a (A), chlorophyll b (B), total chlorophylls (C) and total carotenoids (D) of 7 week old *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values $\pm$ S.E. Different letters indicate significant differences between treatments ($p<0.05$) based on LSD test.

Lipid peroxidation measured as 2-thiobarbituric acid-reactive substances (TBARS) was unaltered by *B. megaterium* inoculation, but it increased when plants were submitted to a salt stress treatment (Figure 2). When plant grew under saline conditions, PGPR inoculation restored the level of TBARS (Figure 2).

Figure 2. Effect of salt (50mM) irrigation on TBARS of 7 week old *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values $\pm$ S.E. Different letters indicate significant differences between treatments ($p < 0.05$) based on LSD. test.

Microarray analyses were conducted to compare treatments with unstressed, non-inoculated plants. Among the 211 up-regulated genes, 129 were related to the salt treatment whereas 42 were linked to *B. megaterium* inoculation and 76 to the interaction between *B. megaterium* and the salt treatment (Supplemental Figure S1). In the same way 228 genes were down-regulated, 137 altered by the salt treatment, 42 by *Bacillus* inoculation and 49 by the combination treatment of *B. megaterium* and salt (Supplemental Figure S1). Comparisons between the control and combination treatments identified 20 up-regulated and 12 down-regulated genes (unknown proteins were not included) (Table 2). Among up-regulated genes, the majority were classified as involved in catalytic activity (55.6 %), followed by those with binding functions (22.2 %), nucleic acid binding transcription (11.1 %) and enzyme regulator activity (11.1 %) (Table 3). The down-regulated genes had transporter activity (42.9 %), translation regulator activity (14.3 %), binding (14.3 %), structural molecule activity (14.3 %) and catalytic activity (14.3 %) (Table 3). Moreover Plant MetGenMap identified 5 pathways significantly changed under the combined

conditions of salt and *B. megaterium* presence: jasmonic acid biosynthesis ($p = 0.0348$), 13-LOX and 13-HPL pathway ($p = 0.0348$), leucopelargonidin and leucocyanidin biosynthesis ($p = 0.0348$), leucodelphinidin biosynthesis ($p = 0.0348$) and flavonoid biosynthesis ($p = 0.0366$). Additional information regarding the altered genes in these pathways is available in the supplemental figures S2, S3, S4, S5 and S6. Also, data of the commonly most up-regulated and down-regulated genes in the three treatments (Salt, *B. megaterium* and *B. megaterium*+salt) compared to unsalted treatment and fold change in gene expression are presented in the supplemental table S1.

Gene identification	Features	Fold change over control (Gene chip)
Up-regulated		
At1g52690	late embryogenesis-abundant protein	18.6
At3g48520	cytochrome P450-like protein	17.4
At1g02400	gibberellin 2-beta-dioxygenase	13.9
At5g09530	periaxin-like protein	13.4
At1g72520	lipxygenase	12.8
At3g01830	calmodulin-like protein	10.8
At1g65400	transmembrane receptor	10.5
At2g42540	cold-regulated protein cor15a precursor	9.4
At3g25780	allene-oxide cyclase activity	7.8
At1g10585	transcription factor activity	7.3
At3g57260	beta-1,3-glucanase 2	6.3
At4g18280	glycine-rich cell wall protein	5.7
At3g55970	leucoanthocyanidin dioxygenase -like protein	4.9
At3g25180	cytochrome P450 monooxygenase	4.8
At5g01540	receptor like protein kinase	4.7
At2g29090	cytochrome P450 monooxygenase	4.7
At4g23140	serine/threonine kinase activity	4.7
At1g61120	S-linalool synthase	4.4
At2g23340	AP2 domain transcription factor	4.3
At4g25810	xyloglucan endo-1,4-beta-D-glucanase	4.3

Down-regulated		
At2g30520	protein with signal transducer activity	17.5
At1g52190	putative peptide transporter	8.6
At1g70830	defense response protein	8.2
At2g02930	putative glutathione S-transferase	6.7
At3g04720	hevein-like protein precursor (PR-4	6.5
At5g57655	D-xylose isomerase (carbohydrate metabolism)	6.4
At2g45470	Arabinogalactan (anchored to membrane)	5.7
At1g31710	copper amine oxidase	5.6
At3g14310	putative pectin methylesterase	5.4
At1g75500	nodulin-like protein	4.9
At2g38120	auxin influx transmembrane transporter activity	4.4
At4g23400	plasma membrane intrinsic protein 1c	4.1

Table 2. *Arabidopsis thaliana* genes most up-regulated and down-regulated specifically in the *B. megaterium*+salt treatment as compared to unsalted treatment and fold change in gene expression. Down-regulated gene fold change is expressed as the reciprocal of the analysis result.

Functional classification	Number	%
Up-regulated		
catalytic activity (GO:0003824)	5	55.6%
binding (GO:0005488)	2	22.2%
nucleic acid binding transcription factor activity (GO:0001071)	1	11.1%
enzyme regulator activity (GO:0030234)	1	11.1%
Down-regulated		
transporter activity (GO:0005215)	3	42.9%
translation regulator activity (GO:0045182)	1	14.3%
binding (GO:0005488)	1	14.3%
structural molecule activity (GO:0005198)	1	14.3%
catalytic activity (GO:0003824)	1	14.3%

Table 3. Functional categorization of up- or down-regulated transcripts in the *B. megaterium*+salt treatment as compared to control treatment.

The effect of salt and *B. megaterium* inoculation on the leaf protein profile in *Arabidopsis* plants was studied using 2-DE. Thirty-five spots were up-regulated due to salt irrigation, 33 induced by the *B. megaterium* inoculation and 42 by the *B. megaterium* and salt combination (Supplemental Figure S7). Studied factors (salt, *Bacillus* and salt+*Bacillus*) down-regulated 6 spots, but none of them were repressed specifically by salt and *Bacillus* combination (Supplemental Figure S7). Among 25 specifically up-regulated spots by the combination treatment, 22 proteins were identified (Table 4). These proteins were classified into different groups according to their presumed biological function: catalytic activity (43.5 %) binding (21.7 %), receptor activity (13 %), transporter activity (13 %), translation regulator activity (4.3 %) and structural molecule activity (4.3 %) (Table 5). The roles of these proteins are discussed in the following section with regard to physiological and biochemical changes observed in *Arabidopsis* inoculated with *B. megaterium* and submitted to salt irrigation.

Table 4. Annotation of identified up-regulated spots in silver-stained 2-DE gels of *Arabidopsis* plants treated with salt irrigation and inoculated with *B. megaterium*. Spot no. represents the number of proteins assigned. Spot volume (%) is an estimation of relative protein abundance. The pI and molecular mass (Mr) values shown are the theoretical and experimental values. SC represents the protein sequence coverage (%) score, which is the Mascot score of the in-solution digestion protocol. PM indicates for each spot, the number of peptide matched according to the results of research on the NCBI nr-protein sequence databases.

No.	Spot % volume		Theor. pI/Mr	SC (%)	PM	Score (P < 0,05 corresponding to score > 51)		Description
	variation	Exp. pI/Mr						
6802	142.38	6.17/52.22	5.95/52.95	57%	36	353.5		Ribulose biphosphate carboxylase large chain (RuBisCO large subunit) [CHAIN 0]
4802	111.53	5.74/56.47	5.95/52.95	26%	15	140.1		Ribulose biphosphate carboxylase large chain (RuBisCO large subunit) [CHAIN 0]
4803	88.62	5.81/58.17	5.95/52.95	21%	10	84.3		Ribulose biphosphate carboxylase large chain (RuBisCO large subunit) [CHAIN 0]
1801	82.52	5.34/58.36	5.25/55.32	36%	15	124.5		ATP synthase subunit alpha, chloroplastic
1803	72.67	5.38/62.26	5.21/65.6	22%	10	75		ATP-dependent zinc metalloprotease FTSH 8, chloroplastic (AtFTSH8) [CHAIN 0]
504	67.3	5.07/33.93	5.71/35.14	69%	15	127.6		Oxygen-evolving enhancer protein 1-1, chloroplastic (OEE1) (MSP-1) [CHAIN 0]
1804	57.35	5.28/58.65	5.25/55.32	24%	9	70.5		ATP synthase subunit alpha, chloroplastic
1806	53.69	5.32/62.26	5.15/68.81	27%	15	136.8		V-type proton ATPase catalytic subunit A (V-ATPase subunit A)
9804	42.53	6.75/68.95	6.17/84.35	26%	14	117.8		5-methyltetrahydropteroyltrimethylglutamate--homocysteine methyltransferase
2804	39.51	5.45/60.94	6.48/63.8	16%	7	69.2		RuBisCO large subunit-binding protein subunit beta, chloroplastic [CHAIN 0]
1808	33.79	5.3/61.91	6.12/74.15	35%	17	130		ATP-dependent zinc metalloprotease FTSH 2, chloroplastic (AtFTSH2) [CHAIN 0]
9710	30.34	6.69/41.48	5.95/52.95	27%	13	110.9		Ribulose biphosphate carboxylase large chain (RuBisCO large subunit) [CHAIN 0]
3803	29.06	5.67/57.48	5.46/53.93	47%	19	179.3		ATP synthase subunit beta, chloroplastic
702	22.02	5.12/44.13	6.47/42.41	44%	14	125.2		Sedoheptulose-1,7-bisphosphatase, chloroplastic (SBPase) [CHAIN 0]
3703	19.72	5.64/47.31	5.93/51.63	20%	6	57.9		Elongation factor Tu, chloroplastic (EF-Tu) [CHAIN 0]
3702	19.67	5.66/49.94	5.46/53.93	35%	13	96.8		ATP synthase subunit beta, chloroplastic

4605	15.64	5.79/38.89	6.18/46.67	28%	9	73.6	Protease Do-like 1, chloroplastic [CHAIN 0]
8903	15.14	6.34/73.55	5.95/52.95	24%	10	90.3	Ribulose biphosphate carboxylase large chain (RuBisCO large subunit)
7703	14.00	6.27/51.79	5.95/52.95	55%	33	299.4	Ribulose biphosphate carboxylase large chain (RuBisCO large subunit) [CHAIN 0]
803	7.60	4.97/63.29	5.26/75.51	14%	8	81.6	Stromal 70 kDa heat shock-related protein, chloroplastic [CHAIN 0]
9902	0.14	6.71/76.16	6.17/84.35	45%	27	237.2	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
7702	0.01	6.23/45.89	7.61/52.5	46%	15	129.8	Monodehydroascorbate reductase, chloroplastic (MDHAR) [CHAIN 0]

Functional classification	Number	%
up-regulated		
catalytic activity (GO:0003824)	10	43.5%
binding (GO:0005488)	5	21.7%
receptor activity (GO:0004872)	3	13.0%
transporter activity (GO:0005215)	3	13.0%
translation regulator activity (GO:0045182)	1	4.3%
structural molecule activity (GO:0005198)	1	4.3%

Table 5. Functional categorization of up-regulated proteins in the *B. megaterium*+salt treatment as compared to control treatment.

3.2.Experiment 2. Effect of salt stress and *B. megaterium* on *CYP94B3* knock-out *Arabidopsis* plants.

One of the most up-regulated genes in PGPR inoculated plants under salt conditions was the cytochrome P-450-like protein (*CYP94B3*). It is involved in jasmonate (JA) metabolism as other up-regulated genes shown by the microarray analysis (lipoxygenase and allene-oxide cyclase). The interest of JA as stress hormone led us to test *CYP94B3* knock-out *Arabidopsis* plants inoculated with *B. megaterium* and grown under salt conditions to reveal the importance of this gene. Under non-stressed conditions no significant changes in rosette dry weight were observed among treatments (Table 6). However, under salt conditions, *B. megaterium* inoculation increased rosette dry weight only in WT plants, but not in *CYP94B3* knock-out ones (Table 6). Moreover, the knock-out plants were the only one that decreased rosette dry weight because of the salt treatment (Table 6). None of the studies factors (genotype, *B. megaterium* inoculation and salt treatment) altered the leaf K⁺ ion content, but Na⁺ was significantly increased in all cases by the salt treatment (Table 6). Inoculation with *B. megaterium* helped to decrease Na⁺ content in WT plants, but not in the knock-out plants, even if the ion levels were enhanced more than 7 times over the steady-state concentration (Table 6).

	Rosette dry weight (g plant ⁻¹)	K (mg Kg ⁻¹ DW)	Na (mg Kg ⁻¹ DW)
WT	0.16 ± 0.02 ab	18142 ± 1011 ab	1399 ± 317 c
WT + Bm	0.16 ± 0.01 ab	18233 ± 709 ab	861 ± 400 c
<i>CYP94B3</i>	0.17 ± 0.02 ab	16744 ± 695 ab	1095 ± 194 c
<i>CYP94B3</i> + Bm	0.14 ± 0.01 bc	17061 ± 732 b	986 ± 89 c
WT + Salt	0.14 ± 0.01 bc	20726 ± 1774 a	8773 ± 273 a
WT + Bm + Salt	0.18 ± 0.02 a	15342 ± 1434 b	7320 ± 1005 b
<i>CYP94B3</i> + Salt	0.12 ± 0.01 c	19062 ± 3441 ab	8167 ± 67 ab
<i>CYP94B3</i> + Bm + Salt	0.14 ± 0.02 abc	18264 ± 1289 ab	8262 ± 1185 ab

Table 6. Effect of salt irrigation on plant rosette dry weight, K content and Na content of 7 week old WT or *CYP94B3* knock-out mutant *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values ± S.E. Different letters indicate significant differences between treatments (p<0.05) based on LSD test.

Total chlorophylls were unaltered due to salt treatment or PGPR inoculation, and the same result was obtained for chlorophyll a (Figure 3). Only *CYP94B3* knock-out mutants showed a lower concentration of chlorophyll b when subjected to salt stress and inoculated with *B. megaterium* (Figure 3). Total carotenoid concentration significantly decreased in WT plants due to the salt treatment (Figure 3).

Figure 3. Effect of salt irrigation (50mM) on chlorophyll a (A), chlorophyll b (B), total chlorophylls (C) and total carotenoids (D) of 7 week old WT and *CYP94B3* knock-out *Arabidopsis* plants inoculated or not with *Bacillus megaterium*. Data represents the mean of 4 values $\pm$ S.E. Different letters indicate significant differences between treatments ($p < 0.05$) based on LSD test.

Monodehydroascorbate reductase (MDHAR) enzyme was shown to be up-regulated according to the proteomic results (Table 4). In order to confirm that enhanced protein content led to higher enzymatic activity, MDHAR activity was measured. MDHAR activity was generally unaffected by the studied factors, but it showed a significant increase in knock-out plants undergoing the combination treatment (Figure 4).

Figure 4. Effect of salt irrigation (50 mM) on TBARS (A), MDHAR activity (B) and anthocyanin (C) content of 7 weeks old WT and *CYP94B3* knock-out *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values $\pm$ S.E. Different letters indicate significant differences between treatments ($p < 0.05$) based on LSD test.

Microarray analysis also showed the alteration of anthocyanin metabolism (leucoanthocyanidin dioxygenase, Table 4). *CYP94B3* knock-out *Arabidopsis* mutants featured less leaf anthocyanin content regardless of the PGPR inoculum when irrigated with water (Figure 4). Salt treatment caused a decrease of anthocyanin content in non-inoculated plants of both genotypes, but the inoculation treatment returned anthocyanin levels to control levels in both genotypes (Figure 4).

PGPR decreased leaf SA concentration in WT plants regardless of irrigation treatment and in *CYP94B3* knock-out mutant plants irrigated with water (Figure 5). Leaf 12-oxo-phytodienoic acid (OPDA) content also declined significantly in water irrigated WT plants after PGPR inoculation. WT plants irrigated with water showed diminished levels of leaf JA after *B. megaterium* inoculation whereas *CYP94B3* knock-out mutants showed significantly increased JA content (Figure 5). Also, mutant plants inoculated with PGPR showed increased JA content when compared to inoculated WT *Arabidopsis* (Figure 5). Under salt stress conditions, leaf JA were greater in *CYP94B3* knock-outs when compared with WT, but the inoculation with *B. megaterium* decreased JA to steady state levels (Figure 5). The *CYP94B3* knock-outs showed significant increases in JA-Ile accumulation in all cases with no differences due to salt treatment or PGPR inoculation (Figure 5).

Figure 5. Effect of salt irrigation (50 mM) on OPDA (A), SA (B), JA (C) and JA-Ile (D) content of 7 week old WT and *CYP94B3* knock-out *Arabidopsis* plants inoculated or not with *B. megaterium*. Data represents the mean of 4 values $\pm$ S.E. Different letters indicate significant differences between treatments ($p < 0.05$) based on LSD test.

3.3.Experiment 3. Effects of increasing salt concentration on *CYP94B3* knock-out *Arabidopsis* plants.

Increasing salt irrigation concentration gradually decreased *Arabidopsis* plant dry matter accumulation in both WT and *CYP94B3* knock-out genotypes (Figure 6). Significantly greater decreases of plant growth of *CYP94B3* knock-out plants compared to WT plants were recorded when irrigated with 50 and 200 mM NaCl (Figure 6).

Figure 6. Effect of increasing salt concentration on plant dry matter accumulation of 7 weeks old WT and *CYP94B3* knock-out *Arabidopsis* plants. Data represents the mean value of 4 values $\pm$ S.E. Different letter indicates significant differences between treatments ($p < 0.05$) based on LSD test.

4. Discussion

The present study deals with the molecular basis of plant salt tolerance and the potential of plant-PGPR association to counteract such abiotic stress. In the first part of the study *Arabidopsis* plants inoculated or not with *B. megaterium* were subjected to a moderate salt stress. Measurements of growth and commonly used parameters of plant metabolism (peroxidation of lipids, chlorophylls and carotenoids) were combined with broad spectrum techniques (microarray data and proteomic characterization) to uncover the salt tolerance after PGPR inoculation. Afterwards, the use of an *Arabidopsis* knock-out line was used to confirm the results. In an attempt to test the robustness of the results, both the wild type and CYP94B3 knock-out plants were grown at increasing salt stress levels.

4.1 Salt stress altered JA metabolism in *Arabidopsis* plants

After 7 weeks, *Arabidopsis* plant growth was enhanced by the inoculation with *B. megaterium* (Table 2) as has been already reported (Nadeem et al. 2007; Kohler et al. 2009). Nevertheless, it is noticeable that higher plant production seems to be inconsistent

with contrasting results after inoculation with PGPR (Marulanda-Aguirre et al. 2008). The *B. megaterium* strain used in the present study showed salt tolerance increases in maize plants and that effect was attributed to higher root hydraulic conductance via aquaporin water transport (Marulanda et al. 2010). In our case, salt treatment did not alter plant dry weight accumulation (Table 1) most probably because salt irrigation was applied for the last and final week of the experiment.

Arabidopsis is considered a typical glycophyte not particularly salt tolerant (Taji et al. 2004) even if its genome contains most of the salt tolerance genes of halophytes (Zhu, 2000). The saline stress was moderate, but enough to increase Na accumulation, as shown in leaf Na accumulation in Table 1. Plants submitted to salt contained 3 times more Na than those irrigated with water, whereas K leaf content remained unchanged among treatments. Na usually competes with K altering metabolic processes in the cytoplasm, such as enzymatic reactions, protein synthesis and ribosome functions (Marschner, 1995) leading to ion toxicity.

Degradation of chlorophylls under salt stress is agreed in the literature as a common response of multiple plant species (Turan et al. 2009; Taffouo et al. 2010). This decrease is primarily a consequence of the action of the enzyme chlorophyllase and the inhibition of chlorophyll synthesis (Santos, 2004). In our study, salt irrigation led to decreases in chlorophyll and total carotenoid content (Figure 1). The degradation of chlorophylls has been often related to oxidative stress. Carotenoids are one of the molecules protecting the photosynthetic mechanisms, scavenging triplet chlorophyll and singlet oxygen (Demming-Adams and Adams, 2002). But beyond this function, carotenoids are known to be involved in preventing the oxidation of membranes, acting as powerful chloroplast membrane stabilizers that partition between light-harvesting complexes (LHCs) and the lipid phase of thylakoid membranes (Demming-Adams and Adams, 2002). This aspect is of interest because some PGPRs have been described to increase membrane stability (Jha and Subramanian, 2014; Abbasi et al. 2013). This feature could explain our results, where *B. megaterium*-inoculated plants grown under salt conditions recovered their chlorophyll and carotenoid concentrations to control levels, and the (Figure 1). The extent of the salt stress can also be estimated by measuring the concentration of TBARS as this parameter is often

used to estimate the oxidative damage (Varghese and Naithani, 2008). In our case, the salt treatment increased oxidative damage as was shown by enhanced TBARS concentration but *B. megaterium* inoculation prevented the rise of lipid peroxidation (Table 2). Plants irrigated with salt and *B. megaterium* showed similar level of TBARS than plants grown in the non saline substrate.

Microarray results obtained specifically under salt stress for PGPR-inoculated plants are presented in Table 2. Regarding their function, the majority of the induced genes had catalytic activity as would correspond to the activation of the salt stress tolerance metabolism, followed by binding proteins (Table 3). It was remarkable that among the genes with putative roles under the saline conditions, three of the most up-regulated were the At3g48520 (cytochrome P450-like protein), At1g72520 (lipoxygenase) and At3g25780 (allene-oxide cyclase) (Table 2).

At3g48520 encodes for *CYP94B3*, a jasmonoyl-isoleucine-12-hydroxylase that catalyzes the formation of 12-OH-JA-Ile from JA-Ile whereas At1g72520, the lipoxygenase 4, is part of the gene family involved in the oxygenation of α -linolenic acid (α -LeA), the initial step in JA synthesis (Wasternack and Hause, 2013) sequentially with cyclopentenone-forming allene oxide cyclase (Otto et al. 2016). These results were confirmed by the significant alteration of jasmonic acid biosynthesis as well as 13-LOX and 13-HPL pathways. JA-Ile is the bioactive form of JA, a key hormone with wide variety of roles from plant reproduction to coordination of plant responses to both biotic and abiotic stresses (Koo et al. 2011). Even though the biosynthesis of JA is well-known, the JA-Ile metabolism and the enzymes involved are poorly understood (Koo et al. 2014). After the JA-Ile peak following the contact with the stressor, there is a decline of its concentration by metabolic turnover (Koo et al. 2009; Miersch et al. 2008). *CYP94B3* together with the *CYP94B1* are responsible for 95% of the catabolism conversion of JA-Ile to 12OH-JA-Ile in *Arabidopsis* leaves (Koo et al. 2014). The knock-out mutants in our study confirmed the hyperaccumulation of JA-Ile (Figure 5) previously reported by Koo with the subsequent reduction in 12-hydroxy-JA-Ile (Koo et al. 2011). This deficient JA-Ile turnover has not been related to confer resistance to fungus infection (Aubert et al. 2015) or to wound-induced growth inhibition (Poudel et al. 2016); however it is still believed that JA-Ile levels perform a key role in the attenuation of

jasmonate responses (Koo et al. 2011; 2014). So it can be hypothesized that a lack of control over JA-Ile concentrations could result in higher plant sensitivity when facing stresses such as saline conditions as is revealed by the microarray analysis. It is surprising that none of the other studied phytohormones, SA, OPDA or even JA, were consistently altered by the mutation (Figure 5). Nevertheless, it needs to be noted that SA leaf concentration was generally decreased after the inoculation with *B. megaterium* (Figure 5). Our results could confirm the general assumption that plant systemic acquired resistance induced by PGPR is mediated by SA (Beneduzi et al. 2012).

At the proteomic level, the effect of the combination treatment confirmed the alteration of plant metabolism revealing the upregulation of 22 spots with significant variations in volume (Table 4). Most of these spots were classified to have catalytic activity (Table 5). It is noticeable the presence of up to 6 rubisco isoforms. Over expression of rubisco has been previously reported by Kandasamy et al. (2009) due to the inoculation with *Pseudomonas fluorescens* as PGPR in rice. The proteomic approach also revealed the alteration of antioxidant defence system though the upregulation of the monodehydroascorbate reductase (Table 4), a ROS-scavenging key enzyme that converts monodehydroascorbate to reduced ascorbate in the ascorbate-glutathione pathway (Noctor and Foyer, 1998). Three spots corresponding to ATP synthase were also induced (Table 4). Previous literature shows that proton pumps can contribute to salt tolerance potentially through regulating the Na compartmentalization in the vacuole, K assimilation, osmotic regulation and antioxidant response (Chen et al, 2015). The only proteomic sign of antioxidant system alteration by PGPR inoculation until now was the MDHAR increase when wheat plants were inoculated with *Bacillus thuringiensis* endured drought stress (Timunsk et al. 2014).

4.2 CYP94B3 knock-out plants confirm the contribution of JA-Ile turnover to salt tolerance

Three of the genes induced by the combination treatment of salt and PGPR inoculation, *CYP94B3*, lipooxygenase and allene-oxide cyclase, are involved in JA metabolism, so we considered of particular interest to focus on the role of JA-Ile oxidation for signal attenuation. We used *CYP94B3* knock-out plants to evaluate the significance of JA-Ile under moderate salt irrigation. Knock-out plants under salt irrigation showed lower growth

and this effect was not reversed by *B. megaterium* inoculation, contrary to WT plants (Table 6). These results indicate the importance of this gene in *Arabidopsis* salt tolerance and the alleviation of this abiotic stress by *B. megaterium* inoculation. Salt treatments showed a similar Na accumulation pattern in WT plants, but the inoculation of WT with *B. megaterium* featured significantly lower Na content. Na concentrations were still elevated when compared to plants not receiving the salt treatment (Table 6). Again, such diminution in Na accumulation by bacterial inoculation was not observed in knock-out plants, which reinforces the crucial role of *CYP94B3* in the induction of salt tolerance by *B. megaterium* inoculation.

Knockout plants inoculated with *B. megaterium* under salt conditions showed a significant rise of MDHAR activity together with the anthocyanin content (Figure 3). *B. megaterium* inoculation increased metabolic activity, which resulted in antioxidant changes that were confirmed with MDHAR activity and anthocyanin as an indicator of stress (Davison et al. 2002). This confirms the proteomics results in which one of the spots corresponded to enhanced MDHAR content (Table 4). Contrasting results were obtained for PGPR inoculation in the case of WT plants. The increase of TBARS was not related to MDHAR activity or reflected in anthocyanin content (Figure 4). Therefore, it is possible that the inoculation with *B. megaterium* increased the stress suffered by the knock-out plants under salt conditions.

In order to confirm the extent of the findings around the sensitivity to salt conditions of the *CYP94B3* knock-out plants, another test was conducted. In this experiment, *Arabidopsis* plants were grown in Petri dishes for 3 weeks under increasing salt concentration in the medium. Although the general trend showed that mutant plants grew less than WT, this result was only significant in the case of plants grown under 50 mM and 200 mM (Figure 6). The fact that the knock-out of *CYP94B3* decreased plant growth under moderate (50 mM) and severe (200 mM) stress conditions reinforces the idea that this gene, and JA-Ile turnover, is important for salt tolerance.

5. Conclusions

In summary, we grew *Arabidopsis* plants and inoculated them with *B. megaterium*. After one week of salt irrigation, the stress was moderate but strong enough to alter plant metabolism. Leaf Na accumulation was concomitant to decreased chlorophyll and carotenoid levels, but PGPR inoculation helped to overcome these adverse effects. Microarray results showed the adjustment of the JA metabolism through the upregulation of *CYP94B3*, a jasmonil-isoleucine-12-hydroxylase, lipoxygenase 4 and allene-oxide cyclase in inoculated plants submitted to salt irrigation, whereas proteomic profiling featured enhanced rubisco and MDHAR content as well as ATP synthase. These results confirmed the extent of saline stress to the antioxidant system and the contribution of proton pumps to salt tolerance. The use of *CYP94B3* knock-out plants allowed us to confirm the contribution of JA-Ile turnover to overcome moderate saline stress. Also evidence of the enhancement of plant salt tolerance by *B. megaterium* is shown. PGPR enhanced carotenoid content of mutant plants submitted to salt treatment as well as MDHAR activity or anthocyanin concentration, allowed saline conditions grown plant to reach similar dry weight than watered ones. Furthermore, inoculation with *B. megaterium* has been proven useful to reveal the alteration of JA-Ile turnover under salt irrigation and to help plants to overcome these stress levels. It was demonstrated that a single gene can modify the response of plants to PGPR inoculation and salt stress.

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