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Characterization of Leaf Transcriptome of Grafted Tomato Seedlings after Rhizospheric Inoculation with *Azospirillum baldaniorum* or *Paraburkholderia graminis*

Federica Caradonia [1,*], Matteo Buti [2], Alessia Flore [1], Roberto Gatti [1], Caterina Morcia [3], Valeria Terzi [3], Domenico Ronga [4], Lionel Moulin [5], Enrico Francia [1,*] and Justyna Anna Milc [1]

1 Department of Life Science, Centre BIOGEST-SITEIA, University of Study of Modena and Reggio Emilia, Via Amendola, n. 2, 42122 Reggio Emilia, Italy
2 Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence, Via Maragliano 77, 50144 Florence, Italy
3 Council for Agricultural Research and Economics-Genomics and Bioinformatics (CREA-GB), Via San Protaso, 302, 29017 Fiorenzuola d'Arda, Italy
4 Pharmacy Department, University of Salerno, Via Giovanni Paolo II n. 132, 84084 Fisciano, Italy
5 IRD, Cirad, University Montpellier, IPME, 911 Avenue Agropolis, 64501, 34394 Montpellier, France
* Correspondence: federica.caradonia@unimore.it (F.C.); enrico.francia@unimore.it (E.F.); Tel.: +39-0522522064 (F.C.); Tel.: +39-0522522041 (E.F.)

Abstract: Inoculation with plant growth promoting rhizobacteria (PGPR) might be a sustainable practice to increase nutrients use efficiency of crops. In order to elucidate the mechanisms underlying the beneficial interaction, an RNA-Seq transcriptional profiling of tomato leaves was performed after roots' inoculation with *Azospirillum baldaniorum* (AB) or *Paraburkholderia graminis* (PG). Overall, 427 and 512 differentially expressed tomato genes were retrieved for AB and PB inoculation, respectively, and in both cases, the number of up-regulated genes exceeded the number of those down-regulated. Expression profiles suggest that the interactions between tomato seedlings and microorganisms are species-specific. The common activated pathways involved genes coding for proteins related to water and nutrients uptake, defense responses to biotic and abiotic stresses and hormonal regulation of fruit-set and ripening. While AB induced genes coding for MYB transcription factors known to be involved in response to biotic and abiotic stresses, PG upregulated 5 genes coding for putative late blight resistance protein homolog. Auxin responsive molecules and gibberellins involved in the fruit-set and early fruit growth in tomato were mainly induced by AB correlating to higher fruit number obtained in a previous field study. On the other hand, ERF transcription factors involved in ripening were induced mainly by PG treatment.

Keywords: rootstock; *Solanum lycopersicum*; plant growth promoting rhizobacteria; transcriptome analysis

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the major horticultural crops worldwide, with an average cropping area of 5 million ha producing ~181 million t of fruits [1]. Tomato fruits are a source of bioactive phenolic compounds, carotenoids (such as lycopene and β-carotene) [2], vitamins (A and B) and minerals [3] that make them an important part of human diet.

In order to guarantee a higher yield and a higher fruit quality, requested to satisfy the increasing demands of a growing population and to enhance the beneficial features of tomatoes, sustainable agriculture practices should be taken in consideration. Practices with low environmental impact can reduce the use of external inputs, such as plant protection products and fertilizers, improve the nutrient and water use efficiency, and mitigate the negative impact of climate change [4].

The exploitation of the great variety of bacterial species dwelling in the soil is a promising and sustainable approach to increase tomato yield and reduce the use of external inputs [5]. Indeed, bacteria can colonize plant root and rhizosphere creating commensalistic, parasitic and mutualistic relationships with host plants [6]. Mutualistic microorganisms can provide a series of beneficial effects on crop growth, health and production quality of pea (*Pisum sativum* L.), maize (*Zea maize* L.), tomato (*Solanum lycopersicum* L.), lettuce (*Lactuca sativa* L.), wheat (*Triticum* spp.), etc. [7]. Some rhizobacteria, such as *Azospirillum* spp. and *Paraburkholderia* spp., are able to improve the macro- and micro-nutrients uptakes by the plants. The root growth stimulation, the improvement of nitrogen fixation and the conversion of insoluble mineral to bioavailable forms are among the main ways in which the nutrients uptake is enhanced by plant growth promoting rhizobacteria–PGPR [8]. Beneficial microorganisms also synthesize or induce plants to produce compounds influencing germination, flowering, and ripening, such as phytohormones [9], and also improve crop tolerance to stresses. *Azospirillum baldaniorum* Sp245 was demonstrated to alleviate the adverse effects of drought stress in purple basil (*Ocimum basilicum* L.) [10]. During chilling stress, *Paraburkholderia graminis* C4D1M improved tomato seedling re-growth and reduced cell membrane injuries in terms of electrolytic leakage and efficiency of photosystem II [11]. Mendes et al. [12] also reported a constant association between suppression activity against *Rhizoctonia solani* on sugar beet plants and the presence in soil of several bacterial phyla, such as *Proteobacteria*, *Firmicutes* and *Actinobacteria*.

As outlined above, the use of PGPR can result in many positive effects on crop performance. However, the underlying molecular mechanisms are not well elucidated, since each beneficial microorganism provides cross-protection against several stresses and, at same time, stimulates plant growth by activating many genes in different plant organs. Understanding the mechanisms of action of beneficial microorganisms is a key requirement for optimizing their use on different crops and environmental conditions, as well as the formulation of bio-based commercial products [13,14]. Transcriptomic studies, based on RNA sequencing, are a good way of defining the molecular mechanisms involved in the interaction between the plant and beneficial microorganisms, as already used for plant-pathogen interplays characterization [15,16]. Several studies examined the tomato interaction with root pathogens [17,18] and, as reported by Zouari et al. [19], tomato could be considered a good model for evaluating the plant-PGPR interaction also in organs distant from the inoculation point.

In a previous experiment, *A. baldaniorum* Sp245 was reported to induce early flowering in grafted tomato seedlings in greenhouse, whereas in open field conditions the same tomato genotype inoculated with either *P. graminis* C4D1M or a bacterial consortium (*P. graminis* and *A. baldaniorum*), an increase of the total yield was observed [20]. In this context, the present study aims to perform an RNA sequencing analysis to highlight differences in the leaf transcriptome of grafted tomato seedlings in responses to *P. graminis* C4D1M or *A. baldaniorum* Sp245 root inoculation.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Microbial Inoculation

The commercial processing tomato genotype 'H3402' (HEINZ, Pittsburgh, Pennsylvania, USA), grafted onto the commercial genotype 'Tomito' (ISI Sementi SpA, Fidenza, Italy), resistant to *Fusarium oxysporum* f. sp. *lycopersici* race 0, *Verticillium albo-atrum* and *Verticillium dahliae* race 0, was used for this experiment based on our previous studies. The seeds were surface sterilized with 1% (*v/v*) sodium hypochlorite and rinsed with sterilized distilled water. Seeds were sown in plateaus filled with sterilized neutral commercial peat (23% organic carbon, 0.5% organic nitrogen and dry apparent density 214 kg m^{-3}, Dueemme S.r.l., Reggio Emilia, Italy) under controlled conditions (25/19 °C day/night temperature regime and ~60% of relative humidity). Coop Habitat (San Vito, Ferrara, Italy), performed grafting of tomato plants as reported in Caradonia et al. [20]. Two weeks after grafting, at four true leaves stage, seedlings were transplanted in pots (6.5 cm × 8.0 cm × 5.5 cm)

filled with neutral peat (23% organic carbon, 0.5% nitrogen, pH 6, electrical conductivity 0.25 dS m^{-1}, and dry apparent density 214 kg m^{-3}; Dueemme S.r.l., Reggio Emilia, Italy). Two PGPR species (*A. baldaniorum* Sp245 and *P. graminis* C4D1M, hereafter AB and PG, respectively) were chosen for this experiment. The bacteria were isolated from soil by CREA Research Center for Genomics and Bioinformatics, identified by PCR amplification of 16S rRNA genes, and stored at −80 °C. After transplanting, bacterial treatments were performed by adding 1 mL of inoculum (10^7 colony forming unit (CFU) mL^{-1}) close to the seedlings' root collars determined according to a preliminary test, as described in Caradonia et al. [11]. Inocula were prepared from a single bacterial colony in 60 mL of tryptone soya yeast extract broth (Liofilchem S.r.l., Teramo, Italy). Culture medium was maintained at 28 °C for 24 h while shaking at 150 rpm; then, suspensions were centrifuged for 4 min at 8000 g, and pellets were washed and suspended in sterilized distilled water. A V-550 UV-VIS spectrophotometer (600 nm) (Jasco Europe, Lecco, Italy) was used to estimate the bacterial concentrations, and sterilized distilled water was added until the concentration of 10^7 CFU mL^{-1} was reached. Seedlings grown in pot without inoculation were used as control. Ten seedlings were considered for each treatment.

After inoculations, seedlings were grown in a greenhouse located at the Department of Life Sciences, University of Modena and Reggio Emilia, with 16/8 h light/dark photoperiod and 25/19 °C day/night temperature.

2.2. Rhizobacteria Rhizosphere Colonization

Six days after treatments the bacterial colonization was verified in each treatment (control; inoculated with AB; inoculated with PG). Three random rhizosphere samples were picked up and placed in sterile tubes containing phosphate buffered saline (PBS). The suspensions were serial diluted and plated onto Petri dishes containing tryptone soya yeast extract agar. Petri dishes were aerobically incubated at 28 °C for 3 days in the dark, colonies were counted and the number of CFU per gram of rhizosphere soil weight was calculated [21].

2.3. RNA Extraction and Illumina Sequencing

Leaf samples were randomly harvested at flowering stage, 35 days after inoculations, from three plants for each treatment (control, inoculated with AB, inoculated with PG), frozen in liquid nitrogen and stored at −80 °C.

Frozen samples were pulverized in a sterile mortar using liquid nitrogen, and the total RNAs were extracted from ground tissues using Nucleospin® RNA plant kit (Macherey–Nagel, Duren, Germany) according to manufacturer instructions, and eluted in 60 µL of RNase-free water. The concentration and the integrity of RNA samples were assessed using Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), respectively. The high-quality RNA samples were sent in dry ice to Novogene company (Sequencing center, Cambridge, UK) for libraries construction and paired-ends sequencing using NovaSeq 6000 s Sequencing System (Illumina, San Diego, CA, USA).

2.4. RNA-Seq Data Handling and Differential Gene Expression Analysis

After assessing the RNA-Seq raw reads quality with FastQC v0.11.9 [22], Trimmomatic v0.36 [23] was used to trim the adapters sequences and remove the low-quality reads. The filtered reads were mapped to tomato reference assembly version SL4.0 [24,25] using HiSat2 v2.2.1 [26]. Reads counts were generated from alignment files using featureCounts v1.6.0 software [27] with default parameters, basing on 'exon' feature and 'transcript_id' meta-feature of ITAG4.0 tomato annotation file retrieved from Sol Genomics website (https://solgenomics.net/, accessed on 30 April 2020).

Differential expression (DE) analyses were carried out using Bioconductor EdgeR v3.28.1 [28]. EdgeR was used to filter out the not expressed or poorly expressed transcripts (a transcript was considered 'active' if reads per million mapping to that transcript was >1

in at least two libraries), normalize the RNA libraries, and do the differential expression analysis with the likelihood ratio test. EdgeR, whose computing approach fits a negative binomial generalized linear model (GLM) to the read counts for each transcript, was used to calculate the Log_2 Fold Change (LFC) of each transcript expression between treated (both AB and PG) and control samples (untreated seedlings). The transcripts with resulting false discovery rate (FDR) lower than 0.05 and LFC lower than −1 or higher than 1 were considered as differentially expressed.

2.5. GO Annotation and Enrichment

GO annotation of the whole SL4.0/ITAG4.0 proteome was obtained through InterProScan mapping using Blast2GO v5.2.5 software [29]. GO enrichment analyses were conducted with GOseq Bioconductor package v1.38.0 [30]. GOseq software is designed to bias the RNA-Seq data by transcripts length, so the median length of the transcripts was calculated with GenomicFeatures Bioconductor package v1.38.2 [31] using the ITAG4.0 annotation file as input. The GO terms with a *p*-value lower than 0.05 were considered as enriched.

2.6. Quantitative RT-Real Time PCR Validation

The differential expression analysis results were validated by carrying out a quantitative reverse transcription PCR (RT-qPCR) of a selected gene set. Nine genes coding for proteins involved in defense, response to abiotic stress, water transport, hormone signaling, or signal transduction were chosen among differentially expressed genes (DEGs) for RNA-Seq data validation.

RNA samples (500 ng per sample), extracted as described in the previous paragraph, were reverse-transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen™, Carlsbad, California, USA) following the instruction manual provided by manufacturer, and the resulting cDNAs were diluted 10 times in nuclease-free distilled water. Oligo Explorer v1.1.2 tool was used to design primers for each gene. Then the primers sequence specificity (no multiple alignments) was verified using the BLAST function against tomato genome v2.4 at Sol Genomics Network website (https://solgenomics.net/, accessed on 23 September 2020). The primers IDs and sequences are listed in Supplementary Table S1.

Quantitative reverse transcription PCR assays were performed using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The elongation factor 1-alpha, commonly used as a housekeeping gene in tomato was used [17,32]. Reactions were carried out in 20 µL containing 10 µL SYBR Green PCR, 2× GoTaq qPCR Master Mix and 100× Reference Dye (Promega Italia S.r.l.), 0.3 µL of each primer, 2 µL of cDNA (1 ng μL^{-1}) and 7.4 µL of water, and performed in triplicate following this cycling protocol: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A melting curve analysis was carried out for each run. The comparative threshold cycle method $2^{-\Delta\Delta Ct}$ [33] was used to analyze the data, and the fold change was calculated as the $\log_2 (2^{-\Delta\Delta Ct})$.

3. Results

3.1. Colonization by Culturable Aerobic Rhizobacteria

Data showed a significantly higher rhizosphere soil colonization in both the treatments ($2.6 \pm 0.4 \times 10^7$ and $2.5 \pm 0.6 \times 10^7$ CFU g^{-1} rhizosphere soil for PG and AB, respectively) in comparison with untreated control ($1.2 \pm 0.2\ 10^7$ CFU g^{-1} rhizosphere soil) at 6 days after inoculation. No differences were found between the two treatments.

3.2. RNA Sequencing and Differential Expression Analyses

Total RNAs were extracted from leaves and sequenced with Illumina NovaSeq 6000 s sequencing system. Overall, 221,891,789 paired 150 bp-long reads were generated, ranging from 23 to 26 million across the nine RNA samples (Table 1). After assessing their quality, RNA-Seq raw reads have been deposited in the ArrayExpress database at EMBL-EBI

(www.ebi.ac.uk/arrayexpress (accessed on 10 January 2022)) under accession number E-MTAB-11398.

Table 1. RNA-Seq data elaboration statistics. For each RNA library the sample properties as well as the number and percentage of paired-end reads survived to trimming and filtering and aligning to tomato SL4.0 reference genome are reported ID, the treatment.

Sample Properties				Trimming and Filtering		Aligning to Genome	
Sample ID	**Treatment [1]**	**Replicate**	**Paired-End Reads**	**No Pairs Survived**	**% Pairs Survived**	**No Aligned Pairs**	**% Aligned Pairs**
T25l_CON	Ctrl	1	26,783,825	25,548,670	95.39%	24,277,920	95.03%
T26l_CON	Ctrl	2	24,651,592	23,369,549	94.80%	21,413,685	91.63%
T27l_CON	Ctrl	3	23,637,298	22,874,761	96.77%	20,835,340	91.08%
T22l_ABA	AB	1	22,012,255	21,065,854	95.70%	19,617,111	93.12%
T23l_ABB	AB	2	27,225,211	26,424,621	97.06%	24,341,237	92.12%
T24l_ABC	AB	3	23,976,755	22,934,673	95.65%	21,820,168	95.14%
T19l_PGA	PG	1	23,263,479	22,361,982	96.12%	21,024,789	94.02%
T20l_PGB	PG	2	26,445,081	25,487,216	96.38%	24,121,624	94.64%
T21l_PGC	PG	3	23,896,293	23,217,583	97.16%	22,004,594	94.78%

[1]. Ctrl: non-inoculated control plants, AB: plants treated with *A. baldaniorum*, PG: plants treated with *P. graminis*.

Adapters sequences and low-quality nucleotides were filtered out, resulting in 95–97% of 'surviving' reads (Table 1). For each RNA library, 91–95% of filtered reads were aligned to the tomato SL4.0 reference assembly, and the number of reads mapping to each predicted transcript was estimated according to ITAG4.0 annotation.

Normalization factors were calculated according to library sizes, and normalized reads counts were used as input data for the differential expression analyses of two comparisons: plants treated with *Azospirillum baldaniorum* (AB) vs. non-inoculated control plants, and plants treated with *Paraburkholderia graminis* (PG) vs. non-inoculated control plants. Detailed results of DE analyses were reported in Supplementary Table S2 and graphically represented in Supplementary Figure S1. According to the above-mentioned thresholds (FDR < 0.05; $|LFC| > 1$), the DE transcripts for the two analyses carried out for this study were reported in Supplemental Table S3, while the resulting numbers of up- and down-regulated transcripts were summarized in Table 2. PG treatment led to a higher number of DE transcripts than AB, moreover more transcripts were up- than down-regulated in both treatments.

Table 2. Summary of the differential expression analysis results.

DE Analysis [1]	Down-Regulated Transcripts	Up-Regulated Transcripts	Total No DE Transcripts
AB vs. Ctrl	184	243	427
PG vs. Ctrl	123	389	512

[1]. Ctrl: non-inoculated control plants, AB: plants treated with *A. baldaniorum*, PG: plants treated with *P. graminis*.

A total of 47 up- and 24 down-regulated transcripts were shared among treatments, while no DE transcripts were found to have opposite modulation after AB and PG treatments (Figure 1).

3.3. Functional Annotation and GO-Enrichment Analyses

Functional annotation and identification of over-represented Gene Ontology (GO) categories among DE transcripts allowed us to identify the GO terms affected by *A. baldaniorum* (AB) and *P. graminis* (PG) treatments in leaves of grafted tomato. In total, 65 and 60 GO terms were identified as significantly enriched for AB and PG treatments, respectively (Table 3). The complete list of enriched GO terms is reported in Supplementary Table S4.

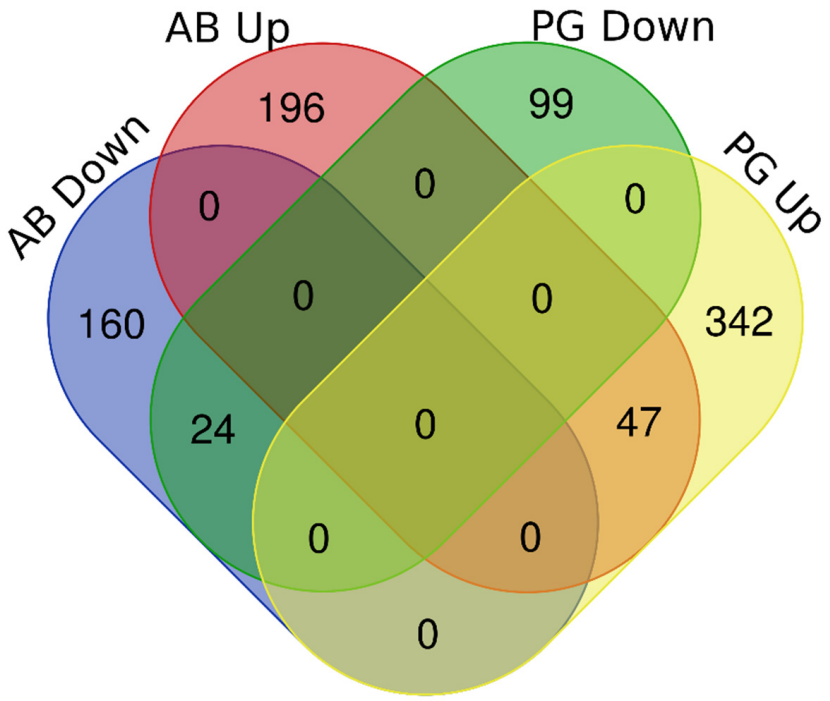

Figure 1. Venn diagram of up- and down-regulated transcripts in AB (*A. baldaniorum* Sp245) and PG (*P. graminis* C4D1M) treatments.

Table 3. Number of enriched GO terms in AB and PG treatments for Molecular Function (MF), Cellular Component (CC) and Biological Process (BP) categories.

Treatment [1]	Molecular FUNCTION	Cellular Component	Biological Process	Total No GO Terms
AB	33	7	25	65
PG	24	6	30	60

[1]. AB: plants treated with *A. baldaniorum* Sp245, PG: plants treated with *P. graminis* C4D1M.

Common (15 GO), AB-specific (50 GO) and PG-specific (45 GO) enriched terms were identified, highlighting a substantially lower number of terms associated to both responses than those specific for either AB or PG. As summarized in Figures 2 and 3, eight enriched biological process-related GO terms were shared among the two treatments (GO:0019464 'glycine decarboxylation via glycine cleavage system'; GO:0009156 'ribonucleoside monophosphate biosynthetic process'; GO:0050482 'arachidonic acid secretion'; GO:0006355 'regulation of transcription, DNA-templated'; GO:0046274 'lignin catabolic process'; GO:0009765 'photosynthesis, light harvesting'; GO:0006529 'asparagine biosynthetic process'; GO:0016567 protein ubiquitination). Moreover, apart from GO:0006355 'regulation of transcription, DNA-templated' in which 26 and 20 DEGs were identified for PG and AB, respectively, all the other terms were represented by single or few DEGs in both treatments. GO:0006355 term included several transcription factor genes. BHLH, MYB and WRKY were the most represented transcription factor families found for AB treatment. On the other hand, in PG treatments, BHLH and MYB, ERF and GRASS were observed to be the most represented gene families (Supplementary Table S3). Another group of highly enriched GO terms in both treatments were those related to photosynthesis such as GO:0009765 'photosynthesis, light harvesting', GO:0015979 'photosynthesis', and GO:0009773 'photosynthetic electron transport in photosystem'.

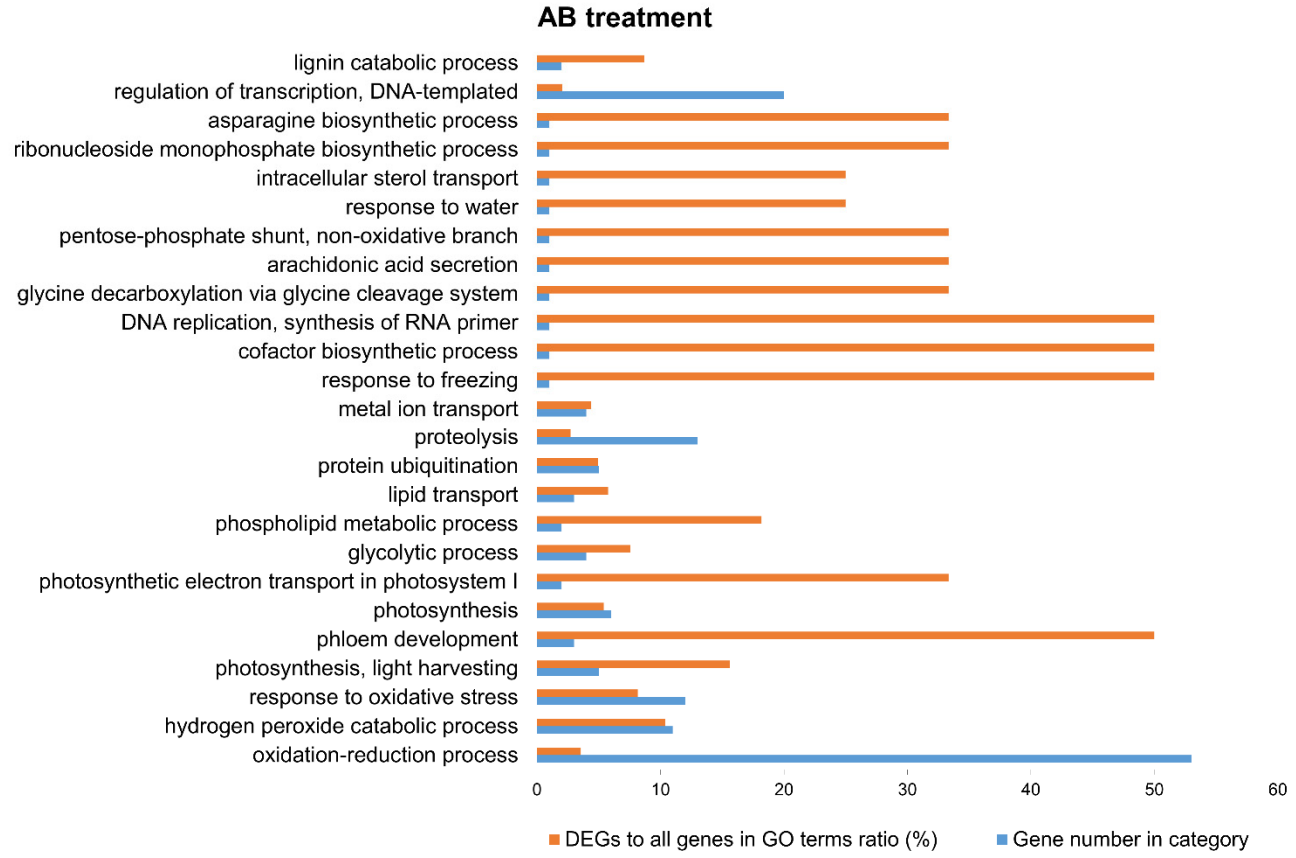

Figure 2. GO enrichment for Biological Process in *A. baldaniorum* Sp245 treatment.

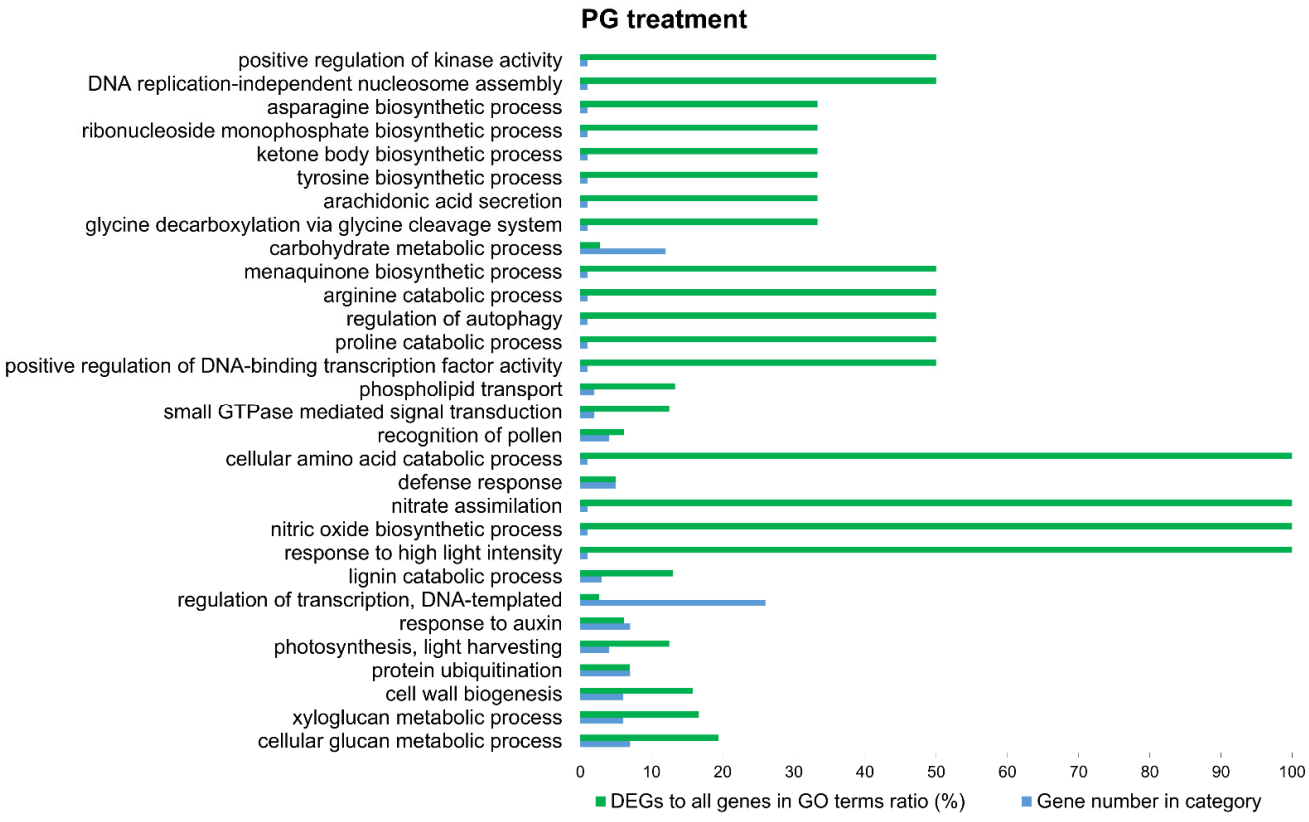

Figure 3. GO enrichment for Biological Process in *P. graminis* C4D1M treatment.

GO terms related to Reactive Oxygen Species (ROS) homeostasis of the cell, such as GO:0055114 'oxidation-reduction process', GO:0042744 'hydrogen peroxide catabolic process' and GO:000697 'response to oxidative stress' were among the most highly enriched categories in the AB treatment. These GO categories included several DEGs coding for ROS-scavenging enzymes, such as peroxidase and polyphenol oxidase (Supplementary File 3). Also GO terms related to response to abiotic stress factors such as water stress were positively modulated in AB treatment. On the other hand, in case of PG treatment, terms related to cell wall biosynthesis and modifications, especially those that occur during fruit ripening and organ abscission (GO:0006073 'cellular glucan metabolic process', GO:0010411 'xyloglucan metabolic process' and GO:0042546 'cell wall biogenesis' and GO:0046274 'lignin catabolic process') were the most highly enriched. GO categories related to response to abiotic and biotic factors, GO:0009733 'response to auxin' and GO:0006952 'defense response', were enriched after PG treatment as well.

DEGs were also used for a functional analysis using MapMan v3.6.0RC1 [34], results of which showed that, while genes related to signaling were slightly modulated (both down and up) after AB treatment, genes related to calcium signaling and receptor like kinase were induced after PG treatment (Supplementary Table S5). A similar pattern was observed for hormone-related genes: while five genes related to auxin and ethylene signaling were modulated in AB, several genes–in particular those involved in ethylene signaling pathways–resulted strongly induced in PG. An induction of genes coding for PR proteins was observed after AB treatment, that could suggest involvement of salicylic acid pathways in tomato seedlings molecular response. Genes involved in the regulation of transcription, protein degradation and transport resulted induced in both the treatments, even if the number of involved genes was often higher in PG. Finally, genes involved in essential functions of the plants such as nutrition and water transport were modulated in both the treatments (Supplementary Table S4).

3.4. Validation of Nine DEGs Using qRT-PCR

A RT-qPCR analysis of nine selected genes was carried out to validate the RNA-Seq data. The expression levels of nine genes are reported in Table 4. The comparison between RNA-Seq and RT-qPCR fold change results showed a similar trend for all the assessed genes, validating the RNA-Seq analysis.

Table 4. Validation of RNA-seq results by qRT-PCR.

Gene ID [1]	Chr [1]	Description [1]	AB		PG	
			RNA-Seq	RT q-PCR	RNA -Seq	RT q-PCR
Solyc10g076240.3.1	10	*Cationic peroxidase 1-like*	6.06	3.55	−0.33	−0.22
Solyc02g078650.4.1	2	*Polyphenol oxidase, chloroplastic-like*	5.33	2.78	1.16	0.74
Solyc01g106605.1.1	1	*Basic form of pathogenesis-related protein 1-like*	8.24	2.11		0.15
Solyc02g062390.3.1	2	*Dehydrin DHN2*	5.05	4.27	2.47	1.65
Solyc06g075650.3.1	6	*Aquaporin TIP1-3-like*	3.18	2.48	2.29	1.52
Solyc03g026280.3.1	3	*AP2 domain CBF protein*	0.55	−0.58	4.41	3.13
Solyc07g056000.2.1	7	*Xyloglucan Endotransglucosylase/hydrolase 2*	2.19	1.81	2.61	2
Solyc05g052040.1.1	5	*Ethylene-responsive transcription factor 5*	0.43	0.03	5.56	1.44
Solyc12g009240.1.1	12	*Ethylene-responsive transcription factor ERF017*	1.95	−0.22	4.72	2.07

[1]. Gene name, chromosome number and functional description derive from the reference genome sequence 'tomato SL 4.0'. AB: *A. baldaniorum* Sp245; PG: *P. graminis.*

4. Discussion

Plant biostimulants, such as PGPR (plant growth promoting rhizobacteria), are important tools of an integrated crop management system, that may help agriculture become more sustainable and resilient. The mechanisms of their beneficial effect may include enhanced nutrient uptake, improvement of biotic/abiotic stress resilience by activation of growth regulators and stress-responsive hormones, oxidative stress reduction and induction of plant defense mechanisms (for a review see e.g., [35]).

In a previous study in greenhouse and open field experiments using grafted tomato plants and different biostimulants, the treatment with *A. baldaniorum* induced flowering stage and increased the number of flowers in greenhouse experiment, while for both AB and PG treatments increased marketable and total yields with higher fruit dry weight, leaf dry weight, and plant total dry weight were observed in open field [20].

In order to understand the molecular mechanisms underlying these interactions, the aim of this study was to profile, for the first time, to the author knowledge, the transcriptomic changes induced in leaves of grafted tomato seedlings root-inoculated with beneficial microorganisms *P. graminis* C4D1M and *A. baldaniorum* Sp245. Interactions between root beneficial microorganisms and plants can improve crop performances inducing molecular and physiologic changes also in organs distant from the point of inoculation such as shoots, leaves and fruits [19,36,37]. Leaves were chosen for this experiment as they play an important role in growth and development of crops, are involved in many processes such as photosynthesis and response to drought stress and represent the first barrier of defense that foliar pathogens meet, during infection of plants. In the present study, grafted tomato was used as a host, since currently, the commercial tomato grafting is widely adopted in the main cropping areas. Data on interactions between *A. baldaniorum* Sp245 or *P. graminis* C4D1M and not grafted plants would not be informative for interactions between these beneficial microorganisms and widely used grafted tomato plants since a recent study has revealed that grafting modifies tomato transcriptome [38] and studies on maize and rice demonstrated that the effects of *Azospirillum* spp. inoculations differed on the strain/cultivar combinations [39,40]. Moreover, RNA transcriptomic profiling studies available in literature focused mainly on effects of microorganisms assessed under a biotic [41,42] or under an abiotic stress factor [43]. Conversely, our strategy was to compare the influence of two beneficial microorganisms on tomato seedlings grown in optimal conditions that may allow to better understand the molecular mechanisms influenced by AB and PG treatments regardless of whether the plants are under stress or not.

The number of DEGs detected after PG and AB treatments was 512 and 427, respectively, and is consistent with those obtained in other studies evaluating the effects of microorganism-plant interactions on gene expression in leaves [36,44].

Only few DEGs were modulated in both treatments, suggesting that the interactions between tomato and microorganisms are species-specific and affect the activity of specific sets of genes, that however, activate and/or involve similar pathways and mechanisms. As it could be expected for PGRP-plant interaction, the shared DEGs included genes involved in nutrients metabolism or activated in response to nutrient starvation, such as Solyc06g062540.3.1, encoding inorganic pyrophosphatase 1-like, and Solyc06g007180.31 encoding asparagine synthetase [glutamine-hydrolyzing]. Asparagine synthetase is responsible for the biosynthesis of asparagine, an amino acid used for protein production and nitrogen assimilation, and a key molecule involved in recycling, transport, and storage of nitrogen in all plant organs [45]. An up-regulation of some inorganic pyrophosphatase genes in both roots and leaves of chickpea plants under nutritional stress has been recently reported [46].

Functional annotation of the DEGs revealed that they coded mainly for proteins involved in water transport, regulation of transcription and hormones synthesis and signaling pathways and those activated in response to oxidative or biotic and abiotic stresses.

In the field experiment of our previous study [20], all the investigated treatments reduced the number of fruits affected by blossom-end rot (BER), a physiological disorder that causes important economic losses. Although BER is mainly associated to the soil concentration of calcium available, it may be influenced also by reduced nutrient and water uptake [20,47]. In the present study, the transcription profiling enabled identification of three differentially expressed aquaporins: Solyc06g075650.3.1 in both treatments, and Solyc06g060760.3.1 and Solyc06g011350.3.1 only in AB treatment. Aquaporins, known as water channel proteins, help plants in the transport of water and other solutes such as glycerol and urea. Aquaporins also regulate the opening and closure of stomata; crucial pro-

cesses for the temperature regulation of leaves and the evaporation of water [48]. Therefore, the induction of aquaporin-coding genes can improve plant performance, especially under drought stress [49]. Other proteins important for drought stress response are dehydrins, in this work Solyc02g062390.3.1 gene that codes for Dehydrin *DHN2* was observed to be induced in both treatments. Dehydrins with a hydrophilic nature can improve hydration and reduce water loss in plants [50]. A study on pepper plants inoculated with *Bacillus licheniformis* K11 showed an induced expression of dehydrin-like protein gene and a higher number of survived plants, compared with the not inoculated control under drought stress [51].

Many studies documented that PGPR can trigger a wide variety of defense mechanisms in plants (e.g., oxidative burst, production of antimicrobial compounds and expression of defense-related genes) [52,53]. In this study, particularly AB treatment modulated the expression of many genes involved in responses to oxidative stress and defense response such as ascorbate oxidase (AO), peroxidase, polyphenol oxidase (PPO), PRs proteins, etc. Oxidative stress occurs when the balance between reactive oxygen species (ROS) production and degradation is broken, leading to an increase of ROS concentration that damages nucleic acids, proteins, and lipids [54]. To respond to the harmful effects of ROS, plants have developed systems involving enzymes such as superoxide dismutase, catalase, ascorbate oxidase, the peroxidases, etc. [55]. AO catalyzes the oxidation of ascorbic acid to monodehydroascorbate, influencing the content of ascorbate and oxygen, and affecting the redox state. Furthermore, this enzyme has a role in the perception of environmental factors and stress responses [56]. In addition, AO was also proposed as relevant in the establishment of mutualistic plant-microbe interactions as its induction was during nodulation in *Lotus japonicus* and during the colonization by an arbuscular mycorrhizal (AM) fungus [57].

Studies on transgenic tomato plants reported that peroxidase (POX) and polyphenol oxidase (PPO) are induced by wounding and pathogen attacks [58,59]. Moreover, induced defense responses of PGPR-treated tomato plants to *Alternaria solani* were shown to be associated with enhanced POX and PPO biosynthesis [60]. Since in the present study, a significant activation of POX and PPO enzymes was observed in plants treated with PGPR without any parallel pathogen infection, this may suggest that these enzymes could be involved also in a sort of defense priming.

Phytophtora infestans (Mont.) de Bary, oomycete causing the disease known as 'Late blight', is one of the main devasting pathogen of potato and tomato. Since only few plant protection products are authorized in Europe against this pathogen for both potato and tomato crops, the control of late blight is a challenge [61] especially in organic farming, where only copper compounds can be applied. Interestingly, the PG inoculation induced the expression of five genes (Solyc05g007630.3.1, Solyc07g049700.1.1, Solyc05g013260.3.1, Solyc09g098100.4.1, Solyc05g005130.3.1) coding for putative late blight resistance protein homolog, while Solyc10g008700.1 coding for MYB49 transcription factor, whose expression was reported to correlate with an increase of resistance of tomato plants to *Phytophtora infestans* [62], was induced by AB treatment. These results may suggest that treatment with beneficial microorganisms AB and PG might induce responses that share mechanisms with those involved in resistance response to *P. infestans*. Furthermore, in a previous study [20], in which the field high moisture conditions allowed the spread of the oomycete *P. infestans* at harvest time, better results were obtained for PGPR treated than for control plants for both morphological parameters and fruit quality traits, suggesting the treatment contrasted the effects of infection.

Therefore, it would be very interesting to investigate in further field and greenhouse trials the use of these beneficial bacteria as an alternative, or to reduce the use of copper compounds against *P. infestans* in organic farming for development of sustainable tomato management with low external inputs.

The expression of many transcription factor genes belonging to MYB and WRKY families, known to be modulated in response to different abiotic and biotic stresses [63,64], was modulated by AB and PG treatments in the present study as well. The unique nomen-

clature for tomato MYB and WRKY transcription factors family adopted and reviewed by Zhao et al. [65] and Huang et al. [63], respectively, was followed. AB treatment induced SlMYB71 (Solyc05g053150.2.1) together with SlMYB49 (Solyc10g008700.3.1) (already mentioned above), known to be also up-regulated along with the fruit development [65], SlMYB41 (Solyc07g054840.4.1) that was reported to affects root architecture and improve tolerance to salinity in tomato plants [66], and SlMYB63 (Solyc10g005550.3.1), a root-specific transcription factor functioning as a node of convergence in the induced systemic resistance and Fe starvation signaling pathways [67]. Among WRKY transcription factors that were reported in literature to show significant induction under stresses of drought, salt and invasion of pathogen implying that these family members might be putative regulators in response to various biotic and abiotic stresses [63], we found SlWRKY43 (Solyc12g042590.2.1) and SlWRKY73 (Solyc03g113120.4.1) induced by AB treatments, SlWRKY41 (Solyc01g095630.3.1) and SlWRKY46 (Solyc08g067340.4.1) upregulated by PG, and SlWRKY6 (Solyc02g080890.3.1) induced by both treatments.

Many signaling molecules are involved in the cross-talks between crops and soil microorganisms. Phytohormones, such as auxins, gibberellins, ethylene, etc., are considered as the main signal molecules in plants [68]. In this study, genes involved in auxins, gibberellins, ethylene, and abscisic acid metabolism and signaling were found to be induced in at least one of the two treatments. Auxins and gibberellins are involved in many aspects of plant growth and development. Mariotti et al. [10] associated the positive modulation of indoleacetic acids (IAA), auxin responsive molecules, and gibberellins with the fruit-set and early fruit growth in tomato. Recently, auxin responsive GH3.1 was proposed as a major player in balancing the auxin synthesis and metabolism, ensuring fruit set in parthenocarpy tomato in any conditions [69]. In the present study, Solyc01g107390 coding for GH3.1 was induced by AB treatment; it is worth noting that in the field experiment [20], higher number of fruits was reported for AB than for PG treatment, the latter anyway being higher than the control not inoculated.

Flower development, fruit ripening, organ senescence, abscission and responses to abiotic and biotic stresses are also modulated by ethylene [70,71]. Ethylene signaling and response, in turn, are regulated by Ethylene Response Factors (ERFs) [72]. The unique nomenclature for tomato ERF transcription factors family, adopted and reviewed by Liu et al. [70], was followed for the genes identified in the present work. Numerous ERFs related to fruit ripening were modulated by PG treatments: SlERF.B4 (Solyc03g093540.1.1), SlERF.B5 (Solyc03g093550.1.1) and SlERF.D4 (Solyc10g050970.1.1) with preferential expression in young unripe fruits that declines at the onset of ripening and, SlERF.B1(Solyc05g052040.1.1) and SlERF.B13 (Solyc08g078190.2.1), known to be upregulated during ripening (Liu et al. 2016). Two other ERFs, SlERF.B2 (Solyc03g093560.1.1) and SlERF.F1 (Solyc10g006130.1.1), reported to be involved in salt and drought tolerance [73] and photosynthesis and growth regulation [74], respectively, were induced by PG treatment as well. AB induced expression of a gene coding for SlERF.C4 (Solyc09g089930) reported in literature to be involved in pathogen resistance and exhibiting high expression in roots, leaves, flowers, and immature fruits [75].

5. Conclusions

Sequencing of RNA extracted from leaves of grafted tomato seedlings, inoculated with two beneficial PGPR and grown under optimal conditions, helped to decipher the mechanisms underlying the plant-beneficial microorganisms' interactions in organ distant from roots. Functional analysis of DEGs enabled identification of common mechanisms modulated by the two treatments that, however, shared only few genes. The common pathways activated by PGPR in the present study involved genes coding for proteins related to water and nutrients uptake, defense responses to biotic and abiotic stresses and hormonal regulation of fruit set and ripening. On the other hand, while AB induced genes coding for different MYB transcription factors known to be involved in response to biotic and abiotic stresses, PG upregulated 5 genes coding for putative late blight resistance

protein homolog. Auxin responsive molecules and gibberellins involved in the fruit-set and early fruit growth in tomato were mainly induced by AB correlating to higher fruit number obtained in a previous field study, while ERF transcription factors involved in ripening were induced mainly by PG treatment. All these results suggest that, as good performance was observed previously in field for plants inoculated with those PGPR applied individually and as consortium, and as the mechanisms they activate seem at least partially complementary, it would be interesting to study their synergic effect on tomato cultivation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102537/s1: Supplementary Figure S1. Scatter plots of mean log2 counts-per-million vs. log2 fold change plots. Transcriptional changes for AB and PG treatments vs. non treated control are represented. AB and PG treatments significant DEGs (FDR < 0.05) are indicated in blue and red, respectively. Supplementary Table S1. Primers (IDs and sequences) used for quantitative reverse transcription PCR assays. Supplementary Table S2. Detailed results of differential expression analysis in two comparisons: plants treated with *Azospirillum baldaniorum* vs. non-inoculated control plants (sheet 'DEA_results_AB'), and plants treated with vs. non-inoculated control plants (sheet 'DEA_results_BG'); Supplementary Table S3. EdgeR differential expression analysis results for all the active transcripts. Supplementary Table S4. GO-enrichment analysis results for differentially expressed transcripts after AB and PG treatment. For each GO term, over- and under-represented *p*-value, number of DEGs, total genes, ontology, and term description were reported. Supplementary Table S5. Results of MapMan 'Biotic stress' response for differentially expressed transcripts after AB and PG treatment.

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Data Availability Statement: The raw reads of the RNA-Seq data presented in this study have been deposited in the ArrayExpress database and are openly available from EMBL-EBI (www.ebi.ac.uk/arrayexpress, accessed on 24 January 2022) under accession number E-MTAB-11398.

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