in Plant Science

frontiers



ORIGINAL RESEARCH published: 05 July 2017 doi: 10.3389/fpls.2017.01159



Transcriptome-Wide Identification of Differentially Expressed Genes in Solanum lycopersicon L. in Response to an Alfalfa-Protein Hydrolysate Using Microarrays

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OPEN ACCESS

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Specialty section:

This article was submitted to Plant Nutrition, a section of the journal Frontiers in Plant Science

Received: 12 February 2017 Accepted: 16 June 2017 Published: 05 July 2017

Citation:

Ertani A, Schiavon M and Nardi S (2017) Transcriptome-Wide Identification of Differentially Expressed Genes in Solanum lycopersicon L. in Response to an Alfalfa-Protein Hydrolysate Using Microarrays. Front. Plant Sci. 8:1159. doi: 10.3389/fpls.2017.01159 An alfalfa-based protein hydrolysate (EM) has been tested in tomato (Solanum *lycopersicon* L.) plants at two different concentrations (0.1 and 1 mL L^{-1}) to get insight on its efficacy as biostimulant in this species and to unravel possible metabolic targets and molecular mechanisms that may shed light on its mode of action. EM was efficient in promoting the fresh biomass and content in chlorophyll and soluble sugars of tomato plants, especially when it was applied at the concentration of 1 mL L^{-1} . This effect on plant productivity was likely related to the EM-dependent up-regulation of genes identified via microarray and involved in primary carbon and nitrogen metabolism, photosynthesis, nutrient uptake and developmental processes. EM also up-regulated a number of genes implied in the secondary metabolism that leads to the synthesis of compounds (phenols and terpenes) functioning in plant development and interaction with the environment. Concomitantly, phenol content was enhanced in EM-treated plants. Several new genes have been identified in tomato as potential targets of EM action, like those involved in detoxification processes from reactive oxygen species and xenobiotic (particularly glutathione/ascorbate cycle-related and ABC transporters), and defense against abiotic and biotic stress. The model hypothesized is that elicitors present in the EM formulation like auxins, phenolics, and amino acids, may trigger a signal transduction pathway via modulation of the intracellular levels of the hormones ethylene, jasmonic acid and abscissic acid, which then further prompt the activation of a cascade events requiring the presence and activity of many kinases and transcription factors to activate stress-related genes. The genes identified suggest these kinases and transcription factors as players involved in a complex crosstalk between biotic and abiotic stress signaling pathways. We conclude that EM acts as a biostimulant in tomato due to its capacity to stimulate plant productivity and up-regulate stress-related responses. Its use in agricultural practices may reduce the need of inorganic fertilizers and pesticides, thereby reducing the environmental impact of productive agriculture.

Keywords: biostimulant, productivity, microarray, hormones, phenols, sugars, defense, signaling

INTRODUCTION

For a long time, the application of synthetic fertilizers and pesticides has been a common practice to boost crop yield (Calvo et al., 2014; Nardi et al., 2016; Posmyk and Szafrańska, 2016). The main purposes of using these chemical additives in agriculture are the improvement of nutrient supply in soil, crop protection and disease control. Unfortunately, these practices are often responsible for chemical and biological degradation of soil, as a result of undesirable levels of accumulated chemicals (Francioso et al., 2000).

In the last years, there has been a rise in attention brought to the area of agricultural biostimulants (Sharma et al., 2014; Brown and Saa, 2015; Posmyk and Szafrańska, 2016). According to the European Biostimulants Industry Council (European Biostimulants Industry Council [EBIC], 2012a), they are defined as products containing substances and/or microorganisms whose function when applied to plants or the rhizosphere in little amounts is to stimulate natural processes, to enhance/benefit nutrient uptake and use efficiency, tolerance to abiotic stress, and crop quality.

Biostimulants pose an innovative solution to an increased world demand for high crop productivity with less unsustainable inputs (Nardi et al., 2016). They do not fall within the regulatory framework of pesticides (European Biostimulants Industry Council [EBIC], 2012a) and operate in plants through different mechanisms than fertilizers, regardless of the presence of nutrients in the products (European Biostimulants Industry Council [EBIC], 2012b). In this respect, the Biostimulant Coalition (2013) in North America specifies that these products are not nutrients.

Biostimulants are manufactured starting from different sources and include complex mixtures of active substances. Therefore, the assignment of specific functions in plants to their individual components is often arduous. du Jardin (2015) divides biostimulants into a few main categories: humic substances (HS), seaweed extracts, protein hydrolizates and microbial inoculants (mycorrhizal fungi and rhizobacteria). Among them, HS and seaweed extracts are the most extensively studied.

HS include humic and fulvic acids, and consist of relatively small molecules of amphiphilic nature, which can form molecular aggregates or supramolecular associations in solution and on mineral surfaces (Dell'Agnola and Nardi, 1987; Wershaw, 1999; Schaumann, 2006). The effects of HS in plants depend on their concentration, molecular weight, chemical-physical properties, as well as on their hormone-like activity (Dell'Agnola and Nardi, 1987; Nardi et al., 1994; Mora et al., 2012; Ertani et al., 2013b; Baglieri et al., 2014; Massa et al., 2016) and capacity to stimulate plant nitrogen (N) assimilation (Quaggiotti et al., 2004). HS generally stimulate root growth, especially in the early stages of plant development (Canellas et al., 2002; Zandonadi et al., 2007; Vaccaro et al., 2009; Mora et al., 2010). They also control nutrient availability via enhancement of root ATPase activity (Maggioni et al., 1987; Nardi et al., 1991; Zandonadi et al., 2010), carbon (C) and oxygen (O_2) exchange between soil and atmosphere, and can accelerate development cycles (Eyheraguibel et al., 2008). A cDNA-AFLP-based transcriptome study indicated that a broad number of genes involved in developmental and metabolic processes, transcription regulation or RNA metabolism are HS-regulated (Trevisan et al., 2011), while a more recent microarray study revealed that molecular targets of HS in plants are genes related to N, C, and sulfur (S) metabolisms (Jannin et al., 2012).

The other wide category of biostimulants encompasses a variety of seaweed extracts, which are employed by virtue of their high content in bioactive compounds. Phytohormones, such as auxins or cytokinins contained in these extracts, are likely responsible for their action in accelerating and improving plant development (Crouch and Van Staden, 1992; Sivasankari et al., 2006; Khan et al., 2009; Roussos et al., 2009). Effects of seaweeds in plant metabolism include enhancement of chlorophyll content (Mancuso et al., 2006; Sivasankari et al., 2006; Spinelli et al., 2010), earlier germination, flowering and fructification (Sivasankari et al., 2006; Roussos et al., 2009), and higher proliferation of secondary roots (Mugnai et al., 2008; Rayorath et al., 2008; Spinelli et al., 2010). Seaweed-based biostimulants can also induce immunity/resistance to pathogens in plants (Joubert and Lefranc, 2008).

Recently, a microarray based-transcriptomic study has evidenced the activation of both nitrogen and sulfur assimilation pathways in *Brassica napus* plants treated with an *Ascophyllum nodosum* seaweed extract (Jannin et al., 2013). Later, the application of this extract to *B. napus* was found to up-regulate the expression of genes coding for specific transporters, like nitrate (NRT1.1 and NRT2.1) sulfate (SULTR1.1 and SULTR1.2), copper (COPT2) and iron transporters (IRT1), or more generic ones (such as NRAMP3), causing the increase in mineral concentrations in the plant (Billard et al., 2014).

Protein hydrolysates represent another notorious category of biostimulants, which consists of amino acids and peptide mixtures. These components are manufactured through chemical and/or enzymatic protein hydrolysis using agroindustrial by-products, from both plant sources (crop residues) and animal wastes (i.e., collagen, epithelial tissues) (du Jardin, 2015). Biostimulants properties of protein hydrolysates are mainly ascribable to their content in low molecular fraction (Quartieri et al., 2002) and free amino acids (Cavani and Ciavatta, 2007).

Previous studies showed that a fabaceae (*alfalfa*) plant derived-protein hydrolysate stimulated maize plant growth by fostering the activity and gene expression of several enzymes involved in nitrogen (N) assimilation and carbon (C) metabolism (Schiavon et al., 2008; Ertani et al., 2009, 2013a), while phenol-containing protein hydrolysates enhanced phenylpropanoid metabolism in the same species (Ertani et al., 2011b). Recently, a metabolic approach highlighted the role of protein hydrolysates in increasing the activity of the light reactions components and Calvin cycle enzymes, and in the promotion of antioxidant accumulation (Ertani et al., 2014).

Despite these described effects of protein hydrolysates in plants, many other still remain to unravel as compared to other classes of biostimulants, especially with respect to the molecular mechanisms triggered by them in aiding plants to overcome stressful conditions. Only recently, a transcriptome study performed in maize plants showed that targets of protein hydrolysates are genes related to cell wall organization, transport processes, stress responses and hormone metabolism (Santi et al., 2017).

Therefore, there is a clear need to enrich our understanding of protein hydrolysate function and mechanism of action in crops in order to develop more efficient materials to apply to plants growing under unfavorable or suboptimal conditions and optimize the industrial processes employed for their manufacturing.

Because transcriptome approaches would be functional to determine the metabolic targets of protein hydrolysates in plants and highlight the signaling pathways involved in the responses of crops to biotic and abiotic stresses, cDNA microarray has been used in this study as a quantitative method for global and simultaneous analysis of plant gene expression to gain knowledge about the expression profiles of genes involved in the responses of the crop tomato (*Lycopersicon esculentum*, cv Microtom) to an *alfalfa*-derived protein hydrolysate.

MATERIALS AND METHODS

Chemical and Spectroscopic Characterization of the Biostimulant

The biostimulant used in the current study, EM, was manufactured by ILSA S.p.A. (Arzignano, VI, Italy) and produced through a fully controlled enzymatic hydrolysis using *alfalfa* (*Medicago sativa* L.) plants. The chemical, physical and spectroscopic characterization of this alfalfa hydrolysate has been previously described by Schiavon et al. (2008) and Ertani et al. (2014).

Experimental Design and Plant Growth

To estimate the effects of EM on tomato plants, a hydroponic experiment was performed. Seeds of tomato (Solanum lycopersicon cv. Microtom) were surface-sterilized by rinsing in 70% (v/v) ethanol for 30-60 s, then in 5% (v/v) sodium hypochlorite (NaClO) for 30 min while rocking on a platform, and washed in distilled water for 5 \times 10 min. The seeds were allowed to germinate and grow for 12 days in half-strength MS (Murashige and Skoog, 1962) agar medium inside a growth chamber with a 14 h light/10 h dark cycle, air temperature of 26/21°C, relative humidity of 70/85% and at a photon flux density (PFD) of 280 mol $m^{-2} s^{-1}$. Germinated seedlings were transferred to 3 L pots (density = 6 plants per pot) and cultivated in a thoroughly aerated Hoagland and Arnon (1950) modified nutrient solution with the following composition (μ M): KH₂PO₄ (80), Ca(NO₃)₂ (1000), KNO₃ (250), MgSO₄ (1000), FeNaEDTA (20), B (4.6), Cl (1.1), Mn (0.9), Zn (0.09), and Mo (0.01). The nutrient solution in each pot was renewed every 3 days to ensure a constant supply of macro- and microelements to plants. After 25 days since the transplant, the EM protein hydrolysate was added to the nutrient solution for 48 h at the following concentrations: 0.1 and 1 mL L^{-1} . These doses and the exposure time were selected based on previous studies screening the most efficient EM concentration in inducing positive physiological responses in plants (Schiavon et al., 2008; Ertani et al., 2009). A group of plants that was not treated with the biostimulant was used as the control. At the end of 48 h, plants were harvested and used for all further molecular and physiological analyses. For fresh weight measurements, 10 plants per treatment were divided into roots and shoots and weighed separately. Samples from the remaining plants were immediately frozen with liquid nitrogen after harvest and kept at -80° C for further analyses.

The experimental design was factorial (species \times biostimulant concentration) with 4 four replicates (1 replicate (=1 pot with 6 plants each) per treatment. All plants collected were representative of the peculiar traits of the species.

Determination of Chlorophyll Content

The quantification of relative chlorophyll concentration was performed through a non-destructive method that uses light transmission through a leaf, at two wavelengths, to determine the greenness and thickness of leaves. Transmission in the infrared range provides a measurement related to leaf thickness, and a wavelength in the red light range is used to determine greenness. The ratio of the transmission of the two wavelengths provides a chlorophyll content index that is referred to as a Soil Plant Analysis Development (SPAD) index (Richardson et al., 2002).

A SPAD (Soil Plant Analysis Development) chlorophyll meter (SPAD-502 model, Minolta Camera Co., Ltd., Osaka, Japan) was used to measure the SPAD index on the last expanded leaf of tomato plants. The determination was carried out on 5 measurements per leaf from 10 plants per experimental condition.

RNA Extraction and Purification

RNA extraction from three biological replicates of leaves and roots was performed using a phenol/chloroform protocol according to Sambrook and Russell (2001). After RNA extraction, DNase treatment was applied (DNase1, Sigma–Aldrich), following the manufacturer's instruction. RNA quality was confirmed by agarose gel electrophoresis, and the concentration and purity of the RNA samples were assessed using a NanoChip Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, United States).

Preparation of Fluorescent Dye-Labeled cDNA and Hybridization

Briefly, 100 ng of purified RNA was reverse transcribed according to the manufacturer's instructions using 200 U of Superscript Reverse Transcriptase III (Life Technologies). Controls and treated samples were compared and, respectively, labeled with fluorescent dyes cyanine 3-CTP and cyanine 5-CTP. After the labeling step, cRNA samples were purified using the RNeasy Mini Kit (Qiagen), and then fragmented to take away secondary structures (specific buffer at 60°C for 30 min) enabling cRNA lengths of between 50 and 200 nucleotides to be obtained and then optimal hybridization with an Agilent 60mer oligonucleotide microarray to be carried out. Thereafter, probe hybridizations were performed at 65°C for 17 h. Each test sample was hybridized using a *Solanum lycopersicon* Gene Expression microarray 4 × 44 K (Agilent Technologies[®]) using a one-color Microarray-Based Gene Expression Analysis (Quick Amp Labeling). All the procedures were performed at CRIBI (Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative, University of Padova, Italy).

Image Acquisition and Bioinformatic Analysis

After probe hybridization, microarrays were scanned with Agilent Scan Control software using default parameters for 4×44 K formats. Data were extracted with Agilent Feature Extraction (FE) program 10.5.1.1 (Agilent Technologies[®]).

Global mean intra-array (Moltiplicatively Detrended) and inter-array (Quantile) normalization was performed across element signal intensity and expression values were transformed into Log_2 ratio of normalized intensities. For annotation of transcripts an annotated probe file was used as a reference, which was generated at EMBL-EBI (Array Express, A-GEOD-8648-Agilent Custom Tomato Gene Expression 4×44 k Array AMADID:19003) and NCBI website.

Significantly up- or down-regulated genes were filtered with fold-change values ≥ 2 or ≤ -2 , respectively, with *q*-value ≤ 0.05 in *t*-test. The program Blast2GO 2.8 was used to perform the gene ontology (GO) analysis and cluster genes based on the biological process.

MapMan and PageMan analyses were done as described in Galla et al. (2009) using the *S. lycopersicon* dataset properly rearranged as input files with the correct genechip identifiers (Agilent) using the pathway dataset Slyc_AGILENT44k _SGN_BUILD2.

Gene sets filtered as explained above were selected for drawing Venn diagrams using the Web-based tool Venn Diagram Generator.

Validation of Gene Expression by Real-Time Quantitative PCR (qRT-PCR)

For quantitative Real-Time PCR experiments, RNA was extracted from three individual samples of leaves and roots of tomato plants grown in hydroponics under the following experimental conditions: control, plus EM 0.1 mL L⁻¹, Se 40 mM. RNA extraction was performed using a phenol/chloroform protocol according to Sambrook and Russell (2001). All the cDNAs were prepared from 3 μ g of RNAs using 200 U of ImProm-IITM Reverse Transcriptase (Promega, Milan, Italy) and oligodT as primers in 20 µl reaction volume. Mixtures were incubated at 37°C for 60 min, 70°C for 5 min, and 4°C for 5 min to stop the RT reaction. Specific primer pairs for the selected sequences are reported in Supplementary Table S1 and tested for their activity at Tm ranging from 58 to 65°C by conventional PCR. qRT-PCR analyses were performed using a thermal cycler 7300 Real-Time PCR System (Applied Biosystem) equipped with a 96 well plates system with the SYBR green PCR Master Mix reagent (Applied Biosystem). Each qPCR reaction (10 µl final volume) contained 1 μ l of diluted cDNA (1:10), 1 μ L of primer couple (10 μ M), and 5 µl of 2× SYBR Green PCR Master Mix according to the manufacturer's instructions. The following thermal cycling profile was used for all PCRs: 95°C for 10 min, 50 cycles of 95°C for 15 s, 60°C for 1 min). The gene expression analysis for each biological replicate was evaluated in two technical replicates (only one set of data is shown in figures). All quantifications were normalized to the actin gene used as housekeeping gene and amplified in the same conditions. Data resulting from qRT-PCR were normalized on the basis of the housekeeping gene by using the $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) and compared to those obtained via microarray. Given the high variation in gene expression, for simplicity data in figures are reported as Log₂ ratio of normalized intensities.

Determination of Total Antioxidant Activity, Phenol and Sugar Compounds

The total antioxidant activity (TAC) was evaluated by measuring the ferric-reducing antioxidant power. The assay was based on the methodology of Benzie and Strain (1996). Ten grams of plant material was homogenized in 20 mL of HPLC grade methanol using an Ultra-Turrax tissue homogenizer (Takmar, Cincinnati, OH, United States) at moderate speed (setting of 60) for 30 s. The ferric-reducing antioxidant power (FRAP) reagent was freshly prepared, containing 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate buffer at pH 3.6. One hundred microliters of the methanol extract was added to 1,900 µL of FRAP reagent and accurately mixed. After leaving the mixture at 20°C for 4 min, the absorbance was determined at 593 nm. Calibration was against a standard curve $(0-1,200 \ \mu g \ mL^{-1}$ ferrous ion) obtained by the addition of freshly prepared ammonium ferrous sulfate. FRAP values were calculated as microgram per milliliter ferrous ion (ferric-reducing power) and are presented as milligram per kilogram of Fe^{2+} Eq (ferrous ion equivalent).

The concentration of total phenols was determined by the Folin-Ciocalteau (FC) assay with gallic acid as calibration standard, using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corp., Columbia, MD, United States). The FC assay was performed by pipetting 200 µL of plant extract (obtained as described above for sugars analysis) into a 10 mL PP tube. This operation was followed by addition of 1 mL of Folin-Ciocalteau reagent. The mixture was vortexed for 20 to 30 s. Eight hundred microliters of sodium carbonate solution (20% w/v) was added to the mixture 5 min after the addition of the FC reagent. This was recorded as time zero; the mixture was then vortexed for 20 to 30 s after addition of sodium carbonate. After 2 h at room temperature, the absorbance of the colored reaction product was measured at 765 nm. The total phenols concentration in the extracts was calculated from a standard calibration curve obtained with different concentrations of gallic acid, ranging from 0 to 600 μ g mL⁻¹. Results were expressed as milligrams of gallic acid equivalent per kilogram of fresh weight (Nicoletto et al., 2013).

For the determination of soluble sugars, leaf and root samples (2 g) were homogenized in water (20 mL) with an Ultra Turrax T25 (IKA, Staufen, Germany) at 13,500 rpm until uniform consistency. Samples were filtered with filter paper (589 Schleicher) and further sieved through cellulose acetate syringe filters (0.45 μ m). The analysis of the extracts was performed

using an HPLC apparatus (Jasco X.LC system, Jasco Co., Tokyo, Japan) consisting of a model PU-2080 pump, a model RI-2031 refractive index detector, a model AS-2055 autosampler and a model CO-2060 column oven. ChromNAV Chromatography Data System was used as software. Sugars were separated on a Hyper-Rez XP Carbohydrate Ca²⁺ analytical column (7.7 mm × 300 mm, Thermo Scientific, Waltham, MA, United States) operating at 80°C. Isocratic elution was performed using water at a flow rate of 0.6 mL/min. The peaks were identified by comparing the retention time with those of standard compounds. To calculate the concentrations in the extract, a calibration curve was drawn for four solutions of known concentration in water.

Elemental Analysis

Quantification of Fe, K, P, and S in leaves of tomato plants was obtained after an acid digestion procedure using a microwave (Milestone Ethos model 1600, Milestone, Shelton, CT, United States). All digestion reactions were carried out in sealed 120 mL Teflon vessels using 500 mg plant material and 10 mL of 30% (v/v) HCl as a solvent. Digested samples were then diluted in 10 mL ultrapure water and assayed via Inductively Coupled Plasma Atomic Emission Spectroscopy (Spectrum Ciros CCD, Kleve, Germany).

Total nitrogen in leaf tissues was detected through a dry combustion procedure using an element analyzer (vario MACRO CNS, Hanau, Germany).

Statistical Analysis

For all determinations with the exception of microarray, the analysis of variance (ANOVA) was performed using the SPSS software version 19.0 (SPSS Inc, 1999), and was followed by pair-wise *post hoc* analyses (Student–Newman–Keuls test) to determine which means differed significantly at p < 0.05 (\pm SD). The number of biological replicates analyzed were three for qRT-PCR and element quantification, 10 for plant growth and SPAD, five for total antioxidant capacity (TAC), phenols and sugars determinations.

For microarray, in each experiment (tissue vs. EM concentration), probes with [Marginal] flag and at least one channel above the background for the three biological replicates were retained. A *t*-test was applied on each filtered gene list according to the following parameters: (i) *t*-test against zero, (ii) Benjamini–Hochberg correction and (iii) *p*-value < 0.05. Only genes whose expression was modified at least by a fold change of 2 (chosen as a threshold) were included in the list of differentially expressed genes (DEGs) (**Table 1** and Supplementary Table S2).

RESULTS

Plant Growth and Chlorophyll Determination

The application of the biostimulant EM to tomato plants at either 0.1 ml L^{-1} or 1 ml L^{-1} concentration led to higher leaf and root fresh biomass production (**Figure 1A**). In leaves, the increase was more pronounced (plus 37%) when plants were supplied with EM

1 ml L^{-1} , while root growth was improved equally (plus 21.4%) by the two EM doses.

The analysis of the SPAD index was conducted to verify the effectiveness of the biostimulant EM in improving tomato plant productivity via enhanced chlorophyll synthesis. The results obtained indicated that SPAD index was increased by EM treatment in a dose dependent manner, being the maximum values recorded in plants added with EM 1 ml L^{-1} (**Figure 1B**).

Transcriptome Analysis Overview

The microarray study was aimed at profiling the EM-responsive genes in leaves and roots of tomato plants treated for 2 days with EM at two different dosages. A total of about 32,000 genes were assayed per each plant tissue × EM treatment. The transcript level of 1938 genes in leaves and 1054 genes in roots of plants supplied with EM 0.1 ml L⁻¹ was significantly altered compared to the control plants, with fold-change values ≥ 2 or ≤ -2 (*q*-value < 0.05) (**Figure 2A**). Based on the same fold change intervals, the transcript abundance of 1687 genes in leaves and 1735 genes in roots of plants supplemented with EM 1 ml L⁻¹ showed significant variation (**Figure 2A**).

Of these genes, 271 and 174 were up-regulated by both EM dosages in leaves and roots, respectively, while 180 and 110 were down-regulated. However, a large number of genes was found to be regulated by a definite EM treatment. Specifically, in leaves the transcript level of 727 and 637 transcripts was increased by EM 0.1 ml L^{-1} and EM 1 ml L^{-1} , respectively, while 760 and 599 were reduced in expression. In roots, the number of differential expressed genes (DEGs) was 774, of which 436 up-regulated and 338 down-regulated, when plants were supplied with EM 0.1 ml L^{-1} . The number of root DEGs was twofold higher in plants added with EM 1 ml L^{-1} and included 1301 up-regulated and 150 down-regulated transcripts.

In the case of sequences that showed a similar trend of regulation between leaves and roots for each test biostimulant dose, we observed that 74 and 269 were up-regulated in plants treated with EM 0.1 and EM1, respectively, while 33 and 28 were down-regulated (**Figure 2A**).

Despite the high number of identified DEGs, only part of them had reported homologs or showed homology to genes coding for predicted proteins (*E*-value < 1.0 E^{-5}) using the blastx program against the plant nr (NCBI) database. These DEGs are listed in **Table 1** (partial list) and Supplementary Table S2 (complete list). The remaining DEGs corresponded to proteins of unknown function or uncharacterized. The fold-change based-distribution of genes significantly altered in expression by EM indicates that in leaves these sequences were mainly within the fold-change range of +2 to +4 when up-regulated, and in the range of -2 to -4 if down-regulated, regardless of the EM dose. In roots, most of sequences fell in the range of +2 to +4, while a minor number was assigned to other ranges (**Figure 2B**).

Based on the biological process, the GO classification of the probes used in cDNA microarray arranged the DEGs in a few prominent categories shared by leaves and roots, with a sequence number >150 each (**Figure 3**). These categories included: organic substance metabolic process, primary metabolic process, cellular metabolic process, single-organism metabolic process, biosynthetic process, nitrogen compound metabolic process, response to stress and regulation of biological process. Other biological processes in which DEGs were entered and accounted for less than 150 sequences each are shown in **Figure 3**.

In general, the total number of sequences belonging to the main biological process categories was higher for leaves compared to roots, especially with respect to organic substance metabolic process (+54%), primary metabolic process (+22%), and cellular metabolic process (+10%). This trend was mainly ascribed to sequences regulated by a definite EM treatment, as the specific distribution of genes up-regulated by both EM dosages in leaves and roots of *S. lycopersicon* plants indicated that sequences involved in these processes are more represented in roots than in leaves, while no significant differences in number of down-regulated sequences were evident between leaves and roots (**Figure 4**).

Gene name	Agilent ID	Fold ch	nange	Annotation		
		EM 0.1 mL L ⁻¹	EM 1 mL L ⁻¹			
		Lea	aves			
Al485516	A_96_p132312	1170.57	2.43	Basic helix-loop-helix (bHLH)		
TA37435	A_96_p011406	265.40	265.31	Aldo/keto reductase		
TA56542	A_96_p126097	177.42	1170.18	expansin		
AK326750	A_96_p120972	176.80	2.63	Phox (PX) domain-containing protein		
AI771499	A_96_p133717	126.68	85.78	AP2 domain-containing transcription factor, putative		
AK329872	A_96_p107139	99.59	15.92	Peroxidase, putative		
BT012835	A_96_p103444	28.46	115.49	Transferase family protein		
TA54953	A_96_p119612	16.83	2.46	Homeobox-leucine zipper		
Al487014	A_96_p131332	16.60	2.58	Putative bzip transcription factor		
AW029915	A_96_p143491	13.75	2.58	Lactoylglutathione lyase		
AK321258	A_96_p171729	11.17	27.52	Cytochrome P450 94A1 (CYP94C1)		
AW443470	A_96_p156756	10.51	2.98	Transferase family protein		
AK323400	A_96_p036591	6.43	33.07	Homeobox-leucine zipper protein 12 (HB-12)		
TA56836	A_96_p127927	4.68	10.69	Zinc finger (Ran-binding)		
M61914	A_96_p171334	4.51	18.34	∟-threonine ammonia-lyase		
AK322433	A_96_p045476	4.07	17.40	Glutathione S-transferase		
TA56114	A_96_p124337	3.45	16.59	CTF2A monooxygenase		
AK326774	A_96_p043686	3.09	10.68	Hydrolase		
BE344500	A_96_p014241	3.08	126.69	Alternative oxidase 1A (AOX1A)		
		Ro	oots			
BF097588	A_96_p181989	7.14	3.72	Cell wall-associated hydrolase		
AK322433	A_96_p045476	6.91	17.89	Glutathione S-transferase		
TA37435	A_96_p011406	6.65	2.69	Aldo/keto reductase family protein		
TA38046	A_96_p181024	6.09	2.43	Heat shock protein 91		
BG130524	A_96_p187884	5.94	2.31	Chitinase		
AK328987	A_96_p151561	5.28	6.98	Calcium-dependent protein kinase 33		
BI933689	A_96_p206279	5.25	2.36	Aldo/keto reductase family protein		
AW041795	A_96_p059781	4.86	3.29	Ribosomal protein L7Ae family protein		
BI923348	A_96_p102244	4.52	2.32	Multidrug resistance-associated protein 6		
DV104033	A_96_p246567	4.09	18.34	Chitinase		
TA50778	A_96_p108952	4.05	5.23	Calcium-dependent protein kinase 33		
AW224326	A_96_p155271	3.94	21.21	Alcohol dehydrogenase 1		
CK468693	A_96_p054591	3.12	11.63	Chromosome chr8 scaffold_23, transcription factor		
BI421662	A_96_p043431	2.73	85.78	Expansin-Like B1		
DB703551	A_96_p232879	2.58	6.42	Kelch repeat-containing protein type 1		
AK328356	A_96_p008426	2.44	9.55	Universal stress protein (USP) family protein		

Annotation is given based on blast search against the plant sequence database. Selection for sequences is based on fold change (>10 and >5 for leaves and roots, respectively, for at least one treatment).



Identification of Metabolic Pathways Regulated by EM

The genes that were identified as differentially expressed in microarray were mapped into functional groups via MapMan in order to gain insight into which gene families and metabolic pathways may represent targets of regulation by EM.

From Figures 5, 6, as well as from Supplementary Table S2, it is evident that some gene families are prominently present. They include defense-related genes [particularly Cytochrome 450 (Cyt450), leucine rich repeat proteins (LRR), heat shock proteins (HSP), aldo/keto reductase, glutathione-S-transferases (GSTs), lactoylglutathione lyase, DNAJ, chitinases, pathogenesis-related protein Bet v, subtilases, DREB2A, hydroxyproline-rich glycoprotein, wound-induced proteins, L-threonine ammonia-lyase, alternative oxidase 1A, syntaxin (SYR1), DC1 domain-containing proteins, thaumatin, ECERIFERUM (CER1), serine carboxypeptidase-like, aspartyl protease, CC-NBS-LRR proteins]; antioxidant-related genes [mainly peroxidases including ascorbate peroxidase (APX), catalases, thioredoxins, hemoglobins, glutaredoxins GRX, dehydroascorbate reductase (DHAR), CTF2A monooxygenases]; transcription factors [e.g., basic helix-loop-helix (bHLH), AP2/EREBPs (APETALA2), homeobox-leucine zipper, bzip, zinc finger (ZFN), pentatricopeptide repeat-containing protein (PPR), WRKY, Myb, Rav, ERF2, chromosome chr8 scaffold_23]; protein kinases [particularly MAPK, MAPKKK, diacylglycerol kinase, calcium and/or calmodulin-domain protein kinases, serine/threonine-protein kinases, CBL-interacting protein kinases, wall-associated kinase 2 (WAK2), cyclin-dependent protein kinases, S-locus lectin protein kinases]; nitrate metabolism genes [particularly nitrate reductase (NR), aspartate aminotransferase (AST), glutamine-dependent asparagine synthetase (ASN1), glutamine synthetase (GS)]; sugar and lipid metabolism-related genes (e.g., lipases, polygalacturonases, pectinesterases, starch synthase, sucrose synthase, cellulose synthase, inositol oxygenases); hormone-related genes

[ethylene forming enzyme (EFE), 1-aminocyclopropane-1carboxylate oxidase (ACO), ein3-binding f-box protein 1, jasmonic acid-amino acid-conjugating enzyme, protein phosphatase 2C (PP2C), ethylene/ auxin/ABA/gibberellinresponsive proteins, S-adenosyl-l-methionine:salicylic acid carboxyl methyltransferase]; photosynthesis-related genes [particularly Ribulose bisphosphate carboxylase, ferredoxin, phytochrome interacting factor 3-like 5 (PIL5), photosystem II 22 kda protein]; development-related genes [mainly expansins, growth-regulating factors 3 (GRF3) and 5 (GRF5), embryo defective proteins, lob domain protein 1]; secondary metabolism-related genes [mainly phenylalanine ammonia lyase (PAL), 2-oxoglutarate-dependent dioxygenase, hydroxy-3-methylglutaryl coenzyme A reductase, isoflavone reductase, terpene synthase, caffeoyl-CoA 3-O-methyltransferase], protein degradation (particularly F-box, ubiquitin and ubiquiting conjugating enzymes, cysteine proteases, AAA-ATPase).

With respect to genes involved in the transmembrane transport of substances, the most represented were those coding for amino acid and peptide transporters, ABC transporters, MATE efflux transporters, tonoplast intrinsic proteins (TIP), ATPases. Other transporters of interest up-regulated under EM treatment were sulfate transporters SULTR 2;1 and SULTR 3;1, nitrate transporter NTR2, ammonium transporter AMT1.1, copper transporters, phosphate transporter PT2, iron-phytosiderophore transporter protein yellow stripe 1 (YS1), potassium channels, sugar transporter, auxin transporter (zinc induced facilitator, ZIFL1), organic cation/carnitine transporters, nodulin MtN21, and glutathione-conjugate transporter MRP4.

A number of GTP-binding proteins involved in protein synthesis and intracellular translocation, as well as UDP glucosyltransferases were also up-regulated by both EM dosages, regardless of the plant organ. Interestingly, two genes, one coding for a polypyrimidine tract-binding protein (PTB) and the other one for a RNA recognition motif (RRM)-containing protein, were strongly up-regulated with a fold change of



visually represent the commonalities among sets of information. In the first two upper diagrams, arrows upward and downward indicate up-regulation and down-regulation, respectively, compared to the control (no EM treatment). The number of differentially expressed (DEGs) relative to a specific ET treatment (EM at 0.1 mL L^{-1} or EM 1 mL L⁻¹) is displayed in the non-overlapping regions of the circles. In the lower two diagrams, the number of sequences commonly or individually up-regulated or down-regulated in leaves and roots at a specific EM dosage **(B)**. Distribution of DEGs in leaves and roots of *S. lycopersicon* based on fold-changes values. The positive and negative numbers on the x-axis represented up- and down-regulation of *S. lycopersicon* genes, respectively.

626 and 32, respectively, in leaves of plants treated with EM 0.1 ml L⁻¹. Both genes play a role in splicing, mRNA stability and translation initiation. Genes coding for RNA recognition motif (RRM)-containing proteins were also identified in leaves of plants supplied with EM 1 ml L⁻¹, however, the fold change values were much lower (barely above 2).

Comparison of DEGs Regulated by Different EM Dosages

The complete list of DEGs that matched with known proteins is reported in Supplementary Table S2, while a partial list of up-regulated genes by both EM concentrations is shown in **Table 1**. Considering the DEGs that are strongly up-regulated (fold change >10) in leaves of *S. lycopersicon* plants by both





EM dosages, we identified only the genes encoding for aldo/keto reductase and AP2 domain-containing transcription factor with a comparable fold change value (Supplementary Table S2). Genes encoding for a bHLH transcription factor, a phox (PX) domaincontaining protein, a peroxidase, a homeobox-leucine zipper protein, a putative bzip transcription factor, a lactoylglutathione lyase were prominently up-regulated by EM 0.1 ml L^{-1} . As an example, the fold changes for the bHLH transcription factor and phox (PX) domain-containing protein were 1170.6 and 176.8, respectively, vs. the 2.43 and 2.63 values determined under EM 1 ml L^{-1} application. However, a number of DEGs was significantly more expressed in leaves of plants treated with the highest EM dose, such as an expansin, a Cytochrome P450 94A1, a homeobox-leucine zipper protein 12 (HB-12), a zinc finger (Ran-binding) protein, a glutathione S-transferase, a CTF2A monooxygenase, an alternative oxidase 1A. The remaining DEGs shared fold change values of comparable magnitude.

With respect to the DEGs whose expression was increased by both EM concentrations in roots, a similar trend for a few of the genes previously mentioned was observed. For instance, the aldo/keto reductase encoding gene displayed a higher fold change value in roots of plants treated with EM $0.1 \text{ ml } \text{L}^{-1}$, while glutathione S-transferase and expansin genes were more strongly up-regulated by EM 1 ml L^{-1} . The genes encoding for chitinases, chromosome chr8 scaffold_23 transcription factor and alcohol dehydrogenase 1 were also more expressed under the EM 1 ml $\rm L^{-1}$ treatment.

In the case of DEGs regulated by a definite EM treatment, 218 and 481 with known function were up-regulated in leaves by EM 0.1 ml L^{-1} and EM 1 ml L^{-1} , respectively. The genes induced by EM 0.1 ml L^{-1} with a fold change >10 included a polypyrimidine tract-binding protein, two embryo defectives (EMB1379 and EMB1303), a lipase class 3, a hemoglobin class 1, an RNA recognition motif (RRM)-containing protein, an inositol oxygenase, a cellulose synthase catalytic subunit, a nodulin MtN21 family protein, a zinc finger (CCCH-type) protein, three peroxidases, a ribose-phosphate pyrophosphokinase 2, a putative kiwellin ripening-related protein precursor, and a MATE efflux family protein. Different DEGs with a fold change >10 were up-regulated by EM 1 ml L^{-1} compared to EM 0.1 ml L^{-1} . Among these are a pathogenesis-related protein Bet v family, an expansin like B1, an In2-1 protein, an oxidoreductase, a late embryogenesis abundant domain-containing a protein syntaxin-related protein (SYR1), a protein kinase, a CAM1 (CALMODULIN 1), a DC1 domaincontaining protein, a 2-oxoglutarate-dependent dioxygenase, a leucine-rich repeat protein, a BETA-TIP (beta-tonoplast intrinsic protein), lob domain protein 1, coatomer protein complex Glutathione S-transferase, UDP-glucoronosyl/UDPglucosyl transferase.



In roots, the fold change determined for genes up-regulated only by EM 0.1 ml L^{-1} was generally lower than 10, with the exception of a leucine-rich repeat protein, whose fold change was 10.2. The other genes that displayed fold change values at least higher than 5 under this EM treatment were an oxidoreductase, a zinc-binding dehydrogenase protein, a PHD zinc finger protein, a protein responsive to abscisic acid 1B (RAB1B), a glycosyl hydrolase. On the contrary, the fold change of genes whose transcript abundance was specifically enhanced by EM 1 ml L^{-1} was higher than 10 for 13 of them, including a zinc induced facilitator-like 1 (ZIFL1), an organic cation/carnitine transporter 2, a Cytochrome P450 94B2, an indolacetic acid (IAA)-amido synthases, a Cytochrome P450 86A7, a LOB domain protein 41, a pentatricopeptide (PPR) repeat-containing protein, a diacylglycerol kinase, a calmodulindomain protein kinase isoform 9 (CPK9), a family II extracellular lipase 1 (EXL1), two alcohol dehydrogenase 2, an hydroxy-3methylglutaryl coenzyme A reductase.

In Supplementary Table S2 the DEGs down-regulated in leaves and roots by both EM 0.1 ml L^{-1} and EM 1 ml L^{-1} are also reported. As evinced from the list of DEGs, some of them fall in the same families of genes that are up-regulated by EM, while others are unique for these groups, like early flowering 6 (ELF6), protodermal factor 1 (PDF1), MCM protein, germin like protein, FtsH protease, which are down-regulated in leaves, and phosphatidylinositol 3- and 4-kinase, defense no death 1 (DND1), which are repressed in roots.

Validation of Gene Expression by qRT-PCR

To validate the results of the cDNA microarray, 18 EM-induced genes (selected based on cDNA microarray data) were analyzed via qRT-PCR in leaves and roots, using primer pairs with amplification efficiencies ranging within 1.96–1.99. All the 18 genes showed similar expression pattern (P < 0.05) in both cDNA microarray and qRT-PCR analysis. Values were expressed as Log₂ (EM treatment – Control) (**Figures 7, 8**).

The correlation coefficient between the fold-changes data obtained via microarray and those obtained via qRT-PCR for each same expression pattern gene was therefore particularly high being $R^2 = 0.97$ and 0.89 for leaves and roots, respectively (values calculated on the average data for EM 0.1 ml L⁻¹ and EM 1 ml L⁻¹, P < 0.05). On this account and since gene expression values between microarray and qRT-PCR can vary within 0.48–0.94 and criteria for the determination of an acceptable validation of microarray results by qRT-PCR are hardly definite (Morey et al., 2006), we believe the microarray data of the current study are validated by qRT-PCR experiments.

Effects of EM on Mineral Content

The product EM was able to promote nutrient accumulation in leaves when applied to plants, regardless of its concentration (**Table 2**). The effect was more pronounced for P and K, as their concentration was about sevenfold higher in EM treated

	Leaves				B EM 0.1 EM 1					
				-	A_96_p031861		Abiotic stress (heat)	A_96_p203664 A 96 p134862		Abiotic stress
Billio Allowing <		EM 0.1	I EM	1	A 96 p052346		Amino acid metabolism	A_96_p109937		Abiotic stress
	A_96_p156	5186		Abiotic stress (heat)	A_96_p020146		Amino acid metabolism	A_96_p109182	_	Abiotic stress (drought/salt)
M. M	A_96_p116	5237		Abiotic stress (heat)	A_96_p095604		Argonaute 4	A 96 p078694	_	Abiotic stress (heat)
Durant Durant<	A_96_p057	7031		Bioric stress (cadmium)	A_96_p188939		Biotic stress	A_96_p049291		Abiotic stress (heat)
Number Number<	A_96_p199	9644		C metabolism	A_96_p176699		Biotic stress (PR proteins)	A_96_p223239		Abiotic stress (heat)
Subso Subso <td< td=""><td>A 96 0150</td><td>0831</td><td>-</td><td>Cell wall</td><td>A_96_p172399</td><td></td><td>Cabrie Cucle</td><td>A_96_p039471</td><td>_</td><td>Abiotic stress (heat) Abiotic stress (heat)</td></td<>	A 96 0150	0831	-	Cell wall	A_96_p172399		Cabrie Cucle	A_96_p039471	_	Abiotic stress (heat) Abiotic stress (heat)
Number Number<	A 96 p126	5097	-	Cell wall modification	A 96 n210459	-	Cell division	A_96_p199789		Biotic stress
Bit Bit <td>A 96 p052</td> <td>2716</td> <td>-</td> <td>Cell wall organization</td> <td>A 96 p172444</td> <td>10.000</td> <td>Cell organization</td> <td>A_96_p000136</td> <td></td> <td>Biotic stress</td>	A 96 p052	2716	-	Cell wall organization	A 96 p172444	10.000	Cell organization	A_96_p000136		Biotic stress
Bible Depresentation (Contactume Foundament) A. Bible	A_96_p051	1666		Cell wall organization	A_96_p095924		Cell wall degradation	A_96_p069444		Biotic stress
Biolog Biolog<	A_96_p194	4619	_	Enzyme familes (Glutathione S-transferases)	A_96_p140762		Cell wall LRR protein	A 96 p035821		Biotic stress (PR proteins, kinase LRR)
Notes Notes <td< td=""><td>A_96_p045</td><td>5476</td><td></td><td>Enzyme familes (Glutathione S-transferases)</td><td>A_96_p043431</td><td></td><td>Cell wall modification</td><td>A_96_p017006</td><td></td><td>Biotic stress (PR proteins)</td></td<>	A_96_p045	5476		Enzyme familes (Glutathione S-transferases)	A_96_p043431		Cell wall modification	A_96_p017006		Biotic stress (PR proteins)
N. M. S.	A_96_p005	5886		Enzyme families (Glutathione 5-transferases)	A_96_p066476		Cell wall synthesis	A_96_p056726		Calvin cylce Rubisco small subunit
Question Particulation Augusta Particulation Particulation<	A_96_01/1	1/29	_	Enzyme families (Cytochrome P450)	A_96_p184239		Chitinase Debudrosscorbate reductare	A_96_p134372		Calvin cylce Rubisco small subunit Cell division
No. No. <td>A 96 p060</td> <td>0146</td> <td></td> <td>Enzyme families (Glutaredoxins)</td> <td>A 96 p209824</td> <td></td> <td>Development</td> <td>A_96_p159666</td> <td></td> <td>Cell division</td>	A 96 p060	0146		Enzyme families (Glutaredoxins)	A 96 p209824		Development	A_96_p159666		Cell division
N. 10.20	A_96_p201	1145		Enzyme families (oxidases)	A_96_p085154	10	Development	A_96_p084119		Cell division
	A_96_p124	1337		Enzyme families (oxidases)	A_96_p239058	1	Development	A_96_p032731	-	Cell organization
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No. 100 No. 100 <t< td=""><td>A_96_p033</td><td>3111</td><td></td><td>Enzyme families (oxidases)</td><td>A_96_p162021</td><td></td><td>Enzyme families (beta 1,3 glucan hydrolases)</td><td>A_96_p059631</td><td></td><td>Cell vescicle transport</td></t<>	A_96_p033	3111		Enzyme families (oxidases)	A_96_p162021		Enzyme families (beta 1,3 glucan hydrolases)	A_96_p059631		Cell vescicle transport
Physical	A_96_p195	5324		Enzyme families (Peroxidases)	A_96_p200099		Enzyme families (beta 1,3 glucan hydrolases)	A_96_p043716	_	Cell vescicle transport
Compose Compose <t< td=""><td>A 96 -049</td><td>139</td><td></td><td>Enzyme families (Peroxidases)</td><td>A_96_p046226</td><td></td><td>Enzyme families (Cytochrome 450)</td><td>A 96 p056646</td><td></td><td>Cell wall modification</td></t<>	A 96 -049	139		Enzyme families (Peroxidases)	A_96_p046226		Enzyme families (Cytochrome 450)	A 96 p056646		Cell wall modification
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	A_96_p074	4234		Enzyme families (UDP-glycosyltransferases)	A_96_p076479		Enzyme families (Glutathione S-transferase)	A_96_p215764		Development
9	A_96_p201	1884		Enzyme fsmilies (Phosphatases)	A_96_p128627		Enzyme families (Oxidoreductases)	A_96_p124427	-	Glutathione transferases
	A_96_p077	7704		Enzyme fsmilies (Phosphatases)	A_96_p093720		Enzyme families (Peroxidases)	A_96_p072125		Glutathione transferases
0.10110 0.101100 0.101100	A 96 p168	0739	-	Hormone metabolism (Auvio)	A_96_0176135		Enzyme families (Peroxidases)	A 96 p108523		Hormone metabolism (Auxin)
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9, 27.00 ••••••••••••••••••••••••••••••••••••	A_96_p006	5481		Hormone metabolism (Ethylene)	A_96_p232449		Enzyme families (Phosphatases)	A_96_p226684		Hormone metabolism (Ethylene)
Number Number Apploy Apploy<	A_96_p227	7009	-	Hormone metabolism (Gibberellin)	A_96_p211504		Enzyme families (UDP Glycosyltransferases)	A 96 p196839		Lipid metabolism
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96, 22379 RAA Regulation of transcription A.96, 22329 Protein degradation (trainer protease) Protein degradation (trainer protease) A.96, 22329 Protein degradation (trainer protease) Protein degradation (trainer protease) A.96, 22329 Protein degradation (trainer protease) Protein degradation (trainer protease) A.96, 22329 Protein degradation (trainer protease) <td< td=""><td>A_96_p086</td><td>5379</td><td></td><td>Redox homeostasis</td><td>A_96_p188164</td><td></td><td>Nucleotide metabolism</td><td>A_96_p191269</td><td></td><td>Protein degradation (cysteine protease)</td></td<>	A_96_p086	5379		Redox homeostasis	A_96_p188164		Nucleotide metabolism	A_96_p191269		Protein degradation (cysteine protease)
96,2239 RNA Regulation of transcription A.98,2127 Protein degradation (Linkipitch-degradation) A.98,21227 Protein degradation (Linkipitch-degradation) A.98,21237 Protein degradation (Linkipitch-degradation) A.98,21237 Protein degradation (Linkipitch-degradation) A.98,21237 Protein degradation (Linkipitch-degradation) A.98,215307 Protein degradation) A.98,215307 Protein degradation (Linkipitch-degradation) A.98,215	A_96_p237	7397		RNA Regulation of transcription	A_96_p222524	1	Protein degradation (cysteine protease)	A_96_p239438		Protein degradation (serine protease)
Sp. 22.22 Protein degradation (Hausteppinon) A. Sp. 23722 Protein degradation (Ubiquitin-dependent) A. Sp. 13222 Protein degradation (Ubiquitin-dependent) Sp. 23131 Protein degradation (Ubiquitin-dependent) A. Sp. 13732 Protein degradation (Ubiquiti	A_96_p232	2959	-	RNA Regulation of transcription	A_96_p232/34		Protein degradation (serine protease)	A 96 p184939		Protein degradation (subtilases)
Augusta RNA Regulation of transcription A 36, 21727 Protein degradation (Ubiputind-egenden) A 36, 21737 Protein modification A 36, 21737 Protein degradation (Ubiputind-egenden) A 36, 21737 Protein modification A 36, 21737 <t< td=""><td>A 96 p166</td><td>274</td><td></td><td>RNA Regulation of transcription</td><td>A 96 p247422</td><td></td><td>Protein degradation (Ubiquitin-dependent)</td><td>A_96_p124222</td><td></td><td>Protein degradation (Ubiquitin-dependent)</td></t<>	A 96 p166	274		RNA Regulation of transcription	A 96 p247422		Protein degradation (Ubiquitin-dependent)	A_96_p124222		Protein degradation (Ubiquitin-dependent)
Sp. 11312 NNA Regulation of transcription A.Sp. 14992 Protein digradation (Ubiquitin-dependent) A.Sp. 13120 Protein digradation (Ubiquitin-dependent) A.Sp. 13320	A 96 n132	312		RNA Regulation of transcription	A 96 p232735		Protein degradation (Ubiguitin-dependent)	A_96_p115297		Protein degradation (Ubiquitin-dependent)
Spe_D132 NNA Regulation of transcription A.Sp. 15131 Protein dynamics Number 3 Sp.D0524 NNA Regulation of transcription A.Sp. 15132 Protein dynamics A.Sp. 15232 Number 3 Sp.D0524 NNA Regulation of transcription A.Sp. 15032 Number 3 Number 3 Sp.D0535 NNA Regulation of transcription A.Sp. 15032 Number 3 Number 3 Sp.D0535 NNA Regulation of transcription A.Sp. 15032 Number 3 Number 3 Sp.D0535 NNA Regulation of transcription A.Sp. 15043 Number 3 Number 3 Sp.D0535 NNA Regulation of transcription A.Sp. 15043 Number 3 Number 3 Sp.D05357 NNA Regulation of transcription A.Sp. 15043 Number 3 Number 3 Sp.D05372 Number 3 Number 3 Number 3 Number 3 Number 3 Sp.D0537 Number 3 Number 3 Number 3 Number 3 Number 3 Sp.D0537 Number 3 Number 3 Number 3 Number 3 Number 3 Sp.D0537 Number 3 <td< td=""><td>A 96 p131</td><td>332</td><td></td><td>RNA Regulation of transcription</td><td>A_96_p147991</td><td></td><td>Protein degradation (Ubiquitin-dependent)</td><td>A 96 p229044</td><td></td><td>Protein post translational modification</td></td<>	A 96 p131	332		RNA Regulation of transcription	A_96_p147991		Protein degradation (Ubiquitin-dependent)	A 96 p229044		Protein post translational modification
96,005121 RNA Regulation of transcription A,56,01526 Protein degradation (Ublquittin-dependent) A,56,01526 Protein indigradation (Ublquittin-dependent)	A_96_p119	9612		RNA Regulation of transcription	A_96_p151351		Protein degradation (Ubiquitin-dependent)	A_96_p177220		Protein synthesis
96, 00510; RNA Regulation of transcription A.96, 01300; Protein adgradation (Ubiquitin-dependent) A.96, 01303; Protein adgradation (Ubiquitin-dependent) A.96, 01302; Protein adgradation (Ubiquitin-dependent) A.96, 01302; RNA Regulation of transcription A.96, 01302; RNA regul	A_96_p096	5214		RNA Regulation of transcription	A_96_p155026		Protein degradation (Ubiquitin-dependent)	A_96_p061251	-	Protein synthesis
96,03502 NA Regulation of transcription A,98,013272 Protein modification A,28,013372 Protein modification A,38,0141 Noteputation of transcription A,38,01424 Noteputation of transcription<	A_96_p061	1761	-	RNA Regulation of transcription	A_96_p113942		Protein degradation (Ubiquitin-dependent)	A_96_p143696		Protein targeting secretory pathway vacuole Protein targeting secretory pathway vacuole
95, 1331/1 Protein Symitability Protein S	A_96_p036	5591		RNA Regulation of transcription	A_96_p108292	in the second se	Protein modification	A_96_p246280	1	Redox
Big Dillson A.S. 201301 Rider (heme) Rider (heme) A.S. 201301 Rider (heme) </td <td>A_96_p133</td> <td>221</td> <td>1</td> <td>RNA Regulation of transcription</td> <td>A 96 p194419</td> <td>Accession in</td> <td>Redox (Glutaredoxin)</td> <td>A_96_p185974</td> <td></td> <td>RNA regulation of transcription</td>	A_96_p133	221	1	RNA Regulation of transcription	A 96 p194419	Accession in	Redox (Glutaredoxin)	A_96_p185974		RNA regulation of transcription
96,055721 RNA regulation of transcription A.96,07076 A.96,07076 A.96,07076 96,005104 RNA Regulation of transcription A.96,07076 RNA regulation of transcription A.96,07076 RNA regulation of transcription 96,07104 RNA Regulation of transcription A.96,07076 RNA regulation of transcription A.96,07076 RNA regulation of transcription 96,07107 RNA regulation of transcription A.96,07076 RNA regulation of transcription A.96,07176 RNA regulation of transcription A.96,07186 RNA regulation of transcription 96,07107 Secondary metabolism A.96,07186 RNA regulation of transcription A.96,07186 RNA regulation of transcription 96,010344 Secondary metabolism A.96,07186 RNA regulation of transcription A.96,07279 Secondary metabolism 96,010344 Secondary metabolism A.96,07208 RNA regulation of transcription A.96,07279 Secondary metabolism 96,010345 Secondary metabolism A.96,07208 Signaling (factum) A.96,07207 Secondary metabolism 96,010345 Secondary metabolism A.96,07208 Signaling (factum) A.96,07208 Signaling (factum) Signaling (factum)	A 96 p038	876		RNA Regulation of transcription	A_96 p150911		Redox (heme)	A_96_p057541		RNA regulation of transcription RNA regulation of transcription
96,091104 RNA Regulation of transcription A.96,0991104 RNA regulation of transcription A.96,099104 RNA regulation of transcription A.96,012564 RNA regulation of transcription A.96,012565 RNA regulation of transcription A.96,012565 Scendary metabolism Scendary metabolism A.96,012565 Scendary metabolism A.96,012565 Scendary metabolism Scendary metabolism	A 96 p018	5721		RNA Regulation of transcription	A_96_p117017		RNA regulation of transcription	A 96 p127937		RNA regulation of transcription
Sig. 1737 av sig. BNA Regulation of transcription A, Sig. 12132 RNA regulation of transcription A, Sig. 121434 Secondary metabolism A, Sig. 121534 Secondary metabolism A, Sig. 121534 Secondary metabolism A, Sig. 121534 Secondary metabolism Sig. 121634 Secondary metabolism Sig. 121634	A_96_p091	104		RNA Regulation of transcription	A_96_p059761		RNA regulation of transcription	A_96_p133137		RNA regulation of transcription
96.p33190 RNA Regulation of transcription A.96.p12464 RNA regulation of transcription A.96.p12169 RNA regulation of transcription A.96.p12166 RNA regulation of transcription A.96.p12166 Scondary metabolism A.96.p02166 RNA regulation of transcription A.96.p02176 Scondary metabolism A.96.p02167 Spalling (alcum) A.96.p02176 Spalling (alcum) Spalling (a	A_96_p176	5374		RNA Regulation of transcription	A_96_p124542		RNA regulation of transcription	A_96_p078504		RNA regulation of transcription
96.p21444 RNA Regulation of transcription A.9b.p21372 RNA regulation of transcription A.9b.p21372 Secondary metabolism A.9b.p21372 Secondary metabolism A.9b.p21372 Secondary metabolism A.9b.p21372 Secondary metabolism A.9b.p21386 RNA regulation of transcription A.9b.p203216 Secondary metabolism 96.p21374 Secondary metabolism A.9b.p10581 Signalling (calcium) A.9b.p203216 Sepalating (calcium) 96.p213246 Secondary metabolism A.9b.p10581 Signalling (calcium) A.9b.p10681 Signalling (calcium) Sepalating (calcium) 96.p213246 Secondary metabolism A.9b.p10581 Signalling (calcium) A.9b.p10780 Signalling (calcium) Sepalating (inase DU726) 96.p123246 Secondary metabolism A.9b.p10581 Signalling (calcium) Signalium (calcium)	A_96_p253	3190		RNA Regulation of transcription	A_96_p184249		RNA regulation of transcription	A_96_p064186		RNA regulation of transcription
99, p352/95 Secondary metabolism A, 95, p00346 RNA regulation of transcription A, 95, p00346 Secondary metabolism A, 95, p00346 Secondary metabolism A, 95, p00346 RNA regulation of transcription A, 95, p00347 Secondary metabolism A, 95, p00346 Signalling (claium) A, 95, p00347 Secondary metabolism A, 95, p00346 Signalling (claium) A, 95, p00346 Signalling (claium) A, 95, p00346 Signalling (claium) A, 95, p00347 Signalling (claium) A, 95, p00347 Signalling (claium) A, 95, p00346 Signalling (claium) A, 95, p00347 Signalling (claium) A, 95, p00347 Signalling (claium) A, 95, p00347 Signalling (claium) A, 95, p00346 Signalling (claium) Signalium (Claium) Signalling (Cl	A_96_p214	1464		RNA Regulation of transcription	A_96_p214529		RNA regulation of transcription	A_96_p012186		Secondary metabolism
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9g. 101244 Secondary metabolism A.96, p151561 Signalling (calcium) A.96, p24267 Algoing (calcium) 96, p013264 Secondary metabolism A.96, p143861 Signalling (calcium) A.96, p243861 Signalling (calcium) Signalling (calcium) A.96, p243861 Signalling (calcium) Signall	A 96 o110	537		Secondary metabolism	A_96_p055126		RNA regulation of transcription	A_96_p097479	-	Secondary metabolism Signalling (calcium)
96,003300 Secondary metabolism A 36, p14380 Signalling (calcium) A 96, p13920 Signalling (calcium) 96,012326 Secondary metabolism A 36, p14380 Signalling (calcium) A 96, p13920 Signalling (calcium) Signalling (calcium) <t< td=""><td>A 96 n103</td><td>1444</td><td></td><td>Secondary metabolism</td><td>A_96_p151561</td><td>No.</td><td>Signalling (calcium)</td><td>A 96 p042851</td><td></td><td>Signalling (calcium)</td></t<>	A 96 n103	1444		Secondary metabolism	A_96_p151561	No.	Signalling (calcium)	A 96 p042851		Signalling (calcium)
36_p21244 Secondary metabolism A 36_p10700 Signalling (receptor kinases) A, 36_p11300 Signalling (receptor kinases) A, 36_p11300 Signalling (receptor kinases) Si	A_95_p038	3306	1	Secondary metabolism	A_96_p146881		Signalling (calcium)	A_96_p117947		Signalling (G-protein)
96.p131372 Secondary metabolism A.96.p13000 Signaling (Einse DU/26) Signaling (Einse DU/26) 96.p131372 Secondary metabolism A.96.p131000 Transport A.96.p131377 Signaling (Einse DU/26) 96.p131372 Signaling (C-protein) A.96.p131300 Transport A.96.p131377 Signaling (Einse DU/26) 96.p24827 Signaling (C-protein) A.96.p131421 Transport (ABC transporters) A.96.p10224 Signaling (Einse DU/26) 96.p34827 Signaling (C-protein) A.95.p10224 Transport (ABC transporters) A.96.p10232 Signaling (Einse DU/26) 96.p34827 Signaling (Lorice-rich repeat receptor-like protein) A.95.p10244 Transport (ABC transporters) A.96.p076364 Signaling (Einse DU/26) 96.p313422 Signaling (Pox (PA) domain containing protein) A.95.p102042 Transport (ABC transporters) A.96.p076396 Signaling (Fox (PA) domain containing protein) A.95.p107634 Transport (ABC transporters) A.96.p078346 Transport (ABC transporters) Signaling (Fox (PA) domain containing protein) A.95.p107646 Transport (ABC transporters) A.96.p078346 Transport (ABC transporters) A.96.p078346 Transport (ABC transporters) Transport (ABC transporters) A.96.p078346	A_96_0212	2464		Secondary metabolism	A_96_p107309		Signalling (receptor kinases)	A_96_p139902		Signalling (kinase DUF26)
96_p50126 Secondary metabolism A_56_p13752 Transport A_56_p13753	A_96_p132	2752	1	Secondary metabolism	A_96_p029251		Sugar metabolism	A_96_p139507		Signalling (kinase DUF26)
96. p057861 Secondary metabolism A, %p. p17929 Transport (ABC transporters) A, 96. p123722 Signalling (Exportein) A, 96. p123727 Signalling (Exportein) A, 96. p12372 Signalling (Exportein) Signalling (Exportei	A_96_p160	0186		Secondary metabolism	A_96_p132062		Transport	A_96_p138737		Signalling (kinase LRR)
signaling (G-protein)	A_96_p067	7861	-	Secondary metabolism	A_96_p197854	1	Transport (ABC transportant)	A_96_p125732		Signalling (kinase LRR)
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9. j13422 Signalling (Light) Inducible platsch-lipid associated protein) A.96 j.137829 Transport (amino acids) A.96 j.075849 Signalling (Phox (Pk) domain containing protein) A.96 j.015820 Transport (amino acids) A.96 j.075849 Signalling (Phox (Pk) domain containing protein) A.96 j.015820 Transport (amino acids) A.96 j.075849 Signalling (Phox (Pk) domain containing protein) A.96 j.015820 Transport (amino acids) A.96 j.026840 Signalling (Phox (Pk) domain containing protein) A.96 j.015836 Wound responsive A.96 j.026846 Yound responsive </td <td>A_96_p248</td> <td>5/27</td> <td></td> <td>Signalling (G-protein)</td> <td>A 96 p024661</td> <td></td> <td>Transport (ABC transporters)</td> <td>A_96_p074664</td> <td>-</td> <td>Signalling (kinase)</td>	A_96_p248	5/27		Signalling (G-protein)	A 96 p024661		Transport (ABC transporters)	A_96_p074664	-	Signalling (kinase)
96.p120972 Signalling (Phox (Pk) domain containing protein) A.96.p12092 Transport (amino acids) A.96.p12092 Signalling (sugrand nutrient physiology) 96.p120972 Signalling (Phox (Pk) domain containing protein) A.96.p12092 Transport (amino acids) A.96.p12092 TCA cycle 96.p125349 Signalling (receptor kinases) A.96.p05396 Wound responsive A.96.p03181 TCA cycle 96.p123491 Xenobiotic detoxification A.96.p12092 Transport (amino acids) A.96.p03181 Transport (AD transporten) 96.p13491 Xenobiotic detoxification A.96.p12082 Wound responsive A.96.p03284 Transport (AD transporten) 4.96.p120801 A.96.p120801 Xenobiotic detoxification A.96.p12469 Transport (AD transporten) 4.96.p120801 A.96.p120801 Xenobiotic detoxification A.96.p12469 Transport (AD transporten) 4.96.p120801 A.96.p126091 A.96.p124092 Transport (AD transporten) Transport (AD transporten) VDP DOWN A.96.p124091 RNA regulation of transcription A.96.p124292 Transport (amino acids) VDP DOWN A.96.p124501 Major CHO metabolism A.96.p012429 Transpo	A 96 o121	422		Signalling (Light) Inducible plastid-linid associated protein)	A_96_p187999		Transport (amino acids)	A_96_p073989		Signalling (light)
96, p.178349 Signalling (receptor kinases) A. 96, p.07846 Transport (amino acids) A. 96, p.028461.2 TCA cycle 96, p.018349 Wound responsive A. 96, p.001816 Wound responsive A. 96, p.001816 TCA cycle 96, p.018349 Wound responsive A. 96, p.001816 Wound responsive A. 96, p.001816 TCA cycle 96, p.018349 Xenobiotic detoxification A. 96, p.001816 Wound responsive A. 96, p.001816 Transport (ABC transporters) A. 96, p.01841 Xenobiotic detoxification A. 96, p.012827 Transport (ABC transporters) A. 96, p.01861 Xenobiotic detoxification A. 96, p.012827 Transport (ABC transporters) View A. 96, p.012801 Xenobiotic detoxification A. 96, p.012827 Transport (ABC transporters) View A. 96, p.012801 Xenobiotic detoxification A. 96, p.01282607 Transport (ABC transporters) UP DOWN A. 96, p.012801 RNA regulation of transcription A. 96, p.012820 Transport (ABC transporters) UP DOWN A. 96, p.016754 Secondary metabolism A. 96, p.012820 Transport (ABC transporters) A. 96, p.016784 Secondary metabolism	A 96 n120	972		Signalling (Phox (Px) domain containing protein)	A_96_p120962		Transport (amino acids)	A_96_p140177		Signalling (sugar and nutrient physiology)
36_0015946 Wound responsive A.96_0001811 LCA (yet participant) 36_0141491 Xenobiotic detoxification A.96_001811 Transport (All Cransporter) 36_0141491 Xenobiotic detoxification A.96_001811 Transport (All Cransporter) 36_0141491 Xenobiotic detoxification A.96_001811 Transport (All Cransporter) 36_0141491 Xenobiotic detoxification A.96_0121601 Transport (All Cransporter) 36_0114191 Xenobiotic detoxification A.96_0121601 Transport (All Cransporter) 36_0114191 Xenobiotic detoxification A.96_0121601 Transport (All Cransporter) 4_05_0126091 Xenobiotic detoxification A.96_0121601 Transport (all Cransporter) 4_05_0126091 RA regulation of transcription A.96_0122607 Transport (all Cransporter) 4_05_0126091 RA regulation of transcription A.96_0202401 Transport (all Cransporter) 4_05_01267341 Secondary metabolism A.96_0200141 Transport (MAT) A.96_002501 Upl metabolism A.96_0001401 Transport (MAT) A.96_012612 Transport (MAT) A.96_00266456 Transport (MAT) A.96_012612 Lipid metabolism A.96_0026400 UDP eyonytransferases A.96_05055131 Lipid metabolism A.96_0021346 </td <td>A 96 p176</td> <td>5349</td> <td></td> <td>Signalling (receptor kinases)</td> <td>A_96_p169764</td> <td></td> <td>Transport (amino acids)</td> <td>A_96_p248612</td> <td></td> <td>TCA cycle</td>	A 96 p176	5349		Signalling (receptor kinases)	A_96_p169764		Transport (amino acids)	A_96_p248612		TCA cycle
96_p143491 Xenobiotic detoxification A_96_p02486 Wound responsive PL_responsive Transport (ARC transporters) A_96_p12669 Xenobiotic detoxification A_96_p02269 Transport (ARC transporters) A_96_p226093 Xenobiotic detoxification A_96_p12292 Transport (ARC transports) Logs(IT/C) A_96_p122692 Transport (amino adds) Logs(IT/C) A_96_p122692 Transport (amino adds) Logs(IT/C) A_96_p122692 Transport (amino adds) A_96_p123691 RNA regulation of transcription A_96_p122407 Transport (amino adds) VP DOWN A_96_p127892 Transport (amino adds) DOWN A_96_p127892 Transport (amino adds) A_96_p127892 Transport (admon/int A_96_p127892 Transport (admon/int A_96_p12789 Transport (admon/int A_96_p12789 Transport (admon/int A_96_p12789 Transport (admon/int A_96_p001786 Transport (MATE) A_96_p001786 Transport (MATE) A_96_p001786 Transport (MATE) A_96_p001786 Transport (admon/int A_96_p001786 UDP g/voy/transferases A_96_p01526 UDP g/voy/transferases A_96_p01526 UDP g/voy/transferases	A_96_p045	5946		Wound responsive	A_96_p009156		Wound responsive	A_96_p031811 A 96_p033501		Transport (ABC transporters)
A 36, 5120393 Xenobiotic detaxification A, 36, 2124693 Transport (ABC transporten) L0g, [T/C) A, 36, 5123093 Xenobiotic detaxification A, 36, 5124592 Transport (ABC transporten) L0g, [T/C) A, 36, 5124093 Biotic stress A, 36, 5124592 Transport (ABC transporten) L0g, [T/C) A, 36, 5126072 Hormone metabolism (Auxin) A, 36, 5124592 Transport (ABC transport (ABC transport) UP DOWN A, 96, 5125072 Hormone metabolism (Auxin) A, 36, 5124607 Transport (ABC transport (ABC transport (ABC transport) UP DOWN A, 36, 512507 Major CHO metabolism A, 96, 5124607 Transport (ABC transport (ABC transport) A, 36, 516754 Secondary metabolism A, 96, 500512 Transport (MATE) A, 36, 5167554 Secondary metabolism A, 96, 500512 Transport (MATE) A, 36, 5167554 Secondary metabolism A, 96, 500512 Transport (MATE) A, 36, 5167554 Secondary metabolism A, 96, 500512 Transport (MATE) A, 36, 5167554 Secondary metabolism A, 96, 500514 Transport (MATE) A, 36, 5167554 Secondary metabolism A, 96, 500506 UDP glyconytransferases A, 36, 5167551 Lipid metabolism A, 96, 500506 UDP glyconytransferases <td< td=""><td>A_96_p143</td><td>3491</td><td></td><td>Xenobiotic detoxification</td><td>A_96_p054486</td><td></td><td>Wound responsive</td><td>A_96_p092854</td><td></td><td>Transport (ABC transporters)</td></td<>	A_96_p143	3491		Xenobiotic detoxification	A_96_p054486		Wound responsive	A_96_p092854		Transport (ABC transporters)
A 56, 22302 Xenobiolic detoxificación A, 56, 212927 Transport (amino acids) L0g,[T/C) A 56, 2024081 Biotic stress A, 96, 212927 Transport (amino acids) A 56, 2024081 Biotic stress A, 96, 2124927 Transport (aminonium) A 56, 2025912 Hormone metabolism (Auxin) A, 96, 2124927 Transport (aminonium) UP DOWN A 56, 2025914 RNA regulation of transcription A, 96, 2026402 Transport (adium) A 56, 2015914 RNA regulation of transcription A, 96, 2016412 Transport (adium) A 56, 2015914 RNA regulation of transcription A, 96, 2016412 Transport (adium) A 56, 2017854 Secondary metabolism A, 96, 2016512 Transport (MATE) A 56, 2018912 Upid metabolism A, 96, 2006476 Transport (MATE) A 56, 2018213 Upid metabolism A, 96, 2006476 UDP givcoyitransferases A 56, 2018216 Upid metabolism A, 96, 201346 UDP givcoyitransferases A 56, 2018216 Upid metabolism A, 96, 201346 UDP givcoyitransferases					A_96_p160861		Xenobiotic detoxification	A_96_p214649		Transport (ABC transporters)
Log_(T/C) A90_(j2408) Blots stress A96_(j24592) Transport (ammonium) UP A96_(j25672) Hormone metabolism (Auxin) A96_(j25672) Transport (ammonium) UP DOWN A96_(j25672) RNA regulation of transcription A96_(j25242) Transport (ammonium) UP DOWN A96_(j25672) RNA regulation of transcription A96_(j2672) Transport (admonium) A96_(j26724) RNA regulation of transcription A96_(j26724) Transport (admonium) A96_(j26734) Secondary metabolism A96_(j26734) Transport (MATE) A96_(j267354) Lipid metabolism A96_(j26734) Transport (intrate) A96_(j267354) Lipid metabolism A96_(j26034) Transport (MATE) A_96_(j263541) Lipid metabolism A96_(j26034) UDP glycoy(transferases) A_96_(j26356) Lipid metabolism A96_(j21346) UDP glycoy(transferases) A_96_(j26356) RNA regulation of transcription A96_(j26044) UDP glycoy(transferases)					A_96_p236093		Xenobiotic detoxification	A_96_p122927	the second second	Transport (amino acids)
A_50_p124272 Hormone metaooism (Auxin) A_50_p05861 Internationality UP DOWN A_56_p02514 RNA regulation of transpirition A_56_p124242 Transport (Caldium) UP DOWN A_56_p124507 Major CHO metabolism A_56_p124242 Transport (Caldium) A_56_p126754 Secondary metabolism A_56_p1024512 Transport (Caldium) A_56_p102797 Transport (Initrate) A_56_p005815 Transport (Initrate) A_56_p1028515 Lipid metabolism A_56_p005826 UDP gixcosystransferases A_56_p005815 Lipid metabolism A_56_p0011346 UDP gixcosystransferases A_56_p005815 Lipid metabolism A_56_p0011346 UDP gixcosystransferases				Log ₂ (T/C)	A_96_p024081		Normone metabolism (Aunie)	A_96_p124592		Transport (ammonium)
UP DOWN A 56 p124507 Major CHO metabolism A, 96 p124242 Transport (Adjon/H A, 96 p127545 Secondary metabolism A, 96 p10812 Transport (Major (Initianic protein) A, 96 p107545 Secondary metabolism A, 96 p00514 Transport (MATE) A, 96 p00526 Transport (Initrate) A, 96 p00526 UDP giveosytransferases A, 96 p0052612 Upid metabolism A, 96 p00216 UDP giveosytransferases A, 96 p0052612 Upid metabolism A, 96 p00136 UDP giveosytransferases					A_96_p126972		BNA regulation of transcription	A_96_p056861 A_96_p124607		Transport (calcium)
UP DOWN A_36_p167854 Secondary metabolism A_96_p10812 Transport (Margi intrinsic protein) A_36_p10797 Transport (nitrate) A_96_p005145 Transport (Margi intrinsic protein) A_36_p10797 Transport (nitrate) A_96_p00526 Transport (Margi intrinsic protein) A_36_p00526 Lipid metabolism A_96_p00266 UDP givcopitransferases A_36_p00526 Lipid metabolism A_96_p002146 UDP givcopitransferases A_36_p005361 Lipid metabolism of transpription A_96_p001346 UDP givcopitransferases					A 96 p124507		Major CHO metabolism	A_96_p124242		Transport (cation/H
A_96_p109797 Transport (nitrate) A_96_p205154 Transport (MATE) A_96_p063545 Upid metabolism A_96_p008266 UDP glyconytransferases A_96_p0163261 Upid metabolism A_96_p008266 UDP glyconytransferases A_96_p0163261 RNA regulation of transcription A_96_p001346 UDP glyconytransferases				UP DOWN	A 96 p167854		Secondary metabolism	A_96_p108512		Transport (Major intrinsic protein)
A_96_p085815 Lipid metabolism A_96_p00520 Up and the point (nitrate) A_96_p082615 Lipid metabolism A_96_p00226 UDP givcosyltransferases A_96_p082616 Lipid metabolism A_96_p082136 UDP givcosyltransferases A_96_p082616 RNA regulation of transcription A_96_p081264 UDP givcosyltransferases					A_96_p109797		Transport (nitrate)	A_96_p205154		Transport (MATE)
A 96 p163261 Upid metabolism A 96 p041346 UDP glycosyltransferases A 96 p064356 RNA regulation of transcription A 96 p081064 UDP glycosyltransferases					A_96_p085815		Lipid metabolism	A 96 p008266		UDP glycosyltransferases
A 96_p064356 RNA regulation of transcription A 96_p081064 UDP glycosyltransferases					A_96_p163261		Lipid metabolism	A_96_p041346	1	UDP glycosyltransferases
					A_96_p064356		RNA regulation of transcription	A_96_p081064	-	UDP glycosyltransferases

FIGURE 5 | (A) Heat Map of representative sequences differentially expressed in leaves of *S. lycopersicon* plants by both EM dosages (0.1 mL L⁻¹ and EM 1 mL L⁻¹). **(B)** Heat Map of representative sequences that showed differential expression in leaves of *S. lycopersicon* treated with a definite EM dosage. Specifically, on the left are the sequences differentially expressed only under EM 0.1 mL L⁻¹, on the right are sequences differentially expressed only under EM 1 mL L⁻¹. The corresponding metabolic pathways associated to the sequences are reported on the right side, while the Agilent codes are shown on the left side of each column. Different tones of blue and red indicate up-regulation and down-regulation, respectively.

			В	M 0.1 -			EM 1	
			A_96_p119187	Abiotic stress		A_96_p073634		Abiotic stress
	Roots		A_96_p193869 A_96_p187919	Abiotic stress Abiotic stress		A_96_p211104		Abiotic stress (drought/salt)
		_	A_96_p121032 A 96 p209074	Abiotic stress (drough Abiotic stress (heat)	t/salt)	A_96_p054796		Abiatic stress (heat)
A i	MO1 EM	1	A_96_p069509	Abiotic stress (heat)		A_96_p128672 A_96_p107864		Amino acid metabolism
		<u>.</u>	A_96_p246522 A_96_p016101	Abiotic stress (heat) Amino acid metabolisi	m	A_96_p094614 A_96_p097229		Amino acid metabolism Amino acid metabolism glutamate
A_96_p188834 A 96_p181024		Abiotic stress (heat)	A_96_p134792 A 96 p171154	Amino acid metabolisi Biotic stress signalling	m	A_96_p015986 A_96_p173669		Amino acid metabolism proline Amino acid metabolism tyrosine
A_96_p099674	14 C	Abiotic stress (heat)	A_96_p127582	C1-metabolism		A_96_p153111 A_96_p153111		Biotic stress Biotic stress
A_96_p223239 A_96_p136212		Abiotic stress (heat) Abiotic stress (heat)	A_96_p219604 A_96_p217964	Cell wall modification Cell wall pectin estera	ses (PME)	A_96_p044341		Biotic stress (PR-proteins)
A_96_p026456 A 96 p246567	-	Abiotic stress (heat)	A_96_p081634	Cell wall pectin estera	ses (PME)	A_96_p032731		Cell organisation
A_96_p187884	7	Biotic stress Biotic stress	A_96_p203739	Cell wall degradation	ses acetyl esterase	A_96_p199019		Cell wall modification
A_96_p115977 A_96_p183174		Biotic stress Cell division	A_96_p213399 A 96 p151431	DNA repair DNA repair		A_96_p229144 A_96_p123682		Cell wall synthesis Cell wall synthesis
A_96_p068421	-	Cell organisation	A_96_p212484	Enzyme families (acyl	transferases)	A_96_p077869 A_96_p200099	-	Enzyme families (phosphatases) Enzyme families (beta 1,3 glucan hydrolases)
A_96_p120607	-	Cell wall modification Development	A_96_p158521 A_96_p062435	Enzyme families (cyto Enzyme families (cyto	chrome P450) chrome P450)	A_96_p138582 A_96_p007546		Enzyme families (cytochrome P450) Enzyme families (cytochrome P450)
A_96_p145236 A_96_p177699		Development Development	A_96_p161731 A_96_p077029	Enzyme families (cyto Enzyme families (cyto	chrome P450) chrome P450)	A_96_p077019 A 96 p163281		Enzyme families (cytochrome P450) Enzyme families (cytochrome P450)
A_96_p246285		DNA.synthesis/chromatin structure.histone	A_96_p031951	Enzyme families (cyto	chrome P450)	A_96_p091460 A_96_p028364		Enzyme families (Glutathione 5 transferance) Enzyme families (Glutathione 5 transferance)
A_96_p152376		Enzyme families (cytochrome P450) Enzyme families (cytochrome P450)	A_96_p075014 A_96_p111867	Enzyme families (cyto Enzyme families (cyto	chrome P450) chrome P450)	A_96_p138797		Enzyma families (Nitrilases)
A_96_p077514 A_96_p034856		Enzyme families (cytochrome P450)	A_96_p124662	Enzyme families (O- m	ethyl transferases)	A_96_p197494		Enzyme families (Oxidases)
A_96_p045476		Enzyme families (glutathione S transferases)	A_96_p218874	Enzyme families (Oxid	ases)	A_96_p033111 A_96_p091654		Enzyme families (Oxidases) Enzyme families (Oxidases)
A_96_p055872		Enzyme families (misc.GDSL-motif lipase) Enzyme families (misc.GDSL motif lipase)	A_96_p014141	Enzyme families (Oxid Enzyme families (Pero	ases) xidases)	A_96_p118762 A_96_p151571		Enzyme families (Peroxidases) Enzyme families (Peroxidases)
A_96_p160891 A_96_p010066		Enzyme families (oxidases) Enzyme families (nernaidases)	A_96_p160381	Enzyme families (Pero	xidases)	A_96_p090874 A_96_p146941		Enzyme families (Peroxidases) Enzyme families (Phosphatases)
A_96_0107614		Enzyme families (UDP glucosyl and glucoronyl transferases)	A_96_p053992	Enzyme families (UDP	glucosyltransferases)	A_96_p031306 A_96_p156096		Enzyme families (UDP glucosyltransferases) Fermentation
A_96_p081064 A_96_p236018	-	Enzyme families (UDP glucosyl and glucoronyl transferases) Enzyme families (UDP glucosyl and glucoronyl transferases)	A_96_p132047 A_96_p215364	Enzyme families (UDP Hormone metabolism	glucosyltransferases) (Auxin)	A_96_p154161		Fermentation
A_96_p155271 A 96 p118662		Fermentation	A_96_p230164	Hormone metabolism	(Auxin)	A_96_p173249		Glycolysis Glycolysis
A_96_p008426		Hormone metabolism (Ethylene)	A_96_p150301	Hormone metabolism	(Auxin)	A_96_p070214		Hormone metabolism (ABA)
A_96_p040196 A_96_p155256		Hormone metabolism (Ethylene) Hormone metabolism (Ethylene)	A_96_p064001 A_96_p216359	Hormone metabolism Hormone metabolism	(Auxin) (Auxin)	A_96_p198919 A_96_p017671		Hormone metabolism (Auxin)
A_96_p079489		Hormone metabolism (Ethylene)	A_96_p162686	Hormone metabolism	(Auxin)	A_96_p107769 A_96_p177974		Hormone metabolism (Avain) Hormone metabolism (Avain)
A_96_p089644		Hormone metabolium (Ethylene) Hormone metabolium (Ethylene)	A_96_p155621 A_96_p147216	Lipid metabolism		A_96_p108522 A_96_p017276		Hormone metabolism (Auxin) Hormone metabolism (Ethylene)
A_96_p019616 A 96 p143326		Hormone metabolism (Ethylene)	A_96_p172219 A 96 p161186	Upid metabolism Lipid metabolism		A_96_p012601 A_96_p245145		Hormone metabolism (Ethylene) Hormone metabolism (Ethylene)
A_96_p188139		Hormone metabolism (Gibberellin)	A_96_p047671	Lipid metabolism		A_96_p030681		Hormone metabolism (Ethylene)
A_96_p008211 A_96_p018746		Hormone metabolism (Gibberellin) Hormone metabolism (Jasmonate)	A_96_p186749 A_96_p064156	Lipid metabolism Lipid metabolism		A_96_p040341		Hormone metabolism (Ethylene)
A_96_p113532 A_96_p189739		Hormone metabolism (Jasmonate)	A_96_p176154	Major CHO metabolist Major CHO metabolist	n	A_96_p201016 A_96_p248092		Hormone metabolism (Jasmonate)
A_96_p225839	a de la companya de la	Lipid metabolism	A_96_p186629	minor CHO metabolisi	m.callose	A_96_p200144 A_96_p007831		Lipid metabolism Lipid metabolism
A_96_p225804 A_96_p167029		Lipid metabolism Major CHO metabolism	A_96_p001431 A_96_p094779	Protein activation Protein activation		A_96_p119457 A_96_p167694		Lipid metabolism Major CHO metabolism
A_96_0206279		minor CHO metabolism	A_96_p067906	Protein postranslation	al modification	A_96_p160346 A_96_p132732		Major CHO metabolism Metal handling-acquisition
A_96_p247297		misc acid and other phosphatases	A_96_p114/02 A_96_p016476	Protein postranslation Protein postranslation	al modification	A_96_p113612 A_96_p225819		Metal handling-acquisition minor CHO metabolism
A_96_p074814 A_96_p066161		misc.acid and other phosphatases Potein targeting (nucleus)	A_96_p219814 A_96_p052751	Protein synthesis Protein synthesis		A_96_p144591		minor CHD metabolism
A_96_p220014		Potein targeting (secretory pathway, vacuole)	A_96_p009126	Protein synthesis		A_96_p113192		Protein (postranslational modification)
A_96_p163051		Protein dargeting (secretory pathway) Protein degradation (cysteine protease)	A_96_p203494 A_96_p175519	Protein synthesis Protein synthesis		A_96_p233959 A_96_p172359		Protein (postranslational modification)
A_96_p028556 A 96 p051021	et al a series de la	Protein degradation (Ubiquitin dependent)	A_96_p176429	Protein synthesis Protein synthesis		A_96_p030001 A_96_p124987		Protein activation Protein degradation (AAA type)
A_96_p228839	the second s	Protein degradation (Ubiquitin dependent)	A_96_p106349	Protein targeting (chic	woplast)	A_96_p044396 A_96_p231399		Protein degradation (AAA type) Protein degradation (subtilases)
A_96_p041071 A_96_p230747		Protein synthesis Protein synthesis	A_96_p213364 A_96_p053521	Protein targeting (nuc Protein targeting (nuc	leus) leus)	A_96_p121427		Protein degradation (subtilases) Protein degradation (Ubiquitin dependent)
A_96_p059781 A_96_0150911		Protein synthesis Redox home	A_96_p160316	Redox (thioredoxin)		A_96_p040721		Protein degradation (Ubiquitin dependent) Redex (diumitaises and ratalases)
A_96_p206574		RNA regulation of transcription	A_96_p185354	RNA regulation of tran	scription	A_96_p201369		Redox (heme)
A_96_p161991 A_96_p054441		RNA regulation of transcription RNA regulation of transcription	A_96_p103199 A 96 p144946	RNA regulation of tran BNA regulation of tran	scription	A_96_p019601	5	Redox (thioredoxin) Redox (thioredoxin)
A_96_p146426		RNA regulation of transcription	A_96_p166274	RNA regulation of tran	scription	A_96_p050501 A_96_p228739		RNA regulation of transcription RNA regulation of transcription
A_96_p017141		RNA regulation of transcription	A_96_p134962	INA regulation of tran	ncription	A_96_p044086 A_96_p044476		RNA regulation of transcription RNA regulation of transcription
A_96_p247132 A 96 p184439		RNA regulation of transcription RNA regulation of transcription	A_96_p022701 A_96_p053506	RNA regulation of tran RNA regulation of tran	scription	A_96_p163836 A 96 p045291		RNA regulation of transcription RNA regulation of transcription
A_96_p038446	-	RNA regulation of transcription	A_96_p193114	RNA regulation of tran	scription	A_96_p214054 A 96_p201224		RNA regulation of transcription RNA regulation of transcription
A_96_p185834	-	RNA regulation of transcription RNA regulation of transcription	A_96_p108822 A_96_p116022	RNA regulation of tran RNA regulation of tran	scription	A_96_p064191		RNA regulation of transcription
A_96_p054591 A_96_p131782		RNA regulation of transcription RNA regulation of transcription	A_96_p230484	RNA regulation of tran	scription	A_96_p150106		RNA regulation of transcription
A_96_p097479		Secondary metabolism	A_96_p151316	RNA regulation of tran	scription	A_96_p246512 A_96_p086494		RNA regulation of transcription
A_96_p188479 A_96_p181189		Secondary metabolism Secondary metabolism	A_96_p113102 A_96_p121252	Secondary metabolism Secondary metabolism	n.	A_96_p196499 A_96_p169004		RNA regulation of transcription RNA regulation of transcription
A_96_p057921 A 96 p130807		Secondary metabolism Secondary metabolism	A_96_p197729	Secondary metabolism		A_96_p141177 A_96_p206539		RNA regulation of transcription Secondary metabolism
A_96_p176424		Secondary metabolism	A_96_p187699	Secondary metabolish Secondary metabolish	n	A_96_p058846 A_96_p260947		Secondary metabolism Secondary metabolism
A_96_p145996 A_96_p108952	-	Secondary metabolism Signalling (calcium)	A_96_p231314 A_96_p191069	Secondary metabolism Signalling (calcium)	n.	A_96_p012961		Secondary metabolism Secondary metabolism
A_96_p151561	2 (A 197	Signalling (calcium)	A_96_p001491	Signalling (calcium)		A_96_p177374		Secondary metabolism
A_96_p138737	·	Signalling (receptor kinases leucine rich repeat II)	A_96_p158126 A_96_p048176	Signalling (calcium) Signalling (calcium)		A_96_p102229 A_96_p146881		Signalling (calcium)
A_96_p134872 A 96 p227069		Signalling (receptor kinases DUF 26) Signalling (receptor kinases extensio)	A_96_p134332	Signalling (light)	inites 1	A_96_p062496 A_96_p065726		Signalling (calcium)
A_96_p001466		Signalling (receptor kinases)	A_96_p219429	Signalling (phosphinos	itides)	A_96_p008131 A_96_p255102		Signalling (G-proteins) Signalling (G-proteins)
A_96_p237427 A_96_p232634		signating (sugar and nutrient physiology) Signalling (sugar and nutrient physiology)	A_96_p118482 A_96_p044186	Signalling (receptor ki Signalling (receptor ki	nases DUF 26) nases DUF 26)	A_96_p163356 A_96_p158141		Signalling (MAP kinases) Signalling (MAP kinases)
A_96_p087979	-	TCA Transport	A_96_p186839	Signalling (receptor kin	nases LRR)	A_96_p061786		Signalling (phosphinositides) Signalling (receptor kinases DUF 26)
A_96_p227439	1 mar 1	Transport	A_96_p226759	Signaling (receptor lo	all associated kinase)	A_96_p021311		Signalling (leucine rich repeat XI) Signalling (sugar and publicated obsold
A_96_p136562 A_96_p202479		Transport Transport	A_96_p157751 A_96_p005563	signalling G-proteins		A_96_p105791 A_96_p227384		Signalling (sugar and nutrient physiology)
A_96_p201344		Transport (ABC transporters)	A_96_p141122	signalling G-proteins		A_96_p113632 A_96_p193269		TCA
A_96_p221699 A_96_p072339	-	Transport (amino acids) Transport (cyclic nucleotide or calcium regulated channels)	A_96_p190354 A_96_p220949	TCA		A_96_p125497 A_96_p112857		TCA TCA
A_96_p229549		Transport (cyclic nucleotide or calcium regulated channels) Transport (cyclic nucleotide or calcium regulated channels)	A_96_p226599	Transport		A_96_p025311 A 96_p143955		Transport Transport
A_96_p179804		Transport (peptides and oligopeptides)	A_96_p101519 A_96_p030701	Transport Transport (ABC transp	orters)	A_06_p212529		Transport
A_96_p016146 A_96_p206324		Transport (peptides and oligopeptides) Transport (peptides and oligopeptides)	A_96_p237387 A 96 p198809	Transport (ABC transp Transport (ABC transp	orters)	A_96_0247757		Transport (amino acids)
A_96_p102244		Transport ABC transporters)	A_96_p068346	Transport (ABC transp	orters)	A_96_p197884 A_96_p115027		Transport (amino acids)
			A_96_p108532 A_96_p058016	Transport (ABC transp Transport (metal)	orters)	A_96_p070104 A_96_p051031		Transport (peptides and oligopeptides) Transport (peptides and oligopeptides)
			A_96_p182089	Transport (metal)		A_96_p016501 A_96_p209869		Transport (peptides and oligipeptides) Transport (phosphate)
			A_96_p116062	Transport (metal) Transport (nucleotide	ι)	A_96_p123757		Transport (potassium)
		Log_(T/C)	A_96_p248192	Transport (peptides an Transport (pe	nd oligopeptides)	A_96_p146486		Transport (sugars)
			and the second sec	I I MILLION Y THE REAL PROPERTY AND		A per care and the		TO BE AND A DECEMBER OF THE ADDRESS

FIGURE 6 | (A) Heat Map of representative sequences differentially expressed in roots of *S. lycopersicon* plants by both EM dosages (0.1 mL L^{-1} and EM 1 mL L⁻¹). **(B)** Heat Map of representative sequences that showed differential expression in leaves of *S. lycopersicon* treated with a definite EM dosage. Specifically, on the left are the sequences differentially expressed only under EM 0.1 mL L⁻¹, on the right are sequences differentially expressed only under EM 1 mL L⁻¹. The corresponding metabolic pathways associated to the sequences are reported on the right side, while the Agilent codes are shown on the left side of each column. Different tones of blue and red indicate up-regulation and down-regulation, respectively.



plants than in the untreated. In the case of S and Fe, maximum values were recorded in leaves of plants treated with EM at the concentration of 1 mL L^{-1} (2 and 3.4 higher than the control, respectively). Nitrogen concentration in plants was increased by about 11%.

Effects of EM on Total Antioxidant Capacity and Content of Phenolic Compounds and Soluble Sugars

The analysis of the TAC in S. *lycopersicon* plants treated with EM at two dosages evidenced the efficacy of the test product to stimulate this parameter (**Table 3**). The degree of TAC improvement by EM 0.1 ml L^{-1} was similar between leaves (+52%) and roots (+58%), and it was generally higher than that caused by EM 1 ml L^{-1} (+115 and +135% in leaves and roots, respectively).

The same trend was observed for the amount of total phenol compounds (**Table 3**), which was more increased following the application to plants of EM at the lower dose (+26 and +34% in leaves and roots, respectively). Indeed, when EM was applied at 1 ml L^{-1} the total phenol content did not appreciably vary in roots, whereas in leaves it was enhanced by +15% compared to the control plants.

With respect to soluble sugars, the content of these compounds was significantly increased in leaves of plants treated with EM 1 ml L^{-1} (+186 and +161% for glucose and fructose, respectively), but no substantial effect was determined by EM 0.1 ml L^{-1} (**Table 3**). Conversely, EM at both dosages was responsible for higher soluble sugar accumulation in roots. EM at 0.1 ml L^{-1} in particular, caused the maximum increase in glucose (+140%) and fructose (+287%) in this tissue.

DISCUSSION

The use of biostimulants in agriculture to improve plant yield by enhancing metabolic processes and resistance to abiotic and biotic stresses has attracted growing interest worldwide (Calvo et al., 2014; Nardi et al., 2016; Posmyk and Szafrańska, 2016). These products do not act on plant metabolism directly; rather, they seem to interact with plant-signaling cascades events that trigger the mitigation of negative plant performance responses associated to environmental stress (Brown and Saa, 2015).

Due to the chemical complexity of biostimulant formulation and their content in multiple bioactive substances, the precise molecular mechanisms though which biostimulants act in plants is of hard unraveling (Bulgari et al., 2015; Nardi et al., 2016). On this account, the current study used cDNA microarray in order to get a better framework of the molecular networks that may be envisioned as potential targets for the development of more efficient protein hydrolysates in the market of biostimulants.

TABLE 2 | Concentration of nitrogen (N), sulfur (S), phosphorus (P), potassium (K), and iron (Fe) in leaves of Solanum lycopersicon plants.

	Ν	S	Р	к	Fe
	% (w/w)		mg k	g ⁻¹	
Control	5.5 ± 0.1a	1405.2 ± 63.2a	736.2 ± 17.4a	$7406.9 \pm 46.3a$	94.5 ± 12.0a
EM 0.1	$6.1 \pm 0.2 b$	$2052.2 \pm 56.2b$	$5420.1 \pm 223.1b$	$47716.2 \pm 502.2b$	$124.6 \pm 21.2a$
EM 1	$6.2\pm0.1b$	$2840.1 \pm 35.1 c$	$4885.7 \pm 225.2b$	$48979.1 \pm 439.8b$	$325.6 \pm 23.5 b$

Different letters along individual columns indicate significant differences between treatments (P < 0.05; $n = 3, \pm SD$).

TABLE 3 Content of soluble sugars (glucose and fructose) and total phenols
(TP), and total antioxidant capacity (TAC).

	Glucose	Fructose	ТР	TAC
	mg g ⁻	¹ FW	mg GA eq kg ⁻¹ FW	mg Fe ² kg ⁻¹ FW
Leaves				
Control	$1.96\pm0.18b$	$2.45\pm0.32c$	$0.36\pm0.02b$	$4.63\pm0.22b$
EM 0.1	$2.09\pm0.33b$	$1.76\pm0.47b$	$0.43\pm0.03a$	$6.40\pm0.31a$
EM 1	$5.61 \pm 0.29a$	$6.41 \pm 0.58a$	$0.41\pm0.02a$	$5.15\pm0.19a$
Roots				
Control	0.51 ± 0.12	0.32 ± 0.10	$0.18\pm0.02a$	$1.35\pm0.12a$
EM 0.1	1.22 ± 0.21	1.24 ± 0.15	$0.23\pm0.03a$	$2.13\pm0.14b$
EM 1	1.11 ± 0.19	0.88 ± 0.17	$0.19\pm0.03a$	$1.82\pm0.30\mathrm{b}$

Different letters along columns, distinctly for leaves and roots, indicate significant differences (P < 0.05) among treatments.

Two different concentrations of the biostimulant EM were tested for their effects in tomato, one of the major crop cultivated globally. The in-depth bioinformatic analysis of all the data produced by microarray highlighted that EM induced some important metabolic activities and cell processes in this plant species. Specifically, a number of genes whose expression showed significant variation in response to EM were involved in plant development, photosynthesis, primary C and N metabolism. The up-regulation of these genes justified the greater leaf and root biomass production of tomato plants treated with EM at both dosages compared to the no-treated plants. In a previous work, the increase of growth parameters in tomato plants treated with plant-derived protein hydrolysates was correlated with the stimulation of nitrogen uptake and assimilation (Colla et al., 2014), and in another study the same product EM was reported to promote N assimilation in maize plants via a coordinated up-regulation of the activity of enzymes and expression of genes implied in carbon C and N metabolism (Schiavon et al., 2008). Similarly, in tomato plants we found that EM increased the expression of N assimilation-related genes coding for NR, aspartate AST, glutamine-dependent asparagine synthetase (ASN1) and GS. Additional N-associated genes involved in the synthesis and turnover of amino acids (e.g., glutamate dehydrogenase, serine decarboxylase, aspartyl protease) and in protein synthesis and modification (particularly translation initiation factors, elongation factors Tu and 1-alpha, aminoacyl-tRNA synthetases, ubiquitin-conjugating enzymes, polyubiquitin) were up-regulated by EM. At the same time, we observed higher transcript accumulation of key genes of the major C metabolism, primarily malate dehydrogenase (MDH), phosphoenolpyruvate carboxylase (PEPC), fumarate dehydrogenase (FDH), and phosphoenolpyruvate carboxylase kinase 2 (PPCK2).

Increased N and Fe accumulation and utilization in leaves can account for enhanced photosynthesis and improved translocation of photosynthates from the sources to the sinks that contribute to the improved plant biomass of plants treated with the protein hydrolysate. In support of this hypothesis, EM promoted N and Fe accumulation in tomato plants. In addition,



EM increased the expression of a sugar transporter in leaves and SPAD index values, the latters functioning as indicators of chlorophyll production and photosynthetic efficiency. It also induced the up-regulation of genes coding for components of the photosynthetic electron transfer chain (e.g., ferredoxin-2, the light-harvesting complex protein LHCA5, a chloroplast ATP synthase chain precursor) and the enzyme Ribulose-1,5bisphosphate carboxylase/oxygenase (RuBisCo), responsible for the process of CO_2 fixation in the Calvin cycle. As a result of this positive effect on photosynthesis, the increase in content of soluble sugars (glucose and fructose) was observed in EM-treated tomato plants. Previous studies reported higher accumulation of sugars (Schiavon et al., 2008; Ertani et al., 2011a) and RuBisCo activity (Ertani et al., 2011a) in maize plants after application of either EM or lignosulfonate-humates.

It is noteworthy that EM in tomato plants stimulated the transcription of a gene coding for a photosystem II



involved in defense, antioxidant activities, plant interactions with organisms, growth and development.

22 kda protein, which is not strictly necessary for efficient light harvesting and photosynthesis, but plays a key role in non-photochemical quenching, a process that preserves the balance between dissipation and utilization of light energy to minimize generation of reactive oxygen species (ROS), thereby preventing plants from photo-oxidative damages.

In addition to improved nutrient use efficiency and photosynthesis, EM influenced the capacity of tomato plants to absorb, translocate and allocate nutrients in different organs by modulating the expression of genes coding for ATPases and proteins that mediate the transport of inorganic elements (e.g., N, S, P, K, Cu, Fe) and organic molecules (mainly amino acids, peptides and sugars) over cells membranes.

EM treatment, for instance, induced higher expression of the nitrate transporter NTP2, which is homolog to the *Arabidopsis thaliana* AtNRT1;4 functioning in leaf nitrate homeostasis (Chiu et al., 2004), and the ammonium transporter AMT1.1. Genes coding for sulfate transporters like SULTR 2;1, which plays a role in xylem loading and root-to-shoot transport of sulfate (Kawashima et al., 2011; Maruyama-Nakashita et al., 2015), and SULTR 3;1, which is chloroplast localized and mediates the entry

of sulfate into the plastids for assimilation into S-amino acids (Cao et al., 2013), as well as the phosphate transporter PT2, the iron-phytosiderophore transporter protein yellow stripe 1 (YS1), potassium channels and copper transporters, were also increased in expression by EM. The up-regulation of nitrate and sulfate transporters, as well as ATPases, by biostimulants was previously described in other plant species (Canellas et al., 2002; Quaggiotti et al., 2004; Jannin et al., 2013). These data indicate that EM can promote the transport of nutrients in tomato plants likely by acting on cell membranes properties.

Consistently with higher expression of nutrient transporters, EM treated-tomato plants exhibited higher foliar accumulation of mineral elements such as N, S, P, K, and Fe, with the most pronounced increase reported for P and K. The effect on mineral nutrition was also reported in a recent study conducted in maize plants using another protein-based hydrolysate (Santi et al., 2017).

The way through which EM modifies the membrane permeability to favor the movement of nutrients may be at least partly ascribed to changes in root architecture shape and development via an auxin-signaling mediated pathway. The EM characterization described in Ertani et al. (2014) reported the presence of IAA (18.5 nmol mg^{-1} C) in the formulation. Previous work showed that auxin-like molecules of microbial origin contained in humic substances could influence plant growth by eliciting auxin-dependent signals that enhance the production of lateral root formation (Trevisan et al., 2010). Several genes encoding for auxin-responsive proteins had significant higher expression in EM-treated tomato plants and might be partly responsible for the observed increase in root biomass. Furthermore, a number of growth regulating factors (GRF3, GRF5), lob domain proteins, and expansins genes were up-regulated by EM. Expansins particularly, mediate cell wall loosening during cell growth and may have a role in improving stress tolerance (Marowa et al., 2016). Expression of expansins was correlated with expression of genes involved in the synthesis (e.g., cellulose synthase) and degradation (e.g., pectinesterases, polygalacturonases, and lipases) of cell wall and membrane structural components.

There are evidences that biostimulants help plants to overcome different biotic and abiotic stress situations (Joubert and Lefranc, 2008; Ertani et al., 2013b). Several EM-responsive genes identified via microarray were implied in detoxification and oxidative stress resistance. Tomato plants treated with EM showed increased TAC of ROS that are usually generated at high levels under stress. Among the genes with a key role in mitigating oxidative stress, the main represented were glutathione peroxidase (GPX), glutathione reductase (GR), GST, peroxidases, thioredoxins, and DHAR. Interestingly, most of these genes are implied in the glutathione/ascorbate detoxifying cycle, thereby suggesting that this pathway may be an important target of the biostimulant mode of action.

Also, EM treatment caused the up-regulation of genes involved in defense systems and plant-organism interactions, perhaps via modulation of the synthesis and signaling of defense hormones [ethylene, jasmonic acid, abscissic acid (ABA), salicylic acid (SA)] by elicitors (phytoactivators) contained in the biostimulant, such as auxins, phenols, amino acids and peptides. Algae extracts from Laminaria digitata, for instance, can induce natural immunity/resistance in plants without exerting any direct antimicrobial activity by virtue of their content in phytoactivators (Joubert and Lefranc, 2008). In support of our hypothesis, the expression of ethylene biosynthetic genes and ethylene/JA/ABA- responsive genes was higher in plants endowed with EM. The hormone signaling pathway elicited by EM likely triggers a cascade of phosphorylation events mediated by a variety of protein kinases (primarily MAPKKK21, CPK28, CRCK3, Pi kinase, LRR kinases, CPK9, WAK2, PEPKR2), which ultimately leads to the transcription of defense-related genes (particularly Cytochrome 450, leucine rich repeat proteins, heat shock proteins, aldo/keto reductase, glutathione-S-transferases, threonine ammonia-lyase and chitinases, lactoylglutathione lyase, DNAJ, pathogenesis-related protein Bet v, subtilases, DREB2A, hydroxyproline-rich glycoprotein, wound-induced proteins, L-threonine ammonia-lyase, alternative oxidase 1A, syntaxin, DC1 domain-containing proteins, thaumatin, ECERIFERUM, ABC transporters). Some of these genes are mainly involved in abiotic stress, like heat shock proteins and

wound-induced proteins, but most of them are important in plant defense against pathogens or herbivores.

A special mention is for ABC transporters, initially identified as transporters involved in detoxification processes, later recognized as crucial for organ growth, plant nutrition, plant development, response to abiotic stresses, pathogen resistance, hormone transport, and interaction of the plant with its environment (Kang et al., 2011). Their up-regulation indicates that a crosstalk of signaling events occurs in plants in response to EM application, which regulates plant primary metabolism, development and defense in plants.

Important part of this crosstalk are EM-responsive genes involved in the secondary metabolism. The hormone ethylene, whose synthesis seems to be stimulated by EM, can positively influence N assimilation and the secondary metabolism associated with the synthesis of phenols and terpenes (Khan et al., 2015). These compounds serve a dual function of deterring invading organisms and attracting pollinators (Lattanzio et al., 2006; Vermerris and Nicholson, 2006; Chen et al., 2011; Zhang et al., 2016). Phenols are also critical for plant development, especially in the synthesis of lignin and pigments, while terpenes include carotenoids, which are important for light harvesting and protection from excess of light radiation. Among the genes identified in tomato plants within this category, the one coding for PAL is of particular interest. PAL is a key enzyme of the phenol biosynthetic pathway and its activity and gene expression was previously shown to increase in response to applications of different biostimulants (Schiavon et al., 2010; Ertani et al., 2011a) and high ethylene levels (Chalutz, 1973). The increase in expression of this gene in tomato plants treated with EM correlated with higher production of phenol compounds they display, thereby providing strong evidence that phenol metabolism is a major target of biostimulants in this species.

Many transcription factors showed variation in expression in response to EM, but the most dramatic change was detected for a basic helix-loop-helix (bHLH) protein, which is known to play a key role in a multiplicity of transcriptional programs related to abiotic stress and plant development (Xu et al., 2014). Among the most largely represented EMresponsive transcription factors identified were AP2/ EREBPs (APETALA2) and WRKY, both involved in abiotic and biotic stress responses and in developmental processes, zinc finger (ZFN) proteins regulating development, growth, stress responses and phytohormone responses (Sakamoto et al., 2004), pentatricopeptide repeat-containing protein (PPR), which facilitate processing, splicing, editing, stability and translation of RNAs (Manna, 2015), bzip proteins, which have a critical role in photomorphogenesis, leaf and seed formation, energy homeostasis, and abiotic and biotic stress responses (Corrêa et al., 2008).

Comparing the effects of different EM concentrations on plant performance and gene expression in tomato, despite EM at 1 mL L^{-1} influenced plant growth, SPAD index and sugars more positively than EM at 0.1 mL L^{-1} , we can conclude that both dosages elicited the main metabolic pathways previously described, thereby suggesting that our data and hypothesized mechanisms for EM mode of action are fairly consistent.

CONCLUSION

The alfalfa-based protein hydrolysate tested in this study showed effectiveness as a biostimulant in tomato plants by enhancing plant productivity via multidirectional mechanisms. Some metabolic pathways can be definitely recognized as targets of EM action in plants, such as N and C primary metabolism, photosynthesis, transport of nutrients, secondary metabolism associated with the synthesis of phenolic and terpene compounds, and developmental processes related to auxin signaling. Furthermore, a number of new genes have been identified in tomato as potential targets of EM, such as those involved in plant-organism interactions, detoxification (glutathione/ascorbate cycle-related, ABC transporters), and defense against abiotic stress. In **Figure 9** a hypothetic model that represents a possible mode of action of EM in plants is depicted.

We conclude that the EM can act as a biostimulant in tomato plants may improve plant productivity and eliciting resistance responses, thereby reducing the need of conventional treatments that employ inorganic fertilizers and pesticides in agricultural practices and impact on the environment.

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AUTHOR CONTRIBUTIONS

AE performed the experiments and wrote the ms. MS analyzed the data and wrote the ms. SN supervised the work and provided financial support.

FUNDING

This research was funded by ILSA S.p.A. (Arzignano, VI, Italy).

AKNOWLEDGMENT

We would like to thank Eric Patterson (Colorado State University) for his precious support in data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01159/ full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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