A DIGE proteomic analysis of wheat flag leaf treated with TERRA-SORB® foliar, a free amino acid high content biostimulant

María José Martínez-Esteso[1], Maria Teresa Vilella-Antón[1], Susana Sellés-Marchart[2], Ascensión Martínez-Márquez[1], Anna Botta-Català[3], Rafael Piñol-Dastis[3], Roque Bru-Martínez[4]

[1]Grupo de Proteómica y Genómica Funcional de Plantas. Dept. Agroquímica y Bioquímica. Universidad de Alicante, Ctra. de San Vicente del Raspeig, s/n, 03690 Alicante, Spain; [2]Unidad de Genómica y Proteómica, SSTTI, Universidad de Alicante, Ctra. de San Vicente del Raspeig, s/n, 03690 Alicante, Spain; [3]Departamento de I+D Fisiología Vegetal, BIOIBERICA, S.A., Plaza Francesc Macià 08029 Barcelona, Spain; [4]IMEM Ramón Margalef, Grupo de Proteómica y Genómica Funcional de Plantas. Dept. Agroquímica y Bioquímica. Universidad de Alicante, Ctra. de San Vicente del Raspeig, s/n, 03690 Alicante, Spain.

ABSTRACT

The flag leaf is the most important source of carbohydrate during wheat kernel filling. Around a 75% of all sugars stored in the kernel come from carbon fixed by this leaf. Terra-Sorb® foliar is an L-α-amino acid-based product from enzymatic hydrolysis for foliar application with a high ratio of free to total amino acids. Previous agronomical studies carried out on grassy, horticultural and tree crops have shown that the application of Terra-Sorb® increases photosynthetic plant activity and chlorophyll content, promotes rapid recovery from stress and improves fruit set.

In this work, we have undertaken a proteomic approach to explore molecular mechanisms potentially involved in the stimulating effect of Terra-Sorb® Foliar on wheat yield when applied in commercial fields. Wheat plants at the flag leaf stage were treated, and a DIGE approach was used to compare the proteomes of treated vs. control plants in four biological replicates.

Thirty-seven protein spots were found to change in abundance (ANOVA p<0.05) out of which 8 were down-regulated and 29 up-regulated in treated leaves. Twenty protein spots (1.2<fold change <1.9) encoded by 11 different genes were successfully identified by nLC-ESI-MS/MS and NCBInr database search.

The deregulated proteins identified were mainly related to the life cycle of Rubisco. Importantly, two proteins involved in the positive regulation of Rubisco activity, namely Rubisco activase, and the large subunit of Rubisco binding protein, were found up-regulated in treated plants, suggesting a better performance of Rubisco. Down-regulated proteins were of metabolic and anti-stress enzymes, including Cu/Zn superoxide dismutase that protects photosystem II from photooxidation.

In conclusion, significant changes were shown to occur in the wheat flag leaf proteome upon Terra-Sorb® Foliar application. The deregulated proteins identified are directly or indirectly involved in the CO_2 fixation which may correlate with the known stimulating effect of Terra-Sorb® Foliar of wheat yield, although further functional experiments are needed to validate the proposed hypothesis.

Keywords: DIGE, leaf, plant biostimulant, proteomics, quantitative, wheat.

Abbreviations

ATP-CF1-A: ATP synthase CF1; **BBCH-scale:** Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie; **CS:** citrate synthase; **DIGE:** differential gel electrophoresis; **EF-Tu:** elongation factor Tu; **EF-Tu Chl:** elongation factor Tu chloroplastic; **GA3PDH:** glyceraldehyde-3-phosphate dehydrogenase; **HSP-90:** heat shock protein 90; **nLC-ESI-MS/MS:** nano-liquid chromatography coupled to tandem mass spectrometry; **Rubisco:** Ribulose-1,5-bisphosphate carboxylase/oxygenase; **RBA:** Rubisco activase; **Rubisco LBP A and B:** Rubisco large-subunit binding protein, subunit alpha and beta; **Rubisco-L:** Rubisco large subunit; **SOD:** superoxide dismutase.

*Corresponding author: Roque Bru-Martínez, Department of Agrochemistry and Biochemistry, Faculty of Sciences, University of Alicante, Ctra. San Vicente del Raspeig s/n, E-03080 Alicante, Spain. E-mail address: Roque.Bru@ua.es

1. Introduction

The exponential intensification of agricultural practices over the last decades, forced a widespread use of fertilizers and pesticides which has caused an increasing impact on the quality of ground and surface waters and food products [1]. Fertilization sources include inorganic salts, mainly chlorures, sulfates, nitrates and organic or synthetic chelants, used to introduce ions through the foliar cuticle layer [2]. Nitrogen fertilization of crops by a high content of nitrates (NO_3^-) leaves behind 50% of nitrogen applied as a residue that can move throughout the soil into groundwater [3]. The rise of nitrates is a potential hazard to human and animal life [4].

Nowadays, healthy food and environmental protection have been top priorities in scientific research. In recent years, innovations in production have been evolving toward low-cost, organic, sustainable, and environmentally friendly systems that must ensure the high yield and quality of crops. Some authors have proposed the use of biostimulants in plant nutrition instead of inorganic fertilizers to improve the nutrient uptake or their utilization by plants [5].

Terra-Sorb® foliar is an L-α-amino acid-based product from enzymatic hydrolysis of proteins of animal origin that has been specially developed by the R&D Department of Bioiberica, S.A. It is composed of 9.3% of free protein amino acids, namely Asp, Ser, Glu, Gly, His, Arg, Thr, Ala, Pro, Cys, Tyr, Val, Met, Lys, Ile, Leu, Phe and Trp which are the most abundant.

In this report, we studied the effect of the TerraSorb® Foliar on wheat (*Triticum aestivum* L.) flag leaf. A critical stage in the development of wheat plants is the emergence of the final leaf called the flag leaf. At this point, the emphasis of management needs to shift to its protection, as the flag leaf is critical for attaining high grain yields. The flag leaf is the most important source of carbohydrate during wheat kernel filling. Around a 75% of all sugars stored in the kernel come from carbon fixed by this leaf [6]. Plants invest an important amount of the photosynthetic energy in biosynthesis of amino acids for protein synthesis. The application of exogenous amino acids via foliar has been shown in previous agronomical studies carried out on grassy, horticultural and tree crops to result in an increase of photosynthetic plant activity and chlorophyll content, promotion of rapid recovery from stress and improvement of fruit set [7, 8, 9].

Proteomics has been shown as a successful approach to analyze the response of plants to external stimulus [10]. In this work, we have undertaken a proteomic quantitative approach based on DIGE technique to explore molecular mechanisms potentially involved in the stimulating effect of Terra-Sorb® foliar on wheat yield when applied in commercial fields.

2. Material and Methods

2.1. Plant material

Leaf samples from wheat (*Triticum aestivum* L.) were sampled at 39 BBCH stage [11] in eight 2 x 10 m plots in an experimental field in Totana (Murcia, Spain). Four plots separated 5 m from control plots were treated with the biostimulant TerraSorb® foliar (Bioibérica S.A). The product was applied at 500 g-ml/ha real doses. After 48h and 72 h of exposure, wheat flag leaves were harvested.

2.2. Protein extraction

Wheat leaves (0.3 g) were pulverized by liquid nitrogen. The frozen powdered was resuspended in 0.5 ml ascorbic acid 10 mM and 0.1 ml protease inhibitor cocktail. Then, 9 ml of extraction buffer (TCA 10% (w/v), 0.07% DTT (w/v)) were added, vortexed and incubated at -20ºC overnight. This homogenate was centrifuged at 15000 x g during 20 min at 4ºC, the supernatant was discarded and the pellet resuspended in wash buffer (0.07% (w/v) DTT in acetone), vortexed and incubated at least 1 h. This procedure was repeated once. Then, washes were performed with ethyl acetate: ethanol (1:2) and then in TCA 10% (w/v) and finally in TCA 10% (w/v) in acetone and finally the pellet was dried at 4ºC. Subsequently the washed pellet was homogenized in 3 ml of extraction buffer (0.7 M sucrose, 0.1M KCl, 0.5M Tris-HCl pH 7.5, 50 mM EDTA, 1% (w/v) PVPP, 1% (w/v) DTT and a cocktail of protease inhibitors) and incubated shaking during 40 min at 4ºC. An equal volume of Tris-saturated phenol pH 7.5 (AppliChem, Darmstadt, Germany) was added and the mixture incubated for 20 min at 15000 x g at 4ºC. The upper phenol phase was recovered and the aqueous phase was submitted to a second phenol extraction. Both phenol phases were pooled and washed twice with an equal volume of phenol washing buffer adjusted to pH 7.0 (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris, 50 mM EDTA, 1% DTT, and a cocktail of protease inhibitors). The recovered phenol was precipitated with 5 vols of 0.1 M ammonium acetate (w/v) in methanol overnight. The precipitate was washed three times in 0.1 M ammonium acetate (w/v) in methanol and twice in 80% acetone (v/v).

2.3. Labeling of proteins with CyDye

The precipitated and air-dried proteins were solubilized in labeling buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS pH 9.0). Insoluble material was pelleted by centrifugation (12000 x g, room temperature, 5 min). The protein concentration in the supernatant was measured using the RCDC method (Bio-Rad, Madrid, Spain).

Two hundred micrograms of protein was diluted to 1 µg/µl with labeling buffer and further cleaned with the Ettan 2D Clean-up kit (GE Healthcare, Madrid, Spain) according to the manufacturer's recommendations. The recovered precipitated protein was solubilized in 30 µl of labeling buffer, the pH was adjusted to 8.5 using NaOH (100 mM), and the protein content was measured again. For labeling of the proteins, 400 pmol of CyDye in 1 µl was mixed with 18 µl of the sample containing 50 µg of protein and incubated on ice for 30 min in the dark. The labeling reaction was terminated by adding 1 µl of 10 mM lysine. Each sample was covalently labeled with a fluorophore, either with Cy3 or Cy5. A mixture containing equal amount of proteins from every sample in the experiment was labeled with Cy2 and used as internal standard.

2.3.1. 2-DE and image scanning

For analytical 2-D DIGE analysis 20 µl each of Cy3-, Cy5-, and Cy2-labeled sample (150 µg of protein) were combined, mixed with 60 µl of sample buffer 1 (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS, 100 mM DTT), and incubated for 10 min on ice. Then, samples were diluted to 150 µl with sample buffer 2 (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS, 50 mM DTT) and submitted by cup-loading to IEF as the first-dimension separation. IPG strips, pH 4-7, 24-cm (GE Healthcare) were swollen overnight in 450 µl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.5% IPG buffer, 0.005% bromophenol blue). IEF was performed at 20ºC on an IPGphor Unit (GE Healthcare/Amersham Biosciences) using the following settings: 3 h at 300 V, 6 h gradient from 300 to 1000 V, 3 h gradient from 1000 to 8000 V, 5 h at 8000 V until an accumulated voltage of 77 kVh was achieved. Strips were equilibrated after focusing in two steps: 30 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS (w/v)) supplemented with 1% DTT followed by 30 min in equilibration buffer supplemented with 1.25% iodoacetamide. SDS-PAGE was carried out as the second-dimension separation in 12.5% acrylamide gels in an Ettan Dalt-six (GE Healthcare/Amersham Biosciences) vertical unit. The separation was run overnight: first step at 80 V, 10 mA/gel, and 1 W/gel for 1 h, second step at 150 V, 12 mA/gel, and 2 W/gel until bromophenol blue line reached to the bottom of the gel. Images of the Cy3-, Cy2-, and Cy5-labeled samples were acquired in a Typhoon 9410 scanner (GE Healthcare/Amersham Biosciences) according to the manufacturer's recommendations.

2.3.2. DIGE image analysis

DIGE images were analyzed using the Progenesis SameSpots v3.0 software (Non-linear Dynamics, Newcastle, UK). First, the images were aligned. The most prominent spots were used to manually assign vectors to the digitized images within each gel and then the automatic vector tool was used to add additional vectors, which were manually revised and edited for correction when necessary. These vectors were used to warp and align gel images with a reference image of one internal standard across and within each gel. After automatic spot detection, spots were manually revised with edit tools for correct detection. Gel groups were established according to the experimental design and spot normalized volume was used to select statistically significant (fold-change, ANOVA, false discovery rate) differentiated spots between conditions analyzed in the experiment. The abundance patterns of the selected spots were analyzed and grouped by hierarchical clustering and assessed by principal component analysis implemented in Progenesis SameSpots. Spot picking and tryptic digestion Preparative 2-D gels loaded with 1.8 mg of protein were used for spot picking. After that 2-DE the gel was stained with the colloidal Coomassie blue G250 method [12], scanned in a transmission-light densitometer (Image Scanner; GE Healthcare), and aligned with the DIGE reference image with SameSpots to outline the spots of interest selected in the previous analysis. The spots were excised using a manual spot picker (The Gel Company, Tübingen, Germany) with a 1.5 mm diameter picker head. Trypsin in-gel digestion [13] was carried out in a Progest (Genomic Solutions, Huntingdon, UK) automatic in-gel protein digestor according to the manufacturer's recommendations for colloidal Coomassie Brilliant blue-stained samples. The gel plugs were extensively washed to remove dye and SDS impurities with 25 mM ammonium bicarbonate, in-gel reduced with 60 mM DTT and S-alkylated with excess iodoacetamide followed by digestion with modified porcine trypsin (Promega, Madison, WI, USA) at 37 ºC for 6 h. Peptides were extracted into ammonium bicarbonate, then into 70% acetonitrile (ACN) and finally into 1% formic acid (FA). Extracted peptides were dried down in a speed-vac bench-top centrifuge and resuspended in 0.1% FA (typically 10 µl).

2.3.3. MS-based protein identification

The tryptic fragments were analyzed by LC-MS/MS using an Agilent 1100 series nano-HPLC system lined on an XCTplus ion trap mass spectrometer (Agilent) equipped with a nano-ESI source. Sample concentration and desalting were performed on a Zorbax 300SB-C18 trap column (0.3 x 5 mm, 5 µm) at 0.3 µl/min while peptide separation was achieved on a Zorbax 300SB-C18 analytical column (75 µm x 15 cm, 3.5 µm) using a 30-min linear gradient of 5-35% ACN containing 0.1% (v/v) FA at a constant flow rate of 0.3 µl/min. MS and MS/MS spectra were acquired in the standard enhanced mode (26 000 m/z per second) and the ultrascan mode (8100 m/z per second), respectively. Mass spectrometer settings for MS/MS analyses included an ionization potential of 1.8 kV and an ICC smart target (number of ions in the trap before scan out) of 400 000 or 150 ms of accumulation. MS/MS analyses were performed
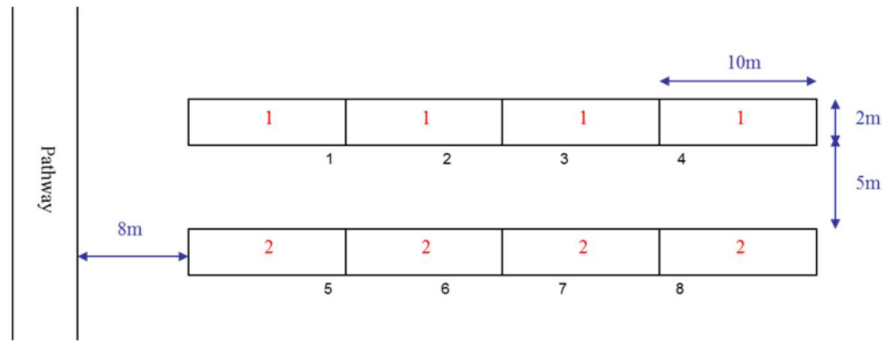
Figure 1. Wheat plants were cultivated in field (Totana, Albacete). This scheme represents an overview of plots disposal where plots 1 untreated a considered as control plants and plots 2 were treated with TerraSorb® foliar. Numbers 1, 2, 5, 6 were plots where plants were harvested two days post-treatment and 3, 4, 7, 8 plots were plots where plants were harvested three days post-treatment.

using automated switching with a preference for doubly charged ions and a threshold of 105 counts and 1.3 V fragmentation amplitude. Each MS/MS spectra dataset (1200 spectra/run) was processed to determine monoisotopic masses and charge states, to merge MS/MS spectra with the same precursor (Δm/z<1.4 Da and chromatographic Δt<15 s) and to select high-quality spectra with the Extraction tool of the Spectrum Mill Proteomics Workbench (Agilent) (SMPW). The reduced dataset was searched against the NCBInr in the identity mode with the MS/MS Search tool of the SMPW using the following parameters: trypsin, up to two missed cleavages, fixed modification S-carbamidomethyl, and a mass tolerance of 2.5 Da for the precursor and 0.7 Da for product ions. Peptide hits were validated first in the peptide mode and then in the protein mode according to the manufacturer's recommended score settings. Briefly, identities interpreted for individual spectra were automatically designated as valid by applying the following scoring threshold criteria to all spectra, protein details mode: protein score > 20, peptide details mode (score, Scored Percent Intensity, delta rank1-rank2) in the following order: peptide charge +2 (> 6, > 60 %, > 2), peptide charge +1 (> 6, > 70 %, > 2), peptide charge +3 (> 8, > 70 %, > 2), peptide charge +4 (> 8, > 70 %, > 2), peptide charge +5 (> 12, > 70 %, > 2) and peptide charge +2 (> 6, > 90 %, > 1). Validation was edited to filter out those peptides with equal scoring in the direct and reversed search and Δmass (|observed - expected|) > 15 ppm. Validated files were summarized in the protein mode to assemble peptides into proteins in order to generate the minimal protein list that best explains the matched peptides. A protein was considered identified with a minimum of two different peptides and a score above threshold marked for the SMPW search engine.

3. Results

3.1. Selection of plant material for differential proteomic analysis

Wheat plants (*Triticum aestivum* L.) were cultivated in field under not controlled conditions in an experimental land in Totana. When wheat plants reached the flag leaf stage (39 BBCH) were exposed to TerraSorb® foliar biostimulant as described in Materials and Methods. After two and three days following foliar spraying treatment according to the scheme in Figure 1, flag leaves were sampled for further proteomic analyses.

Protein extractions were performed from the whole wheat flag leaf without pod considering one leaf as a single individual sample. Four biological replicates were considered in the experiment. Each leaf had an estimated fresh weight of 0.3 g and was processed for protein extraction using a modified method based on the robust phenol/ammonium-methanol precipitation according to Martínez-Esteso et al., (2011) [14]. The amount of protein extracted was statistically independent (ANOVA<0.01) from the applied treatment obtaining a protein yield of 32.4 ± 3.5 mg protein/g fresh tissue for treated samples and 39.9 ± 6.5 mg protein/g fresh tissue for control samples.

Table 1. Experimental design of 2-D DIGE experiment.

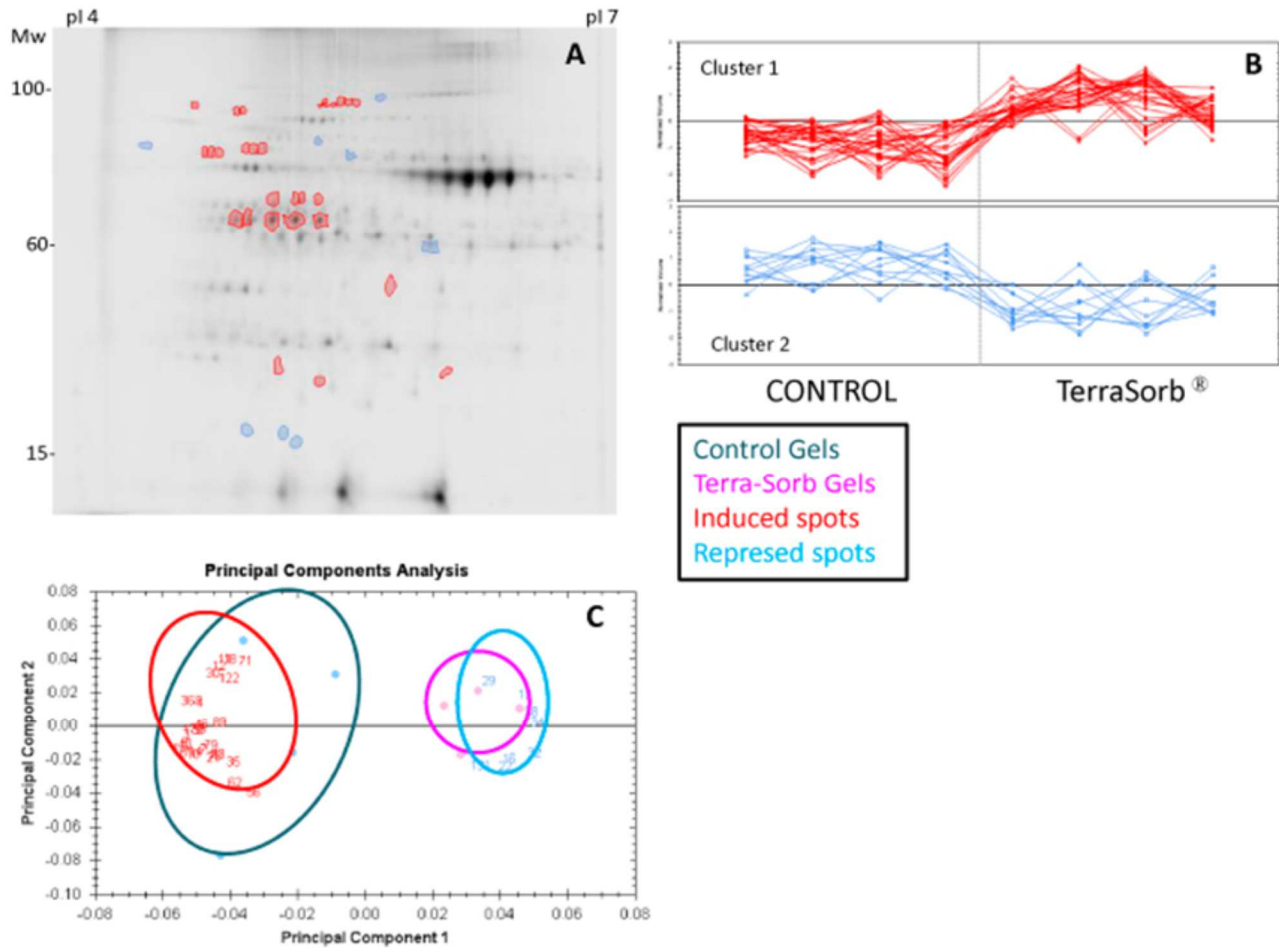| Gel number	Cy2 (Standard)	Cy5 (sample plot/days post-treatment)	Cy3 (sample plot/days post-treatment)
1	Control+treatment	Control plot 1/2	Treatment plot 7/3
2	Control+treatment	Treatment plot 5/2	Control plot 3/3
3	Control+treatment	Treatment plot 6/2	Control plot 4/3
4	Control+treatment	Control plot 2/2	Treatment plot 8/3

Figure 2. 2D DIGE analysis of TerraSorb ® treated vs. Control wheat flag leaves. A) 2D gel reference image showing the location of the selected spots and pertaining to a cluster by a color code. B) Standardized expression profiles of the spots in four replicates across the control and the treatment. C) PCA bi-plot of the two first principal components. Together they both explained 75.49% of the selected spots variability, and the third principal component only explained an additional 9.35%.
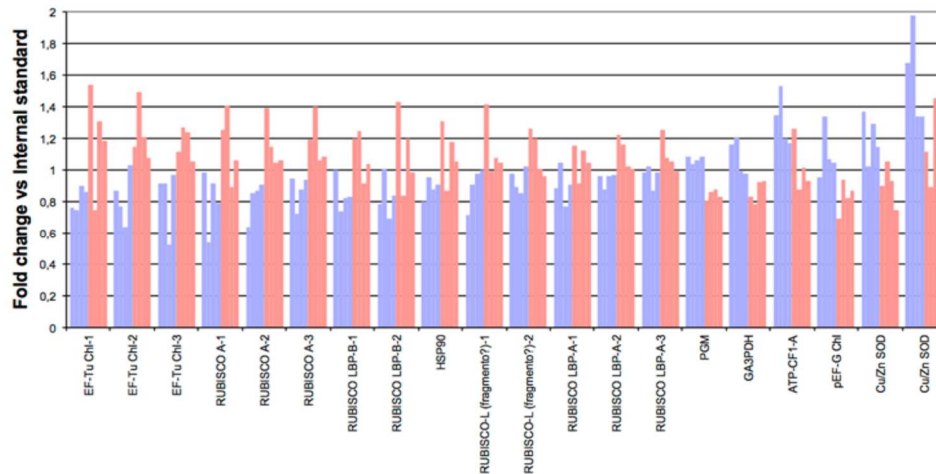

Figure 3. Proteins de-regulated in wheat flag leaf treated with TerraSorb® Foliar. Shown is the relative abundance in each of the four biological replicates of control and treated individuals. NCBInr accession numbers for each protein are: 125540125 EF-Tu Chl, 12643756 Rubisco A, 2493650 Rubisco LBP-B, 556673 HSP90, 548677 Rubisco-L, 134102 Rubisco LBP-A, 32400802 PGM, 15222111 GA3PDH, 14017569 ATP-CF1-A, 1568639 Cu/Zn SOD.

3.2. Protein abundance patterns of wheat flag leaf in response to TerraSorb® treatment

To detect proteins differentially accumulated in the TerraSorb® treatment, 2-D DIGE patterns from control and the corresponding treated samples were compared according to the experimental design shown in Table 1. 2-D DIGE gel maps resolved approximately 918 unique spots across the compared control vs. treatment samples. The comparative analysis by SameSpots software revealed 37 spots (4 % from the number of total spots) above the threshold settings (ANOVA p-value<0.05). Figure 2A shows the reference gel image in which the selected spots are outlined. After a hierarchical clustering analysis of the abundance patterns, the selected spots were classified into two groups (Figure 2B): up-regulated in treatments with TerraSorb® (red), down-regulated in treatment with TerraSorb® (blue). The same color code was used to outline the corresponding spots in the reference gel image (Figure 2A) and the unsupervised PCA bi-plot of spots and gels (Figure 2C). The latter shows a gel grouping (colored dots) that agrees with the experimental design. Twenty-nine of these spots increase their abundance and eight spots shown a decreased accumulation in the treated plants when compared to the controls (Figure 2B). The fold-changes determined were moderate for all the proteins, occurring the highest change of 1.83-fold for the spot 56.

3.3. Identification of differentially expressed proteins

Protein identity was successfully achieved for 26 of the selected spots following a typical protein identification workflow (spot excision, trypsin in-gel digestion, nLC-MS/MS and database search). This number of proteins represents a 70 % from the total of differentially accumulated proteins detected due to TerraSorb® treatment. The twenty-six protein spots were encoded by 13 different genes according to the top hit accession in NCBInr, but in quantitative terms we only considered twenty protein spots whose fold change was above 1.2, which are shown in Table 2. Supplementary File 1 provides details of proteins identification. The values shown in the 'Fold Change' column represent the average increase or decrease of protein abundance related to control with a significance of > 95 % in t-student, and in brackets are given the normalized spots volumes in % of the total spot volume. Those data with a significance level < 95 % are assumed to be due to biological variation and excluded from the analysis. Figure 3 summarizes the results of identification pertaining to the deregulated spots. The identified proteins which abundance increased can be clearly seen in Figure 3 where red bars related to the normalized volume in the treated samples versus internal standard stick out the blue bars corresponding to the control samples. The increased spots are mainly related to the life cycle of Rubisco [15] including six spots of Rubisco large-subunit binding protein, subunit alpha (Spots 8, 11, 12) and beta (Spots 18, 30, 122) (Rubisco LBP A and B) involved in its synthesis and assembly, three spots of Rubisco activase (RBA) (Spots 5, 9, 89) involved in its catalytic activity and two spots of an N-terminal fragment of Rubisco large subunit (Rubisco-L) (Spots 56, 62) which may be related to the degradation of the protein. The rest of increased spots are related to protein synthesis and folding including a spot of heat shock protein 90 (HSP-90) (Spot 71) and three spots of elongation factor Tu (EF-Tu) chloroplastic (Spots 10, 14, 73). The identified proteins which abundance decreased include proteins involved in central metabolism: a spot of phosphoglycerate mutase (PGM) (Spot 32), a spot of glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) (Spot 34) and a spot of ATP synthase CF1 (ATP-CF1-A) (Spot 29), and stress, including two spots of Cu/Zn superoxide dismutase (SOD) (Spots 13, 131). Also, a fragment of a precursor of EF-Tu was found decreased (Spot 22). Most of the spots changing have undergone a moderate increment of its abundance and those proteins down-regulated are spots of medium to low abundance.

4. Discussion

The proteins whose abundance increases upon TerraSorb® treatment carry out functions related to the positive regulation of carbon fixation and protein synthesis. In contrast, proteins whose abundance decreases are involved in functions related to energy metabolism and oxidative stress. The effect of TerraSorb® in the proteome of the flag leaf is clearly different from control samples. Having just the proteomic profile obtained in this experiment, it can be tentatively assigned an explanation at molecular and physiological level. Nevertheless, this general overview suggests that the effects are specific and in consequence related to particular biological processes.

The in bibliographic information about the effect of amino acid-based biostimulants on plant growth at molecular level is scarce. These reports were focused on the expression of a specific set of genes and activity of enzymes being deregulated by the treatment, underlying a targeted approach. Transcripts and activities for the enzymes involved in carbon metabolism (malate dehydrogenase isocitrate dehydrogenase, citrate synthase) and N reduction and assimilation (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, aspartate aminotransferase) were monitored in maize and were found to increase in response to the treatments [5]. In the model plant *Arabidopsis thaliana* the protecting effect against abiotic stresses of an amino acid-based biostimulant product was studied by monitoring the expression of selected genes related to abiotic stress response, in particular salt, drought and low temperature. An increased expression of these stress resistance genes was shown thus concluding that the product stimulates endogenous plant defense response to both biotic

Table 2. Proteins differentially expressed in DIGE of treated and control samples. Selection and quantification of spots of interest was assisted by Progenesis SameSpots. Spots were excised from the gel, trypsin in-gel digested, and the eluted peptides were analyzed by mass spectrometry (ESI-MS/MS). Proteins were identified by MS/MS search against NCBInr protein database using SpectrumMill. aScore: statistical probability of true positive identification of the predicted protein calculated by Spectrum Mill and Mascot Score (*). bSC: percentage of predicted protein sequence covered by matched sequences. cTmw/Emw: molecular mass of predicted protein/molecular mass of protein on gel. dTpI/EpI: pI of predicted protein/pI of protein on gel.

Ref. Spot	Protein Information						Fold Change (Norm. Vol.%)	
Ref. Spot	Accession No.	Protein name	Score[a]	SC (%)[b]	Tmw/Emw[c] (Da)	TpI/EpI[d]	Identified peptides/ matched spectra	Control	Terra-Sorb
73	125540125	translational elongation factor Tu	72.18	10	63685.4/39616	5.74/5.35	4/6	1 (0.051)	1,41 (0.072)
10	125540125	translational elongation factor Tu	37.26	5	63685.4/38898	5.74/5.22	2/2	1 (0.074)	1,37 (0.101)
14	125540125	translational elongation factor Tu	56.93	8	63685.4/39855	5.74/5.26	3/4	1 (0.043)	1,30 (0.056)
18	2493650	RuBisCO large subunit-binding protein subunit beta, chloroplast	291.89	48	53412/55747	4.88/4.99	17/26	1 (0.038)	1,26 (0.048)
122	2493650	RuBisCO large subunit-binding protein subunit beta, chloroplast	369.36	62	53412/54382	4.88/5.05	20/33	1 (0.046)	1,23 (0.057)
8	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	271.12	40	57520.9/52828	4.83/4.81	15/19	1 (0.019)	1,4 (0.026)
12	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	205.89	34	57520.9/52921	4.83/4.77	11/15	1 (0.019)	1,33 (0.026)
11	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	271.12	40	57520.9/53014	4.83/4.74	15/19	1 (0.013)	1,28 (0.017)
56	548677	RuBisCO large subunit. Ribulose bisphosphate carboxylase large chain, N-terminal domain.	48.32	7	52183.6/20446	6.09/5.36	3/11	1 (0.027)	1,83 (0.050)
62	548677	RuBisCO large subunit. Ribulose bisphosphate carboxylase large chain, N-terminal domain.	41.34	7	52183.6/20979	6.09/6.06	3/3	1 (0.005)	1,61 (0.008)
5	12643756	RuBisCO activase A chloroplast precursor	50	7	51074.3/35650	8.04/5.36	3/4	1 (0.442)	1,43 (0.632)
9	12643756	RuBisCO activase A chloroplast precursor	50	7	51074.3/35797	8.04/5.23	3/4	1 (0.718)	1,41 (1.015)
89	12643756	RuBisCO activase A chloroplast precursor	81.61	24	51074.3/35843	8.04/5.10	5/5	1 (0.617)	1,27 (0.787)
71	556673	Heat shock protein 90kDa	238.80	22	88117/73511	4.90/4.67	14/20	1 (0.005)	1,47 (0.007)
32	32400802	phosphoglycerate mutase	51.29	20	29578.7/57149	5.44/5.35	3/5	1 (0.015)	-1,26 (0.012)
34	15222111	glyceraldehyde-3-phosphate dehydrogenase	82.43	17	42847.0/31638	8.16/5.96	5/5	1 (0.146)	-1,27 (0.115)
29	14017569	ATP synthase CF1 alpha subunit	29.01	4	55295.8/51750	6.11/5.52	2/2	1 (0.025)	-1,27 (0.020)
22	125549171	Fragment of putative Elongation factor G, chloroplast precursor (EF-G).	70.54	7	84912.5/55747	5.43/4.43	4/4	1 (0.005)	-1,29 (0.004)
131	1568639	Cu/Zn superoxide dismutase	84.15	25	20323/18765	5.35/5.15	4/6	1 (0.020)	-1,34 (0.015)
13	1568639	Cu/Zn superoxide dismutase	43.65	2	20323/18856	5.35/4.96	2/2	1 (0.008)	-1,48 (0.005)

and abiotic stress factors [16]. Physiological experiments have pointed out that the exogenous application of the amino acid-based biostimulant foliar (BioIberica S.A., Barcelona) improves photochemical leaf efficiency in ryegrass plants grown at 36ºC [17]. A definite cause and effect relationship has not yet been proved and the molecular mechanism that might be related to the improvement of the plant performance after exposure to abiotic stresses is still unknown.

In the present study, the application of a hypothesis-free approach has led to a discovery of deregulated proteins not anticipated, up to date, in plants treated with an amino acid-based biostimulant, thus providing clues on mechanisms of action.

4.1. Positive regulation of Rubisco

Different proteins which have a role in regulating the Rubisco protein were identified. Rubisco activase was found up-regulated by treatment with TerraSorb® foliar in four spots. RBA produces ATP-dependent conformational changes in Rubisco, the enzyme responsible for carbon fixation during photosynthesis, making the inactivated protein re-enter the catalytic cycle. Rubisco can be inactivated during its catalytic cycle by the strong binding of phosphate sugars such as ribulose-1,5-bisphosphate or by the inhibitor 2-carboxyarabinitol 1-phosphate. The activation occurs through a mechanism dependent on ATP in which RBA provokes conformational changes in the Rubisco enzyme which promotes the release of the inhibitory sugar phosphates from the catalytic site [15]. Besides RBA, some polypeptides related to Rubisco regulation were found up-regulated. On the one hand, two subunits of Rubisco LBP, A and B, that form a macromolecular complex that assists to the correct folding of Rubisco. Altogether, these results indicate the need for validation and further study of the role that RBA and Rubisco LBP could have in promoting carbon fixation through the increase of the overall Rubisco activity. On the other hand, we also found that two low molecular weight fragments of Rubisco-L N-terminal domain that are known to fold as a ferredoxin are up-regulated. Although, it has been reported the presence of Rubisco-L fragments with unknown function as a consequence of thermal stress [18], we cannot presently foresee any potential role for this fragment found in the treated wheat plants.

4.2. Protein synthesis stimulation

The elongation factor Tu involved in protein biosynthesis in the chloroplast was detected up-regulated by TerraSorb® foliar treatment. Moreover, HSP-90, a component of the folding machinery of proteins, was detected to be up-regulated. Interestingly, pre-EF-Tu has been demonstrated to reduce thermal aggregation of RBA [19], which is known

to display an exceptional sensitivity to thermal aggregation, and constraints photosynthesis at high temperature [20, 21]. The profiles of EF-Tu and RBA are parallel in the response to the biostimulant TerraSorb® foliar. EF-Tu is thus another target of interest for further studies, as it could play a role beyond protein synthesis in the stabilization of RBA, although other experiments are needed to test this hypothesis.

4.3. Down-regulation of metabolic enzymes and anti-stress proteins

Four proteins were found to be down-regulated: ATP-CF1-A, PGM, G3PDH and a Cu/Zn SOD. For the former three, the level of decrease could be consistent with a lower consumption of the photosynthetic product 3-phosphoglycerate catabolism, that is, to obtain energy for the oxidative processes. Decreased levels of Cu/Zn SOD would be consistent with a lower oxidative state. Cu/Zn SOD is specific and its role is to eliminate superoxide generated by the photoreduction of O_2 in the photosystem I. Although, the reasons for the effect of TerraSorb® in the reduction of oxidative stress at the photosynthetic level cannot be explained by the proteomic data alone obtained in this experiment, the result is consistent with the finding that a biostimulant based on porcine hemoglobin hydrolysate lessened the harmful effects caused by the intense cold and heat treatment in lettuce [8].

5. Concluding Remarks

Our proteomic study provides information about deregulated proteins that were identified in wheat flag leaf after treatment with TerraSorb® foliar. The obtained information provided molecular targets for further validation and a hypothesis for the molecular mechanism underlying the effect of TerraSorb® foliar on wheat flag leaf. However, the confirmation of the effects of this amino acid-based biostimulant at molecular level has shown, that the discovery proteomics approach will need to be included in the future design of targeted studies focused on these biological processes.

6. Supplementary material

Supplementary material 1. Peptide table. For each identified protein a list of matched peptides is given. Besides, charge state and precursor ion m/z data is given for LC–MS/MS analyzed proteins.

Acknowledgements

References

1] B. Hirel, P. Bertin, I. Quilleré, W.Bourdoncle, C. Attagnant, C. Dellay, A. Gouy, S. Cadiou, M. Falque, Plant Physiol. 125 (2001) 1258-1270.

2] G. Melendez, E. Molina, Fertilización foliar: principios y aplicaciones. Universidad de Costa Rica. Centro de Investigaciones Agronómicas. http://www.cia.ucr.ac.cr/docs/ Mem_Foliar_2002.pdf (Accessed May 31, 2013).

3] K. Goulding, Soil Use Manag. 16, (2000) 145-151.

4] K. A. Smith, I. P. McTaggart, H. Tsuruta, Soil Use Manag. 13 (1997) 296-304.

5] M. Schiavon, E. Ertani, S. Nardi, J. Agric. Food Chem. 56 (2008) 11800-11808. DOI: 10.1021/jf802362g.

6] J. Herbert, C. Lee, in: A comprehensive guide to wheat management in Kentucky. (ed). University of Kentucky Cooperative Extension, 2009, (Kentuky) http://www.uky.edu/ Ag/GrainCrops/ID125TOC.html (Accessed May 31, 2013).

7] G.L. Kauffman III, D.P. Kneivel, T.L. Watschke, PGRSA Quart. 33 (4) (2005) 134-141.

8] J. Polo, R. Barroso, J. Ródenas, J. Azcón-Bieto, R. Cáceres, Hort. Technology 16(3) (2006) 483-487.

9] A. Botta, C. Marin, R. Piñol, L. Ruz, E. Badosa, E. Montesinos, Acta Hort. 842 (2009) 721-724.

10] G.K. Agrawal, R. Pedreschi, B.J. Barkla, L.V. Bindschedler, R. Cramer, A. Sarkar, J. Renaut, D. Job, R. Rakwal, J. Proteomics 75(15) (2012) 4588-4601. DOI: 10.1016/j.jprot.2012.03.055.

11] P.D. Lancashire, H. Bleiholder, P. Langeluddecke, R. Stauss, T. van den Boom, E Weber, A. Witzen-Berger, Ann. Appl. Biol. 119 (3) (1991) 561-601. DOI: 10.1111/j.1744-7348.1991.tb04895.x

12] V. Neuhoff, N. Arold, D. Taube, W. Ehrhardt,. Electrophoresis 9 (1988) 255-262.

13] A. Shevchenko, O.N. Jensen, A.V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, A. Shevchenko, H. Boucherie, M. Mann, P Natl Acad Sci USA 93 (1996) 14440-14445.

14] M.J. Martínez-Esteso, S. Sellés-Marchart, D. Lijavetzky, M.A. Pedreño, R. Bru-Martínez, J. Exp. Bot. 62(8) (2011) 2521-2569. DOI: 10.1093/jxb/erq434.

15] Portis, A.R.Jr Photosynth. Res 75 (2003) 11-27.

16] D. Cambri, L. Filippini, F. Apone, S. Arciello, G. Colucci, D. Portoso, In Biostimulators in modern agriculture, ed. H. Gawronska (2008) (Editorial House Wie. Jutra (Warsaw)).

17] G. L. Kauffman III, D. P. Kneivel, T. L.Watschke, Crop Sci. 47 (2007) 261-267.

18] L-F. Zhang, Q. Rui, P. Zhang, X-Y. Wang, L-L. Xu, Plantarum 131 (2007) 64-71. DOI: 10.1111/j.1399-3054.2007.00928.x.

19] Z. Ristic, I. Momcilovic, J. Fu, E. Callegari, B.P. Deridder, J Plant Physiol. 164 (2007) 1564-1571.

20] U. Feller, S. J. Crafts-Brandner, M. E. Salvucci, Plant Physiol. 116 (1998) 539-546.

21] M. E. Salvucci, K.W. Osteryoung, S.J. Crafts-Brandner, E. Vierling, Plant Physiol. 127 (2001) 1053-1064.