Proteomic Analysis of Soybean [Glycine max (L.) Merrill] Roots Inoculated with Bradyrhizobium japonicum Strain CPAC 15

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ABSTRACT: This research intended to analyze the expression pattern of proteins in roots of the Brazilian soybean cultivar Conquista when inoculated with Bradyrhizobium japonicum CPAC 15, a strain broadly used in commercial inoculants in Brazil. At ten days after bacterial inoculation, whole-cell proteins were extracted from roots and separated by 2-D gel electrophoresis. Comparative analysis revealed significant changes in the intensity of 37 spots due to the inoculation (17 up-regulated and 20 down-regulated proteins), identified by MALDI-TOF/TOF-TOF. Identified proteins were associated with COG functional categories of information storage and processing, cellular processes and signaling, metabolism, and also in the “poorly characterized” and “not in COG” categories. Among the up-regulated proteins, we identified sucrose synthase (nodulin-100), β-tubulin, rubisco activase, glutathione-S-transferase, a putative heat-shock 70-kDa protein, pyridine nucleotide-disulphideoxidoreductase and a putative transposase. Proteomic analysis allowed for the identification of some putative symbiotic functions and confirmed the main biological processes triggered in the nitrogen-fixing symbiosis with soybean.

KEYWORDS: rhizobium, 2-D, proteomics, soybean roots, symbiosis

INTRODUCTION

The symbiotic associations of soybean [Glycine max (L.) Merrill] with bacteria belonging to the species Bradyrhizobium japonicum, B. diazoefficiens and B. elkanii have global economic and social importance. Brazil is an outstanding example, as biological nitrogen fixation (BNF) by soybean crops represents a key process in agricultural production systems, resulting in estimated savings of about US$15 billion in N-fertilizers per season.1 The establishment of the symbiosis begins with the exchange of molecular signals between the bacterium and the host plant, involving a succession of complex processes which trigger profound changes in both symbionts. Once the plant recognizes the specific Nod signal produced by the rhizobia, the plant follows a pre-determined developmental pathway to form the nodule. Nodule formation is a highly ordered process that involves the de-differentiation of root cortical cells and their subsequent division to form the mature organ.2–4

Proteomic analysis, which focuses on investigating the cumulative changes and modifications of proteins, helps to acquire a more comprehensive understanding of the responses occurring in host plants under symbiotic conditions.5 Proteomic studies of soybean bradyrhizobia have been reported, including studies with B. japonicum CPAC 15 (= SEMIA 5079),6,7 a strain belonging to the same serogroup as USDA
123 and broadly used in Brazilian commercial inoculants since 1992, due to symbiotic efficiency and competitive-ness characteristics. However, few genomic and proteomic studies have been reported with nodulated soybean, especially with Brazilian genotypes. Recently, Barros de Carvalho and collaborators reported a transcriptional analysis of genes involved in nodulation of soybean cv. Conquista, when inoculated with strain CPAC 15, revealed a variety of transcripts related to primary metabolism, cell-wall modifications, and an antioxidant defense system. Complementing this study, we are now analyzing differential protein expression patterns in the same symbiosis of cultivar Conquista with strain CPAC 15.

**Material and Methods**

**Plant material.** Soybean seeds (cultivar Conquista = MG/BR46) were surface-sterilized and germinated on absorbent paper moistened with sterile distilled water at 22 ± 2°C (in the dark) for three days. Seedlings were transferred to plastic bags containing 200 mL of N-free nutrient solution.

**Inoculum preparation and plant inoculation.** *B. japonicum* strain CPAC 15 (SEMIA 5079) was grown until the exponential phase of growth in yeast manitol broth (YMB). The cells were centrifuged and washed with saline solution (NaCl 0.85%). Aliquots of washed cell suspension were counted in YMB medium, indicating a concentration of 2.27 × 10^7 cells mL^-1. For the inoculated treatment, 1 mL of the inoculum was applied at the base of each radicle of 3-day-old seedlings. The experiment had a fully randomized design with three replicates, each consisting of 20 plants per treatment. Treatments consisted of: soybean roots inoculated with strain CPAC 15 and non-inoculated soybean.

Plants were grown under greenhouse conditions with a 12-h day/night period and mean temperature of 25–28°C/15–18°C (day/night) for ten days. Roots of 13-day-old soybean were then separated from shoots, immediately frozen in liquid nitrogen and stored at −80°C. Effectiveness of inoculation was proven by the inspection of abundant nodule primordia at the harvest. As at this stage only nodule primordia were present, the analyses were performed on whole roots. To confirm that inoculation was successful, we left several plants to grow until flowering stage, when we confirmed abundant nodulation and high production of biomass and N content in the shoots.

**Proteomics analysis.** Whole-cell proteins of soybean roots were extracted from both the inoculated and control treatments following the simplified method described by Rodrigues et al. Total-protein extract was solubilized in isoelectric focusing (IEF) buffer, quantified by the method described by Bradford and mixed with DeStreak buffer (GE Healthcare) at a final concentration of 350 µg, which was employed to rehydrate IGP strips (linear pH 4–7, 13 cm, GE Biosciences). 2-D assays were performed, as described by Rodrigues et al, in triplicate and the gels were analyzed by Image Master 2D Platinum v. 5.0 (GE Healthcare), after being stained with Coomassie Blue PhastGel™ R-350 (GE Healthcare). Well defined spots, present in all three gels, were selected, excised, and processed as described before. Digestion was performed with trypsin (Gold Mass Spectrometry Grade, Promega) at 37°C overnight.

Mass spectra were acquired in a MALDI-TOF-TOF Autoflex Spectrometer (Bruker Daltonics), which was operated in the reflector mode for MALDI-TOF peptide mass fingerprint (PMF) and in the “LIFT” mode for MALDI-TOF-TOF in the fully manual mode, using Flex Control software v. 2.2 and processed using Flex Analysis v. 3.0 (Bruker Daltonics). The PMFs and MS/MS ion spectra generated were searched against the public database NCBI (National Center for Biotechnology Information non-redundant) Viridiplantae, using the Mascot software v. 2.3 (Matrix Science) as previously described. Identifications, available at PRIDE (http://ebi.ac.uk/pride/) with the experiment accession number 14817, were validated only when the MOWSE (Molecular Weight Search) score was significant, and both decoy score and false discovery rates were considered.

**Protein characterization.** A set of bioinformatics tools was used for enhanced characterization of identified proteins. The proteins were fit into COG (Clusters of Orthologous Groups) categories according to their functional inference, using the COGnitor program (http://www.ncbi.nih.gov/COG). Software packages PSORT-B and PSLpred were used for prediction of subcellular localization.

**Results and Discussion**

Thirty-seven differentially expressed spots were analyzed by MALDI-TOF-TOF, resulting in the identification of 37 proteins that are highlighted by their numbers in Figure 1 and listed in Supplementary Table S1.

Seventeen root proteins were up-regulated (increased expression) and twenty were down-regulated (decreased expression), when compared to the control, after inoculation of soybean Conquista with strain CPAC 15 (Fig. 2).

Proteins were distributed into 13 categories according to the functional classification in COG, belonging to four functional groups: information storage and processing (A, L), cellular processes and signaling (M, O, U, Z), metabolism function (C, E, F, G, H), and poorly characterized proteins. Ten proteins were classified as hypothetical/conserved hypothetical and another ten did not fit into any of the categories, being assigned as “not in COG” (Fig. 3).

The most representative category was the “metabolic function,” with 37% of the identified proteins, 43% of which were related to energy production and conversion. Proteins related to cellular processes and signaling comprised 21%, followed by the information storage and processing category with 11%.

Symbiotic nitrogen fixation is an energy-demanding process, and the supply of sucrose to the nodule may limit fixation. In addition, nodule organogenesis requires imported
Roots inoculated with *Bradyrhizobium japonicum* strain CPAC 15

**Figure 1.** 2-D profile of soybean root extract indicating the differentially expressed proteins after inoculation with *Bradyrhizobium japonicum* strain CPAC 15. The molecular weight of protein standards is indicated on the left.

**Figure 2.** Fold change ratio of differentially expressed proteins of soybean roots, after inoculation with *Bradyrhizobium japonicum* strain CPAC 15. See Supplementary Table S1 for more details.

*Proteins only identified in the experimental condition (soybean inoculated with *B. japonicum* CPAC 15). Gel analysis was performed in Image Master 2D Platinum v 5.0 software.

The sucrose synthase (nodulin-100), up-regulated in our study, contributes significantly to the development of the cell wall, among other known functions in nodulation. Its activity increases rapidly during nodule development and declines during senescence. In addition, it is well known that several proteins are involved in plant-cell-wall penetration and cytoskeletal reorganization, given the need for structural modification of the root during the infection process by rhizobia.

Rubisco activase was another up-regulated protein (Supplementary Table S1). This finding is interesting since this enzyme normally accumulates in greening or photosynthetic tissues expressing rubisco. Nevertheless, rubisco activase is a member of a sequence superset of the AAA+ family, which are ATPases associated with diverse cellular activities and include ATP-dependent proteases, membrane fusion, DNA processing, and microtubule severing and trafficking. Some of the AAA+ proteins can also exhibit a classic chaperone activity in preventing the aggregation of denatured proteins and, in some cases, refolding them.

We also identified proteins with potential antioxidant properties. Glutathione-S-transferase and pyridine
Proteomic insights 2013:6

Minor changes in gene expression or redox state of thiols may define the difference between tolerant and susceptible genotypes or species. We also identified an up-regulated putative transposase. By the criterion of inheritance instability, transposable elements have been described in at least 35 mono- and dicotyledonous plant species.

A transcriptomic study performed in conditions similar to those of our study—including the same cultivar and strain—resulted in 3,210 differentially expressed transcripts. Two proteomic spots were used to validate the transcriptional data, represented by a putative glutathione-S-transferase and a sucrose synthase. In accordance with this transcriptional study, our study demonstrated intense metabolic activity during the nodulation process. Amongst the major processes, we highlighted the metabolic pathways of primary metabolism, cell-wall modification, and antioxidant-defense systems.

In our study, proteomics allowed the identification of some putative symbiotic functions and also confirmed the main biological processes triggered in the development of the nitrogen-fixing symbiosis in soybean. Concluding Remarks

By using proteomic tools, we evaluated the protein-expression pattern in roots of Brazilian soybean cultivar Conquista inoculated with Bradyrhizobium japonicum strain CPAC 15, contributing to an understanding of critical events at the cellular level.

It is important to note that data arising from genome and transcriptome studies are not always fully exploitable, since some sequences do not correspond to an assigned function, and direct information is not available about translation or co- and post-translational events of deduced gene products.

In our study, proteomics allowed the identification of some putative symbiotic functions and also confirmed the main biological processes triggered in the Brazilian soybean cultivar Conquista inoculated with B. japonicum strain CPAC 15, contributing to an understanding of critical events at the cellular level.

Concluding Remarks

By using proteomic tools, we evaluated the protein-expression pattern in roots of Brazilian soybean cultivar Conquista inoculated with B. japonicum strain CPAC 15, a strain broadly used in commercial inoculants in Brazil. The analyses allowed the identification of some putative symbiotic functions and also confirmed the main biological processes triggered in the Brazilian soybean cultivar Conquista inoculated with B. japonicum strain CPAC 15, contributing to an understanding of critical events at the cellular level.

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Author Contributions

Conceived of and designed the experiments: EPR, JSSB and MH. Analyzed the data: ART, EPR, JSSB and DFG. Wrote the first draft of the manuscript: ART. Contributed to the writing of the manuscript: ART, EPR, JSSB, DFG, MH. Agreed with manuscript results and conclusions: ART, MH.
Supplementary Data

Supplementary table 1. Proteins identified from roots of soybean cultivar Conquista at 10 days after inoculation with Bradyrhizobium japonicum strain CPAC 15.

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