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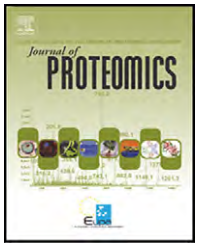

Proteomics reveals differential expression of proteins related to a variety of metabolic pathways by genistein-induced *Bradyrhizobium japonicum* strains

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ABSTRACT

The rhizobia–legume symbiosis requires a coordinated molecular interaction between the symbionts, initiated by seed and root exudation of several compounds, mainly flavonoids, that trigger the expression of nodulation genes in the bacteria. Since the role of flavonoids seems to be broader than the induction of nodulation genes, we aimed at characterizing genistein-induced proteins of *Bradyrhizobium japonicum* CPAC 15 (=SEMIA 5079), used in commercial soybean inoculants in Brazil, and of two genetically related strains grown *in vitro*. Whole-cell proteins were extracted both from induced (1 μM genistein) and from non-induced cultures of the three strains, and separated by two-dimensional electrophoresis. Spot profiles were compared between the two conditions and selected spots were excised and identified by mass spectrometry. Forty-seven proteins were significantly induced by genistein, including several hypothetical proteins, the cytoplasmic flagellar component FliG, periplasmic ABC transporters, a protein related to biosynthesis of exopolysaccharides (ExoN), and proteins involved in redox-state maintenance. Noteworthy was the induction of the PhyR-σ^{EcfG} regulon, recently demonstrated to be involved in the symbiotic efficiency of, and general stress response in *B. japonicum*. Our results confirm that the role of flavonoids, such as genistein, can go far beyond the expression of nodulation-related proteins in *B. japonicum*.

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1. Introduction

Bradyrhizobium japonicum is a rhizobial species capable of establishing symbiotic associations with soybean (*Glycine max*), siratro (*Macroptilium atropurpureum*), mung bean (*Vigna radiata*) and other *Vigna* species. Legume nodulation is a complex multi-step process that requires specific interactions between the symbionts, starting with the exchange of a variety of molecular signals between the host plant and the bacterium. Seed and root exudates comprise a variety of compounds that induce the expression of specific genes in compatible bacteria, generally preceded by a common conserved sequence denominated "*nod* box". Genistein and daidzein are isoflavones found in soybean seeds and root exudates that are primary inducers of nodulation genes of *B. japonicum* [1,2].

It has been shown that the role of flavonoids, such as genistein, can go beyond the expression of nodulation genes. For example, a set of extracellular proteins induced by genistein, including the type-III secretion system, flagellin and hypothetical proteins was previously reported [3,4] and the expression changes were also confirmed at the transcriptomic level [2]. Another study [5], by using RT-qPCR, presented the

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differential expression of *nopP* gene, which may play an important role in the infection process, in cells of a strain of *B. japonicum* incubated with genistein. Interestingly, several non-flavonoid compounds, e.g. jasmonates and phenolic compounds, may also act as inducers of nodulation genes in rhizobial species including *B. japonicum* [6,7]. Altogether, these studies indicate that the molecular signaling pathways between legumes and rhizobia are far from being completely understood.

In the process of rhizobial-strain selection and recommendation for inclusion in inoculants, some characteristics must be taken into consideration, including saprophytic ability in the soil and capacity to nodulate and fix N_2. *B. japonicum* strain CPAC 15 (belonging to the same serogroup as strains SEMIA 566 and USDA 123), obtained in a Brazilian strain-selection program, has a high capacity for adaptation to the stressful environmental conditions of the Cerrados — also known as the Brazilian savannahs — the main soybean-producing area of the country [8]. Its saprophytic and competitive competences against other rhizobial strains have also been broadly demonstrated, accompanied by its high capacity for N_2 fixation, which have resulted in its recommendation for inclusion in commercial inoculants since 1992 [8,9].

Recently, a two-dimensional reference map of whole-cell proteins of *B. japonicum* CPAC 15 was generated, and attempts were made to predict the functions of several expressed hypothetical proteins [10]. In this study, we investigated the effects of genistein in the *in-vitro* expression of *B. japonicum* proteins of strain CPAC 15 and two other genetically related strains, S 370 and S 516, which are characterized by contrasting N_2-fixation capacity [9,11,12]. Identification of proteins produced by this set of strains differentially expressed in the presence of genistein may contribute to our still-poor knowledge of *B. japonicum* proteins induced by soybean flavonoids.

2. Experimental procedures

2.1. Bacterial strains and induction conditions

MB. *japonicum* CPAC 15 is a putative natural variant of strain SEMIA 566, selected for its high saprophytic and environmental stress tolerance capacities and the strain has been broadly used in commercial inoculants in Brazil since 1992; more information about the strain is given elsewhere [8,9]. Strains S 370 and S 516 are also putative variants of SEMIA 566, and previous genetic and symbiotic characterizations of these two strains are available elsewhere [9,11,12]. The three strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection" of Embrapa Soja (http://www.bmrc.lncc.br).

The strains were pre-cultured in 10 mL aliquots of tryptone-yeast extract medium (TY), at 120 rpm and 28 °C, in the dark. The pre-cultures where then transferred to Erlenmeyer flasks containing TY medium and induced or not with genistein (1 μM) dissolved in methanol [3]. In non-induced cultures, methanol was added at the same proportion as in the induced treatment. Cells were incubated until exponential phase of growth was reached (optical density at 630 nm of 0.7–0.8), with low agitation (80 rpm) to avoid the formation of high amounts of extra-cellular polysaccharides, interferents that compromise 2DE gels resolution.

2.2. Preparation of whole-cell extracts of proteins

Cultures were centrifuged at 5000×*g*, at 4 °C and cells were carefully washed with a solution containing 3 mM KCl; 1.5 mM KH_2PO_4; 68 mM NaCl; and 9 mM NaH_2PO_4. Washed cells were resuspended in 600 μL of a buffer containing 10 mM Tris–HCl pH 8.0; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF. Aliquots of 150 μL were stored in an ultrafreezer (−80 °C) until the analyses.

For whole-cell protein extraction, aliquots were resuspended in lysis buffer containing 9.5 M urea; 2% CHAPS; 0.8% v/v Pharmalyte 3–10; and 1% DTT, and submitted to thirty cycles of freezing in liquid N_2 and thawing at 37 °C. The lysates were separated from particulate material by centrifugation at 14,000×*g* for 90 min, at 4 °C. Total-protein concentration was determined by Bradford's method [13].

2.3. 2-D electrophoresis

For IEF, lysates were dissolved with DeStreak buffer (GE Healthcare) to a final concentration of 300 μg of protein and 2% v/v IPGphor in 250 μL of solution. IPG-strips (pH 3–10, 13 cm; GE Healthcare) were rehydrated with the protein solution and covered with cover fluid (GE Healthcare). Loaded strips were submitted to focalization in an Ettan IPGphor IEF system (GE Healthcare) for 1 h at 200 V, 1 h at 500 V, a gradient step to 1000 V for 1 h, a gradient step to 8000 V for 2 h 30 min, and fixed at 8000 V for 1 h 30 min. The final Vh was fixed at 24,800. After focusing, strips were equilibrated first for 20 min in 5 mL of TE buffer (50 mM Tris–HCl pH 8.8; 6 M urea; 30% v/v glycerol; 2% w/v SDS; 0.2% v/v of a 1% solution of bromophenol blue) supplemented with 50 mg DTT and then in TE buffer with 175 mg iodoacetamine, also for 20 min.

The 2-D electrophoresis was performed on a 12% polyacrylamide gel in a Ruby SE 600 Vertical Electrophoresis System (GE Healthcare). The run was carried out for 30 min at 15 mA/gel and 240 min at 30 mA/gel, using the Low Molecular Weight Calibration Kit for SDS Electrophoresis (Amersham Biosciences) as standard. For each strain and condition (induced and non-induced with genistein), the extraction and gel electrophoresis were run in triplicate, therefore with a total of 18 gels. Gels were fixed overnight with an ethanol-acid acetic solution before being stained with Coomassie Blue PhastGel™ R-350 (GE Healthcare), were scanned (ImageScanner LabScan v5.0).

2.4. Gel image analysis

Spots were strictly identified in the high-resolution digitalized gel images and analyzed by Image Master 2D Platinum v 5.0 software (GE Healthcare). Ratios of mean normalized spot volumes were calculated. All selected spots were manually confirmed and statistically evaluated ($p<0.05$) upon Student's *t*-test, using XLSTAT (Addinsoft, France, add-in to Microsoft Excel).

2.5. Sample preparation for MS

Selected spots were excised and processed as previously described [14]. Digestions were done with trypsin (Gold Mass Spectrometry Grade, Promega, Madison, WI) at 37 °C overnight.

2.6. MALDI-TOF/TOF–TOF analysis

Tryptic peptides (0.5 μL) were mixed with saturated solution of HCCA (α-cyano-4-hydroxy-cinamic acid) in 50% acetonitrile, 0.1% TFA (Trifluoroacetic acid). The mixture was spotted onto a MALDI sample plate and allowed to crystallize at room temperature. The same procedure was used for the standard peptide calibration mix (Bruker Daltonics).

For mass spectra acquisition, a MALDI-TOF-MS Autoflex spectrometer (Bruker Daltonics) was operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) in the fully automated mode, or manually in the LIFT mode for MALDI-TOF/TOF, using the FlexControl 3.0 (Bruker Daltonics) software.

2.7. Protein identification

PMFs and MS/MS ions generated were searched against the public database NCBInr (National Center for Biotechnology Information non-redundant), Proteobacteria, using the Mascot software v. 2.3 (Matrix Science). For protein searches, monoisotopic masses were used, considering a peptide tolerance of 150 ppm and allowance of one missed cleavage. When MS/MS was carried out, a tolerance of 0.3 Da was acceptable. Carbamidomethylation of cysteine and oxidation of methionine were considered fixed and variable modifications, respectively.

Identifications were only validated when the Mowse (molecular weight search) score was significant, above the recommended cutoff score. Searches on the decoy database (Mascot) were done and both decoy score and false discovery rates were considered for the identification.

The spectrometry datasets are available at PRIDE (http://ebi.ac.uk/pride/) with the experiment accession number 9769. The number of each protein identified in our study corresponds to the same number of the reference map of *B. japonicum* [10].

3. Results and discussion

3.1. Gel analysis and spot selection

Fig. 1 shows the 2DE profiles of the three *B. japonicum* strains (CPAC 15, S 370 and S 516), induced with genistein, with high congruence of spots between the strains. By using the computer-assisted gel image analysis software, spots were detected between pIs 3 and 10, with molecular masses mainly between 14.4 and 97 kDa. Proteins of experimental molecular masses higher than 97 kDa and highly alkaline had unsatisfactory resolution and, therefore, were not considered in the analysis.

The volume of each spot was normalized as percent of the total volume of all spots detected in the gel. This procedure was followed for all eighteen gels and the values generated for each spot were compared between induced and non-induced treatments, for each strain; only spots showing statistically significant differences ($p<0.05$) between genistein-induced and -non-induced cells were selected (Fig. 2). A significant difference indicated that the genistein acted in a specific mechanism, resulting in changes in protein abundance.

At the proteomic level, in addition to the same 2DE pattern, we have also found, for the great majority of the proteins, high congruence in the intensity of induction by genistein for all three strains. This observation does not mean that the levels of expression for a given protein are similar for all three strains, but rather that the rates of induction, evaluated by the relative volumes (%vol) are similar. The detection of differential expression in different strains confirms a more trustworthy result, given the equivalence of the data of the same protein in each strain [15].

3.2. Proteins of *B. japonicum* strains induced by genistein

For the identification, the score given for all spots is the Mowse (Molecular Weight Score), given by Mascot software, are available on PRIDE database (accession number 9769). Mowse is based on probability and gives a score that informs how the theoretical values are statistically related to the experimental values. Indeed, the Decoy score (also given in Mascot search results) was analyzed to eliminate the false positives.

Even using the Proteobacteria database for the search, the great majority of our best and significant hits provided an exact match with proteins inferred from the *B. japonicum* USDA 110 genome (Table 1), which means that the occurrence of false positives are almost impossible. The Mowse and Decoy scores are being considered more intuitive for protein searches than e-value, because they are more independent of the database size. Also, in the case of Mowse score, the statistical weight is given for each individual peptide match, and not for the whole sequence as the e-value.

In *B. japonicum*, genes related to the symbiosis, including nodulation and N_2 fixation are located in a symbiotic island [16]. However, in a transcriptomic study with *B. japonicum* induced by genistein *in vitro*, only 16% of the up-regulated clones were located in this island [17]. The results from our study support that genistein may be also capable of inducing genes located outside the symbiotic island and related to other metabolic pathways. The characterization of these proteins is important to understanding how broad is the genistein influence on the metabolism of *B. japonicum* as a whole. When compared to the control treatment with methanol, genistein resulted in over-expression of forty-seven proteins, comprising diverse functional groups, as shown on Table 1.

All proteins identified were of cytoplasmic and periplasmic origin (data from PsortB software analysis), as the extraction method favored release of soluble proteins. Although the main known role of flavonoids is the induction of nodulation genes, no proteins related to nodulation genes were identified in our study, probably because of the growth stage of the cells.

Nodulation genes are highly expressed during the early log phase, but seem to decrease to undetectable levels during

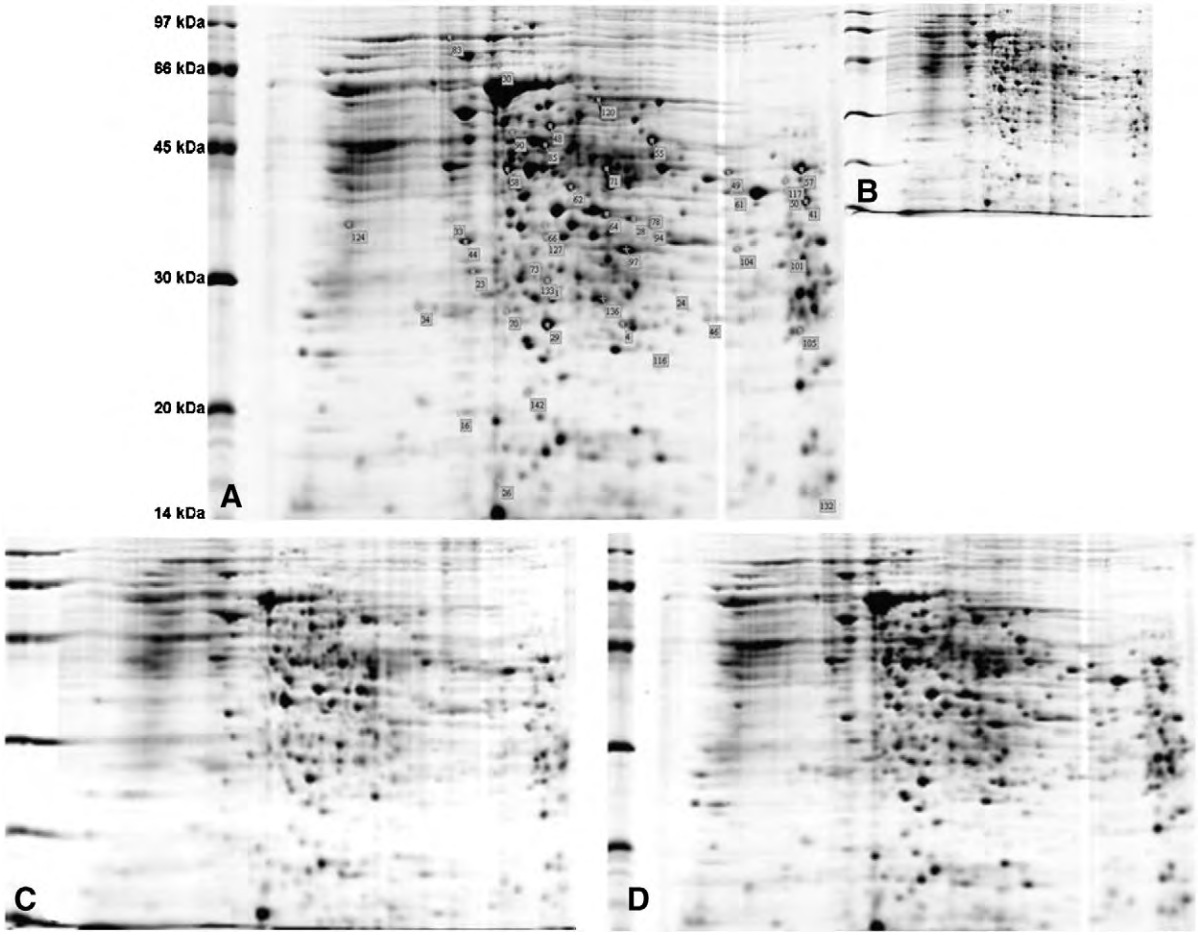

Fig. 1 – CPAC15 strain 2DE reference profile of whole cell extract indicating the differentially expressed proteins after genistein induction (A) in relation to the control treatment (B). Strains S370 (C) and S516 (D), both genistein induced, showed a high degree of congruence in the spots profile.

middle and late log phases — the stage of the cells in our study — probably as a result of a sensitive feedback regulation mechanisms [18]. Under this condition, the *nod* genes are repressed by a cascade initiated by the regulator NswB, triggering induction of NolA that activates NodD2, which then represses the *nod* genes. This repression of *nod* genes has been demonstrated both *in vitro* and *in planta* [18]. This may also explain the difficulty to detect nodulation-related proteins in other proteomics experiments in *Rhizobium leguminosarum* [19] as well as in planta in *B. japonicum* [20].

Six other differentially expressed proteins were not included in our description, as they did not fulfill the statistical requirements. The great majority of the proteins identified in our study were detected in the three strains; however, with four of them the values of induction were not statistically significant for all strains ($p<0.05$), as listed on Table 1. One example was for the amino acid-binding protein Bll2909 and the hypothetical protein Bll2738, which were found to be induced in the three strains, but not significantly in S 370. Another exception was the 50S ribosomal protein L25/general stress protein Ctc (Bll7441), which was highly expressed in the presence of genistein only by strain S 516.

3.2.1. *Induced proteins related to chemotaxis, EPS biosynthesis and cysteine metabolism*

In our study the synthesis of a flagellar protein was upregulated by genistein, confirming previous functional genomics assays [2,4]. We identified the differential expression of FliG (Blr7000) (Fig. 1), a cytoplasmic flagellar component implicated in motility by switching the direction of flagellar rotation, related to chemotaxis. Chemotaxis towards genistein by *B. japonicum* strains is still controversial, since the flavonoid is not the main rhizobial chemo-attractant released in soybean exudates [21]. A microarray experiment showed that the main group of genistein-inducible genes was represented by flagellar proteins [2], which were detected in the secretome analysis [3].

Another protein that was induced by genistein was an UTP-glucose-1-phosphate uridylyltransferase, product of the *exoN* (blr1499) gene, and involved in the initial steps of exopolysaccharide (EPS) biosynthesis. The effect of flavonoids, including genistein, in the induction of EPS synthesis was also demonstrated in *Sinorhizobium fredii* [22]. Although the role of EPSs on symbiotic interaction is still not well defined, various biological functions have been assigned to them, such as

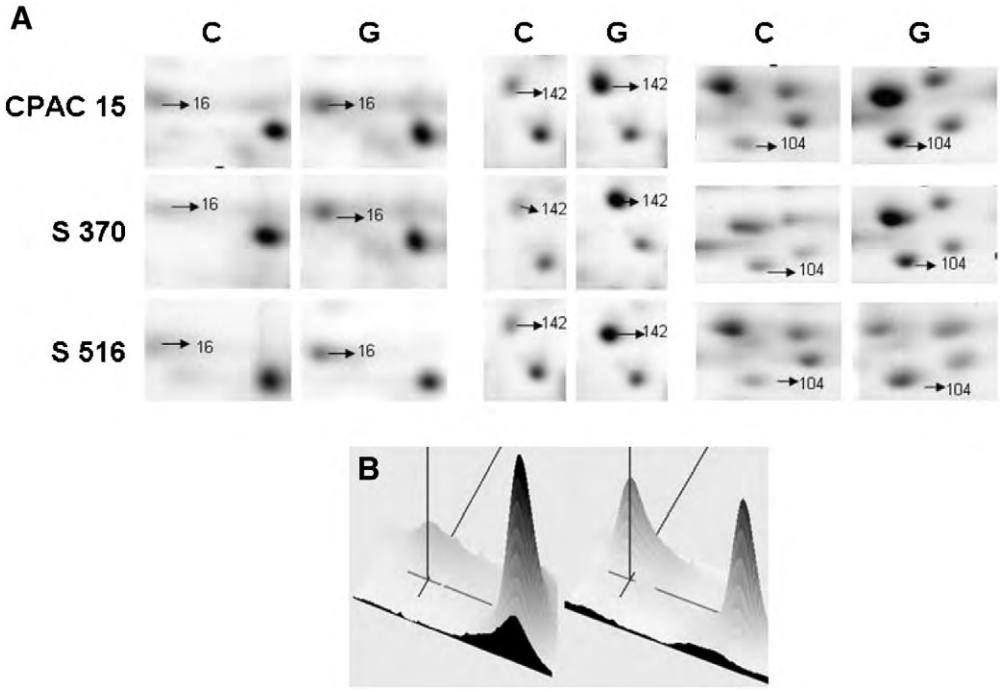

Fig. 2 – (A) Close-up views of selected spots in CPAC 15, S 370 and S 516 strains, differentially expressed in the presence of genistein (G) in relation to the control treatment (C). Spots are also marked at Fig. 1 and described in Table 1. (B) Three-dimensional view showing the degree of induction of extracytoplasmic function sigma-factor protein (σ^{EcfG})(Spot 16).

protection from desiccation, attachment to roots, infection-thread formation, and suppression of plant-defense responses [23]. In addition, rhizobial EPSs seem to act as signaling molecules that generate specific developmental responses on the host [24]. In *S. meliloti exoN* mutants, EPS production was not inhibited, but there was a significant decrease in the biosynthesis of succinoglycan exopolysaccharide type 1 (EPS1) [24].

Another important protein for the establishment of the symbiosis identified in our study was the GTP-binding protein TypA. This protein plays several roles in a wide range of bacterial species; besides being involved in stress adaptation, it is required also for both pathogenic and symbiotic interactions [25].

Proteins involved in redox-state maintenance, also important for bacteria/host-plant interactions, such as electron-transfer flavoprotein alpha and beta subunits (Blr1378 and Blr1377) and thioredoxin (Blr0594), were also up-regulated by genistein. The same proteins were up-regulated in the proteome of *Gluconacetobacter diazotrophicus* co-cultivated with sugarcane plantlets [26].

Finally, three periplasmic ABC-transporter amino acid-binding proteins (Blr5676, Bll1058 and Blr4446) were differentially expressed in the presence of genistein. ABC transporters, which represent one of the largest superfamilies of active membrane transport proteins (MTPs), have a highly conserved ATPase domain that binds and hydrolyzes ATP, supplying energy for the uptake of a variety of nutrients and for the extrusion of drugs and metabolic wastes [27]. Rhizobia, including *B. japonicum*, are known for their abundance of ABC transporters [28,29], also confirmed at the proteomics level [10,30], and in this study we found that some were induced by genistein.

3.2.2. *Differential expression of the PhyR-σ^{EcfG} regulon*

In a proteomics study, it has been demonstrated that the two-component response regulator PhyR is responsible for the epiphytic adaptation of *Methylobacterium extorquens* to *Arabidopsis thaliana* [31]. Later, it was shown that PhyR is also involved in a general stress-response mechanism for both *M. extorquens* [32] and *B. japonicum* [33,34] and, in a more complex system control, in *S. meliloti* [35]. So, it was recognized that PhyR is a member of a signaling cascade, acting together with an extracytoplasmic function sigma-factor protein (σ^{EcfG}, Blr7797), involved in both general stress response and symbiotic efficiency [34]. Our work showed that these two proteins were significantly induced in the presence of genistein (Table 1).

It is also noteworthy that *B. japonicum* mutants lacking PhyR or σ^{EcfG} were impaired in nodulation ability [34]. Although the PhyR/σ^{EcfG} regulatory system is not fully characterized, it may well act at the early stages of symbiosis development, revealing greater complexity and more features to the role of flavonoids released by the host plant.

3.3. *Hypothetical proteins differentially expressed in the presence of genistein*

The detection of a protein in a proteomics experiment may allow the removal of the tag "hypothetical," if a function can be assigned according to its biochemical features. The differential expression of this class of proteins may provide helpful

Table 1 – Identified proteins of *Bradyrhizobium japonicum* whole cell extracts differentially expressed after the induction with genistein. Matched peptides masses and MS/MS combined results are available in PRIDE (http://ebi.ac.uk/pride/) under the experiment accession number 9769.

Spot number [a]	Locus ID	Protein description	MW (T/E)	pI (T/E)	Fold change ratio [b]		
					CPAC 15	S 370	S 516
4	bll7441	50S ribosomal protein L25/general stress protein Ctc [c]	24.6/25	9.30/7.57	1.42±0.18	1.29±0.15	1.65±0.11
16	blr7797	RNA polymerase sigma factor (σ^{EcfG})	19.9/19	5.3/5.16	2.00±0.19	1.97±0.09	1.86±0.12
21	bll5360	Putative beta-lactamase precursor	31.4/28	8.5/6.58	1.81±0.05	1.97±0.06	1.92±0.13
23	bll7795	Two-component response regulator PhyR	29.6/29	5.04/5.52	1.72±0.08	1.77±0.03	1.89±011
24	mll3725	Hybrid sensory histidine kinase (*Mesorhizobium loti*)	153.6/27	5.11/8.19	1.98±0.02	1.87±0.06	1.81±0.09
26	bll4288	Two-component response regulator	16.1/16	5.29/5.70	2.41±0.21	2.58±0.12	2.50±0.2
28	blr1499	UTP-glucose-1-phosphate uridylyltransferase	32.1/34	6.62/7.75	1.54±0.05	1.76±0.05	1.65±0.06
29	bll4944	ATP-dependent Clp protease proteolytic subunit	23.5/24	5.70/6.56	2.23±0.16	2.21±0.11	2.31±0.19
30	blr0540	GTP-binding tyrosin phosphorylated protein TypA	67.1/69	5.49/6.90	2.08±0.05	2.17±0.12	2.11±0.12
31	blr7000	Flagellar motor switch protein G	40.4/31	4.88/5.86	1.98±0.10	1.81±0.08	2.19±0.11
33	blr0594	Thioredoxin	33.5/34	5.19/5.22	1.79±0.12	1.70±0.03	1.69±0.04
34	blr0676	Heat shock protein	21.6/27	4.84/4.75	1.57±0.05	1.53±0.02	1.64±0.05
41	bll6137	Quinone oxidoreductase	34.3/37	8.84/9.86	2.30±0.20	2.11±0.14	1.99±0.09
44	blr1378	Electron transfer flavoprotein alpha subunit	32.1/32	5.14/5.38	1.99±0.08	1.80±0.10	1.89±0.05
45	blr1377	Electron transfer flavoprotein beta subunit	26.4/27	8.86/9.73	1.89±0.08	1.89±0.12	2.00±0.06
46	BRADO2303 (blr37305)	Two component sensor kinase for C4-dicarboxylate transport (*Bradyrhizobium* sp. ORS278)	67.3/25	7.74/8.63	1.87±0.05	1.82±0.07	1.67±0.03
48	bll6899	ABC transporter substrate-binding protein	51.9/45	6.97/7.08	1.64±0.13	1.61±0.11	1.62±0.08
49	bll0733	ABC transporter glycerol-3-phosphate-binding protein	48.2/40	7.68/8.96	1.79±0.08	1.78±0.11	1.67±0.12
50	bll1523	Glyceraldehyde-3-phosphate dehydrogenase	35.8/38	7.77/9.45	1.63±0.07	1.66±0.08	1.78±0.08
55	bll7416	Aspartate aminotransferase	44.4/44	6.04/7.95	1.64±0.12	1.62±0.09	1.63±0.13
57	blr5676	ABC transporter substrate-binding protein	40.0/41	8.90/9.46	1.80±0.12	1.91±0.14	1.87±0.12
58	blr2922	ABC transporter amino acid-binding protein	48.4/40	6.35/6.03	1.60±0.01	1.65±0.08	1.66±0.07
61	bll4453	Cysteine synthase A	37.2/38	7.57/8.93	1.73±0.06	1.74±0.06	1.60±0.02
62	bll1058	ABC transporter substrate-binding protein	39.7/37	6.62/6.91	1.86±0.11	1.68±0.08	1.61±0.08
64	bll2909	Amino acid binding protein [d]	36.0/34	7.59/7.34	1.70±0.10	1.36±0.15	1.62±0.09
66	blr4446	ABC transporter amino acid-binding protein	36.8/33	6.21/6.57	1.64±0.11	1.62±0.11	1.77±0.12
70	blr0495	Isopropylmalate isomerase small subunit	22.7/26	5.52/5.97	1.79±0.16	1.63±0.10	1.71±0.12
71	bll5953	ABC transporter substrate-binding protein	41.8/39	7.71/7.39	1.75±0.09	1.66±0.08	1.82±0.12
73	blr4884	ABC transporter substrate-binding protein	37.6/40	8.72/9.68	2.56±0.32	2.47±0.21	2.41±0.29
78	BBta_6849 (blr7115 [e])	Phosphoribosylaminoimidazole carboxylase ATPase subunit (*Bradyrhizobium* sp. BTAi1)	40.1/35	6.17/7.91	1.62±0.12	1.60±0.05	1.64±0.10
83	bll0779	Polynucleotide phosphorylase/polyadenylase	78.3/86	5.47/5.31	1.82±0.14	1.77±0.15	1.73±0.14
85	bll5945	S-adenosylmethionine synthetase	43.6/41	5.88/6.58	1.70±0.06	1.66±0.04	1.60±0.04
90	bll3808	3-oxoacyl-(acyl carrier protein) synthase II	44.8/45	5.56/6.12	1.81±0.13	1.90±0.19	1.92±0.20
94	bll6223	3-hydroxybutyryl-CoA dehydrogenase	30.3/33	6.44/7.94	1.94±0.14	1.87±0.17	1.64±0.10
97	blr3940	Putative propionyl-CoA carboxylase beta chain	57.1/31	8.35/7.55	1.72±0.09	1.62±0.05	1.62±0.08
101	bll5333	Putative dehydrogenase	38.3/32	9.52/9.62	1.85±0.15	1.76±0.17	1.89±0.12
104	blr1091	ABC transporter phosphate-binding protein	36.3/31	7.68/9.05	2.12±0.22	2.07±0.21	1.93±0.11
105	bll4865	Putative beta-type carbonic anhydrase	25.9/24	8.26/9.85	1.90±0.08	1.87±0.14	1.91±0.07
116	blr7568	TrpR binding protein [d]	20.8/23	6.97/8.01	1.58±0.12	1.51±0.14	1.63±0.10
117	blr2780	NAD-dependent alcohol dehydrogenase	37.8/39	8.05/9.67	1.70±0.09	1.67±0.07	1.72±0.07
120	bll7437	Hypothetical protein (Transcriptional regulation of fatty acid biosynthesis)	17.4/53	7.93/7.27	1.85±0.14	1.73±0.12	1.77±0.10
124	blr7528	Hypothetical protein (Uncharacterized conserved protein, putative periplasmic ligand-binding sensor protein)	28.0/35	4.22/3.95	1.92±0.15	1.96±0.12	1.89±0.15
127	bll2738	Hypothetical protein (No related data) [d]	14.0/21	11.33/6.25	1.83±0.05	1.48±0.27	1.71±0.09
132	blr2474	Hypothetical protein (Identical to Bll5191)	17.0/15	9.62/9.83	1.64±0.09	1.76±0.08	1.67±0.10
133	blr2865	Hypothetical protein (Methionyl-tRNA synthetase)	28.0/28	6.25/6.46	1.64±0.08	1.79±0.07	1.80±0.08
136	blr5933	Hypothetical protein (Fumarylacetoacetate hydrolase)	25.5/26	6.60/7.19	3.00±0.22	3.21±0.32	2.89±0.15
142	bll6649	Hypothetical protein (PRC-barrel like protein)	18.0/20	6.74/6.28	2.63±0.18	2.46±0.19	2.69±0.15

[a] According to the reference map of *B. japonicum* CPAC 15 [10].
[b] The fold change ratio of one protein was obtained from the protein abundance ratios between the genistein and control treatments. SD 2 means the standard deviation of protein ratios of the spot.
[c] Genistein induction was statistically significant ($p<0.05$) only for S 516.
[d] Genistein induction was statistically significant ($p<0.05$) only for CPAC 15 and S 516.
[e] High degree of similarity 1 with locus shown in brackets of the *B. japonicum* USDA 110 genome.

and new information and deeper understanding of uncharacterized aspects of a given biological system.

Recently, in the proteomic reference map of *B. japonicum* strain CPAC 15, the functional inference of twenty-six hypothetical proteins by the utilization of several bioinformatics tools was successfully done [10]. Seven of these hypothetical proteins were found in our study to be induced by genistein (Table 1).

Transcription induction of Bll6649 was previously reported in bacteroids of *B. japonicum* [36,37]. A functional inference for this protein [10] showed similarity to a PRC-barrel, with two membrane-related domains. Despite PRC derivates from "bacterial photosynthetic reactions," the domain is also involved in the processing of rRNA [38]. In addition, Bll6649 is a putative member of the PhyR/σ^{EcfG} regulon [34], which also figured in our sample set of genistein-induced proteins.

The down-regulation of Blr2474 in bacteroids, in comparison to aerobically grown cells was verified before [37] and our study presented that the expression of this hypothetical protein is significantly induced by genistein. In the complete genome of *B. japonicum*, there are two identical copies of this protein, the other one being Bll5191. Blr2474 is very similar to the reported symbiotically induced *S. meliloti* protein Nex18 [39], which possesses a fascilin domain, characteristic of some surface-associated proteins involved in cell adhesion, an essential initial step in root infection by rhizobia.

A probable fumarylacetoacetate (FAA) hydrolase (Blr 5933) analog to the product of *hmgB* gene was also induced by genistein. This protein is involved in the catechol pathway and was described in a comparative proteomic study of *B. japonicum* under free-living and nodule-residing conditions [20].

3.4. *Difference between strains S 370 and S 516*

In a previous study, the genomes of strains S 370 and S 516 were compared by representational difference analysis (RDA) [12], and twenty-four gene sequences were identified as unique to S 370. None of these sequences was identified in our study, which may be attributed to two factors. First, only genistein-induced proteins were identified in our study. Second, when the sequences obtained in the RDA study were analyzed with software designed to predict cellular sublocation, we found that the majority corresponded to extracellular/membrane proteins, involved with transport. On the other hand, in our comparative proteomic analysis, we focused on the whole-cell content, which excludes very hydrophobic proteins and the secretome.

Strains CPAC 15, S 370 and S 516 are natural variants of the same parental strain SEMIA 566 and, therefore, are genetically highly similar [8,11], as reflected in the 2DE profiles (Fig. 1). Despite variations in spot volumes between the strains (data not shown), the relative volumes (%vol), which are considered in differential expression analysis, were similar in all three strains for the great majority of the proteins, resulting in congruent results.

All forty-seven proteins described in this study were also detected in a proteomic reference map built from whole-cells of *B. japonicum* strains CPAC 15, S 370 and S 516 grown under *in vitro* conditions, where 148 spots were identified as cytoplasmic and periplasmic proteins belonging to diverse metabolic pathways [10].

3.5. *Concluding remarks*

It is well known that genistein induction plays a key role in the establishment of the symbiosis with soybean plants, through the induction of several genes in free-living *B. japonicum*. Here we report that differential protein-expression responses to genistein can go far beyond their roles in activating nodulation and type-III secretion-system genes. The induced proteins identified in our study are involved in a variety of metabolic pathways, including the interesting signaling cascade of PhyR-σ^{EcfG}, functionally related to stress response and with symbiosis by a still unknown mechanism; the proteins in our study were constitutively expressed *in vitro* and significantly stimulated in the presence of genistein.

We did identify several nodulation-related proteins; however, no one is actually encoded by a nodulation (*nod*, *noe* and *nol*) gene. We attribute the results to the growth conditions, since the nodulation genes are mainly expressed in early growth phase stage.

Transcriptomics and proteomics characterizations are not always correlated, especially if the time of induction and compounds are distinct, which are important features in expression analysis. Mimicking the conditions of induction of a previous proteomic experiment focused on genistein-inducible extracelullar proteins [3], we now add important knowledge for a more global comprehension of the metabolic responses of *B. japonicum* to genistein. Indeed, our findings correlate well with a recently published article about daidzein-induction effects in the proteome of *B. japonicum* strains [40].

It is also noteworthy that the characterization of the proteins in our study was confirmed in three genetically related strains, adding robustness to our results, and confirming that the forty-seven proteins identified were induced by genistein.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2011.10.032.

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Supplemental Table 1: Statistical evaluation of the identified proteins of *Bradyrhizobium japonicum* whole cell extracts differentially expressed after the induction with genistein.

Spot ID	Protein locus	Protein description	T test[1]		
			CPAC 15	S 370	S 516
4	bll7441	50S ribosomal protein L25/general stress protein Ctc[2]	0,5675	0,0620	0,0015
16	blr7797	RNA polymerase sigma factor (σ^{EcfG})	0,0025	0,0006	0,0028
21	bll5360	Putative beta-lactamase precursor	0,0046	0,0332	0,0012
23	bll7795	Two-component response regulator PhyR	0,0112	0,0005	0,0068
24	mll3725	Hybrid sensory histidine kinase *(Mesorhizobium loti)*	0,0041	0,0176	0,0005
26	bll4288	Two-component response regulator	0,0074	0,0014	0,0129
28	blr1499	UTP-glucose-1-phosphate uridylyltransferase	0,0143	0,0033	0,0214
29	bll4944	ATP-dependent Clp protease proteolytic subunit	0,0072	0,004	0,0032
30	blr0540	GTP-binding tyrosin phosphorylated protein	0,0010	0,0338	0,0046
31	blr7000	Flagellar motor switch protein G	0,0002	0,0019	0,001
33	blr0594	Thioredoxin	0,007	0,043	0,0012
34	blr0676	Heat shock protein	0,012	0,029	0,034
41	bll6137	Quinone oxidoreductase	0,0289	0,008	0,006
44	blr1378	Electron transfer flavoprotein alpha subunit	0,0039	0,0012	0,0052
45	blr1377	Electron transfer flavoprotein beta subunit	0,0032	0,0054	0,0019
46	BRADO2303	Two component sensor kinase for C4-dicarboxylate transport (*Bradyrhizobium* sp. ORS278)	0,0092	0,0066	0,0032
48	bll6899	ABC transporter substrate-binding protein	0,0043	0,0007	0,0033
49	bll0733	ABC transporter glycerol-3-phosphate-binding protein	0,0083	0,0013	0,0012
50	bll1523	Glyceraldehyde-3-phosphate dehydrogenase	0,0004	0,0001	0,0055
55	bll7416	Aspartate aminotransferase	0,0067	0,0006	0,008
57	blr5676	ABC transporter substrate-binding protein	0,019	0,023	0,017
58	blr2922	ABC transporter amino acid-binding protein	0,0056	0,0098	0,0067
61	bll4453	Cysteine synthase A	0,0089	0,0012	0,0019
62	bll1058	ABC transporter substrate-binding protein	0,0065	0,0015	0,0038
64	bll2909	Amino acid binding protein[3]	0,0234	0,0656	0,0087

66	blr4446	ABC transporter amino acid-binding protein	0,0056	0,0007	0,0003
70	blr0495	Isopropylmalate isomerase small subunit	0,0032	0,0068	0,0005
71	bll5953	ABC transporter substrate-binding protein	0,0036	0,0367	0,0298
73	blr4884	ABC transporter substrate-binding protein	0,0024	0,0011	0,0015
78	BBta_6849	Phosphoribosylaminoimidazole carboxylase ATPase subunit (*Bradyrhizobium* sp. BTAi1)	0,0009	0,0016	0,0006
83	bll0779	Polynucleotide phosphorylase/polyadenylase	0,0227	0,0334	0,0187
85	bll5945	S-adenosylmethionine synthetase	0,0013	0,0041	0,0011
90	bll3808	3-oxoacyl-(acyl carrier protein) synthase II	0,0003	0,0056	0,0009
94	bll6223	3-hydroxybutyryl-CoA dehydrogenase	0,0229	0,0143	0,0098
97	blr3940	Putative propionyl-CoA carboxylase beta chain	0,0001	0,0017	0,0027
101	bll5333	Putative dehydrogenase	0,0002	0,0002	0,0014
104	blr1091	ABC transporter phosphate-binding protein	0,0043	0,0042	0,0003
105	bll4865	Putative beta-type carbonic anhydrase	0,0018	0,0036	0,0057
116	blr7568	TrpR binding protein[3]	0,0128	0,0057	0,0143
117	blr2780	NAD-dependent alcohol dehydrogenase	0,0087	0,0216	0,0223
120	bll7437	Hypothetical protein (Transcriptional regulation of fatty acid biosynthesis)	0,0012	0,0143	0,0041
124	blr7528	Hypothetical protein (Putative periplasmic ligand-binding sensor protein)	0,0342	0,0056	0,0033
127	bll2738	Hypothetical protein (No related data)[3]	0,0256	0,0748	0,0011
132	blr2474	Hypothetical protein (Identical to Bll5191)	0,0015	0,0011	0,0024
133	blr2865	Hypothetical protein (Methionyl-tRNA synthetase)	0,0093	0,0008	0,0004
136	blr5933	Hypothetical protein (Fumarylacetoacetate hydrolase)	0,0045	0,0018	0,0012
142	bll6649	Hypothetical protein (PRC-barrel like protein)	0,0036	0,0052	0,0033

[1] p-value of the Student t-Test, null hypothesis being that there was no change in expression between control and genistein conditions.

[2] Genistein induction was statistically significant ($p<0.05$) only for S 516

[3] Genistein induction was statistically significant ($p<0.05$) only for CPAC 15 and S 516