A comparative proteomic analysis of the early response to compatible symbiotic bacteria in the roots of a supernodulating soybean variety

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ABSTRACT

To reveal the processes involved in the early stages of symbiosis between soybean plants and root nodule bacteria, we conducted a proteomic analysis of the response to bacterial inoculation in the roots of supernodulating (En-b0-1) and non-nodulating (En1282) varieties, and their parental normal-nodulating variety (Enrei). A total of 56 proteins were identified from 48 differentially expressed protein spots in normal-nodulating variety after bacterial inoculation. Among 56 proteins, metabolism- and energy production-related proteins were upregulated in supernodulating and downregulated in non-nodulating varieties compared to normal-nodulating variety. The supernodulating and non-nodulating varieties responded oppositely to bacterial inoculation with respect to the expression of 11 proteins. Seven proteins of these proteins was downregulated in supernodulating varieties compared to non-nodulating variety, but expression of proteasome subunit alpha type 6, gamma glutamyl hydrolase, glucan endo-1,3-beta glucosidase, and nodulin 35 was upregulated. The expression of seven proteins mirrored the degree of nodule formation. At the transcript level, expression of stem 31 kDa glycoprotein, leucine aminopeptidase, phosphoglucomutase, and peroxidase was downregulated in the supernodulating variety compared to the non-nodulating variety, and their expression in the normal-nodulating variety was intermediate. These results suggest that suppression of the autoregulatory mechanism in the supernodulating variety might be due to negative regulation of defense and signal transduction-related processes.

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1. Introduction

The interaction between rhizobia and leguminous plants is nonpathogenic, and leads to the development of root nodules colonized by rhizobia that reduce atmospheric nitrogen into ammonia for use by the plant [1]. The induction of bacterial nodulation genes by flavonoid compounds released by leguminous plants causes the synthesis of lipochitooligosaccharide nodulation factors [2]. The rhizobia enter the deformed root hairs by an endocytosis-like mechanism and are sur-
rounded by a new subcellular structure called an infection thread, which is derived from the plasma membrane of the root-hair cells [3]. The infection thread extends to cortical cells in which cell division had already been re-initiated by unknown signals before the infection thread made direct contact. Cortical cell division gives rise to the formation of the nodule primordium, including the nodule meristem [4]. Finally, bacterial cells are endocytosed from the infection thread into a host-derived membrane, where they differentiate into bacteroids [5] and a new root organ called a nodule develops [6]. The nodule provides a low-oxygen, high-nutrient environment suitable for nitrogen fixation [7].

The endosymbiotic rhizobia convert mineral nitrogen to ammonia that is then supplied to the plant for incorporation into amino acids and ultimately proteins, in exchange for a supply of photosynthates from the plant. This symbiotic interaction allows legumes to grow and produce protein-rich seeds even in nitrogen-poor soil. Nodule formation and bacterial infection are strictly controlled by the plant [8]. To ensure that the symbiotic interaction with rhizobia is mutually beneficial, legumes employ regulatory mechanisms to control the number of nodules produced. The nodulation of a normal soybean plant is restricted by a systemic feedback regulation mechanism called “autoregulation of nodulation,” in which nodule formation on one part of the root systemically suppresses subsequent nodule formation in other root regions [9]. Autoregulation of nodulation is accomplished by a local-signaling plant hormone as well as through systemic signaling.

Autoregulation reduces the nodule density and the size of the nodulation zone [10]. Rhizobia trigger the production of an autoregulation of nodulation signal that systemically regulates nodulation [11]. The rhizobia-induced signals are transported from the roots to the leaves. Perception of the autoregulation of nodulation signal leads to the production of an inhibitory factor that suppresses nodulation events in the root [12]. Disturbances in autoregulation of nodulation lead to the development of excessive root nodules, as well as a nitrate-tolerant nodulation phenotype [13,14].

In the early stages of the interaction with legumes, rhizobia trigger root hair deformation, depolarization of the plasma membrane [15], cytoskeletal rearrangement [16], calcium spiking [17], and division of cortical cells [18]. A metabolic shift in the plant’s cells is required to support the formation of additional cell wall and plasma membrane components [19,20]. The expression of genes and proteins involved in plant defense responses also changes during symbiosis [21,22], nutrient exchange [23], and signal transduction [24]. Symbiosis cannot be fully understood without complete knowledge of the processes that occur during the early stages; however, little is known about autoregulation, which occurs during the early stage of symbiosis. To close this research gap, this study investigated the early stages of autoregulation of nodulation in supernodulating and non-nodulating varieties of soybean. Root proteins from soybean varieties inoculated with Bradyrhizobium japonicum were analyzed by proteomic approach based on two-dimensional polyacrylamide gel electrophoresis (2-DE) in combination with mass spectrometry (MS). Candidate genes that are potentially involved in autoregulation were analyzed at the transcript level using semiquantitative reverse-transcription real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Bacteria cultures

B. japonicum MAFF 211342 bacterial strain was obtained from the Genebank at National Institute of Agrobiological Science (Tsukuba, Japan) and was grown at 30 °C for 3 days in yeast manniitol broth medium containing 3.67 mM K2HPO4, 0.81 mM MgSO4, 1.71 mM NaCl, 50 mM mannitol, 40 mM CaCl2, and 0.04% yeast extract adjusted to pH 6.8. Before inoculation, B. japonicum cells were centrifuged for 10 min with 2,000 × g and washed and diluted with sterile water to OD600 = 0.1.

2.2. Plant growth and treatment

Three soybean (Glycine max L.) varieties were used in this study; supernodulating (Eb-b0-1), and the nonnodulating (En1282) varieties, and their parental variety (Enrei) [25]. Seeds were sterilized in 2% sodium hypochlorite, rinsed several times with sterile water and germinated on silica sands under white fluorescent light (600 μmol m−2 s−1, 16th light period) in a growth chamber (Sanyo, Tokyo, Japan) maintained at 25 °C and 70% relative humidity. For inoculation, B. japonicum suspension was applied to 3-day-old seedlings to be completely covered with inoculum. Application of water without B. japonicum was used as mock inoculation. The morphological parameters (fresh weight of shoot, root and nodules, length of shoot and root and number of nodules) were measured at 10 days after inoculation. For proteome analysis, roots were collected at 2 days after inoculation, immediately frozen in liquid nitrogen and stored at −80 °C for subsequent procedures. Inoculation experiments were repeated three times.

2.3. Protein extraction

A portion (500 mg) of roots was ground to powder in liquid nitrogen by using mortar and pestle. The powder was transferred to acetone containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol. After vortexing, the suspension was sonicated for 5 min and then incubated for 45 min at −20 °C. After incubation, the suspension was centrifuged at 9000 × g for 20 min at 4 °C. The supernatant was discarded and resulting pellet was washed with acetone containing 0.07% 2-mercaptoethanol twice. The resulting pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA). Dried samples were resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 5% 3-{(3-cholamidopropyl)dimethylammonium}-1-propanesulfonate, and 2 mM tributylphosphine by vortexing for 1 h at 25 °C. The suspension was centrifuged at 20,000 × g for 20 min at 25 °C. Supernatant was collected as protein extract. Protein contents were determined using the Bradford method [26] with bovine serum albumin as the standard.

2.4. Two-dimensional polyacrylamide gel electrophoresis

For 2-DE, protein samples (400 μg) were prepared in a final volume of lysis buffer containing 8 M urea, 2 M thiourea, 5% 3-{(3-cholamidopropyl)dimethylammonium}-1-propanesulfonate, 2 mM tributylphosphine and 0.4% Bio-Lyte pH 3/10 (Bio-Rad,
Hercules, CA, USA), and were directly loaded into a focusing tray. The immobilized pH gradient strips (3-10NL, 11 cm, Bio-Rad) were rehydrated for 14 h at 50 V. Isoelectric focusing (IEF) was carried out with the Protean IEF Cell (Bio-Rad) at following conditions: 250 V for 15 min with a linear ramp, 8000 V for 1 h with a linear ramp, and finally 8000 V at 35,000 V/h with a rapid ramp at 20 °C. After IEF, the strips were equilibrated with equilibration buffer I containing 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 130 mM dithiothreitol for 30 min, and then in buffer II containing 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 135 mM iodoacetamide for 30 min. Equilibrated strips were placed onto 15% SDS-polyacrylamide gels with 5% stacking gels and then sealed with 1% agarose. Electrophoresis in the second dimension was performed at a constant current of 35 mA. The gels were stained with Coomassie brilliant blue (CBB).

2.5. Gel image analysis

Images of 2-DE were analyzed using a GS-800 calibrated densitometer scanner (Bio-Rad) and the position of individual proteins on gels was evaluated with PDQuest software (version 8; Bio-Rad). The isoelectric point (pI) and molecular mass of each protein were determined using 2-DE standard marker (Bio-Rad). The amount of protein in a spot was estimated using the PDQuest software with local regression model normalization. For comparative analysis, qualitative and quantitative analyses were performed using PDQuest software.

2.6. Peptide preparation for mass spectrometry analysis

After CBB staining and destaining, gel pieces of protein spots were excised from 2-DE gels. Proteins were reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH4HCO3 for 30 min. The gel pieces were digested in 100 mM NH4HCO3 solution containing 1 pM trypsin (Wako, Osaka, Japan) at 37 °C over-night. The tryptic peptides were extracted three times from the gel pieces with 0.1% trifluoroacetic acid in 50% acetonitrile. The tryptic peptides were extracted three times from the gel pieces with 0.1% trifluoroacetic acid in 50% acetonitrile. After the tryptic peptides were excised from 2-DE gels. Proteins were reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH4HCO3 for 30 min. The gel pieces were digested in 100 mM NH4HCO3 solution containing 1 pM trypsin (Wako, Osaka, Japan) at 37 °C over-night. The tryptic peptides were extracted three times from the gel pieces with 0.1% trifluoroacetic acid in 50% acetonitrile. These procedures were performed on DigestPro (Intavis Bioanalytical Instruments AG, Cologne, Germany). The peptide solution obtained was desalted on NuTip C-18 pipet tips (Glygen, Columbia, MD, USA) and eluted in 0.1% formic acid. Desalted peptide solution was analyzed by MS.

2.7. Protein identification by nano-liquid chromatography-tandem MS

A nanospray LTQ XL Orbitrap MS (Thermo Fisher Science, San Jose, CA, USA) was operated in data-dependent acquisition mode with the installed XCalibur software. Using an Ultimate 3000 nanoLC (Dionex, Germering, Germany), peptides in 0.1% formic acid were loaded onto a 300 μm ID×5 mm C18 PepMap trap column. The peptides were eluted from the trap column and their separation and spraying were done using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a nano-capillary column (NTTC-360/75-3, Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. Full scan mass spectra were acquired in the Orbitrap over 150–2000 m/z with a resolution of 15,000. The three most intense ions above the 1000 threshold were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35% after accumulation to a target value of 1000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of peptides. Acquired MS/MS spectra were converted to individual DTA files using BioWorks software (version 3.3.1) (Thermo Fisher Science). The following parameters were set to create a list of peaks: parent ions in the mass range with no limitation, one grouping of MS/MS scans, and threshold at 100.

Soybean genome sequences were downloaded from the Department of Energy (DOE) database [27] (Phytozone, version 7.0, http://www.phytozone.net/soybean) and converted into FASTA format. The resulting peptide sequence data were used to search this database using the MASCOT search engine (Matrix Science, London, UK). Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme, and one missed cleavage was allowed. Parameters for search were peptide mass tolerance 10 ppm, fragment mass tolerance 0.2 Da, maximum missed cleavages 1, peptide charges +1, +2, +3. The proteins with at least five matched peptides in the Mascot search results with more than 15% sequence coverage were considered. The ion scores with greater than 22 were significant for soybean genome sequence databases (P<0.05). The Mowse score of more than 21 peptides from the MS data were significant with P<0.05. The positives matches were searched against the NCBI protein database (http://www.ncbi.nlm.nih.gov) by BLASTP algorithm for updated annotation and identification of homologous proteins.

2.8. RNA extraction and qRT-PCR

Frozen soybean roots (200 mg) were ground to powder in liquid nitrogen with mortar and pestle. Total RNAs were extracted from the powder using an RNasy Plant Mini kit (Qiagen, Valencia, CA, USA) and treated with RNase-free DNase I during extraction. Portions (1 μg) of total RNAs isolated from each sample were reverse-transcribed into cDNAs in a 20 μL reaction volume using iScript™ cDNA synthesis kit (Bio-Rad) according to manufacturer’s protocol. qRT-PCR was performed on cDNA mixtures corresponding to 25 ng of total RNA for specific primers and 0.0025 ng of total RNA for 18S rRNA primers in a 10 μL reaction volume using iQ™ SYR Green Supermix (Bio-Rad) on a MyiQ™ single-color real-time PCR detection system (Bio-Rad). The PCR conditions were as follows: 95 °C for 3 min, then 45 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The quantity of each transcript was calculated using a standard curve of a dilution series of pUC19 plasmid. RNA levels were normalized using soybean 18S rRNA (Accession number: X02623) as an internal control. The primers were designed using Primer3 web-interface (http://frodo.wi.mit.edu/primer3/). In the case of soybean genes excluding 18S rRNA, primers were picked from predicted 3’ untranslated regions (Supplementary Table 1). Specificities of primers were checked by melting curve analysis and agarose gel electrophoresis of amplified DNA fragments.
Fig. 1 – Morphological effects associated with *B. japonicum* inoculation in shoots and roots of variable nodulation soybean varieties. Seeds of non-nodulating (En1282), normal-nodulating (Enrei), and supernodulating (En-b0-1) soybean varieties were germinated for 3 days, and either inoculated with bacteria (+, black column) or mock-inoculated (−, white column). Shoots, roots, and nodules were collected 10 days after inoculation and the shoots and roots were photographed (A). The bars show “1 cm” and arrows show the positions of nodules. Values for the fresh weight of shoots, roots, and nodules, root length, and number of nodules are indicated in (B). Each value represents the mean ± SE of 10 seedlings. Letters denote significant differences between mock- and bacteria-inoculated samples according to Duncan’s test (P < 0.05).
2.9. Statistical analyses

The statistical significance of the results was evaluated with the Student’s t-test when only two means were compared or with two-way ANOVA followed by Duncan’s multiple comparisons test otherwise. Data analyses and graphical representations were performed using Microsoft Office Excel 2007, a language and environment for statistical computing and graphics [28].

3. Results

3.1. Growth characteristics of soybean varieties with different nodulation levels in the presence or absence of rhizobacteria

In order to examine the relative effects of nodulation level on plant growth, non-nodulating (En1282), normal-nodulating (Enrei), and supernodulating (En-b0-1) soybean varieties...

Fig. 2 – 2-DE pattern of proteins in soybean roots involved in a symbiotic interaction with compatible bacteria. Seeds of the Enrei variety were germinated for 3 days and inoculated with B. japonicum. Two days after inoculation, proteins were extracted from the roots, separated by 2-DE, and stained with CBB (A). Roots from non-inoculated soybean seedlings were used as controls. Open circles indicate spots with altered expression. Upward and downward arrows indicate upregulation and downregulation, respectively. The differentially expressed protein spots were quantified using PDQuest software and plotted as relative intensities (B). Each value represents the mean ± SE of the relative protein intensity determined from gels of three biological replicates. The means were compared using the Student’s t-test and significantly different values are indicated by asterisks (**P<0.01). Standard errors are denoted by error bars. White and black columns show control and inoculated samples, respectively.
were inoculated with *B. japonicum* on 3 days after imbibition, and their growth and nodulation level were measured on 10 days after inoculation (Fig. 1). For the En1282 and En-b0-1 varieties, there was no significant difference in the length and fresh weight of the shoot (Fig. 1A and B). There was also no significant difference in the length of the roots between the En1282 and En-b0-1 varieties, but their roots were significantly shorter than those of the Enrei variety (Fig. 1A and B). The root fresh weight was greater in the Enrei variety than in the other varieties (Fig. 1A and B).

The presence of *B. japonicum* had no significant effect on either the weight or length of the roots (Fig. 1B). After inoculation with *B. japonicum,* the number of nodules increased significantly in the En-b0-1 variety, but no nodules formed in the En1282 variety. The number of nodules formed in the En-b0-1 variety was more than 10 times greater than the number formed in the Enrei variety, and the weight of the nodules in the En-b0-1 variety was three times greater than that of the Enrei variety (Fig. 1B).

### 3.2. Differential expression of proteins during symbiotic interaction between normal-nodulating soybeans and nodule-forming bacteria

A proteomic approach was used to identify proteins involved in the early stages of the response of soybeans to infection with symbiotic bacteria. Three-day-old normal-nodulating Enrei seedlings were inoculated with *B. japonicum* and the roots were collected 2 days after the inoculation. Proteins were extracted from the roots and separated using 2-DE. In total, 439 protein spots were detected upon CBB staining (Fig. 2A). The relative intensities of all spots from three independent biological replicates were analyzed using PDQuest software. Statistical analysis of comparisons between samples from mock- and bacteria-inoculated plants revealed that the intensity of 25 protein spots significantly changed after inoculation (*P* < 0.05), indicating differential expression. Fourteen protein spots (56%) were upregulated and 11 spots (44%) were downregulated (Fig. 2B). Six of the differentially expressed spots (86%) were upregulated more than 2-fold, while only one spot (14%) was downregulated by more than 2-fold (Fig. 2B).

### 3.3. The regulation of nine protein spots differs between En1282 and En-b0-1 plants during symbiosis

All 25 of the differentially expressed protein spots observed in roots of inoculated Enrei variety plants were also detected in the roots of En1282 and En-b0-1 variety plants (Fig. 3). Inoculation of the roots of En1282 plants led to a change in the expression of 21 of the 25 protein spots (84%), similarly to the Enrei plants. In the case of En-b0-1 plants, the intensity of 11 spots (44%) changed upon inoculation. Interestingly, the expression levels of spots 5, and 17 increased in the En1282 and Enrei plants upon inoculation, but those in the En-b0-1 decreased. On the other hand, the expression level of spot 8 decreased in the En1282 and Enrei, and increased in En-b0-1 (Fig. 3).

Next, the root proteomes of En-b0-1 and En1282 variety seedlings were characterized to identify proteins associated with the early stages of autoregulation of nodulation. A
total of 46 protein spots that were differentially expressed \((P<0.05)\) after inoculation with bacteria were detected (Supplementary Fig. 1). The expression of 23 of these protein spots had been shown to be altered in samples from the Enrei cultivar. Of the remaining 23 protein spots, the expression level of nine spots (nos. 26, 30, 31, 35, 36, 37, 40, 41, and 46) changed significantly only in root samples from En1282 plants, while the expression of five spots (nos. 29, 39, 44, 47, and 48) changed significantly only in En-b0-1 samples. The expression of nine of the remaining protein spots changed significantly in samples from both En1282 and En-b0-1 plants (Fig. 4), and the level of change in expression was similar in both varieties for three of these protein spots. Spots 27, 33, 38, and 42 were upregulated in En1282 and downregulated in En-b0-1 plants after inoculation. Conversely, the expression of spots 28 and 32 was downregulated in En1282 and upregulated in En-b0-1 plants. Out of a total of 48 protein spots, the expression of at least nine was under opposite regulation in the non-nodulating and nodulating varieties (Supplementary Fig. 2).

3.4. Proteins involved in metabolism and energy production are the primary B. japonicum-responsive proteins in all varieties

The 2-DE protein spots were analyzed using MS to identify proteins that are differentially expressed during the early stages of autoregulation of nodulation that follow inoculation with B. japonicum. The MS-determined peptide sequences were searched against the soybean genome sequence database (http://www.phytozome.net/soybean), and sequence matches were subjected to homology searching against the nucleotide sequence database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). In the case of peptide sequences that produced no matches to homologous proteins in searches using the soybean genome database, those sequences were searched against the NCBI nonredundant bacterial database. Of 48 protein spots analyzed using MS, four produced no identifications. A total of 56 proteins were identified from the remaining 44 spots, and these identifications included an unknown protein and six bacterial proteins (Tables 1 and 2).

The biological functions of the identified proteins were assigned on the basis of the classification scheme described by Bevan et al. [29]. Information obtained from the Universal Protein Resource (http://www.uniprot.org) and Phytozome databases were used for the functional classification. In all soybean varieties tested, most of the proteins identified were classified as being involved in metabolism and energy production, though the level of expression and direction of regulation (i.e., up or down) of some of these proteins differed between varieties (Fig. 5A, B, and C).

All identified proteins were also analyzed with WoLF PSORT prediction (http://wolfpsort.org), ESLpred (http://
www.imtech.res.in/raghava/eslpred), and Subloc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) to predict their subcellular localization. In all varieties, more than 50% of the differentially expressed proteins were cytoplasmic. Most of the remaining proteins were predicted to be localized in the plastids and mitochondria (Fig. 5D, E, and F).

3.5. Expression of genes encoding candidate autoregulation of nodulation proteins

Of the 11 protein spots that were regulated in opposite directions between the En1282 and En-b0-1 varieties, the expression level of four spots (nos. 17, 33, 38, and 42) correlated with nodule formation. Both the level of expression of these proteins and the number of nodules formed differed in the En1282 and En-b0-1 varieties. In the Enrei variety, the expression level of these proteins and the number of nodules formed were intermediate between the supernodulating and non-nodulating varieties. Thus, these four soybean proteins are candidates for being involved in autoregulation of nodulation.

To examine the expression of these proteins at the mRNA level, we analyzed their corresponding genes using qRT-PCR (Fig. 6). The expression pattern of the genes for three candidate proteins (stem 31 kDa glycoprotein, leucine aminopeptidase, and phosphoglucomutase) generally corresponded with the observed change in protein level, with the exception that some transcript levels in Enrei variety were not intermediate between the transcript levels in En1282 and En-b0-1 varieties.

4. Discussion

*B. japonicum* infection causes root nodule formation in leguminous plants such as soybean. To study the early response(s) of soybeans to *B. japonicum* infection, the root proteins of supernodulating, normal-nodulating, and non-nodulating soybean varieties were analyzed using a proteomic approach. Out of 56 proteins identified, six were of bacterial origin. Among the bacterial proteins, glycosyltransferase, TetR family transcriptional regulator, and ATP synthase were upregulated, while translational elongation factor Tu, SOS response transcriptional repressor, and AAA ATPase were downregulated during the early stages of the symbiotic interaction between *B. japonicum* and the normal-nodulating Enrei variety. F0F1 ATP synthase, a subtype of prokaryotic ATPase/synthase [30] and key enzyme in energy metabolism, was upregulated during the symbiotic interaction. Exopolysaccharides act as signals to plant hosts to initiate infection thread formation [31], and their biosynthesis requires glycosyltransferases, an enzyme that transfers nucleotide diphospho-sugars to growing polysaccharide chains [32]. Little is known about the role of TetR family transcriptional regulators, but these proteins are believed to be involved in
In competition with other bacteria in the soybean rhizosphere [33].

The upregulation of glycosyltransferase and TetR family transcriptional regulator observed in this study might enhance polysaccharide-mediated signaling and transcriptional regulation in the bacterial partner during the early stages of legume-rhizobium symbiosis [34].

One of the three bacterial proteins that were downregulated during the symbiotic interaction, AAA ATPase, is involved in a wide range of processes in prokaryotes and eukaryotes, including symbiotic nitrogen fixation [35,36]. Bacterial translation elongation factor Tu is a component of pathogen-associated molecular patterns [37]. Similarly to a previous report [38], in this study expression of translation elongation factor Tu was downregulated during symbiosis. The SOS response DNA repair system is induced upon exposure of bacteria to DNA damaging agents and arrests DNA replication and cell division [39]. This mechanism requires the function of several genes regulated by SOS response transcriptional repressors [40]. The observed decrease in the expression of SOS response transcriptional repressor during the early stage of symbiosis might be related to a shift in DNA synthesis in the bacterial cells.

The expression level of 19 of the 50 plant proteins identified changed as a result of bacterial inoculation in Enrei plants. Energy production-related proteins involved in the glycolytic pathway and tricarboxylic acid cycle were among those induced by B. japonicum inoculation. The upregulation of ATPase beta subunit suggests that inoculation with rhizobia leads to enhanced ATP production in soybean cells. Another upregulated protein, phosphoglucomutase, catalyzes the formation of the glycolytic entry molecule glucose-6-phosphate from glucose-phosphate produced from glycogen through the action of phosphorylase [41].

Table 1 – Proteins differentially expressed in the roots of soybean variety Enrei during symbiosis with compatible bacteria.

<table>
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<tr>
<th>Spot no.</th>
<th>Homologous protein</th>
<th>Accession no.</th>
<th>Score</th>
<th>Cov. (%)</th>
<th>M.P.</th>
<th>Blast score</th>
<th>Mr (kDa)</th>
<th>Ratio</th>
<th>p value</th>
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<td>1</td>
<td>Glycosyltransferase, group 1</td>
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<td>146</td>
<td>23</td>
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<td>38.1/7.1</td>
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<td>2</td>
<td>Serine Hydroxymethyltransferase</td>
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<td>207</td>
<td>18</td>
<td>8 (5)</td>
<td>859</td>
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<td>43.1/7.1</td>
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<td>8</td>
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<td>20.4/7.2</td>
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<td>12</td>
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<td>32.8/4.7</td>
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<td>0.03</td>
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<td>13</td>
<td>gag-pol polyprotein</td>
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<td>155.9/9.3</td>
<td>39.0/4.8</td>
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<td>14</td>
<td>NAD-dependent sorbitol dehydrogenase</td>
<td>BAF75466.1</td>
<td>140</td>
<td>16</td>
<td>5 (2)</td>
<td>645</td>
<td>39.3/6.27</td>
<td>40.7/5.6</td>
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<td>Phosphomonomutase</td>
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<td>11</td>
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<td>25.3/5.9</td>
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<td>d-3-phosphoglycerate dehydrogenase</td>
<td>X002525010.1</td>
<td>261</td>
<td>13</td>
<td>6 (2)</td>
<td>708</td>
<td>63.4/8.57</td>
<td>32.6/5.9</td>
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<td>17</td>
<td>31 kDa glycoprotein</td>
<td>P10743.1</td>
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<td>32</td>
<td>8 (4)</td>
<td>500</td>
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<td>29.8/5.8</td>
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<td>127</td>
<td>14</td>
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<td>47.1/5.5</td>
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<td>17</td>
<td>5 (4)</td>
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<td>41.4/6.4</td>
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<td>35.3/5.6</td>
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<td>F0F1 ATP synthase subunit beta</td>
<td>YP_679666.1</td>
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<td>16</td>
<td>6 (1)</td>
<td>915</td>
<td>54.7/7.72</td>
<td>44.2/6.4</td>
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<td>23</td>
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<td>513</td>
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<td>5 (1)</td>
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<td>877</td>
<td>59.5/5.80</td>
<td>52.5/4.9</td>
<td>2.23</td>
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a) Spot no, spot number as given in Fig. 2.
b) Accession no, accession number according to the NCBI database.
c) Score, ions score of identified protein using soybean genome sequence databases.
d) Cov., sequence coverage, the proteins with less than 10% sequence coverage was excluded from the result.

M.P. e Blast score, The score of the high-scoring segment pair (HSP) from that database sequence.

f) Blast score, The score of the high-scoring segment pair (HSP) from that database sequence.

g) Exp., experimental.

h) Theo., theoretical; Mr, molecular weight; pi, isoelectric point.

i) The ratio of the ratio of change in abundance of protein spots compared to the control analyzed by LSD test.

j) p value, indicates the significance of up- or down-regulation of spots according to the t-test through analysis of variance.

k) ND, Non-identified.
carbon metabolism (NAD-dependent sorbitol dehydrogenase). The induction of NAD-dependent sorbitol dehydrogenase is linked to activation of glycolysis, which includes the biosynthesis of phosphoenolpyruvate, a compound important for the synthesis of organic acids in bacteroids and substrates for nitrogen assimilation [43]. Carbohydrates are also required, not only as a source of energy for nitrogen fixation, but also as a source of carbon skeletons for ammonium assimilation and its export from nodules [44]. Our results suggest that the increased expression of metabolism-related proteins during early symbiosis could be due to a general requirement for metabolites used for various biosynthetic pathways and for energy production in response to B. japonicum infection.

Colebatch et al. [44] reported that only a specific root region, the area in which root hairs rapidly elongate, is susceptible to infection, and that nodules in this zone normally inhibit subsequent nodule formation via an autoregulatory mechanism. An important goal of our study was to identify
the proteins involved in autoregulation and the control of nodule initiation in soybeans. Forty-eight differentially regulated proteins that are potentially involved in this system were found. Our results show that 11 proteins are regulated in the opposite direction after bacterial inoculation in supernodulating and non-nodulating varieties of soybean. These proteins are involved in metabolism, protein destination, and disease resistance/defense.

To limit the number of candidate proteins to those involved in the autoregulation mechanism, we took into account the expression level of each protein and how its expression correlated with the nodulation level in the normal-nodulating Enrei variety. Three criteria were defined for selecting candidate proteins involved in autoregulation: i) the level of protein expression in non-inoculated plants should be in the same range among the varieties tested, ii) the level of protein expression in inoculated normal-nodulating variety plants must be intermediate between supernodulating and non-nodulating variety plants, and iii) the level of expression of selected proteins must correlate with the extent of nodulation. Among the 11 proteins that were regulated in opposite directions upon inoculation in the non-nodulating and supernodulating varieties, four

Fig. 5 – Classification of proteins that are differentially expressed during the early stages of symbiosis with B. japonicum. The functions of differentially expressed proteins identified in non-nodulating (A), normal-nodulating (B), and supernodulating (C) variety soybeans were assigned using the classification scheme described by Bevan et al. (1998). Proteins were classified into nine groups and there were categorized as either unclear or unclassified. The localization-based classification of proteins identified in non-nodulating, normal-nodulating, and supernodulating variety soybeans is shown in D, E, and F, respectively. Identified proteins were also classified according to the subcellular localization predicted by WoLF PSORT prediction (http://wolfpsort.org/), ESLpred (http://www.imtech.res.in/raghava/eslpred/), and Subloc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm). White and black columns represent upregulated and downregulated proteins, respectively.
were selected as candidates for being involved in the autoregulation mechanism: peroxidase, phosphoglucomutase, leucine aminopeptidase, and stem 31 kDa glycoprotein.

The expression of stem 31 kDa glycoprotein decreased significantly during the early stage of symbiosis in supernodulating variety plants, and increased in the non-nodulating and normal-nodulating varieties. Low molecular weight vegetative storage proteins such as stem 31 kDa glycoprotein are lysine-rich and are classified as metabolism-related proteins that play a role in nitrogen storage [45]. Methyl jasmonate, which is involved in plant responses to a variety of external stimuli such as wounding and pathogen attack, can alter the expression of low molecular weight vegetative storage proteins [46]. It has been demonstrated that symbiotic interactions require temporal and spatial activation of different defense mechanisms in infected plants [47]. The observed downregulation of stem 31 kDa glycoprotein during the early stage of symbiosis in supernodulating variety plants might therefore be related to the suppression of defense mechanisms.

An important signaling even occurring during early stages of symbiosis is the modulated production/accumulation of reactive oxygen species (ROS) [48]. Peroxidase acts primarily as a ROS-scavenging enzyme [49], altering the level of ROS in roots after rhizobia inoculation [50]. In this study, expression of a peroxidase isozyme was induced in non-nodulating soybeans and suppressed in supernodulating plants 2 days after inoculation, which is consistent with the reported increase in peroxidase expression in a supernodulating mutant during the early stages of symbiosis [38]. Therefore, the early response to B. japonicum inoculation might include the induction of a peroxidase isozyme associated with hydrogen peroxide signaling in roots.

Phosphoglucomutase plays a key role in glycogen metabolism, which is enhanced during early rhizobia-legume symbiosis [51]. In this study, phosphoglucomutase expression was upregulated in non-nodulating and normal-nodulating variety soybeans 2 days after inoculation, but downregulated in supernodulating soybeans, suggesting that the autoregulation mechanism demands a high level of energy, and that deficiencies in the energy supply system may be related to deficiencies in autoregulation and the appearance of the supernodulating phenotype.

Previous studies have suggested that a proper ratio of auxin to cytokinins in roots is critical for the initiation of cortical cell division, nodule formation, and the regulation of nodule numbers [52]. Changes in the auxin level or distribution are believed to play two important roles during nodulation: i) the inhibition of auxin transport leads to a reduction in the auxin:cytokinin ratio and allows cell division to begin during the initial step of symbiosis, and ii) increases in the auxin level inhibit later cell divisions [53], a process that can be considered part of the autoregulation mechanism. Inhibition of auxin transport interferes with hormone balance, and can thus induce additional nodules on the root [54]. In our study, the expression of leucine aminopeptidase, which is an auxin transport receptor [55], was
upregulated in non-nodulating plants but was downregulated in supernodulating plants. This result suggests that the observed induction of leucine aminopeptidase expression in non-nodulating and Enrei variety plants 2 days after inoculation is associated with inhibition of cell division occurring later, whereas downregulation of the expression of this enzyme in supernodulating plants is related to a decrease in auxin transport and subsequent suppression of the autoregulation mechanism.

Changes in the expression of mRNAs for stem 31 kDa glycoprotein, leucine aminopeptidase, and phosphoglucomutase occurring early after B. japonicum inoculation in En1282 and En-b0-1 plants correlated well with changes in expression at the protein level. Opposing regulation of mRNAs for stem 31 kDa glycoprotein, leucine aminopeptidase, and phosphoglucomutase in En1282 and En-b0-1 plants was confirmed at multiple time points during the early stages of the response to B. japonicum inoculation (Fig. 6). In contrast, the mRNA expression for the four candidate autoregulation of nodulation proteins in the Enrei variety was not intermediate between the En1282 and En-b0-1 varieties. In addition, the expression of peroxidase as measured at the mRNA level did not follow the change observed at the protein level, indicating that the amount of a protein expressed does not always correlate with the level of gene expression as measured by the level of mRNA [56].

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.09.022.

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References


identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol 2004;136:3159–76.


