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# *Medicago truncatula* Root Nodule Proteome Analysis Reveals Differential Plant and Bacteroid Responses to Drought Stress<sup>1,2</sup>[W][OA]

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Drought is one of the environmental factors most affecting crop production. Under drought, symbiotic nitrogen fixation is one of the physiological processes to first show stress responses in nodulated legumes. This inhibition process involves a number of factors whose interactions are not yet understood. This work aims to further understand changes occurring in nodules under drought stress from a proteomic perspective. Drought was imposed on *Medicago truncatula* 'Jemalong A17' plants grown in symbiosis with *Sinorhizobium meliloti* strain 2011. Changes at the protein level were analyzed using a nongel approach based on liquid chromatography coupled to tandem mass spectrometry. Due to the complexity of nodule tissue, the separation of plant and bacteroid fractions in *M. truncatula* root nodules was first checked with the aim of minimizing cross contamination between the fractions. Second, the protein plant fraction of *M. truncatula* nodules was profiled, leading to the identification of 377 plant proteins, the largest description of the plant nodule proteome so far. Third, both symbiotic partners were independently analyzed for quantitative differences at the protein level during drought stress. Multivariate data mining allowed for the classification of proteins sets that were involved in drought stress responses. The isolation of the nodule plant and bacteroid protein fractions enabled the independent analysis of the response of both counterparts, gaining further understanding of how each symbiotic member is distinctly affected at the protein level under a water-deficit situation.

One of the best studied N<sub>2</sub>-fixing symbioses is the one established between certain members of the Leguminosae family and soil bacteria, collectively termed rhizobia. This symbiotic interaction results in the formation of a unique plant organ, the root nodule, to which the plant supplies reduced carbon for the bacteroids (differentiated form of bacteria) in exchange for fixed nitrogen.

In the last decade *Medicago truncatula*, which establishes a symbiosis with *Sinorhizobium meliloti*, has

emerged as a useful model legume for molecular and genetic studies (Barker et al., 1990; Cook et al., 1997) and has been the focus of extensive research at both transcript and protein level. The proteomic characterization of different plant organs, as well as cell cultures, has been carried out (Mathesius et al., 2001; Gallardo et al., 2003; Watson et al., 2003, 2004; Catalano et al., 2004; Valot et al., 2004; Lei et al., 2005). Detailed proteomic characterization of *S. meliloti* both as free-living cells and bacteroid has been described elsewhere (for a comprehensive review, see Djordjevic, 2004). However, little is known about the plant proteome in the *M. truncatula* root nodule. One of the closest approaches was carried out by Bestel-Corre et al. (2002) in which they compared the root proteome when inoculated with symbiotic bacteria and arbuscular mycorrhizal fungi. In a similar manner, Natero et al. (2000) analyzed the symbiotic interaction between *Medicago alba* and *S. meliloti*, but the microbial counterpart was the main focus of the study.

Therefore, in the first part of this work the soluble plant proteome in nodules of *M. truncatula* in symbiosis with *S. meliloti* was characterized. The approach chosen was a proteomic technique based on two-dimensional (2D) liquid chromatography separation of peptides in complex mixtures. Proteomic studies are traditionally carried out using 2D gel electrophoresis techniques (2D-PAGE). However, despite the fact that it was pioneered nearly 37 years ago (Kaltschmidt and

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Wittmann, 1970), 2D-PAGE has some restrictions: automation, reproducibility, and quantification are still major fields of development. To address some of these limitations, alternative gel-independent separation methods have been developed. One of them is the direct identification of proteins from complex protein mixtures based on peptide separation via liquid chromatography columns, followed by tandem mass spectrometry (LC/MS/MS). In this process, complex protein mixtures are enzymatically digested into peptides and subsequently loaded onto chromatography nanocolumns with high resolution capacity. Depending on the number of columns, liquid chromatographic separation is generally one dimensional (1D) or 2D (Link et al., 1999; Washburn et al., 2001; Koller et al., 2002; Wienkoop et al., 2004; Wienkoop and Weckwerth, 2006; Zhang et al., 2006).

The second part of this work is the quantitative analysis of the nodule proteome under a water-deficit situation. N<sub>2</sub>-fixing legumes are especially sensitive to water deficit and other environmental stresses, with drought being one of the major environmental factors affecting plant productivity (Boyer, 1982; Zahran, 1999). Under this stress, symbiotic nitrogen fixation (SNF) is one of the physiological processes to first show stress responses in nodulated legumes, occurring before the decrease in CO<sub>2</sub>-photosynthetic assimilation rates (Durand et al., 1987). Several hypotheses have been proposed by different research groups to explain the decline in nitrogen fixation during drought: (1) regulation through the control of carbon flux within nodules, mainly due to down-regulation of Suc synthase (SuSy; González et al., 1995; Gordon et al., 1997; Arrese-Igor et al., 1999); (2) regulation based on the internal oxygen levels within nodules (Durand et al., 1987; Diaz del Castillo et al., 1994; Serraj and Sinclair, 1996); and (3) nitrogen feedback regulation (Serraj et al., 2001; King and Purcell, 2005). However, to date, the molecular mechanisms responsible for these physiological responses are not yet understood. To further characterize this complex regulation process, the variation in the protein profiles of control and water-stressed nodules was analyzed. Symbiotic root nodules are complex structures, where plant and bacteria cells are in close proximity. Therefore, special care was taken to ensure minimal cross contamination during the separation of the plant and bacteroid fractions. This enabled the independent analysis of the response of both symbiotic partners, gaining further understanding of how each symbiotic member is distinctly affected by drought.

## RESULTS AND DISCUSSION

### Separation of Plant and Bacteroid Fractions of *M. truncatula* Root Nodules

Most of the published proteomic studies include a protein extraction step based on tissue homogeniza-

tion using liquid nitrogen. However, when this is applied to nodule tissue, a significant level of cross contamination among plant and bacteroid protein fractions is observed (Natera et al., 2000; Bestel-Corre et al., 2002). As the separation of plant and bacteroid fractions in nodules was one of the aims of this study, we initially evaluated the degree of protein cross contamination between the fractions when comparing homogenization using liquid nitrogen to homogenization using ice-cold extraction buffer (adapted from Saalbach et al., 2002) followed by centrifugation of the extract to pellet bacteroids. The level of contamination between the fractions was first estimated by western-blot analysis employing antibodies against the MoFe protein of the bacteroid nitrogenase complex (NifDK; Fig. 1). Second, plant fractions were analyzed via LC/MS/MS and peptide mass spectra were matched against the *S. meliloti* protein database to detect bacterial proteins present in the putative plant fraction. While maintaining total protein content in nodules ( $26.2 \pm 2.3$  mg protein/g fresh weight nodule), the ratio of bacteroid to plant fraction protein content was 1:2 when ice-cold buffer was used, whereas the ratio decreased to 1:7 when liquid nitrogen was employed. Furthermore, LC/MS/MS analyses showed the presence of bacterial origin proteins in the putative plant fraction after liquid nitrogen homogenization, representing almost 40% of the total peptides detected in the bacteroid fraction. However, when homogenization buffer was employed, the presence of contaminating bacterial origin peptides was reduced, representing

**Figure 1.** Influence of the extraction method on bacteroid protein contamination in the plant fraction of *M. truncatula* root nodules. Homogenization using liquid nitrogen was compared to homogenization using an extraction buffer (adapted from Saalbach et al., 2002). Plant and bacteroid fractions were separated by centrifugation and aliquots (10 µg of protein) were loaded onto a SDS gel. Immunoblot was performed using antibodies against bacterial NifDK as a marker for bacteroid contamination. Fractions 1 and 2 are the plant and bacteroid fractions obtained when using homogenization buffer. Fractions 3 and 4 are the plant and bacteroid fractions obtained when nodules were homogenized using liquid nitrogen. Molecular mass markers are shown on the left. Predicted values for *S. meliloti* NifD and NifK are 56.5 and 57.6 kD, respectively.

only 3% of the total bacteroid peptides detected. This reduction was not due to protein degradation, as total nodule protein content was maintained, but to a different distribution of the protein among the fractions. Taking all these data together, both approaches indicated that liquid nitrogen was the key factor involved in the unwanted breakage of bacteroid cells and, consequently, responsible for the bacterial protein contamination in the plant fraction. Once the method for minimal cross contamination was established, two types of analyses were performed. A flow diagram of the experimental work is represented in Figure 2.

#### Plant Protein Identification in N<sub>2</sub>-Fixing *M. truncatula* Root Nodules

The plant fraction of *M. truncatula* root nodules was collected as outlined above and soluble protein was extracted. To identify a large number of nodule plant proteins, a double separation step was performed according to a previous analysis of *Arabidopsis* (*Arabidopsis thaliana*) leaf proteome (Wienkoop et al., 2004). First, using anion-exchange FPLC the initial plant extract was fractionated into nine subfractions. Then protein from each of the nine subfractions was precipitated, enzymatically digested, and separated using 2D-LC/MS/MS (Fig. 2). The obtained mass spectra were searched against The Institute for Genomic Research (TIGR) Medicago Gene Index release 8.0 database (now located at <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago>) using stringent criteria as described in "Materials and Methods." As a result, 377 unique plant proteins were identified, representing the largest nodule plant proteomic analysis reported so far. This result confirms the usefulness of novel liquid chromatography-based methods for the large-scale identification of proteins in complex tissues such as N<sub>2</sub>-fixing nodules. The complete list of identified proteins, ordered by functional groups, is presented in Supplemental Table S1. Some of the most relevant identified proteins are discussed below.

The list of identified proteins was functionally classified using the Mapman program (Fig. 3), which classifies genes/proteins based on gene ontology and functional classification databases (Gene Ontology Consortium and Kyoto Encyclopedia of Genes and Genomes database; Thimm et al., 2004; Usadel et al., 2005). In contrast to previously reported large-scale proteome analyses, in which a large number of unknown proteins are often reported, surprisingly, this study contains a reduced portion of proteins with unknown function. Although the reason behind remains unclear, this result may be due to the fact that in nodules most of the high abundant and detectable proteins have been characterized, becoming a suitable source for physiological interpretation.

Following classification, the largest functional group comprises proteins involved in amino acid metabolism and protein synthesis and degradation. Apart

**Figure 2.** Diagram of the experimental work flow. Two types of analyses have been performed: The *M. truncatula* plant nodule proteome was profiled using FPLC and 2D-LC/MS/MS and a quantitative study of plant and bacteroid responses to drought stress at the protein level was carried out using 1D-LC/MS/MS. SCX, Strong cation exchange; RP, reverse phase.

from the known enzymes involved in nitrogen assimilation in nodules, several proteins related to the biosynthetic pathway of sulfur-containing amino acids were detected. This is particularly interesting taking into account the limited information available on sulfur metabolism in legume nodules. Uptake of sulfur from the soil occurs almost exclusively in the form of sulfate and the sole entry step for the metabolism of sulfate is the formation of adenosine 5'-phosphosulfate,

**Figure 3.** Functional classification of the identified proteins in the 2D-LC/MS/MS analysis of *M. truncatula* nodule plant fraction. Proteins were functionally classified using Mapman, which is based on Gene Ontology Consortium and Kyoto Encyclopedia of Genes and Genomes database. OPP, Oxidative pentose pathway.

by ATP sulfurylase (Saito, 2004). A nodule ATP sulfurylase (TC100144) was identified that had a close similarity to a plastidic enzyme recently reported to be abundantly expressed in soybean (*Glycine max*) roots (Phartiyal et al., 2006). In addition, several enzymes were identified that are involved in the biosynthesis of sulfur-containing amino acids, such as Met synthase, Cys synthase, or Cys desulfurase. Notably, this analysis enabled the detection of a considerable number of enzymes involved in the biosynthesis of *S*-adenosyl-L-Met (SAM), a primary methyl-group donor and also a precursor of metabolites such as ethylene, polyamines, and vitamin B1 (Amir et al., 2002). SAM is synthesized from Met and ATP by the enzyme SAM synthetase, for which several isoforms have been identified in this analysis. These findings suggest that  $N_2$ -fixing root nodules play an important, so far overlooked, role in plant sulfur assimilation, especially the biosynthesis of sulfur-containing amino acids and regulatory compounds such as SAM.

For proteins related to energy production, the analysis allowed the almost complete identification of enzymes of the glycolytic pathway and tricarboxylic acid cycle. In nodules Suc can be hydrolyzed to monosaccharides by SuSy or alkaline invertase (Morell and Copeland, 1985; Sturm et al., 1999). In this analysis a SuSy isoform was identified (TC100410) that is known to be the nodule-enhanced isoform in *M. truncatula* nodules (Hohnjec et al., 2003), as well as a putative  $\beta$ -fructofuranosidase/alkaline invertase (TC106886) recently reported to be expressed in *Lotus japonicus* during nodule development (Flemetakis et al., 2006).

The next largest functional class of proteins is involved in redox state control and defense against biotic and abiotic stress. Besides the dominating presence of

leghemoglobin (Lb), a key protein controlling the internal oxygen concentration within the nodule, a set of enzymes involved in antioxidant defense were also found. These included members of the ascorbate/glutathione cycle, such as mono- and dehydroascorbate reductase and glutathione reductase, reflecting the active antioxidant defense that occurs within nodules (for review, see Matamoros et al., 2003). Several pathogen-related (PR) proteins were also detected, including a member of the PR-10 family, PR10-1, which has been reported as constitutively expressed in *M. truncatula* roots (Mathesius et al., 2001; Watson et al., 2003) and up-regulated upon pathogen infection in roots and leaves (Gamas et al., 1998; Colditz et al., 2004, 2005).

Furthermore, this proteomic analysis allowed for the identification of several proteins known to be involved in signaling processes in  $N_2$ -fixing nodules. Two calmodulin-like proteins (CaML) were identified, corresponding to proteins CaML 2 and 6b (for which amino acid sequence information is provided in Supplemental Table S4). A third protein (CaML 4) was also detected but, as only one single peptide was found, it was not included in the protein identification list. A group of six of these calmodulin-like proteins were considered to be specifically expressed in *M. truncatula* root nodules in the in silico transcript analysis carried out by Fedorova et al. (2002). These putative calmodulin-like proteins were further characterized in a recent study by Liu et al. (2006) in which, using specific antibodies, the authors identified CaML 2 and 5 based on single amino acid peptide sequences. The detection of two of these low abundance calmodulin-like proteins in this study is further evidence for the potential of nontargeted liquid chromatography-based techniques.

### Quantitative Proteomic Analysis of *M. truncatula* Root Nodule

To evaluate the physiological effect of the imposed water deficit, two parameters were measured: nodule water potential ( $\Psi_w$ ) and apparent nitrogenase activity (ANA), which was evaluated as  $H_2$  evolution (Fig. 4, A and B). In drought-stressed plants,  $\Psi_w$  showed a slight decline on day 3 ( $-1.07 \pm 0.08$  MPa), but the decrease was only statistically significant on day 6 ( $-2.42 \pm 0.29$  MPa). Values for control plants remained constant over the course of the experiment ( $-0.81 \pm 0.05$  MPa). In parallel, drought stress caused a 30% and 70% reduction in ANA on days 3 and 6, respectively, when compared to control plants. These results are in agreement with several previous studies (for review, see Zahran, 1999).

Following physiological characterization, nodules were analyzed to obtain an overview of changes at the protein level. Extracts were separated into plant and bacteroid fractions and independent analyses were carried out. The obtained mass spectra of the plant and bacteroid fractions were searched against the TIGR *M. truncatula* Gene Index release 8.0 database (<http://compbio.dfc.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago>) and the *S. meliloti* strain 1021 Genome Project protein database (<http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/index.html>). This resulted in the identification and quantification of 90 nodule plant proteins and 97 bacteroid proteins within the different samples. A selection of the relatively most abundant proteins found in every fraction is shown in Tables I and II, together with graphs showing their relative abundance during the drought treatment. The complete list of proteins together with their spectral count values is included as Supplemental Tables S2 and S3 for plant and bacteroid analysis, respectively. Also provided is the amino acid sequence information obtained from the mass spectra, including a selection of the different peptides found per protein and their Sequest-identification scores (Supplemental Tables S4 and S5).

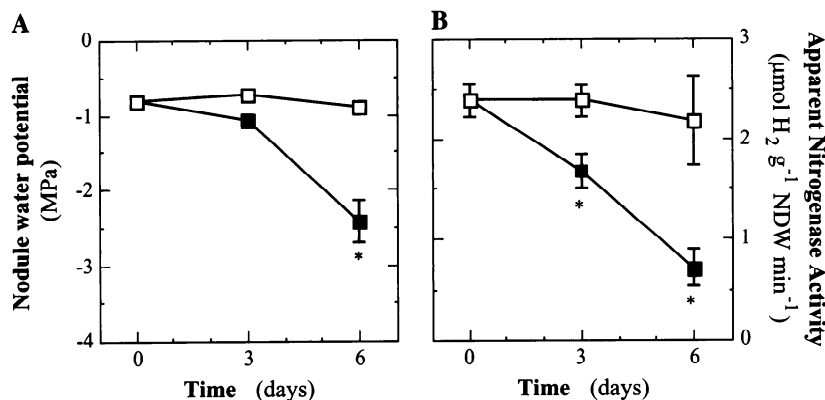
One of the main problems when dealing with large complex datasets is extracting the important variables in an unsupervised way that allows for the interpre-

tation of the results. In our case, just considering the plant fraction, we are dealing with around 100 proteins measured, two treatments, at two time points, and five biological replicates per sample. For analyzing these multifactorial and multivariate data sets statistical techniques are needed. In this study, MetaGeneAlyse was used, a Web-based service that provides the integrated analysis of data sets containing genetic, proteomic, or metabolite information (Daub et al., 2003; Scholz et al., 2004). The independent component analysis (ICA) is a statistical tool that allows for rapid sample classification based on such multivariate data. Each point in the ICA graph represents the whole protein profile of one biological sample. Samples showing similar behavior in their protein profile are grouped together. In this work the ICA graphs for plant and bacteroid protein analysis (Fig. 5, A and B) show a clear separation between control and drought samples along the IC01 axis. ICA allows not only for the visualization of the data but also for the extraction of the most statistically relevant proteins showing the strongest influence on the separation of the samples. These proteins showing a statistically relevant response to water stress are represented in the top section of Tables I and II for plant and bacteroid proteomic analysis, respectively. When both plant and bacteroid protein data sets are integrated, there is a clear improvement in sample pattern recognition (Fig. 5C), in agreement with previous studies (Morgenthal et al., 2005; Weckwerth and Morgenthal, 2005).

### General Down-Regulation of Nodule Plant Metabolism under Water Deficit

Five nodule plant proteins showed a statistically relevant variation during drought stress: Met synthase, SuSy, Asn synthetase (AS), Lb, and the transcriptional eukaryotic elongation factor-2 (eEF-2), with the relative content of all of them decreasing in the drought samples (see the top section of Table I). This occurred at day 3 for most of the proteins, when nitrogen fixation had only declined by 30% compared to control plants (Fig. 4B).
















The plant protein showing the strongest response to drought stress was identified as Met synthase








**Figure 4.** Effect of drought stress on nodule water potential (A) and SNF as a measure of ANA (B) in *M. truncatula* 'Jemalong' plants. Control plants are represented in white, drought-stressed plants in black. For each parameter, an asterisk represents statistically significant differences with the corresponding control value at  $P \leq 0.05$ . Bars indicate the SE ( $n = 6$  biological replicates).

**Table I.** Quantitative changes in the protein profile of *M. truncatula* nodule plant fractions during drought

TC (tentative consensus) code represents the identifier code of tentative consensus sequences retrieved from the Medicago Gene Index from TIGR release 8.0. Protein identity is preceded by Swiss-Prot accession number in parentheses. Proteins found to be statistically involved in the plant response to drought based on ICA are represented in bold in the top section of the table. A selection of the relatively most abundant proteins found in the plant analysis is listed below. Control samples are shown in white, drought samples are symbolized with a striped pattern. First and third columns represent the average peptide ion count values of control nodules at day 3 (C3) and day 6 (C6), respectively. Second and fourth columns represent average values of drought-stressed nodules at day 3 (D3) and day 6 (D6), respectively. Bars represent  $\pm$  SE ( $n = 5$  biological replicates). For the complete set of proteins, see Supplemental Table S2.

TC Code	Spectral Count C3, D3, C6, D6	Protein Identification
<b>TC106598</b>		(Q71EW8) Methionine synthase
<b>TC100410</b>		(Q9T0M6) SuSy
<b>TC100391</b>		(O24483) Asparagine synthetase
<b>TC100393</b>		(P19252) Asparagine synthetase
<b>TC106593</b>		(Q42928) Lb
<b>TC93936</b>		(Q9ASR1) EF-2
TC106592		(P27992) Lb 1
TC106579		(P28010) Lb
TC106578		(P27993) Lb 2
TC106518		(P34922) Glyceraldehyde-3-P DH
TC100309		(Q6RIB7) Enolase
TC93931		(Q9SBR9) Basic blue protein
TC106729		(O04998) Glutamine synthetase
TC106707		(P29828) Protein disulfide-isomerase precursor
TC100150		(O04855) Carbonic anhydrase

**Table I.** (Continued.)

TC Code	Spectral Count C3, D3, C6, D6	Protein Identification
TC100432		(O81262) Early nodule-specific protein
TC107133		(Q08480) Adenylate kinase B
TC93945		(O04275) F1 ATPase
TC107339		(Q9FES4) ENOD18 protein
TC107075		(Q02735) Phosphoenolpyruvate carboxylase

(TC106598), which is involved in both the de novo synthesis of Met and in the regeneration of the methyl group SAM. In plants it has been estimated that about 20% of the Met is incorporated into proteins while 80% is converted to SAM (Giovanelli et al., 1985). SAM functions as a primary methyl-group donor and, most interestingly, as a precursor for metabolites such as ethylene (Adams and Yang, 1977). Thus, Met occupies a central position in cellular metabolism: as a protein constituent, in the initiation of mRNA translation, and as a component of the regulatory molecule SAM. Although it is assumed that Met synthesis, accumulation, and consumption are under stringent regulatory control (Hesse and Hoefgen, 2003; Hesse et al., 2004), the molecular and biochemical characterization of plant Met synthase is still limited. Very little is known about the role of Met synthase in  $N_2$ -fixing nodules, and to our knowledge this is the first report in which an abiotic stress is linked to a decrease on the relative content of this protein. Furthermore, it may be speculated that a decline in the levels of Met synthase may cause a reduction in SAM content in drought-stressed nodules.

The second highest impact on the separation of control and drought samples is attributed to TC100410, which corresponds to nodule-enhanced SuSy (Hohnjec et al., 2003). Root nodules are highly specialized sink tissues in which at least one SuSy isoform is strongly induced (Morell and Copeland, 1985) and reduction in SuSy activity is considered to be one of the key factors responsible for the inhibition of SNF during drought (González et al., 1995; Gordon et al., 1997; Gálvez et al., 2005). The fact that this study also identified a decline in this protein as part of the plant response to water deficit highlights the usefulness of proteomics as an unbiased approach to the detection of stress markers.

The next proteins found to be involved in the plant response to water deficit were identified as two isoforms of AS (TC100391, TC100393). In temperate-climate legumes, fixed nitrogen is mainly exported from nodules

**Table II.** Quantitative changes in the protein profile of *S. meliloti* bacteroid fractions during drought

Accession numbers as retrieved from *S. meliloti* strain 1021 Genome Project protein database. Proteins found to be statistically involved in the bacterial response to drought based on ICA are represented in bold in the top section of the table. A selection of the relatively most abundant proteins found in the bacterial analysis is listed below. Control samples are shown in white, drought samples are symbolized with a striped pattern. First and third columns represent the average peptide ion count values of control nodules at day 3 (C3) and day 6 (C6), respectively. Second and fourth columns represent average values of drought-stressed nodules at day 3 (D3) and day 6 (D6), respectively. Bars represent  $\pm$  SE ( $n = 3$  biological replicates). For the complete set of proteins, see Supplemental Table S3.

Accession No.	Spectral Count C3, D3, C6, D6	Protein Identification
<b>SMc01428_AA-cspA2</b>		Transcription regulator cold shock protein
<b>SMc01770_AA-glyA1</b>		Serine hydroxymethyltransferase
<b>SMc01350_AA-gatB</b>		Glutamyl-tRNA amidotransferase subunit B
<b>SMc01309_AA-rplC</b>		50S ribosomal protein L3
SMc00913_AA-groEL1		GroEL1 chaperonin
SMc02857_AA-dnaK		Heat shock protein 70 chaperone
SMa0825_AA-nifH		NifH Nitrogenase Fe protein
SMa0827_AA-nifD		NifD nitrogenase Fe-Mo $\alpha$ -chain
SMa0829_AA-nifK		NifK nitrogenase Fe-Mo $\beta$ -chain
SMc01311_AA-tufA		EF Tu
SMc02479_AA-mdh		Malate dehydrogenase
SMc02480_AA-sucC		Succinyl-CoA synthetase $\beta$ -chain
SMa0124_AA-groEL3		GroEL3 chaperonin
SMc02501_AA-ATPD		ATP synthase $\beta$ -chain

**Table II.** (Continued.)

Accession No.	Spectral Count C3, D3, C6, D6	Protein Identification
SMc02087_AA-gltA		Citrate synthase
SMc02481_AA-sucD		Succinyl-CoA synthetase $\alpha$ -chain
SMc02483_AA-sucB		Dihydroipoamide succinyl transferase component of 2-oxoglutarate DH complex (E2)
SMc01312_AA-fusA1		EF G
SMc01906_AA-hrm		Histone-like protein
SMc03846_AA-acnA		Aconitate hydratase
SMc00531_AA		ATP-binding cassette transporter ATP-binding protein
SMc01285_AA-rpoA		DNA-directed RNA polymerase $\alpha$ -chain
SMa0810_AA-fixU		FixU nitrogen fixation protein

to the rest of the plant as Asn, which is synthesized by the concerted action of two enzymes, Asp aminotransferase and AS (Vance et al., 1994). In alfalfa (*Medicago sativa*) and soybean nodules AS has been described in detail (Huber and Streecher, 1985; Shi et al., 1997), while information about this enzyme in nodules from *M. truncatula* is mostly restricted to transcriptomic data (Györgyey et al., 2000; Carvalho et al., 2003; Colebatch et al., 2004; Tesfaye et al., 2006). In this work AS showed a decrease in level while other enzymes involved in the nitrogen assimilation pathway in nodules, such as Asp aminotransferase or Gln synthetase (GS), showed no response. Interestingly, a possible role of phosphorylation as a posttranslational regulatory mechanism of GS activity in *M. truncatula* nodules has recently been reported (Lima et al., 2006). Whether this type of regulation takes place during drought stress is a question for further exploration.

Lbs are symbiotic oxygen carrying proteins found at millimolar concentrations in the cytoplasm of bacteroid-containing nodule cells. They are essential for the control of low internal oxygen while maintaining an adequate supply for bacteroid respiration (Appleby,



**Figure 5.** Graphical representation of ICA results on the nodule proteome during drought. Plant analysis is represented in A, bacteroid analysis in B. C represents the result of the integration of both plant and bacteroid data sets. C3 and D3 stand for control and drought at day 3 of treatment. C6 and D6 represent control and drought at day 6 of treatment. The ICA graphs allow for sample pattern recognition. Each point in the graph represents the whole protein profile of one biological sample.

1984; Ott et al., 2005). In this proteomic analysis, the relative content of Lb was by far the highest compared to other nodule plant proteins and up to eight Lb isoforms, some of them sharing a high sequence similarity, were detected and quantified. Drought caused a significant decrease in one Lb isoform (TC106593). Lb has been suggested to play a role during abrupt water stress in nodules, whereas gradual or moderate water deficit did not affect Lb content (González et al., 2001; Marino et al., 2006). In this study, although the drought treatment applied can be considered as gradual, the LC/MS/MS technique allowed for the quantification of small variations in protein content in an isoform-specific manner.

Finally, a protein with high similarity to eEF-2 (TC93936) was highlighted by the statistical analysis. eEF-2, formerly known as aminoacyltransferase II, catalyzes the translocation of tRNAs, facilitating the movement of the ribosome relative to the mRNA during protein synthesis (Proud, 1994). In plants, homolog genes have been cloned in sugar beet (*Beta vulgaris*) and Arabidopsis (Vogel et al., 1999; Guo et al., 2002) but little is known about the role of this EF in legume nodules. Guo et al. (2002) reported that Arabidopsis mutants in this gene failed to induce the transcription of cold-responsive genes. Furthermore, they showed that this EF was involved in new protein synthesis during cold stress. The decline in the relative content of the eEF-2-like protein in nodules subjected to drought stress may be related to a down-regulation of de novo protein synthesis under water-limiting conditions.

Water-deficit stress causes a well-documented decline in SNF in  $N_2$ -fixing legumes (Arrese-Igor et al., 1999; Zahran, 1999; Serraj et al., 2001), which is also shown for nodulated *M. truncatula* plants in this study (Fig. 4B). It can be inferred from the proteomic analysis that the plant response in nodules involves a global reduction of protein biosynthesis and a down shift of cellular carbon, nitrogen, and sulfur metabolism, thus reducing the energy-demanding process of nitrogen

fixation. Altogether, this study shows a clear integration of both physiology and proteome analyses, with the added value of identifying which specific proteins, up to the level of isoforms, are first affected by water deficit.

#### **Bacteroid Up-Regulation of Protein Biosynthesis as an Adaptation Mechanism to Drought Stress**

Although proteomic characterization of *S. meliloti* bacteroid has been previously described (Djordjevic, 2004), some of the 97 proteins identified in this comparative analysis (Supplemental Table S3) deserve special mention. Several chaperonin proteins, including GroEL, GroES, and heat shock proteins, essential to the establishment of the symbiosis (Yeh et al., 2002), were detected. Indeed, the largest spectral count number corresponded to a member of the GroEL family, assigned to GroEL1 (Djordjevic et al., 2003). The second most abundant category was comprised of proteins related to SNF, predominantly components of the nitrogenase complex, such as NifD, NifH, and NifK. It is generally accepted that bacteroids assimilate very little fixed ammonia, which is mainly exported to the host plant (Brown and Dilworth, 1975; Vance et al., 1994). However, this study identified several nitrogen assimilation enzymes that have been described as absent or present at very low levels in bacteroids, such as GS (GlnA), nitrogen regulatory proteins PII (GlnB) and PIIA (PtsN), and urease accessory protein (UreE). This is consistent with the recent hypothesis that an active amino acid cycling between both partners occurs in nodules rather than a simple export of  $NH_4^+$  from bacteroids to the host plant cell (Lodwig et al., 2003).

In a similar manner to the plant analysis, bacterial fractions from control and water-stressed nodules were separated, and changes at the protein level were analyzed. Table II shows a summary of the relative variation in protein abundance, estimated as spectral counts during the time course of the drought treatment. ICA

highlighted the influence of at least four bacteroid proteins on the discrimination between control and drought nodule samples (top section of Table II), with all of them showing an increase in relative abundance in the drought-stressed samples.

The first one appears to be a transcription regulatory protein (CspA2; SMC01428), a homolog to the major cold-shock protein in *Escherichia coli* CspA (Jones and Inouye, 1994). CspA proteins act as RNA chaperones that are able to bind RNA without apparent sequence specificity, thus preventing the formation of secondary structures that prohibit translation at low temperatures (Jiang et al., 1997). O'Connell and Thomashow (2000) first described and characterized one of these cspA genes in *S. meliloti*, currently annotated as SMC04319, while searching among the annotated sequences of the *S. meliloti* genome reveals up to eight different proteins belonging to the CspA family. Three of these were identified in this proteomic analysis, and CspA2 appears to be specifically involved in the bacteroid response to drought, possibly by facilitating the translation of mRNAs involved in the adaptation to stress.

The bacterial protein showing the second highest response to drought was identified as a chromosome-encoded Ser hydroxymethyltransferase (GlyA1; SMC01770). GlyA1 levels remained constant in bacteroids from control nodules, whereas there was a 4-fold increase in protein content in nodules under water deficit. This enzyme, which catalyzes the reversible conversion of Ser into Gly and the transfer of a one-carbon unit to tetrahydrofolate, was shown to be essential for SNF as glyA mutants of *Bradyrhizobium japonicum* are unable to establish an effective symbiosis (Roszbach and Hennecke, 1991). Under osmotic stress, *S. meliloti* has been reported to accumulate osmoprotectant compounds such as Gly betaine (Smith et al., 1988), which the bacteria can use as both an osmoprotectant and a source of carbon and energy (Talibart et al., 1997). However, it is difficult to explain the increase in GlyA1 in drought-stressed bacteroids as no other enzymes related to the biosynthesis or catabolism of Gly betaine were detected in this proteomic analysis. Further investigations may help to elucidate its role in bacteroids from water-stressed nodules.

The other two identified bacteroid proteins that responded to drought stress are related to protein biosynthesis. One of these is a putative glutamyl-tRNA amidotransferase (GatB; SMC01350) that catalyzes the biosynthesis of glutamyl-tRNA, an intermediate in RNA translation (Gagnon et al., 1996). 50S ribosomal protein L3 (RplC; SMC01309) was also found to be more abundant in drought-stressed bacteroids. Under stress conditions, protein biosynthesis and ribosomal genes are reported to be repressed due to slower cell growth. However, the observed increases of glutamyl-tRNA amidotransferase and ribosomal protein RplC suggest that bacteroids from nodules experiencing water deficit may temporarily activate protein biosynthesis as a stress adaptation mechanism.

### Symbiotic Partners Respond Simultaneously, But Differently, to Drought Stress

The relative influence of each symbiotic partner on the regulation of nitrogen fixation in the legume-*Rhizobium* symbiosis is a subject of debate. It has been suggested that the massive amplification of bacterial numbers in the nodule leads to the proteome being dominated by bacterial proteins (Djordjevic et al., 2003). To further investigate the relative influence of both micro- and macrosymbiont proteomes on the nodule response to drought, both protein data sets were combined and an integrative ICA was carried out (Fig. 5C). After data normalization, the integrative statistical analysis suggested that both plant and bacteroid fractions respond simultaneously to water deficit and with similar statistical significance at the protein level. However, while there was a general down-regulation of proteins related to plant metabolism, bacteroid cells were up-regulating protein biosynthesis, probably as an adaptation to the water deficit imposed. The simultaneous proteomic analysis of both symbiotic partners has been shown to be a useful tool which, together with transcriptomic developments such as the dual-genome chip *M. truncatula*-*S. meliloti* (Barnett et al., 2004), will help to further understanding of this intriguing symbiotic interaction.

### CONCLUSION

The identification of 377 plant proteins using 2D-LC/MS/MS has confirmed the efficacy of liquid chromatography-based methods for proteomic analysis in complex tissues such as legume nodules. This profiling can now serve as a nodule proteome database for future targeted protein studies. The independent analysis of the nodule plant and bacteroid fractions confirmed the role of SuSy as a key enzyme involved in drought stress and also identified new marker enzymes such as plant Met synthase and bacteroid Ser hydroxymethyltransferase. This study has highlighted several novel avenues for further investigation to better understand the complexity of the SNF response to drought stress.

### MATERIALS AND METHODS

#### Biological Material, Growth Conditions, and Drought Treatment

*Medicago truncatula* 'Jemalong A17' plants inoculated with *Sinorhizobium meliloti* strain 2011 were grown in 1 L pots with a mixture of vermiculite:perlite (5:2, v/v) as substrate under controlled environmental conditions (14-h day/10-h night; 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity; 22°C/16°C day/night temperature; 70%–60% relative humidity). Plants were watered with nutrient solution (Evans, 1981) containing 0.25 mM ammonium nitrate for the first 4 weeks to improve plant performance during the initial development stage. During the following weeks, nutrient solution was nitrogen free. When plants were 10 weeks old they were randomly separated into two sets: control and drought stressed. Control plants were supplied daily with nutrient solution to field capacity whereas drought stress was imposed to the other group by withholding

water/nutrients for either 3 or 6 d. Since cell expansion is the first physiological process affected by drought, nutrient deficiency is implausible during this short study period. Water-stressed plants and their corresponding controls were harvested at day 3 and day 6 after the onset of drought to obtain mild and severely drought-stressed plants. Nodule water potential ( $\Psi_w$ ) and ANA measurements were carried out. The water potential of detached nodules was measured in C52 sample chambers connected to a Wescor HR-33T dewpoint hygrometer (Wescor). ANA was measured as  $H_2$  evolution of intact plants in an open flow-through system under  $N_2:O_2$  (79%:21%, v/v) according to Witty and Minchin (1998) using an electrochemical  $H_2$  sensor (Qubit System). Following this, root nodules were collected, frozen in liquid nitrogen, and stored at  $-80^\circ C$  for further analysis. Four types of nodule samples were analyzed: control plants at day 3 (C3), drought-stressed plants at day 3 (D3), control plants at day 6 (C6), and drought-stressed plants at day 6 (D6).

### Protein Extraction

Nodules (0.1 g fresh weight) were homogenized in a mortar and pestle with an ice-cold extraction buffer (25 mM MES, 450 mM mannitol, 7 mM  $Na_2EDTA$ , 7 mM  $CaCl_2$ , 5 mM  $MgCl_2$ , 20 mM ascorbic acid, 10 mM dithiothreitol, pH 7.2; according to Saalbach et al., 2002). Homogenates were centrifuged at 2,000g at  $4^\circ C$  for 15 min and supernatants were collected as nodule plant fractions. Pellets were washed twice with extraction buffer to avoid plant protein contamination and subsequently sonicated for bacteroid disruption. Sonicated fractions were further centrifuged at 10,000g at  $4^\circ C$  for 15 min and supernatants were collected as nodule bacteroid fractions. Soluble proteins were precipitated overnight at  $-20^\circ C$  after adding 20 volumes of acetone containing 0.07% (v/v) 2-mercaptoethanol. Pellets recovered by centrifugation were rinsed with cold acetone solution, air dried, and resuspended in 0.6 mL of solubilization buffer (8 M urea, 0.1 M  $NH_4HCO_3$ , pH 8.5).

### Immunoblot Analysis

Plant and bacteroid protein extracts (20  $\mu g$ ) were separated by SDS-PAGE on 10% (w/v) polyacrylamide gels. Gels were transferred onto nitrocellulose membranes and blocked overnight with 5% (w/v) nonfat milk powder in Tris buffer saline (TBS). Polyclonal antibodies raised against nitrogenase complex components NifDK were incubated for 1 h at 1:10,000 dilution in TBS. After primary antibody incubation, membrane was washed with Tween-TBS and further incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:20,000; Sigma). Cross-reacting protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (NBT/bCIP, Sigma) as substrates.

### FPLC

For FPLC analyses 0.6 g fresh weight nodule was homogenized as described above and 5 mM phenylmethylsulfonyl fluoride proteinase inhibitor was added to the nodule homogenate. The plant protein fraction was sterile filtered using a 0.45 mm filter (Schleicher and Schuell) and the FPLC run was performed as previously described (Wienkoop et al., 2004). Briefly, a total of 25 mg nodule plant protein was loaded onto a 1-mL Resource Q column (Amersham-Pharmacia Biotech) equilibrated with 10-column bed volumes of buffer A (50 mM Tris-HCl, pH 8.0). The column was washed with buffer A until  $A_{280}$  reached baseline level. Bound proteins were eluted with a 50-mL linear gradient from 0 to 500 mM NaCl in buffer A at a flow rate of 3 mL  $min^{-1}$ . Nine protein fractions of 2 mL were collected and protein was precipitated as described above.

### In-Solution Protein Digestion

Digestion was carried out according to Washburn et al. (2001). Aliquots containing 500  $\mu g$  of protein were digested for 5 h at  $37^\circ C$  with sequencing-grade endoproteinase Lys-C (1:100, v/v, Roche) in solubilization buffer. Samples were then diluted in buffer containing 2 M urea, 10% (v/v) acetonitrile, 0.1 M  $NH_4HCO_3$ , and 1 mM  $CaCl_2$  (pH 8.5). Proteins were further digested overnight at  $37^\circ C$  with Poroszyme immobilized trypsin beads (1:10, v/v, Applied Biosystems). After centrifugation for beads removal, the obtained peptide mixtures were desalted using SPECC18 columns according to the manufacturer's instructions (Varian). Finally, desalted digest solutions were dried and pellets stored at  $-20^\circ C$  until use.

### Liquid Chromatography/Mass Spectrometric Analysis

Prior to the mass spectrometric measurement, protein digest pellets were dissolved in 5% (v/v) formic acid. Protein samples (200  $\mu g$ ) were loaded and concentrated on a precolumn. For quantitative 1D analyses, samples were loaded onto a 50 cm silica-based C18 reverse phase monolithic column with 100  $\mu m$  i.d. (manufactured in the lab of Prof. Nabuo Tanka, Kyoto). For 2D analyses, protein digests obtained from the FPLC fractions were first loaded onto a 3.5  $\mu m$  i.d. strong cation exchange column (Agilent) and successively eluted, via four salt steps (0%, 3%, 10%, and 100% 0.5 M ammonium bicarbonate in 2.5% [v/v] acetonitrile and 0.1% [v/v] formic acid) onto a precolumn. Elution of the peptides was performed using a 2 h gradient from 100% solvent A (2.5% [v/v] acetonitrile, 0.1% [v/v] formic acid in water) to 100% solvent B (99.9% [v/v] methanol, 0.1% [v/v] formic acid in water) with a flow rate of 300 nL  $min^{-1}$ . Eluting peptides were analyzed with an LTQ mass spectrometer (Thermo Electron) operated in a data-dependent mode. Each full MS scan was followed by three MS/MS scans, in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation using a normalized collision energy of 35%. The temperature of the heated capillary and electro spray voltage were  $150^\circ C$  and 1.8 kV, respectively.

### Database Search

After mass spectrometric analyses, DTA files were created from raw files and were then searched against different databases for protein identification using Bioworks 3.2 software featuring the Sequest search algorithm. For the plant fractions, the *M. truncatula* Gene Index release 8.0 EST database from TIGR (<http://compbio.dfc.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago>) was employed. For bacteroid fractions, the protein database of the *S. meliloti* strain 1021 Genome Project (<http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/index.html>) was used. Automatic analysis of SEQUEST results was performed using DTASelect (Tabb et al., 2002) and a list of identified proteins was obtained using the following criteria: normalized difference in correlation score ( $\Delta C_n$ ) of at least 0.08, peptides with a +1 charge state were accepted if they had a cross correlation ( $X_{corr}$ ) of at least 2.0, 2.2 for +2 charge state peptides, and  $X_{corr} > 3.5$  for +3 charged peptides, with at least a required identification of two different peptides per protein. For mass tolerances LTQ default settings were being used (precursor ion tolerance 1.4 amu; peptide tolerance 1.5 amu). For quantitative analysis, peptide modifications were not accepted and for comparison among treatments the cumulative sum of recorded peptides per protein, called spectral count, was used according to Liu et al. (2004). Spectral count is a semiquantitative measure for tracking changes in protein abundance in complex samples, based on the cumulative sum of recorded peptide spectra that can be matched to a given protein. The validity of this label-free quantification method, compared to others such as peak integration, has been widely demonstrated (Cox et al., 2005; Old et al., 2005; Wienkoop et al., 2006; among others).

### Statistical Analysis

Statistical ICA was carried out using MetaGeneAlyse (<http://metagenealyse.mpimp-golm.mpg.de>). A distance matrix was written and subsequently uploaded to the MetaGeneAlyse server. First, principal component analysis was applied as a preprocessing step for dimensionality reduction to a set of three principal components and visualization of relevant variances. ICA was then applied to this reduced data set for the evaluation of those covariant protein sets that were involved in the response of the plant and bacteroid fractions to water deficit and could be used to differentiate between the samples. The extracted independent components were then ranked by kurtosis as a measure of the influence of a specific protein on the separation of control and drought samples. For more details see the user manual downloadable at the Web site and also Scholz and Selbig (2007).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table S1.** List of identified plant proteins in *M. truncatula* root nodules.

**Supplemental Table S2.** Quantitative proteomic analysis of the plant fraction of *M. truncatula* root nodules during drought stress.

**Supplemental Table S3.** Quantitative proteomic analysis of the bacteroid fraction of *M. truncatula* root nodules during drought stress.

**Supplemental Table S4.** Summary of the amino acid sequence information and Sequest identification scores of the plant fractions of *M. truncatula* root nodules.

**Supplemental Table S5.** Summary of the amino acid sequence information and Sequest identification scores of the bacteroid fractions of *M. truncatula* root nodules.

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