A proteomic approach reveals new actors of nodule response to drought in split-root grown pea plants

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ABSTRACT

Drought is considered the more harmful abiotic stress resulting in crops yield loss. Legumes in symbiosis with rhizobia are able to fix atmospheric nitrogen. Biological nitrogen fixation (SNF) is a very sensitive process to drought and limits legumes agricultural productivity. Several factors are known to regulate SNF including oxygen availability to bacteroids, carbon and nitrogen metabolisms; but the signalling pathways leading to SNF inhibition are largely unknown. In this work, we have performed a proteomic approach of pea plants grown in split-root-system where one half of the root was well-irrigated and the other was subjected to drought. Water stress locally provoked nodule water potential decrease that led to SNF local inhibition. The proteomic approach revealed 11 and 7 nodule proteins regulated by drought encoded by *P. sativum* and *R. leguminosarum* genomes respectively. Among these 18 proteins, three proteins related to flavonoid metabolism, two to sulphur metabolism and three RNA-binding proteins were identified. These proteins could be molecular targets for future studies focused on the improvement of legumes tolerance to

drought. Moreover, this work also provides new hints for the deciphering of SNF regulation machinery in nodules.

ABBREVIATIONS

SNF: Symbiotic Nitrogen Fixation, Nase: Nitrogenase; CCoACOMT: Caffeoyl-CoA O-Methyltransferase; GRP: Glycine-Rich Protein; IMP: Inosine-5´-monophosphate; MDH: Malate Dehydrogenase; MS Mass Spectrometry; ROS: Reactive Oxygen Species; SAM: S-adenosyl-L-Met; SS Sucrose Synthase; SRS: Split-Root System.

INTRODUCTION

Abiotic stresses provoke the major losses in agricultural output. Among them, drought is considered to be the one that causes the most important crops yield loss (Boyer, 1982; Mittler and Blumwald, 2010). Moreover, global warming associated with climate changes will substantially increase drought conditions in the next 25 years. In fact, United Nations estimate that in 2025 1.8 billion people will be living in countries or regions with absolute water scarcity and two-thirds of the world population could be under conditions of water stress (FAO, 2007).

Legumes, mainly because of the high protein content of their grains, are some of the most important crops worldwide. Legumes have the ability to feed from atmospheric N_2, through symbiosis with soil bacteria that gives rise to the development of a new organ, the nodule, where the nitrogen fixation process (SNF) is set up (Oldroyd *et al.,* 2011). Nitrogenase enzyme complex (Nase) within the bacteroids is the ultimate responsible of reducing the N_2 to ammonia. SNF is a highly-energy demanding process that involves significant respiration rates in the nodules, which must be finely-regulated because of the high susceptibility to oxygen of Nase (Oldroyd *et al.,* 2011). Indeed, SNF is an extremely sensitive process to environmental fluctuations, being the carbon burden and the need for extremely low-oxygen levels key aspects contributing to the high sensitivity of the process (Arrese-Igor *et al.,* 2011). In these context, three main regulatory mechanisms have been explored in the last decades: 1) regulation through the carbon flux within nodules, through a rapid nodule sucrose synthase (SS) down-regulation (González et al., 1995); 2) nitrogen feed-back regulation (Serraj *et al*., 2001) and 3) a regulation based on an interaction between the internal nodule oxygen concentration and reactive oxygen species (ROS) homeostasis (Diaz del Castillo and Layzell., 1995; Naya *et al*., 2007). However, it is still a matter of debate which of these (C, O or N) is responsible of SNF regulation or whether they act in a co-operative way.

To improve symbiotically grown legumes tolerance to drought, a more precise understanding of the factors limiting and regulating the response of SNF under abiotic stresses is needed to find new potential molecular targets to modify or select. In this sense, "omics" represent an enormous powerful tool to find targets in complex mixtures of biological samples such as a whole-protein extract of a legume-nodule where the cohabitation of rhizobial bacteroids and plant cells takes place. Many proteomic studies have been performed to study legumes biology; however, the majority of them have been performed in non-symbiotic legumes (fed with external N). The proteomic studies of mature nitrogen-fixing nodules are scarce and mainly in the model plant *Medicago truncatula* (Bestel-Corre *et al*., 2002; Oger *et al*., 2012). Similarly, many proteomic approaches have been done in plant subjected to abiotic stresses, including drought (Roy *et al*., 2011), but again the study of nitrogen-fixing legumes under these conditions is mainly focused in *M. truncatula* (Larrainzar *et al*., 2007; Larrainzar *et al*., 2009). Proteomics approaches in nodules of economically important legumes leading with abiotic stresses or drought is extremely limited and to our knowledge is restricted to soybean under drought (Gil-Quintana *et al*., 2013).

In the last few years, the local or systemic origin of nitrogen fixation control under abiotic stresses have been addressed by the use of split-root system approaches for different legume species and stresses. For instance, *M. truncatula* (Gil-Quintana *et al*., 2013a), soybean (Quintana *et al*., 2013b) and pea (Marino *et al*., 2007) under drought and *M. truncatula* exposed to Cd (Marino *et al*. 2013). In these works, the alteration of nodule metabolic pathways under drought has been shown to be mainly locally affected. Similarly, it has been shown that the alteration of ROS homeostasis, associated to nodule response to abiotic stresses, seems to be locally regulated. However, the signalling cascades leading to SNF inhibition are still in its infancy. Thus, in this work, we have performed a comparative proteomic approach of nodules from pea plants grown in split-root system (SRS) where half of the root system of pea plants was irrigated at field capacity, while the other half was water deprived to try to get further knowledge on the molecular control of SNF regulation under drought. The identification of new potential actors related to nodule functionality might be useful to further select plant genotypes with an increased tolerance to drought.

MATERIALS AND METHODS

Experimental procedures and growth conditions

Pea seeds (*Pisum sativum* L. cv. Sugar-lace provided by Bonduelle SA, Milagro, Spain) were sterilised and grown as described in Marino et al., (20007). Four week-old plants

were irrigating to field capacity to one root-side (C) and withholding water/nutrients the other root-side (D) during seven days. Nodules were harvested, immediately frozen in liquid nitrogen and stored at –80ºC for analytical determinations.

Water relations

Nodule water potential was determined by a psychrometer Wescor HR-33T (Wescor Inc. 5500, Logan, UT, USA). Four nodules per split-root were collected and confined in C52 sample chambers for at least 1 h until temperature and vapour equilibration was reached.

Water content (WC) was calculated with the following formula. WC = (FW-DW)/FW, where FW and DW mean fresh weight and dry weight respectively.

Nitrogen fixation determination

NF was measured as apparent Nase activity, determining hydrogen evolution as described in Zabalza et al., (2008).

N content determination

Total root N content was determined with a NC 2500 Elemental Analyzer (Carlo Erba, Milan, Italy). Briefly, two to three milligrams of powdered plant material from each sample was separately packed in tin capsules. N content was determined by flash combustion at 1020ºC. The resulting combustion gases pass through a reduction furnace into the chromatographic column using helium as carrier gas and detected by thermal conductivity. Acetanilide was used as internal standard.

Protein extraction

Nodules (~0,3 g) were homogenised in a mortar and pestle with 1,2 mL of lysis buffer as described in Farinha *et al.* (2011). Protein content was assayed by the Bradford method (Bradford *et al*. 1976).

For the determination of the protein content from the plant and the bacteroidal fractions separately, fresh nodules were homogenised with lysis buffer (Farinha et al., 2011) and centrifugated at low-speed 2000 g to avoid bacterial disruption. The supernatant was kept for plant-fraction protein determination. The centrifugation pellet was washed three times with lysis buffer and boiled during 10 min with KOH 2M. It was centrifuged again and the protein content of the supernatant quantified by the Bradford assay (Bradford *et al*. 1976).

Western blotting

Immunoelectrophoresis was performed as described in Marino *et al.*, (2013). The antibodies used were α-SS (1:5000) and α-NifD (1:2000) as primary antibodies and goat anti-rabbit horseradish peroxidase conjugate as secondary antibody (1:50000, Sigma-Aldrich).

2D Electrophoresis

Samples containing 0.9 mg of protein, which corresponds 100-150 mg of nodules, were analysed as previously described (Farinha *et al*., 2011; Valdes *et al*., 2013) For each biological nodule sample, two biological replicates and three experimental replicates were performed (in Supporting Figure S2 are shown the 12 master gels performed). The first dimension was run onto pH 4-7, 18 cm immobilized pH gradient (IPG) strips (Immobiline DryStrips, GE Healthcare). For the second dimension, the strips were loaded on SDS-PAGE 12 % polyacrylamide gels (26x20x0.1cm).

Image and data analysis

2-DE gels were analysed as described in Irar *et al*. (2010) by the use of the ImageMaster ™ 2-D Platinum 5.0 Software (GE Healthcare). In order to compare the nodules proteome of the different samples, automatic spot matching was established between synthetic gel images. Careful visual inspection was performed to confirm correct spot matching.

Statistical analyses

The statistical evaluation of proteins expression differences among treatments was performed as previously described (Jorrin-Novo, 2009; Farinha et al., 2011). Spots showing a quantitative variation or their relative spot volume (≥ Ratio 1.5) and positive GAP (Statistical parameters ImageMaster™ 2-D Platinum 5.0 Software) were selected as differentially expressed. Significant protein abundance was validated by Student's t-Test ($p<0.05$).

Protein identification

Proteins were identified at the Proteomics Platform of Barcelona Science Park, University of Barcelona (a member of ProteoRed network) by MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems) or LC-ESI-QTOF (Q-TOF Global, Micromass-Waters) mass spectrometers as previously described (Irar et al., 2010). The obtained results were submitted for database searching in a MASCOT search engine against non-redundant NCBI and Swissprot databases. The search parameters were: Oxidation of methionine

(variable modification), carbamidomethyl of cysteine (fixed modification) and 1 missed cleavage. Peptide tolerance was 200 ppm and 0.25 Da, respectively for MS and MS/MS spectrum. Peptide charge was +1 for MALDI identified proteins and +2, +3, +4 for ESI identified proteins). See Supporting Table S1 for the proteins identification dataset.

RESULTS AND DISCUSSION

Drought effects on nodules of pea plants in split-root

Environmental stresses induce a rapid inhibition of SNF process in legume nodules. Several works in the last few years have dealt with the question whether this inhibition is driven at the nodule level (local) or by a signal of shoot origin (systemic). In this sense, local SNF control under water stress has been shown in *M. truncatula* (Gil-Quintana *et al*., 2013a), pea (Marino *et al*., 2007) and soybean (Gil-Quintana *et al*., 2013b). The local control of SNF under Cd has also been recently reported in *M. truncatula* (Marino *et al*. 2013). In pea, SNF control was shown to be related with a rapid down-regulation of SS, enzyme that hydrolyses the sucrose coming from the photosynthesis to ultimately feed the bacteroids mainly with malate for its energy obtaining (Gálvez et al., 2005). This SS inhibition was associated to a cell redox imbalance (Marino *et al*., 2007). However, the nodule metabolic adjustment leading to SNF inhibition seems to be species specific since for instance in *M. truncatula* subjected to Cd the local SNF control was also associated to changes in cell redox homeostasis but not to SS inhibition, as it has been also reported under drought (Naya et al., 2007). Indeed, SNF inhibition in *M. truncatula* under Cd was associated to a control involving leghemoglobin-O_2-ROS-Nase. Thus, nodulated pea plants were grown in an SRS and subjected one part of the split-root to water deprivation whereas the other half root was maintained in optimal conditions. In agreement with previous results (Marino *et al*., 2007), in this study, the SRS allowed having in the root system of the same plant, nodules with a reduced water potential (-1,1 MPa) in the droughted-half whilst the nodules of the control-half had a water potential typical of well-irrigated plants, around -0.6 MPa (Fig. 2A). Nodule biomass in the droughted half root (11.54 ± 1.15 mg of nodules dry weight) was reduced respect to the control half root (18,46 ± 1.92 mg of nodules dry weight). This difference was due to a growth arrest of the nodules, since nodule water content was similar among control (87,1 ± 1,2 %) and stressed nodules (86,0 ± 0,2 %) what indicates the still mild degree of the imposed water stress. We checked that SNF was actually inhibited in the droughted-half and we confirmed a reduction close to the 50% in Nase activity (Fig. 3). According to the mild

water stress, although SNF was inhibited, roots total N content did not still change between the control and the treated halves (data not shown).

SS and Nase are among the most sensitive nodule metabolism components to environmental stresses in the plant and bacteroid respectively (Arrese-Igor et al., 2011). We performed a western blot for these two components and we observed that both SS and Nase (NifD) were reduced in the droughted half of the root, with their content representing 38 and 85% of control nodules respectively (Fig. 2C). However, the degree of this 7-day water deprivation stress was moderate since total protein content of both the plant fraction and the bacteroids did not vary between both halves of the root (Fig. 2B). Moreover, the stronger drought effect observed on SS compared to NifD suggests, together with the overall down-regulation of the identified plant proteins compared to the rather up-regulation of bacteroidal proteins (see below), that drought is perceived first in the nodule plant fraction, as it has been already shown in grain legumes under abiotic stresses (Arrese-Igor et al., 2011).

Proteome analysis of nodules under two water regimes within the same plant

To further understand the SNF regulation we sought for new actors involved in nodule response under drought by performing a comparative proteomic study among nodules of both sides (control and drought) of the splitted-root of pea plants. To estimate the changes in protein abundance between the two nodule proteomes, we used normalized spot volume or volume percentage (individual spot volume / all spots volume within a given gel) using the ImageMaster software. The pattern of spot distribution was similar between the 2DE-gels along the separation range *pI* 4-7 and molecular mass 14-66 KDa (Fig. 4A). However, the number of spots was different between control and water-stressed nodules, 1056 and 1179 spots respectively (Fig. 4). When comparing the proteome of control nodules versus nodules under drought, the percentage of spot matching was 81% (906 spots; Fig. 4B). Moreover, we looked for spors with fold-change ratios ≥ 1.5 ($p<0.05$) between control and drought nodule proteomes (Fig. 4). About 2.3% of the proteins (21 spots) shared by the control and the water-stress treatments registered significant differences in accumulation. In order to determine the variation among nodule proteins the linear relationship between CV and normalized spot volumes was calculated. The correlation (Student's t-test, $p<0.01$) between technical replicates was of 0.97 and 0.98 for control and drought treatments respectively, while between biological replicates was somewhat lower, 0.83 and 0.86 for control and drought treatments respectively (Table 1). The scatter plots generated by the Image Master 2D Platinum 5.0 software is provided in Supporting Figure S1. Regarding biological replicates, the correlation

between the less abundant proteins was higher than that observed among high abundant proteins (Supporting Fig. 1). Besides, the average CV was lower between technical replicates (0.20-0.22) when compared with biological replicates (0.34-0.39) (Table 1).

The analysis of the 2-DE gel images representing the nodule proteome of the two halves of the split-root, revealed 21 protein spots with quantitative differences in their accumulation (Fig. 4). Out of these, eighteen were identified by MS/MS, eleven encoded by the *P. sativum* genome (P1-11, Table 2) and seven by *R. leguminosarum* (B1-7, Table 3). Five out of the 18 identified spots (P7, B1, B2, B3 and B4) were significantly accumulated in the drought treatment and thirteen (P1, P2, P3, P4, P5, P6, P8, P9, P10, P11, B5, B6 and B7) in the control treatment. Interestingly, while there was a general down-regulation of plant proteins, four out of the seven bacteroidal proteins identified were up-regulated. This pattern has been previously observed in *M. truncatula* root nodules under drought suggesting a different adaptative mechanism to drought of both symbionts (Larrainzar et al., 2007).

Identified plant proteins were mainly down-regulated by drought

This comparative proteomic approach allowed identifying, both in the plant fraction and in bacteroids, proteins regulated by drought related to sulfur (S) metabolism, specifically components of the S assimilation pathway involved in methionine metabolism (Supporting Table S2; Fig. 5; Tables 2, 3). S is one of the six macronutrients necessary for plant growth, in particular is required for cysteine and methionine synthesis together with a variety of secondary metabolites. Plants, in contrast to animals, are able to assimilate S from the inorganic S present in the soil. Sulfate is first incorporated into cysteine for the posterior synthesis of other S-containing compounds. S assimilation pathway appears to be connected with drought stress response, not only for the production of the well-known sulfur containing glutathione and choline-O-sulfate but, for instance, it has been shown that some abscisic acid functions are sulfate-dependent (Ernst *et al*., 2010). Overall, it has been suggested that S assimilation must be tightly regulated under this stress so the plant can cope with "competing interests" between the different metabolic pathways important for plant survival under drought (Chan *et al*., 2013). Methionine adenosyltransferase (S-adenosylmethionine synthethase 2; EC 2.5.1.6; spot P2, Fig. 4, Table 2) which is in charge of the synthesis of S-adenosyl-L-Met (SAM), a primary methyl-group donor and a precursor of important metabolites as polyamines, or ethylene (Amir *et al*., 2002), has been found to be down-regulated by drought in the nodule plant partner (Fig. 5A). In the bacterial partner, the S-adenosyl-L-homocysteine hydrolase enzyme (EC 3.3.1.1; spot B2, Fig. 4, Table 3),

responsible for the reversible conversion of S-adenosyl-L-homocysteine into adenosine and homocysteine increased *ca*. 4 times in response to drought (Fig. 5B). We might speculate that the response of these two enzymes could provoke a decrease in SAM content in response to drought. Interestingly, in a proteomic study of *M. truncatula* nodules subjected to drought only five proteins were identified showing a significant down-regulation during drought stress (Larrarinzar *et al*., 2007). Out of those five proteins, Methionine synthase was the one showing the strongest response. S-adenosylmethionine synthethase and Methionine synthase down-regulation have also been found associated with nodule ageing (Matamoros *et al*., 2013). Both in nodule natural senescence and in stressed-induced senescence ROS over-production has been reported (Marino *et al*., 2009). In a proteomic approach during *M. truncatula S-meliloti* symbiosis, both plant and microsymbiont S-adenosylmethionine synthethase was found sulfenylated in mature nodules (Oger *et al*., 2012). Overall, ROS produced in nodules could be involved in S-metabolism regulation. Finally, it has been shown that SNF in *Trifolium repens* was drastically reduced in S-deficient plants in relation, among others, with Nodule O_2 and Nase (Varin *et al*., 2010)

Three out of the eleven proteins identified from the host plant belong to the flavonoid biosynthesis pathway. Two chalcone flavonone isomerases (spots P6 and P8; Fig. 4, Table 2) and the Caffeoyl-CoA O-Methyltransferase enzyme (spot P4; Fig. 4, Table 2). Chalcone flavones isomerases catalyze the conversion of chalcone into a flavonone in different steps of flavonoid biosynthesis. The Caffeoyl-CoA O-Methyltransferase (CCoACOMT) converts caffeoyl-CoA into feruloyl-coA. Interestingly, coupled to this reaction SAM is transformed into S-adenosyl-L-homocysteine. As stated above, SAM production is surely impaired because of the S-adenosylmethionine synthethase down-regulation which would secondarily limit the substrate availability for CCoACOMT proper functioning. CCoACOMT function has been mainly associated to lignin biosynthesis (Guo *et al*., 2001). In the *M. truncatula cra1* mutant showing compact root architecture, CCoACOMT was the most down-regulated gene and this mutant presented reduced lignin content and changes in flavonoid pattern. *cra1* mutant did not present any morphological nodule phenotype, although its functionality was not investigated (Laffont *et al*., 2010). In tissues other than nodules CCoACOMT has generally been found to be induced upon abiotic stresses for example in soybean roots (Yamaguchi *et al*., 2010), sunflower seeds (Fulda *et al*., 2011) or in rice leaves (Salekdeh *et al*., 2002). Overall, it seems that CCoACOMT has a nodule specific regulation and that in lignifications processes might be related to plant response upon environmental changes.

Besides, in the plant partner, 3 glycine-rich proteins (GRPs) were also down-accumulated under drought stress (Fig. 5A). These three GRPs belong to the Class IV of GRPs also known as RNA-binding GRPs (GR-RBP-s) because besides the glycine-rich domain, they present a RNA-recognition motif (RRM) (Lorkovic *et al*., 2010). GRPs have been associated to a wide range of different functions in plant cells including cell wall structure, plant defence, cell elongation, abiotic stress response or plant flowering and development (Mangeon *et al*., 2010). It has been determined that the expression of GRPs is regulated by a number of external stimuli including drought (Sachetto-Martins *et al*., 2000). The *Arabidopsis thaliana* genome encodes eight GR-RBPs. The GRP corresponding to the spot P11 (Fig. 4; Table 2), shows an 84% of identity with *Arabidopsis* GRP7. Interestingly GRP7 was found to be repressed by ABA and osmotic stress (Cao *et al*., 2006). One of the functions that have been attributed to AtGRP7 is the regulation of stomata opening and closure under stress conditions (Kim *et al*., 2008). However, the mechanisms of how GR-RBPs contribute to plant responses under stresses are largely unknown. Moreover, RBPs are also known to regulate gene expression in different ways including alternative splicing. A substantial fraction (similar to 30%) of plant genes is alternatively spliced, and many plant genes undergo alternative splicing in response to a variety of stresses and might be important for stress adaptation (Reddy *et al*., 2007). In this context, the GRP-target RNA interactions and the GRP-mediated regulation of RNA metabolism and folding in post-transcriptional gene regulation are not fully understood but could be essential for nodule, and plants in general, response to abiotic stresses.

We also identified a GroEL-like chaperone ATPase, a proteasome subunit and the 12-oxophytodienoic acid 10,11-reductase were also identified in the plant fraction and were down-regulated by drought stress (Fig. 4, 5A; Table 2). The 12-oxophytodienoic acid 10,11-reductase (EC 1.3.1.42) is an enzyme of the jasmonic acid biosynthesis pathway. Jasmonates are ubiquitous stress-signalling compounds of plants, finding this enzyme down-regulated could mean that although the 7-day drought stress treatment was slight to provoke nitrogen fixation inhibition without generating a general plant breakdown but it was strong enough to induce a down-regulation of JA pathway.

Among all the differentially expressed proteins encoded by the pea genome only one of them was induced by drought (Figure 5A, spot P7). The nodule lectin PsNLEC-1 whose content doubled in the nodules subjected to drought. Lectins are nonenzymatic carbohydrate-binding proteins that specifically recognize diverse sugar structures and mediate a variety of biological processes, including signalling cascades, cell to cell communication, molecules

transport or enzymes posttranslational regulation (Kijne *et al*., 1997.). Legume lectins function associated to the nitrogen fixing-process is still unclear but they are thought to have a role in promoting the aggregation of rhizobia in infectable root hairs (Diaz *et al*., 1995), in stimulating mitotic activity in root cortical cells (Brewin and Karakailsky, 1997) and in the infected zone as a transient N reserve (Law, 1996). PsNLEC-1 has been shown to be localized in the symbiosome compartment of infected cells and in the vacuole (Dahiya *et al*., 1997), and it has been suggested that it might play a role in nodule development (Dahiya *et al*, 1998; Bolaños *et al*., 2001). Plant lectins have also been proposed to play a role in abiotic stresses response (Jiang *et al*., 2010, Babosha, 2008). For instance in Arabidopsis 89 genes out of 199 lectin genes were regulated by different abiotic stresses, and 62 out of this 89 were regulated by drought (Jiang *et al*., 2010). The functional role of lectins under abiotic stresses is unclear and much remains to be learned about their function but it seems that they might be acting in the signalling cascades downstream the stress perception (Jiang *et al*., 2010). Interestingly, the pea lectin receptor PsLecRLK was induced upon salt and drought exposure, further evidencing a potential role of lectins in pea signal transduction in response to drought (Joshi *et al*., 2010). Moreover, it has been suggested that lectins could be playing a role in the protection of cells against oxidative stress (Babosha *et al*., 2008), which has been already been shown to occur in pea nodules under drought (Marino *et al*., 2007).

Microsymbiont proteome response to drought

From bacterial origin we identified seven proteins differentially expressed, three down-regulated and four up-regulated (Fig. 5B). Malate dehydrogenase (MDH) content slightly increases in bacteroids in response to drought. Sucrose is the main carbohydrate provided by the plant to the nodules, sucrose is then hydrolysed to dicarboxylic acids to provide with carbon and energy to the bacteroids. In this work, SS content is reduced by water stress (Fig. 2C) and it has been previously shown that SS inhibition leads to nodule malate content depletion (Marino *et al*., 2006, Marino *et al* 2007). Thus, we could speculate that an early response to malate limitation could be MDH induction in the bacteroids. In bacteroids of common bean nodules MDH was also shown to be induced upon mild salt stress which also provoked malate content diminution in the nodules; however, a more severe stress inhibited MDH activity (Ferri *et al*., 2000).

Inosine-5´-monophosphate (IMP) dehydrogenase (EC 1.1.1.205) content in the microsymbiont was doubled upon drought exposure. This enzyme catalyzes the NAD^+ dependent conversion of IMP to Xanthine monophosphate in the purine metabolism pathway.

There is not much evidence of the potential role of this enzyme under stress conditions. IMP dehydrogenase induction could be related to NADH regeneration of great importance to support antioxidative machinery in stress conditions and also to store nitrogen in the form of purines which is not being exported from the nodule due to water stress.

Although plants mainly supply carbon skeletons to bacteroids, and in return they receive ammonia the metabolic exchange is more complex. In fact, effective N_2 fixation by *R. leguminosarum* bv *viciae* bacteroids requires either one of two broad-specificity amino acid ABC transporters (AAP and BRA) (Lodwig et al., 2003). This phenomenon is named symbiotic auxotrophy because bacteroids become dependent of amino acids supply from the plant (Prell *et al*., 2009). Preventing branched-chain amino acid uptake by nodule bacteria leads to amino acid starvation, failure to fully develop, reduced size, and endoreduplication of chromosomes and provokes a nitrogen starvation phenotype when inoculated on pea (*Pisum sativum*) plants (Lodwig *et al*., 2003). In the present work, we have found a component of the ABC transporter AAP, the General L-amino acid-binding periplasmic AAPJ protein (spot B4; Fig. 4, Table 3). This component was induced in pea nodules in response to drought. The role of these transporters in relation to abiotic stresses remains to be elucidated. However, Taté *et al*., (2012) suggested a putative role of glutathione in the regulation of amino-acid cycling in bacteroids. Thus the reduction in glutathione content in nodules subjected to drought (Marino *et al*., 2007) might be linked to the induction of the AAPJ protein in this work.

Among the three down-regulated proteins, NifH, the Fe-component the Nase complex, was identified (spot B5; Fig. 4; Table 3). This is in agreement with the western blot showing NifD, the MoFe-component of Nase, down-regulation in the droughted half-root (Fig. 2C). The high sensitivity of Nase under environmental stresses has been previously reported, among others, in *M. truncatula* under Cd (Marino *et al*., 2013) and *M. sativa* under drought (Naya *et al*., 2007). Besides the biological relevance of NifH down-regulation, it represents also a very good internal control of the validity of the proteomic assay. The other two down-regulated proteins identified in *R. leguminosarum* bacteroids where an ATPase and a thioredoxin family protein (Fig. 4, Table 3). ATPase down-regulation could reflect the reduction in bacteroid respiration related to carbon limitation due to SS down-regulation (Fig. 2C). Thiorredoxins family is composed by thioredoxins but also by glutaredoxins and peroxiredoxins. They are small redox proteins that play a role in redox signaling and they are associated to a wide range of cellular processes. In legume bacteroids, these proteins have been shown to be required for optimal nodule development and nitrogen fixation activity (Frendo *et al*., 2013)

In conclusion, the identification of 11 *P. sativum* and 7 *R. leguminosarum* proteins regulated by drought in nodules of pea plants grown in split-root provides new potential targets for future studies focused on legumes drought tolerance improvement. Moreover, the overall down-regulation of the identified plant proteins compared to the rather up-regulation of bacteroidal proteins suggests an enhanced sensitivity of the nodule plant fraction to the stress. Finally, this study highlights the importance of S-metabolism in water stress conditions and to our knowledge provides the first hints of GR-RBPs potential role in legume nodule responses under stress conditions and opens a new avenue for the implication of post-transcriptional gene regulation in nitrogen fixation regulation.

AUTHOR CONTRIBUTIONS

SI and DM performed experiments; SI, DM, CA and EMG designed research and analysed data; DM wrote the paper with the cooperation of the rest of the authors.

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SUPPORTING INFORMATION

Figure S1. Linear regression of protein amounts between 2-DE gel replicates from the control and droughted halves of the pea split-root system. The linear relationship between spot normalized volumes (% Vol.) is shown for triplicate gels running three biological replicates.

Figure S2. 2D master gels from nodules of control (C) and droughted (D) halves of the split-root system.

Table S1. Identification of plant and bacteroid proteins.

Table S2. Quantification data for the identified protein spots.

TABLE LEGENDS

Table 1. Comparison of the analytical and biological variation across *P. sativum* nodules separated by 2-DE. Average values of coefficient of correlation (r) and coefficient of variation (CV) obtained from pairwise comparison between normalised protein spot volumes across distinct gel replicates are shown (two independent protein extractions were performed and 2-DE gels were run in triplicate).

Table 2. Summary of the *P. sativum* nodule plant fractions differentially regulated proteins in response to drought.

Table 3. Summary of the *P. sativum* nodule bacteroids differentially regulated proteins in response to drought.

FIGURE LEGENDS

Figure 1. *P. sativum* plants grown in split-root-system leading to a root system divided in two equal parts.

Figure 2. A) Nodule water potential in control and water stressed *P. sativum* plants grown in split-root system. B) Nodule plant-fraction and bacteroidal protein content and C) protein content of sucrose synthase (SS) and a nitrogenase component (NifD) of control and water stressed nodules of *P. sativum* plants grown in split-root system. Each bar represents mean ± SE for n=6. Asterisk (*) represents significant differences ($p \leq 0.05$).

Figure 3. A) Nitrogen fixation in control and water stressed nodules of *P. sativum* plants grown in split-root system. Each bar represents mean ± SE for n=6 Asterisk (*) represents significant differences ($p \leq 0.05$).

Figure 4: Representative 2-DE gel images of P. *sativum* nodule proteome from the control (C) and droughted (D) halves of the split-root system revealed protein spots with quantitative differences in their accumulation. A) Comparison of total proteins of control and drought treatment. The selected spots are indicated in the gels. With arrows and in red are represented unidentified spots. B) Venn diagrams showing the mean number of matching spots between C (blue) and D (green) provenances after synthetic gel comparisons using the ImageMaster Platinum software.

Figure 5. Analyses of protein spots with significant differences between control and water stressed nodules of *P. sativum* plants grown in split-root system. A) Identified proteins from the nodule plant fraction B) Identified proteins from the nodule bacteroids. Quantitative variation of protein abundance was validated by Students's t-test ($P<0.05$). Data are shown as mean ± SE of two biological replicates.

Table 1. Comparison of the analytical and biological variation across *P. sativum* nodules separated by 2-DE. Average values of coefficient of correlation (r) and coefficient of variation (CV) obtained from pairwise comparison between normalised protein spot volumes across distinct gel replicates are shown (two independent protein extractions were performed and 2-DE gels were run in triplicate).

	Control	Drought
Technical replicates	CV = 0.20 *r* = 0.97	CV = 0.22 *r* = 0.98
Biological replicates	CV = 0.39 *r* = 0.83	CV = 0.34 *r* = 0.86

Table 2. Summary of the *P. sativum* nodule plant fractions differentially regulated proteins in response to drought.

Spot	Protein name	MS method	Score	Coverage (%)	Theor Mw / pI	Exper Mw/pI	Gene ID	Species
P1	GroEL-like chaperone, ATPase	MALDI-TOF-TOF	92	11	61.4 / 6.3	63.2 / 5.2	gi|92882356	*M. truncatula*
P2	S-adenosylmethionine synthase 2	ESI-QUAD-TOF	100	8	43.6 / 5.6	44.9 / 7.3	gi|127046	*D. cariophyllus*
P3	12-oxophytodienoic acid 10, 11-reductase	MALDI-TOF-TOF	290	24	41.0 / 5.7	42.0 / 5.7	gi|40645349	*P. sativum*
P4	Caffeoyl-CoA O-methyltransferase	ESI-QUAD-TOF	216	23	26.9 / 5.3	29.7 / 4.9	gi|86110327	*M. truncatula*
P5	RNA-binding glycine-rich protein 3	ESI-QUAD-TOF	83	5	27.0 / 5.8	25.3 / 4.6	gi|502149339	*C.arietinum*
P6	Chalcone-flavonone isomerase 1B-1	MALDI-TOF-TOF	91	9	25.1/5.9	24.4 / 5.4	gi|357444973	*M. truncatula*
P7	Nodule lectin (PsnLec-1)	MALDI-TOF-TOF	126	9	28.9 / 4.8	24.4 / 5.4	gi|75319593	*P. sativum*
P8	Chalcone--flavonone isomerase	ESI-QUAD-TOF	133	13	25.1 / 7.1	23.3 / 5.1	gi|729104	*P. sativum*
P9	Proteasome subunit beta type-6-like	ESI-QUAD-TOF	287	26	25.2 / 5.1	23.2 / 4.8	gi|502132065	*C. arietinum*
P10	RNA-binding glycine-rich protein (RGP-1c)	ESI-QUAD-TOF	59	15	16.9 / 6.6	15.1 / 5.0	gi|502149239	*C. arietinum*
P11	RNA-binding glycine-rich protein (RGP-1b)	ESI-QUAD-TOF	64	12	14.8 / 5.5	14.5 / 5.0	gi|469071	*N. sylvestris*

Table 3. Summary of the *P. sativum* nodule bacteroids differentially regulated proteins in response to drought.

Spot	Protein name	MS method	Score	Coverage (%)	Theor Mw/pI	Exper Mw/pI	Gene ID	Species
B1	Inosine 5'-monophosphate dehydrogenase	ESI-QUAD-TOF	588	26	52.2 / 6.1	54.1 / 6.1	gi|116250620	*R. leguminosarum*
B2	S-adenosyl-L-homocysteine hydrolase	ESI-QUAD-TOF	468	19	50.1 / 5.4	50.0 / 5.4	gi|86355699	*R. etli*
B3	Malate dehydrogenase	MALDI-TOF-TOF	191	19	33.8 / 5.5	36.2 / 5.4	gi|116254171	*R. leguminosarum*
B4	General L-amino acid-binding periplasmic protein AAPJ precursor	MALDI-TOF-TOF	123	29	36.1 / 5.1	36.2 / 5.4	gi|116251960	*R. leguminosarum*
B5	NifH	ESI-QUAD-TOF	204	18.0	25.9 / 5.2	24.8 / 4.7	gI89475634	*R. leguminosarum*
B6	Thioredoxin family protein	ESI-QUAD-TOF	234	45	16.9 / 4.9	17.8 / 4.7	gi|116250810	*R. leguminosarum*
B7	ATPase	ESI-QUAD-TOF	117	27	16.5 / 5.2	16.5 / 5.1	gi|489636556	*R. leguminosarum*

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Figure 2

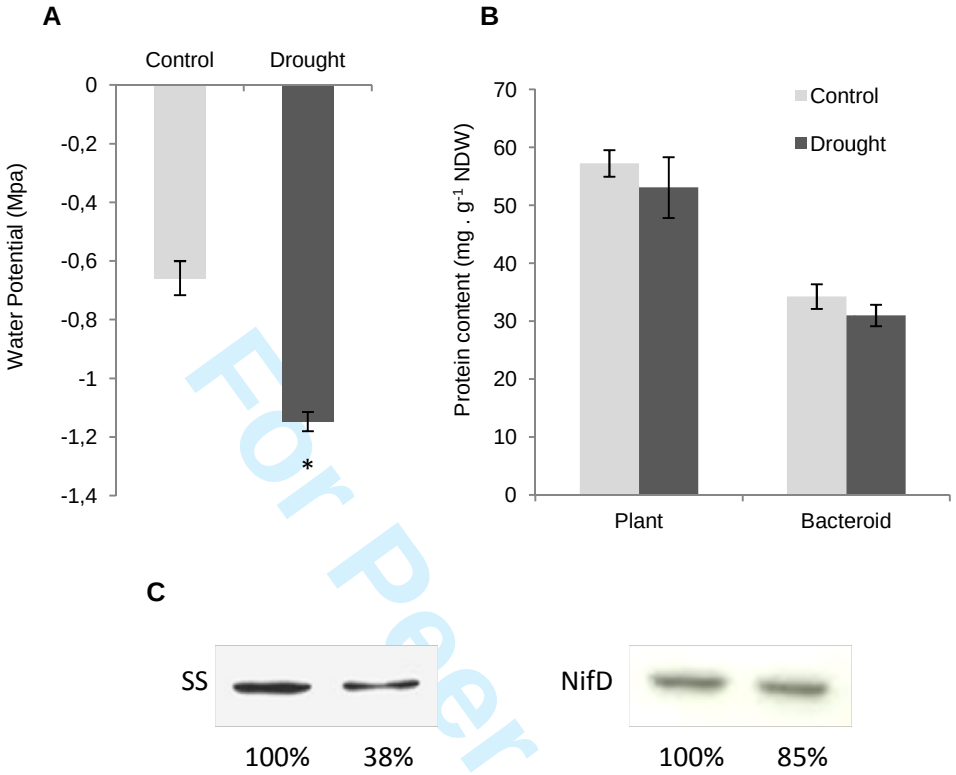

Figure 3

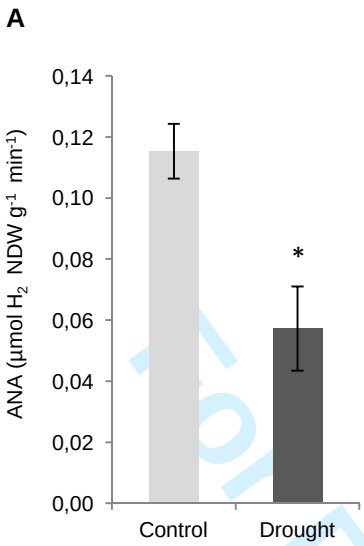

Figure 4

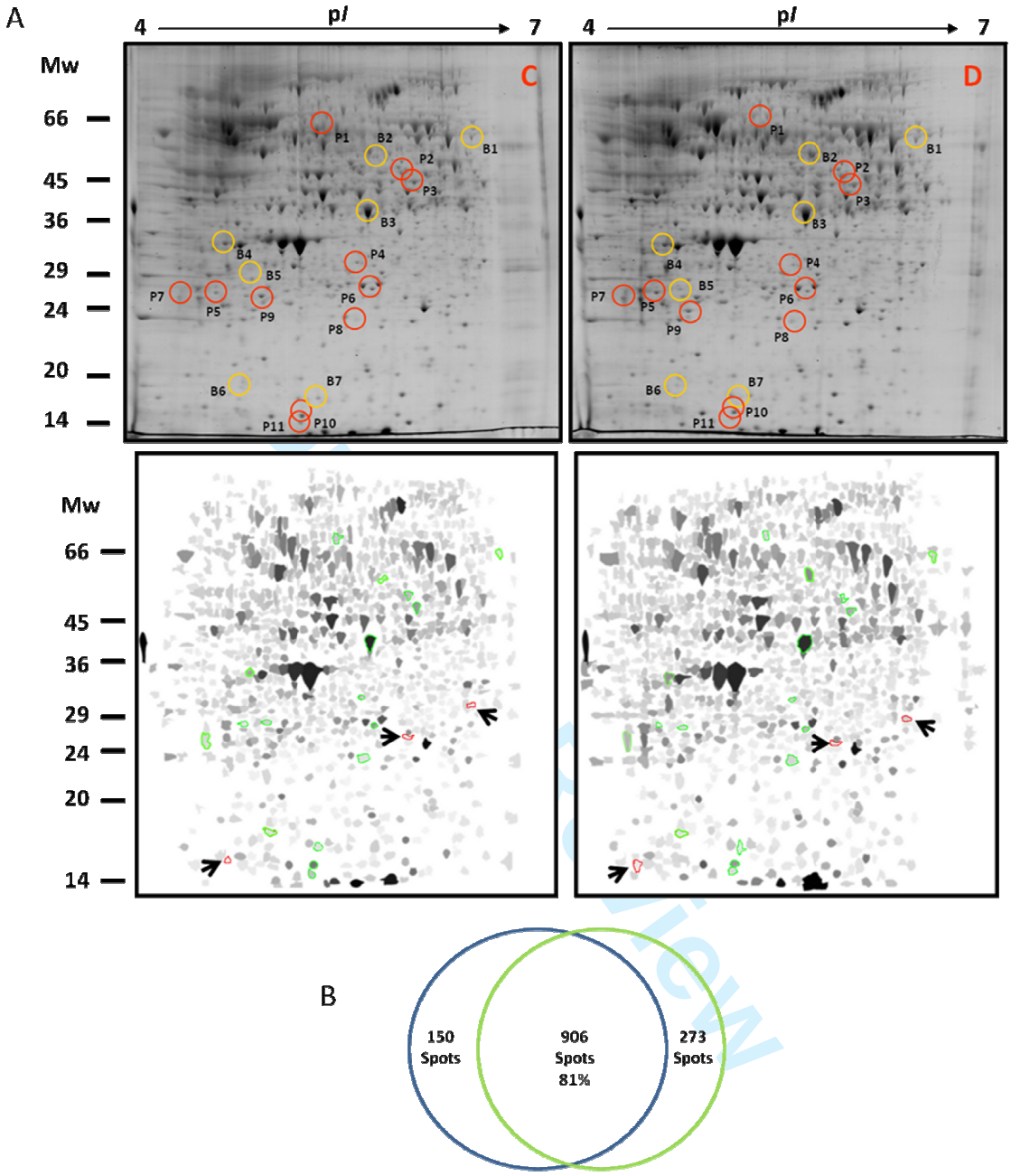
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Figure 5