Research Paper

Concerted changes in N and C primary metabolism in alfalfa (Medicago sativa) under water restriction

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Abstract

Although the mechanisms of nodule N2 fixation in legumes are now well documented, some uncertainty remains on the metabolic consequences of water deficit. In most cases, little consideration is given to other organs and, therefore, the coordinated changes in metabolism in leaves, roots, and nodules are not well known. Here, the effect of water restriction on exclusively N2-fixing alfalfa (Medicago sativa L.) plants was investigated, and proteomic, metabolomic, and physiological analyses were carried out. It is shown that the inhibition of nitrogenase activity caused by water restriction was accompanied by concerted alterations in metabolic pathways in nodules, leaves, and roots. The data suggest that nodule metabolism and metabolic exchange between plant organs nearly reached homeostasis in asparagine synthesis and partitioning, as well as the N demand from leaves. Typically, there was (i) a stimulation of the anaplerotic pathway to sustain the provision of C skeletons for amino acid (e.g. glutamate and proline) synthesis; (ii) re-allocation of glycolytic products to alanine and serine/glycine; and (iii) subtle changes in redox metabolites suggesting the implication of a slight oxidative stress. Furthermore, water restriction caused little change in both photosynthetic efficiency and respiratory cost of N2 fixation by nodules. In other words, the results suggest that under water stress, nodule metabolism follows a compromise between physiological imperatives (N demand, oxidative stress) and the lower input to sustain catabolism.

Key words: Alfalfa, C/N, drought, metabolomic, nodule, proteomic.

Introduction

It is widely accepted that drought causes a major restriction of N2 fixation efficiency in nodules of legumes (Serraj and Sinclair, 1996; Serraj et al., 1997; González et al., 1998; Larraínzar et al., 2007; Naya et al., 2007; Larraínzar et al., 2009). In fact, it is roughly estimated that water restriction in legume plantations may cause a reduction of up to 17 Gt N year−1 of global N2 fixation (Burns and Hardy, 1975). Several mechanisms responsible for the decrease in nodule activity under water-limited conditions have been proposed in the literature (see, for example, Naya et al., 2007; Aranjuelo et al., 2011), and all involve nitrogenase (Nass, the enzyme responsible for N2 conversion to ammonia) as the key target of water restriction. First, a build-up of amino acid (or other nitrogenous compounds) pools in nodules may cause
a feedback inhibition on $N_{\text{ase}}$ activity (Serraj et al., 1999, 2001; King and Purcell, 2005). This effect on amino acid content has been described recently in alfalfa nodules in a previous study (Aranjuelo et al., 2011). However, despite the accumulation of glutamine and asparagine, the nature and the intrinsic mechanisms of such an inhibition are unknown. Secondly, $N_{\text{ase}}$ activity may be limited by ATP availability due to the restriction of bacteroid respiration (González et al., 1998; Arrese-Igor et al., 1999; Erice et al., 2011). The latter is believed to be caused by a lower carbon input in nodules, which in turn results from the lower photosynthetic activity of source organs (leaves) and a decrease in sucrose synthase (which cleaves sucrose into fructose-6-phosphate and UDP-glucose) activity in nodules. Indeed, nodule metabolism has been described (Vance and Heichel, 1991) to be conditioned to carbon (sugars) provision from leaves since the transfer of photosynthates from leaves to nodules is very rapid (Voisin et al., 2003) and represents up to 50% of total photosynthetic CO$_2$ fixation. Furthermore, ~60% of sugars delivered to nodules are consumed by respiration to sustain ATP synthesis (which is in turn needed by the $N_{\text{ase}}$-catalysed reaction). Thirdly, both the increase in O$_2$ permeability of the nodule surface and the decrease in the nodule respiration rate may increase the dissolved O$_2$ mole fraction in bacteroids, thereby inhibiting $N_{\text{ase}}$ and possibly causing oxidative stress (Rubio et al., 2002; Naya et al., 2007; Becana et al., 2010). In fact, drought stress induces the expression of genes involved in detoxification of reactive oxygen species (ROS), such as those encoding Cu/Zn-superoxide dismutase and cytosolic glutathione reductase (Naya et al., 2007).

Taken as a whole, published data suggest that changes in nodule respiration rate and primary carbon metabolism seem to be at the heart of the physiological response of nodules to drought. Nevertheless, a better understanding of these metabolic effects of water restriction would require an integrated investigation of metabolites (metabolomics) and enzymatic activities (activitomics). Under drought conditions, many metabolic pools to vary) are still uncertain. However, the metabolism of other organs is likely to be critical to sustain nodule metabolism under water restriction: (i) in a previous study conducted with alfalfa (Medicago sativa L.) plants, leaf metabolism revealed by proteomics, and metabolic profiles appeared to be tightly linked to nodules (Aranjuelo et al., 2011). Furthermore, the data obtained suggest that a decrease in shoot N demand was involved in the accumulation of nodule amino acids and a decrease in $N_{\text{ase}}$ activity. In fact, (ii) root metabolism is also likely to influence nodule activity since roots are a major storage organ in which the remobilization of sugars and nitrogenous compound is crucial to regrowth and stress survival (Avic et al., 1996; Volenc et al., 1996). As a working hypothesis, and according to what was observed in previous studies, it was expected that proteins involved in N$_2$ fixation and assimilation would be inhibited by drought. Furthermore, nodule respiration, together with the oxidative stress machinery, was expected to be affected by drought.

As an aid to clarifying these aspects of nodule biology, the effect of drought on nodule proteins and metabolites in alfalfa was examined using proteomic and metabolomic analyses, respectively. In addition, the metabolism, the photosynthetic rate, and respiration of leaves, as well as root metabolism and respiration were followed. The results suggest that changes in protein abundance, catabolic pathways, and amino acid synthesis occur in nodules and that the decrease in N$_2$ fixation activity is homeostatic to match both nodule respiration and leaf photosynthesis.

Materials and methods

Experimental design

The experiment was conducted with N$_2$-fixing (nodulating) alfalfa (Medicago variety) grown in 7 litre plastic pots filled with silica sand (0.7 mm). One plant per pot and eight plants per treatment were sown in this study. Plants were grown in controlled-environment chambers (Conviron E15, Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 25/15 °C (day/night) with a photoperiod of 14 h at 400 µmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux density (PPFD). During the second, third, and fourth week after sowing, all the plants were inoculated (once per week) with Sinorhizobium meliloti strain 102F78. To ensure that the sole N source was N$_2$ fixed by nodules, the plants were irrigated with an N-free Hoagland nutrient solution (Hoagland and Arnon, 1950). The plants were watered twice a week with the nutrient solution and also with deionized water so as to avoid salt accumulation in pots. After 3 months, one half of the plants were maintained under optimal water availability conditions while the other half were subjected to drought by stopping watering. Suppression of irrigation was maintained for 7 d. Water withholding for 7 d induced a severe water stress in the plants (Naya et al., 2007).

After 7 d, the water status and $N_{\text{ase}}$ activity were determined and gas exchange measurements were carried out. Then the different organs were harvested, and nodules, root, and leaf samples were immediately frozen with liquid nitrogen and stored at −80 °C for the further proteomic and metabolomic analyses.

Nitrogenase activity

$N_{\text{ase}}$ activity was measured in detached nodules using the acetylene (C$_2$H$_2$) reduction method (Hardy et al., 1973). Use of this method has been debated (Minchin et al., 1994; Vessey, 1994). Acetylene has been suggested to induce a decline in $N_{\text{ase}}$ activity by decreasing the resistance to O$_2$ diffusion into the infected zone of the nodules, thereby inhibiting $N_{\text{ase}}$ activity (Witty et al., 1984). However, as observed by Minchin et al. (1994), in cases where acetylene is greatly modified, the results might be acceptable. Nodules were enclosed in a 1 litre glass flask into which 100 ml of C$_2$H$_2$ was added. The flask was incubated at room temperature for 10 min, before the N$_2$ decrease induced by exposure to excess acetylene. Afterwards, eight samples of 5 ml were withdrawn from the flask and the ethylene content in

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Gas exchange and chlorophyll fluorescence determinations

Gas exchange measurements were carried out with the LiCor 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, NE, USA) on healthy and fully expanded apical leaves under conditions similar to growth conditions (400 µmol m⁻² s⁻¹ PPFD, 25 °C, 380 µmol mol⁻¹ CO₂, 21% O₂). Photosynthetic CO₂ assimilation (A) was determined using equations developed by von Caemmerer and Farquhar (1981). Stomatal conductance (gs) was determined as described by Harley et al. (1992). Nodule and root respiration were determined using an external cuvette connected in parallel to the sample air hose of the LiCor 6400 (Aranjuelo et al. 2009). Mesophyll conductance (gₑ) was determined according to Pons et al. (2009). The intracellular CO₂ mole fraction (Cᵢ) was determined according to Long and Bernacchi (2003).

Proteomic analyses

Nodule samples (200 mg fresh weight) were ground in a mortar using liquid nitrogen and re-suspended in 2 ml of cold acetone containing 10% trichloroacetic acid (TCA). After centrifugation at 16 000 g for 3 min at 4 °C, the supernatant was discarded and the pellet was rinsed successively with methanol, acetone, and phenol solutions as previously described by Wang et al. (2003). The pellet was stored at −20 °C or immediately re-suspended in 200 µl of R2D2 rehydration buffer [5 M urea, 2 M thiourea, 2% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, 2% N-decyl-N,N-dimethyl-3-ammonio-1-propanesulphonate, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine, 0.5% IPG buffer (GE Healthcare, Uckfield, UK), pH 4–7] (Mechin et al., 2003). Total soluble protein (TSP) concentration was determined with the method of Bradford (1976) using bovine serum albumin as a standard. Two-dimensional electrophoresis was performed as described by Aranjuelo et al. (2011).

Gels from four independent biological replicates were used, and the analysis of gels was performed as previously described by Aranjuelo et al. (2011). After detection of protein spots using silver staining (Aranjuelo et al., 2011), pictures of the 2-D gels were acquired with a dedicated automated system (MultiPROBE II, PerkinElmer). Gel fragments were subsequently incubated twice for 15 min in a H₂O/CH₃-CN solution to allow the extraction of peptides. Peptide extracts were then dried and dissolved in a starting buffer, made up of 3% CH₃CN and 0.1% HCOOH in water, for chromatographic elution. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and analysed with an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonik, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (Matrix Science, London, UK) search engine as previously described by Desclós et al. (2008). Once the proteins were identified, their presumed biological function was determined according to Bevan et al. (1998).

Metabolic analyses

Gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) was performed on a LEICO Pegasus III with an Agilent 6890N GC system and an Agilent 7893 automatic liquid sampler. The column was an RTX-5 wintrerga-Guard (30 m×0.25mm id + 10 m integrated guard column) (Restek, Evy, France). Leaf, root, and nodule samples (20 mg of powder from freeze-dried material) were ground in a mortar in liquid nitrogen, and then in 2 ml of 80% methanol, to which ribitol (100 µmol l⁻¹) was added as an internal standard. Extracts were transferred to 2 ml Eppendorf tubes, and centrifuged at 10 000 g and 4 °C for 15 min. Supernatants were transferred to fresh tubes and centrifuged again. Several aliquots of each extract (0.1, 3×0.2, and 0.4 ml) were spin-dried under vacuum and dried at 80 °C until analysis. Methoxyamine was dissolved in pyridine at 20 µg ml⁻¹, and 50 µl of this mixture was used to dissolve the dry sample (from the 0.2 ml aliquot, see above). Following vigorous mixing, samples were incubated for 90 min at 30 °C with shaking. A 80 µl aliquot of N-methyl-N-(trimethyl-silyl) trifluoroacetamide (MSTFA) was then added, and the mixture was vortexed, and incubated for 30 min at 37 °C with shaking. The derivatization mix was then incubated for 2 h at room temperature. Before loading into the GC autosampler, a mix of a series of eight alkanes (chain lengths: C₉-C₉) was included. Analyses were performed by injecting 1 µl in splitless mode at 230 °C as injector temperature. The chromatographic separation was performed in helium as a gas carrier at 1 ml min⁻¹ in the constant flow mode and using a temperature ramp ranging from 80 °C to 330 °C between 2 min and 18 min, followed by 6 min at 330 °C. The total run time per injection was 30 min. Ionization was by electron impact at 70 eV, and the MS acquisition rate was 20 spectra s⁻¹ over the m/z range 80–500. Peak identity was established by comparison of the fragmentation pattern with MS available databases (NIST), using a match cut-off criterion of 700/1000, and by retention index (RI) using the alkanes series as retention standards. The integration of peaks was performed using the LEICO Pegasus software. Because automated peak integration was occasionally erroneous, integration was verified manually for each compound in all analyses.

Statistical analyses

Data were examined by one-factor analysis of variance (ANOVA) (Fig. 1). Differences were considered to be significant when P < 0.05. All values shown in the figures and tables are means ±SE (n=4).

Results

Physiological parameters

Leaf photosynthesis was followed during the experiment so as to check whether the water restriction applied did cause
a physiological response. The main parameters are shown in Table 1. After 7 d of water restriction, the water potential of leaves decreased from −14 MPa to −38 MPa and photosynthesis decreased nearly 2.5-fold to 7.6 μmol m⁻² s⁻¹. The decrease was mainly caused by changes in stomatal and internal conductance, which both decreased to the same extent (~2.5 fold). The internal-to-external CO₂ mole fraction ratio (C/C_a) also decreased in droughted plants. The photochemical yield of PSII was also strongly affected by water restriction, indicating that photosynthetic electron sinks were less abundant and promoted non-photochemical quenching of chlorophyll fluorescence (not shown). In the dark, the maximum photochemical yield of PSII was unchanged, indicating that the photosynthetic apparatus per se was not altered by drought. Leaf night respiration (CO₂ evolution in darkness) decreased 1.5-fold. The respiration-to-assimilation (R_i/A) ratio thus changed from 11% (control) to ~20% under drought, demonstrating a clear change in the carbon balance of the leaf. Nodule and root respiration (CO₂ evolution) was affected by water restriction (decreased 2-fold), suggesting that catabolism was down-regulated and/or that CO₂ uptake [phospho(enol)pyruvate carboxylase (PEPc) activity] increased, thereby causing a decrease in the net CO₂ production.

Metabolomic patterns

Targeted metabolomic analyses were carried out on control and droughted plants and led to the relative quantitation of 88 metabolites. Here, the quantitation was normalized to the dry weight (DW) of samples so as to avoid any discrepancy due to changes in relative water content under drought conditions. Using an ANOVA (two factors), 32 metabolites were affected significantly by water restriction (Fig. 1). Although overall the results obtained in this study are the same as those obtained in a previous study (Aranjuelo et al., 2011), there are some differences in asparagine, glutamate, aspartate, and pinitol content. As shown in Supplementary Table S1 available at JXB online, the tendencies were the same, while a larger variability masked the statistical significance in the present data set. The hierarchical clustering yielded two major groups of metabolites: (i) hexose, pentose metabolites, and alanine (lower group in Fig. 1) that decreased under water restriction; and (ii) sucrose, myo-inositol, and organic acids (two upper groups in Fig. 1) that increased under water restriction. Taken as a whole, water restriction seemed to promote sucrose (and raffinose) synthesis and the production of downstream metabolites (osmolytes such as proline), with little effect on sap metabolites involved in nitrogen assimilation, such as asparagine, which is the major chemical species exported by nodules in indeterminate legumes. In fact, amongst export-related nitrogenous compounds, only glutamine appeared to be a significantly affected metabolite (in Fig. 1). Leaf starch content was lower in drought (60.7 μmol g⁻¹ DM) than in control (591.0 μmol g⁻¹ DM) plants.

Nevertheless, carrying out a two-factor ANOVA cloaks disparities between organ response patterns. An organ-specific statistical analysis was carried out in order to examine metabolites that specifically responded to drought in each organ (Table 2). Clearly, the metabolic pattern obtained was very different in leaves, roots, and nodules. In leaves, water restriction caused an increase in sucrose and sucrose-derived compounds and a decrease in glutamine and asparagine. This suggests that the exchange of nitrogenous metabolites between organs was impeded by water restriction. In roots, there was a significant increase in glutamate (and its derivatives pyro-glutamate and proline) and in the organic acids malate and fumarate (and its isomer maleate), indicating an increased metabolic flux through PEPc. This was also the case in nodules. Furthermore, there was a significant increase in 2-oxoglutarate (2OG), which is the precursor of glutamate. Several metabolites related to redox control also accumulated: this was the case for nicotinate (NADH metabolism), gluconate (involved in NADPH production via the pentose phosphate pathway), ascorbate, and threonate (ascorbate metabolism).

Table 1. Photosynthetic and respiratory parameters of alfalfa plants grown under controlled and drought conditions. Photosynthetic CO₂ assimilation under growth conditions (A), stomatal and internal conductance (g_s and g_m, respectively), chloroplastic CO₂ concentration (C_a), intercellular and ambient CO₂ ratio (C_i/C_a), maximal and actual PSII photochemical yield (F_v/F_m and ΔF/ΔF_m, respectively), and leaf, root, and nodule respiration (CO₂ evolution).

| Variable          | Units | Control        | Drought       | P*  |
|-------------------|-------|----------------|---------------|-----|
| A                 | μmol m⁻² s⁻¹ | 18.8 ± 1       | 7.6 ± 0.8    |    |
| g_s               | mol m⁻² s⁻¹  | 0.62 ± 0.028   | 0.25 ± 0.07   |    |
| g_m               | mol m⁻² s⁻¹  | 0.20 ± 0.01    | 0.07 ± 0.01   |    |
| C_a               | μmol mol⁻¹   | 201.62 ± 56.59 | 113.56 ± 18.06 |    |
| C_i/C_a           | dl        | 0.78 ± 0.03    | 0.51 ± 0.03   |    |
| F_v/F_m           | dl        | 0.79 ± 0.04    | 0.78 ± 0.02   |    |
| ΔF/ΔF_m           | dl        | 0.59 ± 0.08    | 0.30 ± 0.06   |    |
| R_leaf            | μmol m⁻² s⁻¹ | −2.2 ± 0.4     | −1.5 ± 0.5    |    |
| R_root            | nmol g DW⁻¹ s⁻¹ | −49.9 ± 12.3  | −22.6 ± 1.1   |    |
| R_without         | nmol g DW⁻¹ s⁻¹ | −480 ± 3      | −228 ± 31    |    |

Asterisks indicate a significant difference between conditions (P < 0.05).

dl, dimensionless.

Nitrogenase activity

Nₐₖₑ activity of nodules (assayed with acetylene) is shown in Fig. 2. Water restriction had a clear effect on N₂ fixation, which decreased nearly 2-fold (Fig. 2A). However, when expressed relative to photosynthesis (Nₐₖₑ activity assayed with acetylene divided by the photosynthetic rate: Nₐₖₑ/A), nitrogenase activity was rather similar under control and water-restricted conditions (no significant difference; Fig. 2B). The respiratory cost of N₂ fixation (CO₂ evolution rate of nodules divided by Nₐₖₑ activity) was perfectly equal under both conditions (Fig. 2C). In other words, this indicates that under water-restricted conditions, the catabolic activity of nodules...
decreased to match the metabolic imperatives (ATP and C-skeletons) of N₂ fixation.

Nitrogenous compounds

The content of TSP is shown in Fig. 3A. Water restriction caused only modest changes in TSP content, with a small but significant increase in leaves and a significant decrease (protein degradation) in nodules. The ratio of nitrogenous metabolites to their deamminated/deamidated counterparts is shown in Fig. 3B. Most visible (outside the 0.5–1.5 area) changes were an increase in the glutamate/glutamine ratio in roots and nodules, a large increase in the 2OG/glutamate ratio in roots, and an increase of the pyruvate/alanine ratio in leaves. Additionally, in nodules, there was a decrease in pyruvate/alanine and aspartate/asparagine ratios (arrows). The apparent mass action ratio (without ATP) of asparagine synthetase, [asparagine][glutamate]/[aspartate][glutamine], was therefore almost unchanged in nodules under water restriction (drought-to-control ratio ~0.8). Therefore, metabolite ratios indicate that presumably glutamine synthesis was slowed down (glutamate/glutamine ratio higher) while asparagine synthesis was maintained at a rather similar rate. In nodules, urea and polyamines (putrescine) decreased under water restriction (not shown), suggesting that nodule metabolism favoured asparagine (which remained constant) as a transport metabolite.

Nodule proteome

Nodule proteins were extracted and separated on a gel, and most abundant proteins were identified by mass spectrometry. Amongst proteins that changed significantly upon water restriction (P < 0.05), 11 were identified (Table 3; Fig. 4). Since the protein content changed under water restriction (Fig. 3A), two types of quantitation were done: the relative content was normalized to the sample protein content (usual quantitation procedure) or to the DW, and statistics were computed on the basis of both. The abundance of the 11 proteins changed with respect to both DW and protein, while seven changed on a DW basis and only one changed on a protein basis. Several enzymes involved in carbon primary metabolism varied significantly: the abundance of the NADP-dependent malic enzyme decreased, while that of
Table 2. Metabolites that change significantly (P < 0.05) under drought in each organ. The statistical analysis was carried out with the different organs considered independently.

| Metabolism                        | Leaf | Root | Nodule |
|-----------------------------------|------|------|--------|
| Glutamate metabolism              |      |      |        |
| 2-OH-gluatrate                    | ↑    |      | ↑      |
| 2-Oxo-gluatrate                   |      | ↑    |        |
| Pyro-gluatrate                    | ↑    | ↑    | ↓      |
| Glutamine                         | ↓    |      | ↑      |
| Glutamat                          | ↑    | ↑    |        |
| Proline                           |      | ↑    | ↑      |
| Organic acid metabolism           |      |      |        |
| Glycemic acid                     | ↓    | ↑    | ↑      |
| Fumaric acid                      | ↑    |      | ↑      |
| Maleic acid                       | ↑    | ↑    |        |
| Malic acid                        | ↑    | ↑    |        |
| Sugar metabolism                  |      |      |        |
| Galactinol                        | ↑    |      | ↑      |
| Raffinose                         | ↑    | ↑    | ↑      |
| Sucrose                           | ↑    | ↑    | ↑      |
| Pentose metabolism                |      |      |        |
| Glucicic acid                     | ↑    |      | ↑      |
| Xylose 1                          | ↓    |      |        |
| Xylose 2                          | ↓    |      |        |
| N metabolism and amino acids      |      |      |        |
| Asparagine                        | ↓    |      |        |
| β-Alanine                         | ↑    |      |        |
| Alanoin                           | ↓    |      |        |
| Glycine                           | ↑    | ↑    |        |
| Nicotinic acid                    | ↑    |      |        |
| O-Acetylsersine                   | ↓    |      |        |
| Putrescine                        |      | ↓    |        |
| Tryptophan                        | ↑    |      |        |
| Glucose-derived acids             |      |      |        |
| Ascorbic acid                     | ↑    | ↑    | ↑      |
| Threonic acid                     | ↑    | ↑    |        |
| Lipid metabolism                  | ↑    |      |        |
| Glycerol                          | ↑    |      |        |
| Glycerol 3P                       | ↓    |      |        |
| Linoleic acid                     |      |      | ↑      |

Xylose 1 and 2 refer to two distinct xylose derivatives in metabolomics GC-TOF-MS analyses. Upward- and downward-pointing arrows indicate an increase and a decrease in the metabolite content, respectively.

PEPc increased. The abundance of methionine synthase was found to decrease, as was that of monodehydroascorbate reductase. Two heat-shock proteins (HSP70 and HSP70-like) were found to be less abundant.

Discussion

Deleterious effects of water availability on nodule performance have been previously described in alfalfa (Díaz del Castillo and Layzell, 1995; Schulze, 2004; Naya et al., 2007; Larraínzar et al., 2009; Aranjuelo et al., 2011). In most of these studies, however, nodule activity is examined regardless of the implication of other plant organs (roots and leaves). Here, nodule metabolism was characterized, but the consequences of water restriction on photosynthesis and primary metabolism of roots and leaves were also investigated.

Nitrogen and carbon metabolism in nodules

The present data show that drought impeded N\textsubscript{2} fixation by its deleterious effect on nodules (Fig. 2), and this was accompanied by changes in primary carbon and nitrogen metabolism. Metabolomic analyses indicate that the asparagine content remained more or less constant (insignificant decrease) while glutamine synthesis decreased, probably reflecting the decrease in glutamine synthetase due to the lower NH\textsubscript{3} input by N\textsubscript{fix}. Probably, therefore, there was homeostasis of asparagine synthesis, as indicated by the more or less unchanged mass action ratio of the reaction (Fig. 3). [It is nevertheless recognized that the decrease in the aspartate/asparagine ratio (Fig. 3) may have also arisen due to the consumption of aspartate so as to produce, for example, β-alanine (significant metabolite in Table 2) via aspartate decarboxylation.] In addition, there was a lower content of urea and putrescine, suggesting that the proportion of asparagine (usually ~60% of transported N) in exported nitrogenous compounds increased. Thus, asparagine appeared to be a preferred metabolic product (Fischinger et al., 2010). There was also a small but visible decrease in soluble proteins, suggesting that some proteolysis and protein catabolism might have occurred (Fig. 3A).

Nevertheless, there was a clear decrease in the rate of CO\textsubscript{2} evolution by nodules, suggesting that catabolism was down-regulated under water restriction. The imbalance between the lower provision of C skeletons by the Krebs cycle and demand for glutamine and glutamate to sustain asparagine synthesis was probably compensated for by (i) protein remobilization (Fig. 3A) and (ii) the involvement of the anaplerotic pathway. In fact, it was found that malate and maleate accumulated and, furthermore, the relative abundance of PEPc increased (Table 3). Accordingly, the abundance of the malic enzyme decreased. The crucial role of PEPc in metabolic fluxes to sustain glutamate and glutamine synthesis has been demonstrated (Lawrie and Wheeler, 1975). It is shown here that under water restriction, this pathway is up-regulated (Fig. 4, bottom).

The decrease in the respiration rate (CO\textsubscript{2} evolution) in nodules may have two causes: first, an increase in PEPc fixation (enhanced CO\textsubscript{2} fixation led to a lower apparent CO\textsubscript{2} evolution rate); and secondly, the lower activity of glycolysis and the Krebs cycle. Both causes are likely. PEPc activity was indeed greater (see above). As observed by Fischinger et al. (2010), nodule N\textsubscript{2} fixation is linked to nodule CO\textsubscript{2} fixation by PEPc. This agrees with the present study, in which drought conditions increased PEPc abundance, presumably promoting N\textsubscript{2} fixation. Nodule metabolism is believed to be limited by carbohydrate availability (González et al., 1998; Arrese-Igor et al., 1999). However, although drought caused the rate of photosynthesis to decrease (Table 1), sucrose tended to be more abundant in nodules. In contrast, there was a depletion
C and N metabolism in droughted alfalfa

(Fig. 1, though insignificant in roots) in hexoses, which may have caused a lower glycolytic input. The change in the sucrose/hexose ratio possibly arose due to an altered sucrose synthase activity. The preferential partitioning of triose phosphates to reduced molecules with osmoregulant capacity such as glycerol, which was found to accumulate (Table 2), may also explain the lower commitment of carbon to glycolysis (pyruvate production) and respiration. Similarly, pyruvate was more committed to alanine synthesis than to the Krebs cycle (Fig. 3). Such an increase in sugar alcohols (glycerol, galactinol, and myo-inositol), together with the increase in amino acid (proline), sugars (sucrose and raffinose), and organic acids (fumaric acid and malate) may be linked to osmoregulation and homeostasis of the nodule and leaf.

Fig. 2. Nitrogenase ($N_{\text{ase}}$, nmol N$_2$ g DW$^{-1}$ s$^{-1}$) activity in nodules (A), $N_{\text{ase}}$ photosynthetic yield $N_{\text{ase}}$/A (B; mol N$_2$ fixed g nodule DW$^{-1}$ s$^{-1}$ per mol CO$_2$ fixed g leaf DW$^{-1}$ s$^{-1}$, i.e., mol N$_2$ mol$^{-1}$ CO$_2$) and respiratory cost of $N_{\text{ase}}$ activity $R_{\text{nodule}}$/$N_{\text{ase}}$ (C; mol CO$_2$ mol$^{-1}$ NH$_3$). $R_{\text{nodule}}$ used for calculations is from Table 1. $N_{\text{ase}}$ activity was measured using the acetylene assay. In A, the difference between control and drought is significant (*P < 0.05). In B and C, there is no significant difference between treatments.

Fig. 3. Total soluble proteins (A) and metabolic ratios (B) in leaves, roots, and nodules under control and drought conditions. In A, significant differences under drought are indicated with an asterisk ($P < 0.05$). In B, the drought-to-control quotient of the metabolite ratios is indicated on the x-axis. To facilitate reading, the steady line (no changes) is indicated with a broken line. Most visible changes in nodules are indicated with arrows. Source data are from metabolomic analyses (see also Fig. 1).
Concerted metabolic changes in other organs

The effects of drought on metabolic pathways discussed below are represented in Fig. 5. As a consequence of the inhibition of photosynthesis under water restriction (Table 1), leaves 'compensated' for lower CO₂ fixation rates by a lower export activity and/or a more pronounced remobilization of starch (Lee et al., 2008). Both hypotheses are likely, since (i) the abundance of proteins with respect to dry weight was found to increase (Fig. 3) and starch was less abundant; and (ii) the content in raffinose increased significantly.

Leaf nitrogenous metabolites were also affected by water restriction, probably due to the inhibition of N₂ fixation and/or a more pronounced renobilization of starch (Lee et al., 2008). Both hypotheses are likely, since (i) the abundance of proteins with respect to dry weight was found to increase (Fig. 3) and starch was less abundant; and (ii) the content in raffinose increased significantly.

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Proteins that vary significantly (P < 0.05) under drought in alfalfa nodules. The spot number represents the number assigned to the spot on the gel (see Supplementary Material at JXB online for a typical picture of the gel). The ratios indicated here refer to the drought-to-control ratio of the protein abundance estimated using the spot volume normalized to dry weight (DW) or total soluble proteins (Prot). An asterisk indicates proteins for which the identification was performed using 3–4 accurate fragments (accurate mass) blasted against the Medicago database. ‘Unknown’ indicates that no satisfactory match was found in the protein database or that several equally probable, unrelated proteins were found. Spots numbered above 500 correspond to poorly abundant proteins (small or very small spots even under control conditions).

| Spot no. | Name                          | Trend    | Ratio (DW) | Ratio (protein) | Score (%) |
|---------|-------------------------------|----------|------------|-----------------|-----------|
| Proteins significant on both DW and protein bases |
| 11      | Alpha 1,4-glucan protein synthase | Increase | 3.77       | 2.80            | 45*       |
| 12      | Disulphide isomerase           | Decrease | 0.27       | 0.20            | 50*       |
| 14      | HSP70-like                     | Decrease | 0.28       | 0.21            | 25*       |
| 15      | PPR protein                    | Decrease | 0.28       | 0.21            | 25*       |
| 19      | Unknown                        | Decrease | 0.32       | 0.24            | –         |
| 21      | MDHA reductase                 | Decrease | 0.34       | 0.25            | 44        |
| 28      | Lipoxigenase                   | Increase | 2.48       | 1.84            | 62        |
| 29      | NADP-ME                        | Decrease | 0.40       | 0.30            | 56        |
| 32      | HSP70                          | Decrease | 0.49       | 0.36            | 425       |
| 556     | Unknown                        | Decrease | 0.53       | 0.39            | –         |
| Proteins significant on a DW basis only |
| 33      | Methionine synthase            | Decrease | 0.50       | 0.37            | 158       |
| 34      | Unknown                        | Decrease | 0.56       | 0.41            | –         |
| 544     | Unknown                        | Decrease | 0.52       | 0.39            | –         |
| 562     | Unknown                        | Decrease | 0.53       | 0.40            | –         |
| 742     | Unknown                        | Decrease | 0.64       | 0.48            | –         |
| 787     | Unknown                        | Decrease | 0.66       | 0.49            | –         |
| Proteins significant on a protein basis only |
| 35      | PEP-carboxylase                | Increase | 1.75       | 1.30            | 809       |
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[2OG], decreased only slightly (drought-to-control quotient of ~0.8) in drought.

**N$_2$ fixation regulation and yield**

Under water-limited conditions, several reasons may explain why N$_{ase}$ activity decreases (see the Introduction). The involvement of feedback effects by nitrogenous compounds has been proposed (Hartwig et al., 1994; Serraj et al., 1998b, 2001; Schulze, 2003; King and Purcell, 2005; Aranjuelo et al., 2011). According to these studies, the lower above-ground N demand leads to the accumulation of N$_2$ fixation products in nodules, with the resulting inhibition of N$_{ase}$ activity. Ureides and free amino acids are candidate metabolites involved in such an inhibition (King and Purcell, 2005; Larraínzar et al., 2009). Here, no significant increase in ureides was observed (although allantoin increased), but a significant accumulation of glycine and β-alanine was seen in nodules. In leaves, the contents of glutamine, asparagine, and putrescine were much lower under water restriction (Table 2). Such a change in the leaf to nodule N balance strongly suggests that a lower above-ground nitrogen demand/consumption and/or a lower export of nitrogenous compounds have contributed to their build-up in nodules (Fig. 5).

Oxidative stress has also been shown to impede nodule N$_{ase}$ activity (Gogorcena et al., 1995; Iturbe-Ormaetxe et al., 2001; Becana et al., 2010). Several studies demonstrated that the quenching of ROS can be impeded by drought, thereby causing oxidative damage (Gogorcena et al., 1997; Iturbe-Ormaetxe et al., 2001; Naya et al., 2007). Here, it was found that both ascorbate and threonate accumulated in nodules, suggesting that an oxidative stress occurred. Surprisingly,
however, drought altered the content of monodehydroascorbate reductase (Table 3). A lipoxygenase was found here to be more abundant and was perhaps associated with the oxidative response of lipid metabolism or signal transduction (Egert and Tevini, 2002).

Although the main cause of the decrease in Nase activity remains somewhat undefined (feedback inhibition versus oxidative stress), it should be noted that Nase activity nearly matched leaf photosynthetic rate and perfectly matched nodule respiration (no difference compared with control conditions in Fig. 2B and C). At the cellular level, the respiratory cost of Nase activity (CO₂ evolved by nodules per unit of Nase activity in Fig. 2) is mainly associated with the demand for ATP (cofactor of the reaction) and C skeletons to assimilate evolved ammonia into glutamate and glutamine. Theoretical estimates of the respiratory cost of Nase-catalysed NH₃ production (based on ATP and C skeleton requirements) have been found to be near 2.4 mol CO₂ mol⁻¹ NH₃ (for a specific discussion and a review of experimental results, see Atkins et al., 1978), and here a CO₂ evolved-to-Nase activity ratio of 2.7 was found under both control and water-limited conditions. It should be noted that in Fig. 2, total nodule respiration was used to calculate the respiratory cost and thus maintenance respiration seemed to be a modest component of whole nodule respiration (>0.3 units, i.e. 11% only of the respiratory cost). In other words, carbon primary metabolism in nodules

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**Fig. 5.** Most visible changes in carbon primary metabolism of leaves, roots, and nodules of *Medicago sativa* under drought. This figure is a tentative summary of the present results. Thick and broken arrows represent enhanced and repressed pathways, respectively. GABA, γ-aminobutyrate; G3P, 3-phosphoglyceraldehyde; Mal, malate; PEP, phosphoenolpyruvate; PEPc, PEP carboxylase; OA, organic acids; OAA, oxaloacetate; SSA, succinic semialdehyde; TCAP, tricarboxylic acid pathway; 2OG, 2-oxoglutarate.
(catabolism) was mostly (~90%) associated with sustaining N\textsubscript{ass} activity in both control and water-restricted conditions.

**Conclusions and perspectives**

N\textsubscript{2} fixation by nodules under water-limited conditions appears to result from a compromise between metabolic imperatives (to sustain the nitrogenase-catalysed reaction) and the tendency of altered plant-scale metabolic exchanges (sucrose, glutamine, asparagine, and ureides) to exert a down-regulation. Furthermore, the increase in key intermediates such as 2-OG and pyruvate (precursor of amino acids) reflects both the synthesis of amino acids with osmotic capacity such as proline and the production of organic acids. It is further possible that a lower leaf N demand (as reflected by the depletion in leaf amino acids) and the increase in nodule amino acid content in droughted plants reflects the feedback inhibition exerted by nitrogenous compounds on N\textsubscript{2} fixation activity within nodules.

It is nevertheless recognized that the present results do not distinguish between bacteroid and plant cell metabolites since nodules were analysed as a whole. Thus alterations in the exchange between nodule plant cells and *Sinorhizobium* cells may also contribute to the observed metabolic patterns. Furthermore, metabolic fluxes could certainly be examined more carefully using \textsuperscript{13}C labelling and \textsuperscript{13}C tracing by nuclear magnetic resonance (NMR) fluxomic techniques, and this approach to reveal a novel role of Brassica napus drought 22 kD/\textsubscript{24}O protein–dye binding. Analytical Biochemistry 72, 248–254.

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**Supplementary data**

Supplementary data are available at *JXB* online. Table S1. Metabolomic analysis of leaf, root, and nodules under drought and control conditions.
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