Journal : EXBOTJ

Article Doi : 10.1093/jxb/ers367

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

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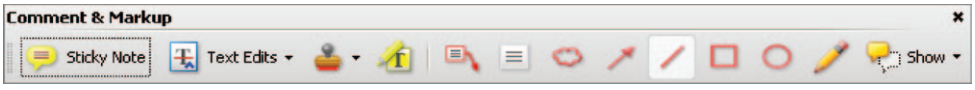

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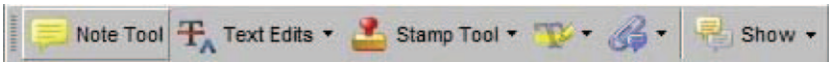

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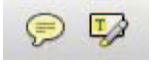

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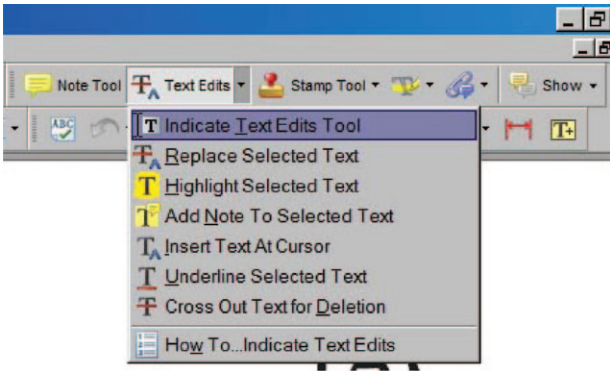

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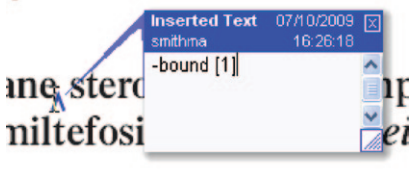

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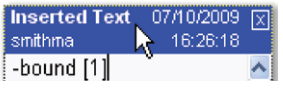

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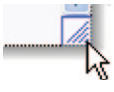

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RESEARCH PAPER

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

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Received 22 November 2012; Revised 22 November 2012; Accepted 4 December 2012

Abstract

Although the mechanisms of nodule N_2 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 N_2-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 N_2 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 N_2 fixation efficiency in nodules of legumes (Serraj and Sinclair, 1996; Serraj *et al*., 1997; González *et al*., 1998; Larrainzar *et al*., 2007; Naya *et al*., 2007; Larrainzar *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 N_2 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 (N_{ase}; the enzyme responsible for N_2 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_{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_{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_{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_{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 metabolites, such as hexoses, are believed simply to accumulate (Muller *et al.*, 2011, and references therein). Further, minor sugars (e.g. trehalose and mannitol), amino acids (e.g. proline), and organic acids (e.g. malate, fumarate, and isocitrate) also appear to accumulate under water restriction. Although recent publications include a more detailed metabolomic characterization (Larrainzar *et al.*, 2009; Aranjuelo *et al.*, 2011; Kang *et al.*, 2011), metabolite patterns and their coordinated changes between plant compartments are unclear. Furthermore, within nodules, only N_{ase} activity is usually determined, with no further information about the effect of drought on other nodule proteins and enzymatic activities. Thus, the influence of drought on nodule metabolic pathways and associated changes in metabolite exchange between nodules and other plant organs (which may also cause some 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_{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 (Avice *et al.*, 1996; Volenec *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 (*Magali* 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_{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_{ase} activity was measured in detached nodules using the acetylene (C_2H_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_{ase} activity by decreasing the resistance to O_2 diffusion into the infected zone of the nodules, thereby inhibiting N_{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_2H_2 was added. The flask was incubated at room temperature for 10 min, before the N_{ase} decrease

induced by exposure to excess acetylene. Afterwards, eight samples of 5 ml were withdrawn from the flask and the ethylene content in the samples was quantified using a Fractovap 4200 (Carbo Erba Strumentazione, Milan, Italy) gas chromatograph equipped with a hydrogen flame ionization detector and a Poropak R30/100 column (2 m×1/8 id). Analyses were carried out at 90 °C (45 °°C detector and injector) with He as a carrier gas at a flow rate of 25×ml min^{-1}. This protocol, which gives acetylene reduction activity, is believed to provide a good estimate of relative N_{ase} activity (Streeter, 2003; King and Purcell, 2005).

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} CO_2, 21% O_2). 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 (g_m) was determined according to Pons *et al*. (2009). The intracellular CO_2 mole fraction (C_c) was determined according to Long and Bernacchi (2003). Plants were dark-adapted for 50 min before dark respiration (R_D) measurements (Nogués *et al*., 2004).

Fluorescence parameters were measured with a fluorescence chamber (LFC 6400–40; LI-COR) coupled to the Li-Cor 6400. Light-adapted variables included steady-state fluorescence yield F, maximal fluorescence F'_m, variable fluorescence F'_v, and the quantum yield of photosystem II photochemistry $\Phi_{PSII}=(F'_m-F)/F'_m$. Leaves were then dark-adapted for 20 min and F_o (minimum fluorescence), F_m (maximum fluorescence), F_v (variable fluorescence (F_m–F_o), and F_v/F_m [maximum quantum yield of PSII photochemistry, $(F_m-F_o)/F_m$] were measured.

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 rehydratation buffer [5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-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, Saclay, France), 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 the ProXPRESS 2D proteomic Imaging System and analysed using Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). The molecular mass (M_r) and isoelectric point (pI) were calculated using Progenesis SameSpots software (Nonlinear Dynamics) calibrated with commercial molecular mass standards (precision protein standards pre-stained, Bio-Rad) run in a separate marker lane on the 2-D gel.

Proteins were identified by ESI-LC MS/MS (electrospray ionization-liquid chromatograpy tandem mass spectrometry). Excised spots were washed several times with water and dried for a few minutes. Trypsin digestion was performed overnight with a dedicated automated system (MultiPROBE II, PerkinElmer). Gel fragments were subsequently incubated twice for 15 min in a H_2O/CH_3CN solution to allow the extraction of peptides. Peptide extracts were then dried and dissolved in a starting buffer, made up of 3% CH_3CN 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 Daltonic, 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 Desclos *et al*. (2008). Once the proteins were identified, their presumed biological function was determined according to Bevan *et al*. (1998).

Metabolomic analyses

Gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) was performed on a LECO Pegasus III with an Agilent 6890N GC system and an Agilent 7683 automatic liquid sampler. The column was an RTX-5 w/integra-Guard (30 m×0.25mm id + 10 m integrated guard column) (Restek, Evry, 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 stored at –80 °C until analysis. Methoxyamine was dissolved in pyridine at 20 mg 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: C_{10}–C_{36}) 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 alkane series as retention standards. The integration of peaks was performed using the LECO 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 CO_2 mole fraction ratio (C_i/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 (CO_2 evolution in darkness) decreased 1.5-fold. The respiration-to-assimilation (R_{leaf}/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 (CO_2 evolution) was affected by water restriction (decreased 2-fold), suggesting that catabolism was down-regulated and/or that CO_2 uptake [phospho*enol*pyruvate carboxylase (PEPc) activity] increased, thereby causing a decrease in the net CO_2 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 dowstream 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 CO_2 assimilation under growth conditions (A), stomatal and internal conductance (g_s and g_m, respectively), chloroplastic CO_2 concentration (C_c), intercellular and ambient CO_2 ratio (C_i/C_a), maximal and actual PSII photochemical yield (F_v/F_m and $\Delta F/F_m'$, respectively), and leaf, root, and nodule respiration (CO_2 evolution).

Variable	Units	Control	Drought	
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 ± 0.09	0.07 ± 0.01	*
C_c	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	*
$\Delta F/F_m'$	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_{nodule}	nmol g DW^{-1} s^{-1}	–480 ± 3	–228 ± 31	*

Asterisks indicate a significant difference between conditions ($P < 0.05$).
dl, dimensionless.

Nitrogenase activity

N_{ase} activity of nodules (assayed with acetylene) in shown in Fig. 2. Water restriction had a clear effect on N_2 fixation, which decreased nearly 2-fold (Fig. 2A). However, when expressed relative to photosynthesis (N_{ase} activity assayed with acetylene divided by the photosynthetic rate: N_{ase}/A), nitrogenase activity was rather similar under control and water-restricted conditions (no significant difference; Fig. 2B). The respiratory cost of N_2 fixation (CO_2 evolution rate of nodules divided by N_{ase} activity) was perfectly equal under both conditions (Fig. 2C). In other words, this indicates that under water-restricted conditions, the catabolic activity of nodules

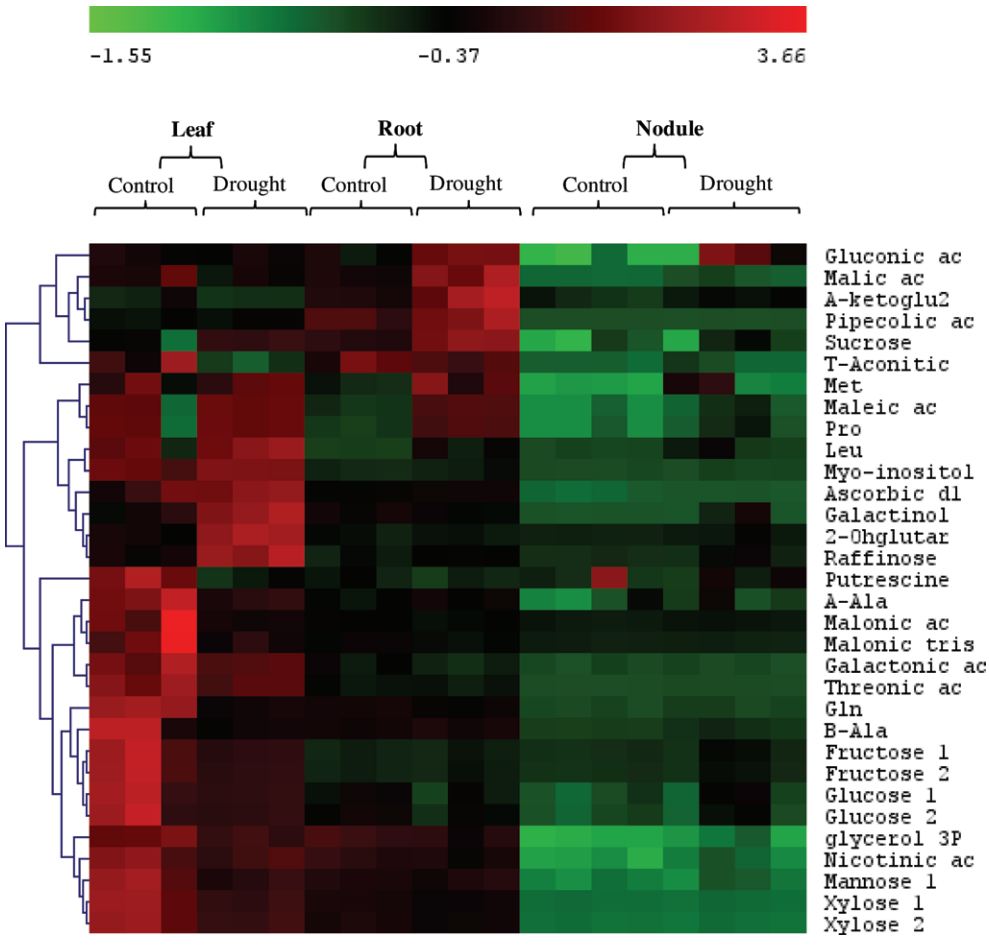

Fig. 1. Metabolomic analysis of leaf, root, and nodules under drought and control conditions. The figure shows only metabolites that vary significantly ($P < 0.05$) under drought conditions using a two-factor ANOVA [treatment (drought versus control) and nature of the organ (leaf, root, nodule), resulting in six groups]. The list of metabolites that vary significantly in each organ, using organ-specific statistics is shown in Table 2.

decreased to match the metabolic imperatives (ATP and C-skleletons) of N_2 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 deaminated/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 rectriction (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.

	Leaf	Root	Nodule
Glutamate metabolism			
2-OH-glutarate	↑		
2-Oxo-glutarate			↑
Pyro-glutarate		↑	
Glutamine	↓		
Glutamat		↑	
Proline		↑	↑
Organic acid metabolism			
Glyceric acid		↓	
Fumaric acid		↑	
Maleic acid		↑	↑
Malic acid		↑	↑
Sugar metabolism			
Galactinol	↑		
Raffinose	↑		
Sucrose	↑	↑	
Pentose metabolism			
Gluconic acid		↑	
Xylose 1		↓	
Xylose 2		↓	
N metabolism and amino acids			
Asparagine	↓		
β-Alanine			↑
Allantoin	↓		
Glycine		↑	↑
Nicotinic acid			↑
o-Acetylserine		↓	
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 (Diaz del Castillo and Layzell, 1995; Schulze, 2004; Naya *et al.*, 2007; Larrainzar *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_2 fixation by its deleterious effect in 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_3 input by N_{ase}. 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_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_2 evolution) in nodules may have two causes: first, an increase in PEPc fixation (enhanced CO_2 fixation led to a lower apparent CO_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_2 fixation is linked to nodule CO_2 fixation by PEPc. This agrees with the present study, in which drought conditions increased PEPc abundance, presumably promoting N_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

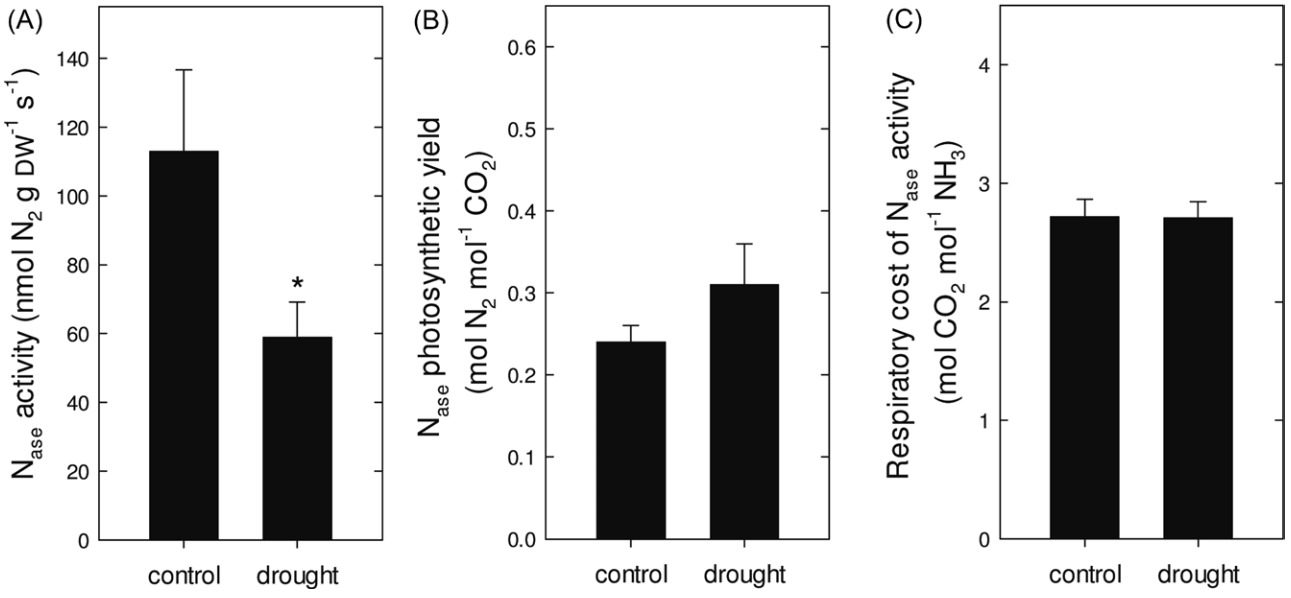

Fig. 2. Nitrogenase (N_{ase}, nmol N_2 g DW^{-1} s^{-1}) activity in nodules (A), N_{ase} photosynthetic yield N_{ase}/A (B; mol N_2 fixed g nodule DW^{-1} s^{-1} per mol CO_2 fixed g leaf DW^{-1} s^{-1}, i.e. mol N_2 mol^{-1} CO_2) and respiratory cost of N_{ase} activity R_{nodule}/N_{ase} (C; mol CO_2 mol^{-1} NH_3). R_{nodule} used for calculations is from Table 1. N_{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.

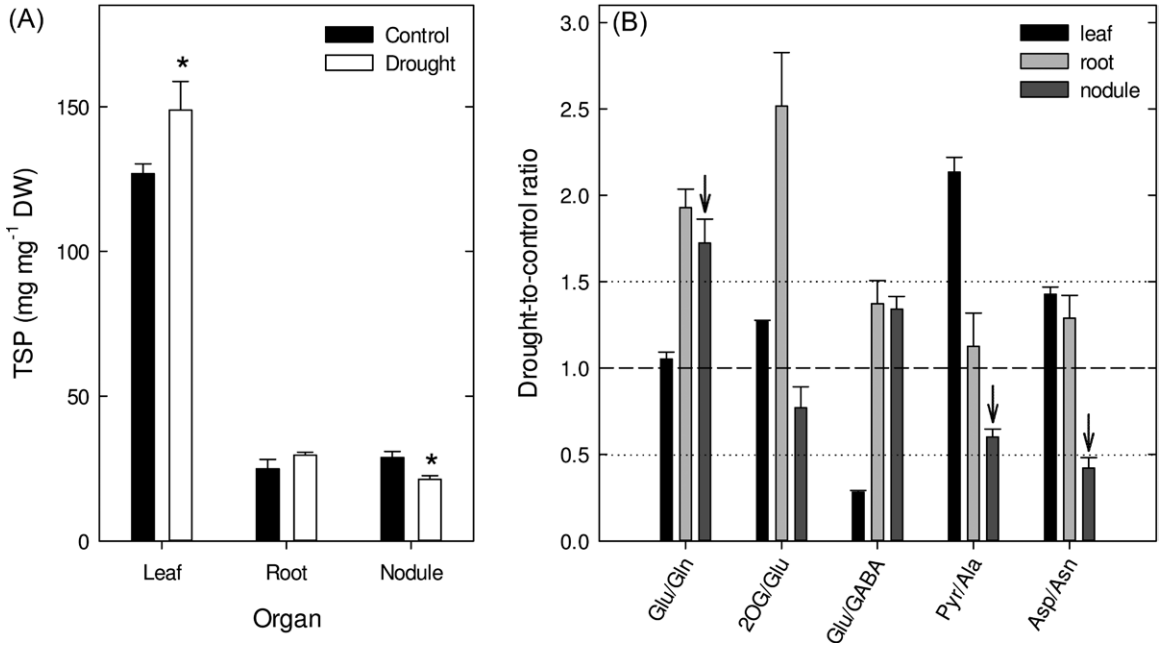

Fig. 3. Total soluble proteins (A) and metabolic ratios (B) in leaves, roots, and nodules under control and drought conditions. In A, significant differences under drought are indicated with an asterisk ($P < 0.05$). In B, the drought-to-control quotient of the metabolite ratios is indicated on the *x*-axis. 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).

(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

Table 3. 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	Lipoxygenase	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

water potential (Streeter *et al*., 2003; Valliyodan and Nguyen, 2006; Zhang *et al*., 2011).

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 photosynthetis under water restriction (Table 1), leaves 'compensated' for lower CO_2 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 on N_{ase} activity: leaf glutamine, asparagine, allantoin, and putrescine, which derive partly from nodule N_2 fixation, decreased. The depletion of glutamine in leaves might also stem from the synthesis of proline, which originates from glutamate and is probably associated with the stabilization of protein structure (Schobert and Tschesche, 1978) and osmoregulation (Irigoyen *et al*., 1992; Larrainzar *et al*., 2009; Joshi *et al*., 2010; Kang *et al*., 2011). The present study also suggests that glutamate metabolism itself was altered in leaves, with more glutamine, and 2-hydroxyglutarate and γ-aminobutyric acid (GABA); for the latter metabolite, this is insignificant. Further, there was a clear enhancement of the GABA pool compared with glutamate (lower glutamate/GABA ratio, Fig. 3) and, accordingly, the pyruvate/alanine ratio decreased (GABA is deaminated by pyruvate to yield alanine+succinic semialdehyde). In other words, the GABA shunt was clearly stimulated in leaves. This agrees with the well-recognized induction of GABA synthesis under stressful conditions (Serraj *et al*., 1998*a*; Bouché and Fromm, 2004; Shelp *et al*., 2009; Sulieman, 2011). However, there was no visible enhancement of the GABA shunt in other organs under the present experimental conditions.

As observed in nodules, in roots there was probably a lower respiratory input (Table 1) of C skeletons, which was compensated for by the anaplerotic pathway (PEPc fixation), with a significant increase in malate and fumarate (Table 2). Roots have been described to be the main storage organ in alfalfa (Volenec *et al*., 1996), in which amino acids and soluble proteins represent the largest soluble nitrogenous fractions (Hendershot and Volenec, 1993). In contrast to leaves, in which the amino acid content decreased under drought conditions, the build-up of glutamate, proline, pyro-glutamate, glycine, and tryptophan suggests that roots represented a preferential N sink compared with leaves (Fig. 5, dashed-dotted arrows). Interestingly, there was a clear change in deaminated/deamidated metabolites compared with their counterparts (the glutamate/glutamine and 2OG/glutamate ratios increased; Fig. 3) but, as a result, the apparent mass action ratio of glutamate synthase, $[glutamate]^2/[glutamine]$

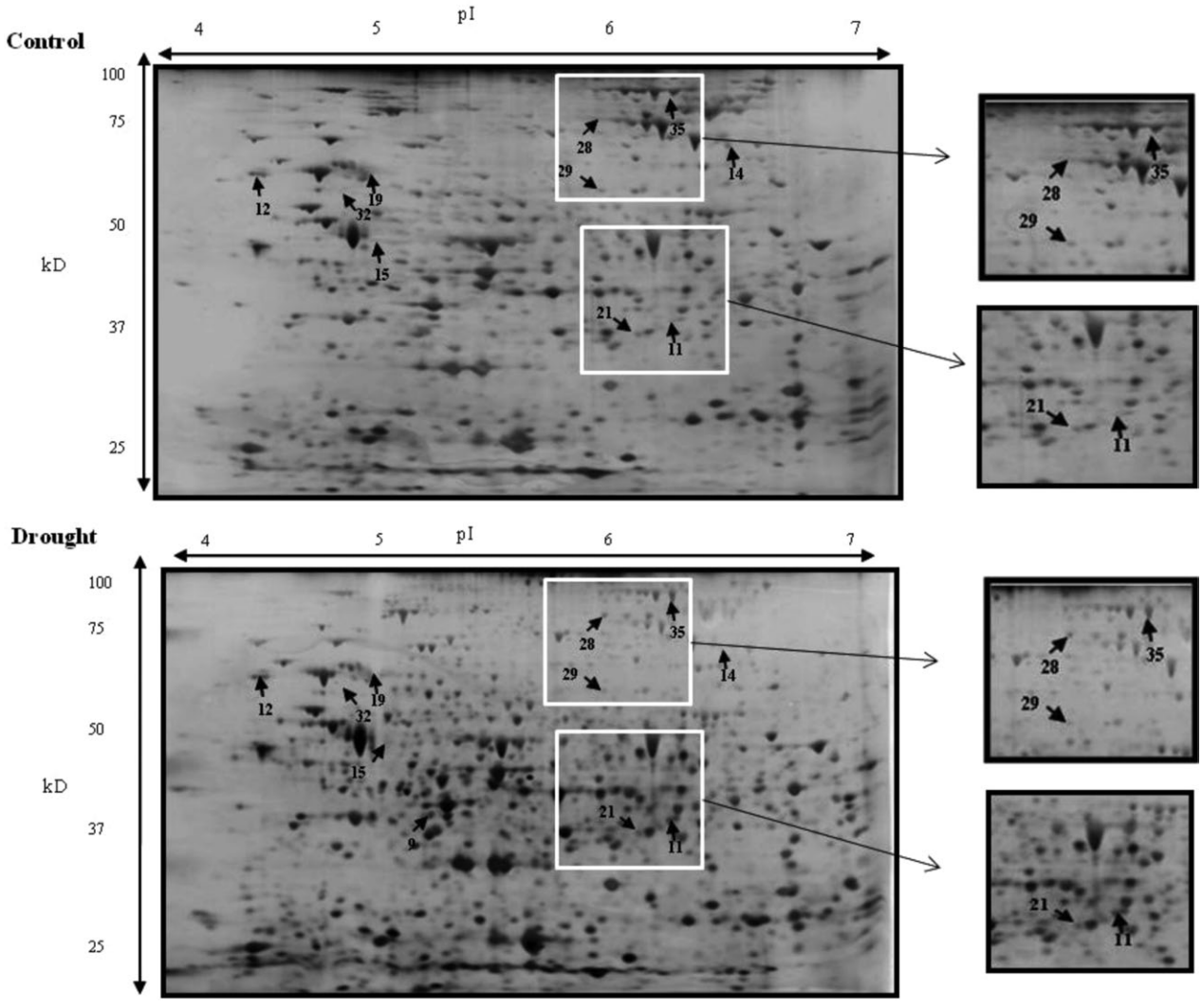

Fig. 4. Silver-stained two-dimensional gel electrophoresis of proteins extracted from alfalfa (*Medivago sativa* L.) nodules exposed to control versus drought conditions. In the first dimension, 125 µg of total protein was loaded on a 18 cm IEF strip with a linear gradient of pH 4–7. The second dimension was conducted in 10% polyacrylamide (w/v) gels (20 × 20 cm) (for details see the Materials and methods). The gel image analyses was conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 17 proteins (marked by arrows) with significantly different expression depending on the water treatment.

[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.*, 1998*b*, 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; Larrainzar *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,

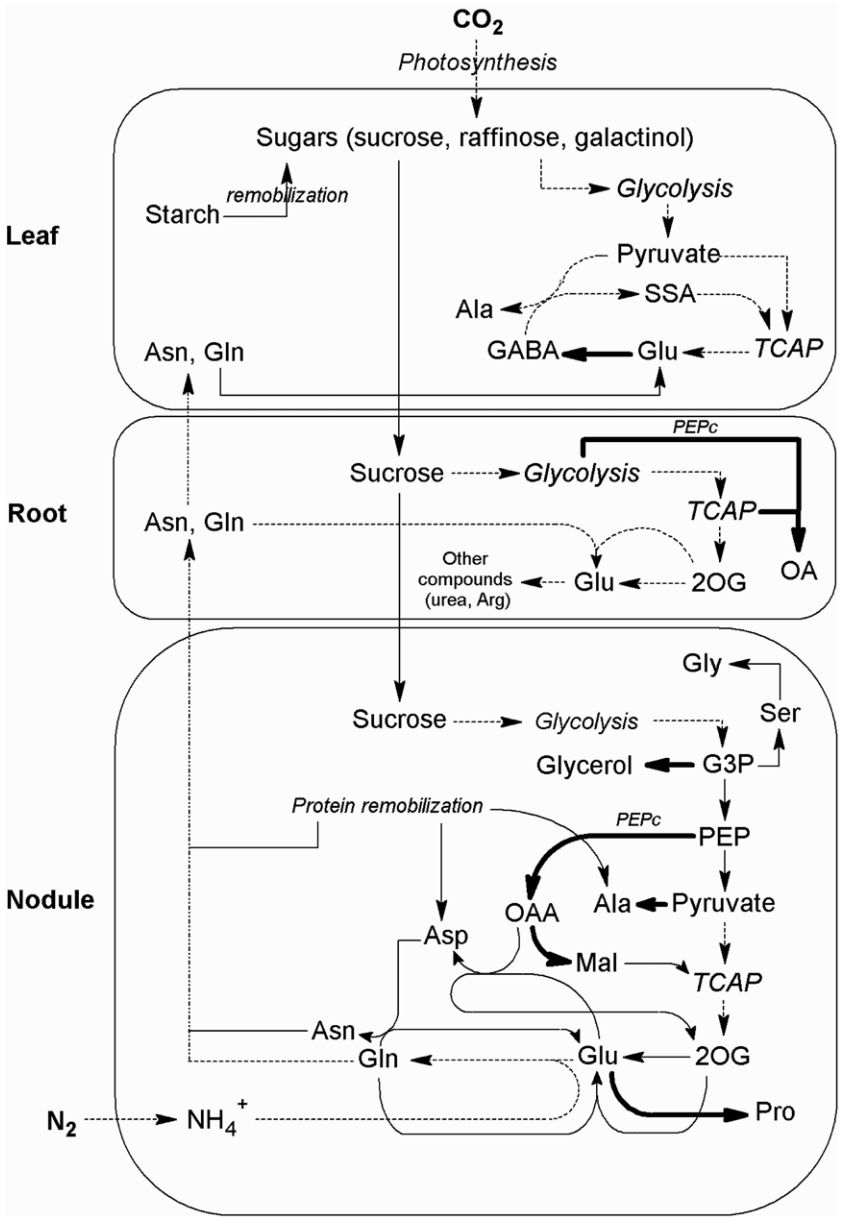

Fig. 5. Most visible changes in carbon primary metabolism of leaves, roots, and nodules of *Medicago sativa* under drought. This figure is a tentative summary of the present results. Thick and broken arrows represent enhanced and repressed pathways, respectively. GABA, γ-aminobutyrate; G3P, 3-phosphoglyceraldehyde; Mal, malate; PEP, phospho*enol*pyruvate; PEPc, PEP carboxylase; OA, organic acids; OAA, oxaloacetate; SSA, succinic semialdehyde; TCAP, tricarboxylic acid pathway; 2OG, 2-oxoglutarate.

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 N_{ase} activity remains somewhat undefined (feedback inhibition versus oxidative stress), it should be noted that N_{ase} 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 N_{ase} activity (CO_2 evolved by nodules per unit of N_{ase} 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 N_{ase}-catalysed NH_3 production (based on ATP and C skeleton requirements) have been found to be near 2.4 mol CO_2 mol^{-1} NH_3 (for a specific discussion and a review of experimental results, see Atkins *et al*., 1978), and here a CO_2 evolved-to-N_{ase} 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

(catabolism) was mostly (~90%) associated with sustaining N_{ase} activity in both control and water-restricted conditions.

Conclusions and perspectives

N_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_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 *Sinohizobium* cells may also contribute to the observed metabolic patterns. Furthermore, metabolic fluxes could certainly be examined more carefully using ^{13}C labelling and ^{13}C tracing by nuclear magnetic resonance (NMR) fluxomic techniques, and this should be addressed in the future. An understanding of the effectiveness of legumes in producing nitrogenous compounds (and proteins) requires an assessment of all aspects of plant performance, especially respiratory losses and remobilization processes. Here, it is suggested that water-limited conditions enhanced protein remobilization in nodules and a coordinated change in Krebs cycle activity. A more specific examination of protein dynamics by ^{15}N pulse–chase labelling should provide relevant data to understand the nitrogen economy of legumes in dry environments.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Metabolomic analysis of leaf, root, and nodules under drought and control conditions.

Acknowledgements

This study was supported in part by European research projects PERMED and OPTIMA and the Spanish Ministry of Science and Technology PALEOISOCROP (CGL2009-13079-C02). IA was the recipient of a Ramón y Cajal contract sponsored by the Spanish Ministry of Economy and Competitiveness.

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