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Drought stress provokes the down-regulation of methionine and ethylene biosynthesis pathways in Medicago truncatula roots and nodules

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Short running title:

S metabolism in drought-stressed *M. truncatula*

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ABSTRACT

Symbiotic nitrogen fixation is one of the first physiological processes inhibited in legume plants under water-deficit conditions. Despite the progress made in the last decades, the molecular mechanisms behind this regulation are not fully understood yet. Recent proteomic work carried out in the model legume *Medicago truncatula* provided the first indications of a possible involvement of nodule methionine (Met) biosynthesis and related pathways in response to water deficit conditions. To better understand this involvement, the drought-induced changes in expression and content of enzymes involved in the biosynthesis of Met, S-adenosyl-L-methionine (SAM) and ethylene in *M. truncatula* root and nodules were analyzed using targeted approaches. Nitrogen-fixing plants were subjected to a progressive water deficit and a subsequent recovery period. Besides the physiological characterization of the plants, the content of total sulfur, sulfate and main S-containing metabolites was measured. Results presented here show that S availability is not a limiting factor in the drought-induced decline of nitrogen fixation rates in *M. truncatula* plants and provide evidences for a down-regulation of the Met and ethylene biosynthesis pathways in roots and nodules in response to water deficit conditions.

Key-words:

Drought, *Medicago truncatula*, symbiosis, nodule, methionine, ethylene, proteome.

INTRODUCTION

Water deficit is considered the most limiting factor on plant growth and crop productivity worldwide. Plants facing water-deficit conditions experiment changes in an array of processes at the physiological, developmental and molecular level, leading to growth arrest and reduction of photosynthetic rates, through complex regulatory networks (Bray, 1997; Flexas & Medrano, 2002; Chaves *et al.* 2003; Hirayama & Shinozaki, 2010). Symbiotic nitrogen fixation in legume plants is particularly sensitive to water deficit conditions. In fact, inhibition of nitrogen fixation has been shown to precede that of photosynthesis in drought-stressed legumes (Durand *et al.* 1987; Djekoun & Planchon, 1991). In the last decades, several hypotheses have tried to explain the observed drought-induced decline in nitrogen fixation rates. Although initial studies suggested that O_2 limitation in the symbiotic root organs, i.e. nodules, was responsible for the observed inhibition of nitrogen fixation (Durand *et al.* 1987; Hunt & Layzell, 1993), subsequent works presented evidences showing that O_2 was not the only factor involved (Del Castillo *et al.* 1994; Del Castillo & Layzell, 1995). Regulation mediated by an N signal has received much attention in recent years. This hypothesis was built on the observation that ureides and certain amino acids accumulate in leaves and nodules of drought-stressed legume plants (de Silva *et al.* 1996; Purcell *et al.* 1998; Serraj *et al.* 1999; Vadez *et al.* 2000; King & Purcell, 2005; Ladrera *et al.* 2007; Sulieman *et al.* 2010). Nevertheless, recent results obtained using a split-root system have shown that a local amino acid accumulation occurs prior to any measurable decline in nitrogen fixation rates, thus downplaying the role of N feedback mechanisms (Gil-Quintana *et al.* 2013a; 2013b). The hypothesis of a C-mediated regulation was proposed upon the observation that drought provokes an inhibition of sucrose synthase activity, an accumulation of sucrose and a decrease in the content of malate, the main C source transported into the symbiosome (Udvardi *et al.* 1988), concomitant with the reduction

of nitrogen fixation levels in several grain legume species (González *et al.* 1995; Gordon *et al.* 1997; Ramos *et al.* 1999). This regulatory mechanism, however, does not fully explain changes occurring in forage legumes such as alfalfa (*Medicago sativa* L.) and barrel medic (*Medicago truncatula* Gaerth.) when subjected to water-deficit conditions (Naya *et al.* 2007). Indeed, high-throughput proteomic analysis of drought-stressed *M. truncatula* nodules suggested, for the first time, the possible involvement of S metabolism in response to water deficit (Larrainzar *et al.* 2007; 2009). Two were the main new insights gained from these analyses: i) several enzymes involved in S metabolism were identified in nitrogen-fixing nodules; ii) using relative quantification techniques, a reduction in the protein content of methionine synthase (MetS) and S-adenosyl-L-methionine synthetase (SAMS) was detected in nodule samples from drought-stressed plants.

Unlike animals, plants and microorganisms are able to assimilate S from inorganic forms present in soils, mostly sulfate. Several enzymes are involved in the early S assimilation pathway, namely ATP sulfurylase, adenosine 5'-phosphosulfate (APS)-reductase, sulfite reductase and *O*-acetylserine (thiol) lyase (OASTL, also known as Cys synthase; Logan *et al.* 1996; Hell *et al.* 2002; Wirtz & Hell, 2006). The *de novo* synthesis of methionine (Met) requires the activity of cystathionine γ-synthase, cystathionine β-lyase and MetS (Thompson *et al.* 1982; Ravanel *et al.* 1995; Ravanel *et al.* 1998). Met can be then incorporated into proteins, adenosylated to form S-adenosyl-L-methionine (SAM) by SAMS or methylated to form S-methyl Met (Saito, 2004). SAM is the main one-carbon methyl donor both in prokaryotes and eukaryotes, essential for the regulation of the biosynthesis of Asp-related amino acids and precursor of secondary compounds such as polyamines and the plant hormone ethylene (Roje, 2006).

The observation that the levels of enzymes involved in Met and SAM synthesis were reduced in drought-stressed *M. truncatula* nodules (Larrainzar *et al.* 2007; 2009)

led us to hypothesize that ethylene production may be consequently affected, with possible implications in drought-stress signaling and regulation of nitrogen fixation. In the present work, we test this hypothesis by analyzing the effects of drought on the expression and content of enzymes involved in the biosynthesis of Met, SAM and ethylene in *M. truncatula* plants subjected to a progressive water deficit and a subsequent recovery period. Expression and absolute protein quantification data are accompanied by a detailed characterization of the water deficit imposed at the physiological level. Water withholding experiments also limit nutrient supply to the plant. To discard the possibility that the observed reduction in S metabolism was due to a limitation in S, instead of being a consequence of the drought stress *per se*, the content of total S, sulfate and the main S-containing metabolites in roots and nodules -i.e. the antioxidant glutathione and homoglutathione, GSH and hGSH- were also examined.

MATERIALS AND METHODS

Plant growth conditions

M. truncatula "Jemalong A17" plants were grown in 1 L pots with a mixture of vermiculite:perlite (5:2, v/v) as substrate under controlled environmental conditions (14-h day/10-h night; 400 µmol m^{-2} s^{-1} light intensity; 22°C/16°C day/night temperature; 70 to 60% relative humidity). After sowing, seedlings were inoculated with *Ensifer* (previously named *Sinorhizobium*) *meliloti* 2011, strain that, under our growth conditions, does not lead to nitrogen-stressed plants when grown exclusively under symbiotic conditions. Plants watered with nutrient solution (Evans & Moore, 1981). For the first 4 weeks, solution was supplemented with 0.25 mM ammonium nitrate in order to improve plant performance during the initial development stage. During the following weeks, nutrient solution was not supplemented with a nitrogen source.

Drought stress and recovery treatments

Ten weeks after planting, plants were randomly separated into two groups: control and drought-stressed. Control plants were supplied with nutrient solution to field capacity daily, whereas drought stress was imposed to the other group by withholding water/nutrients. Drought-stressed plants and their corresponding controls were harvested at day three and day six after the onset of drought in order to obtain mild and severely drought-stressed plants. Watering was resupplied to a subset of severely drought-stressed plants for two, three or four days, to obtain different levels of recovery. Root and nodule samples were collected, frozen in liquid N_2 and stored at -80 ºC for further analysis. Plants recovered for three and four days were employed for physiological characterization of the plant response to rewatering. Six types of samples were analyzed: control plants at the beginning of the experiment (C0), control plants at day three (C3), drought-stressed plants at day three (D3), control plants at day six (C6), drought-stressed plants at day six (D6), plants rewatered for two days (DR) and their respective controls (CR).

Measurement of nodule water potentials, apparent nitrogenase activity and net photosynthesis

Water potential of freshly detached nodules was measured in C52 sample chambers connected to a Wescor HR-33T Dew Point Microvoltmeter (Wescor, USA). Apparent nitrogenase activity (ANA) was estimated as H_2-evolution of intact plants in an open flow-through system under N_2:O_2 (79%:21%, v/v) using an electrochemical H_2 sensor (Qubit System Inc., Canada) as previously described(Witty & Minchin, 1998). The H_2 sensor was calibrated with high purity gases (Praxair, Spain) using a gas mixer (Air Liquide, Spain) flowing at the same rate as the sampling system (500 mL min^{-1}). Net

150 photosynthesis was measured using a portable infrared gas analyzer (LI-6200, Li-Cor,
151 USA).
152
153 **Determination of total S and sulfate**
154 Total leaf, root and nodule S was measured by combustion of plant dry material
155 using an elemental analyzer Flash EA1112 (Thermo Finnigan, USA. Components of the
156 combustion mixture (N_2, CO_2, H_2O and SO_2) were separated by a chromatographic
157 column and detected by thermal conductivity.
158 For the determination of soluble sulfate, frozen nodules were homogenized to a fine
159 powder in liquid N_2 using a mortar and pestle. 1.5 mL of 10% (w/v) trichloroacetic acid
160 was added and the homogenate was centrifuged for 10 min at 1,750 g and 4ºC. The
161 aqueous phase was washed three times with diethyl ether saturated with water. The
162 diethyl ether solution was discarded and the aqueous phase was purged with helium for
163 2 min and subsequently filtered (0.45- µm pore size). Sulfate levels were determined by
164 ion chromatography in a DX-500 system (Dionex Ltd., UK) by isocratic separation
165 using Dionex IonPac AG11 and AS11 columns according to the manufacturer's
166 instructions.
167
168 **Determination of low molecular-mass antioxidant compounds**
169 GSH, GSSG, hGSH and hGSSG were extracted from frozen nodules and analyzed
170 by high-performance capillary electrophoresis in a Beckman Coulter P/ACE system
171 5500 (Beckman Coulter Inc., USA) associated with a diode array detector, as previously
172 described (Zabalza *et al.* 2007). GSH and hGSH were directly measured from plant
173 extracts after calibration with purified GSH (Sigma-Aldrich, Spain) and hGSH (kindly
174 provided by Dr. Manuel Becana, EEAD-CSIC, Zaragoza). In order to obtain total GSH
175 and hGSH pools, samples were treated with dithiothreitol. GSSG and hGSSG levels

were calculated as the difference between the total pools and their respective reduced forms (Davey *et al.* 2003).

RNA extraction and gene expression analysis

Real-time quantitative PCR (qRT-PCR) analyses were done following the MIQE Guidelines (http://www.rdml.org/miqe). Nodule samples (~ 0.1 g fresh weight, FW) were homogenized using liquid N_2 and total RNA was extracted by the TRIzol method according to the manufacturer's instructions (Invitrogen, Spain). Extracted RNA was subsequently quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Thermo Fisher Scientific, USA). Samples with a 260/280-absorbance ratio >1.8 were used for analyses. Two µg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random primers in the presence of 40 units of recombinant RNaseOUT ribonuclease inhibitor (Invitrogen, Spain). qRT-PCR was performed on a light cycler ABI Prism 7000 SDS (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems, USA). Each reaction was performed using 5 µL of a 1:2 (v/v) dilution of the original reverse transcription mixture with 0.3 µM of each primer in a total reaction volume of 25 µL. Primers used for qRT-PCR were designed using the Primer Express software (Applied Biosystems, USA) and are listed as supporting information (Table S1). Reactions were incubated for 2 min at 50ºC and 10 min at 95ºC, followed by 60 cycles of 15 s at 95ºC and 1 min at 60ºC. At the end of the PCR cycles, a melting curve analysis (from 65ºC to 95ºC) was performed to confirm the accuracy and specificity of the amplified product. Reactions were performed in triplicate and three biological replicates were used for each treatment. Expression values were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), applying the geometric mean of three reference genes: *M. truncatula* 18S, beta-actin and elongation factor-1 alpha (Supporting Information Table S1).

202

203 **Protein extraction and absolute quantification**

204 Nodule samples (~ 0.1 g FW) were homogenized in ice-cold mortar and pestle using
205 the following homogenization buffer (6 mL per g FW): 50 mM MOPS, pH 7, 10 mM
206 dithiothreitol, 10 mM 2-mercaptoethanol, 1 mM EDTA, 20 mM KCl, and 5 mM $MgCl_2$.
207 One µL of plant protease inhibitor cocktail (Sigma-Aldrich, Spain) was added to each
208 sample. Homogenates were centrifuged for 15 min at 2,000g and 4°C. After
209 centrifugation, supernatants were collected as nodule plant fractions. Protein was
210 quantified using a Bradford-base dye-binding assay (Bio-Rad, Spain) with bovine serum
211 albumin as a standard.

212 For absolute protein quantification, ^{13}C, ^{15}N-labeled peptides specific for each
213 protein were synthesized (Thermo Electron, Ulm, Germany). Protein samples were
214 digested and quantified as described in Larrainzar *et al*. 2009). Absolute quantification
215 of a protein was performed by comparing the peak area of the internal standard peptide
216 with the corresponding native peptide (Wienkoop *et al.* 2008). Peptides used are listed
217 in Supporting Information Table S2.

218

219 **RESULTS**

220 **Drought produces a decline in nitrogen fixation prior to a reduction in**
221 **photosynthesis rates in *M. truncatula***

222 To provide the physiological framework for the drought and subsequent recovery
223 treatments, nodule water potential (Ψ_w), net photosynthesis and apparent nitrogenase
224 activity (ANA), measured as hydrogen evolution in intact plants, were determined
225 during the time course of the experiment (Fig. 1). Nitrogen fixation estimated as ANA
226 provides a more accurate estimation of nitrogen fixation rates when using hup- rhizobial

strains than the commonly widespread acetylene reduction assay (Minchin *et al.* 1983; 1986).

Drought stress provoked a progressive decline in nodule Ψ_w, significant already at day three, producing values of -2.42 ± 0.29 MPa at day six (Fig. 1a). Two days of rewatering were sufficient to restore nodule Ψ_w, reaching values close to those of control plants (-0.56 ± 0.04 MPa). Although photosynthesis was not significantly affected by drought at early stages (-14%), six days of water deficit provoked a sharp reduction of net photosynthetic rates, reaching values of 4.51 ± 0.49 µmol CO_2 $s^{-1}cm^{-2}$ (Fig.1b). After two days of rewatering, photosynthesis rates were only 17% lower than those of control plants, showing a full recovery by day three. In contrast, four days of rewatering were necessary to fully alleviate the drought-induced decline in nitrogen fixation rates (-75% at day six). These results show that in *M. truncatula* nitrogen fixation is largely affected by drought stress, even more than other physiological parameters such as photosynthesis.

In terms of plant biomass parameters, under our experimental conditions average plant dry weight is 4.16 ± 0.24 g, while average nodule dry biomass is 0.066 ± 0.003 g, values which typically correspond to ~300 nodules per plant. During the treatment neither total plant nor nodule dry biomass showed significant differences (Supporting Information Figure S1 a and b). In contrast, as expected during a water-deficit experiment, nodule water content significantly and progressively declined in drought-stressed plants, reaching control values two days after rewatering (Figure S1c) and following the same trend observed for nodule water potential (Fig. 1a).

Given that water nodule Ψ_w was already restored after two days of rewatering, subsequent analysis were carried out with samples at this time point of recovery.

Total S and sulfate accumulate in drought-stressed *M. truncatula* roots but not in nodules

After physiological characterization of the drought and recovery treatments, we assessed the variations in the content of total S and soluble sulfate in both root and nodule tissues (Fig. 2). In nodule samples, neither the levels of S nor the content of sulfate showed significant variations during the treatment (Fig. 2a and c). In contrast, the total content of S in roots experiencing water deficit increased to more than two-fold that of control plants as early as day three (Fig. 2b). As soon as water was resupplied, though, total root S levels declined. The levels of sulfate in roots mimicked the trend observed for total S, showing an accumulation in roots during the water-deficit period and reaching control values after rewatering (Fig. 2d).

GSH levels decline in *M. truncatula* nodules under water-deficit conditions

Next, the levels of the main S-containing antioxidants present in nodules, GSH and hGSH, were quantified. Both the reduced form and the total pool of the antioxidants were measured. Drought caused a reduction in the levels of GSH (Fig. 3a) and the GSH+GSSG pool (Fig. 3b), a trend that was reversed by rewatering. This drought-induced decline in the content of GSH is characteristic of oxidative stress responses, during which GSH is used to scavenge reactive oxygen species (Dalton *et al.* 1986; Matamoros *et al.* 1999). In contrast, with hGSH, only a minor reduction was observed at D6, while the total hGSH+hGSSG pool showed no statistical differences during the experiment (Fig. 3c and d).

SAMS and ACO are transcriptionally down-regulated in drought-stressed nodules

In previous proteomic studies, we identified the drought-induced decline in the levels of two enzymes involved in S metabolism in *M. truncatula* nodules: MetS and

SAMS, corresponding to the Medicago Gene Index 8.0 tentative consensus (TC) sequences TC106598 and TC106384, respectively (Larrainzar *et al.* 2007; 2009). Because TC numbers are subjected to continuous update, we performed a BLAST search to identify the corresponding protein sequences as annotated in the International *M. truncatula* Genome Annotation Group (IMGAG, Mt3.5 version 4). MetS was assigned to Medtr7g086300, while SAMS did not retrieve any 100% match, thus, it was named using the GenBank ID: BT148742.1. To analyze the effects of water-deficit in the expression level of these and downstream enzymes involved in the biosynthesis of ethylene, real-time quantitative PCR (qRT-PCR) measurements were carried out (Fig. 4). Analysis included upstream genes involved in Met biosynthesis pathway, cystathionine γ-synthase (Medtr7g011230) and cystathionine β-lyase (contig_8476_2), along with the most highly expressed 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS, Medtr7g079080) and oxidase (ACO, Medtr6g092620) genes in nodules (as inferred from the *M. truncatula* Gene Expression Atlas; http://mtgea.noble.org/v2; Benedito *et al.* 2008).

Neither the transcript levels of cystathionine γ-synthase nor those of cystathionine β-lyase showed significant changes during the drought/recovery treatments (Fig. 4). Interestingly, these two genes presented a similar expression pattern, in line with a co-regulation of expression in genes encoding subunits of an enzymatic complex. The levels of MetS transcripts showed a trend to decline under water-deficit conditions, without reaching the established 0.5-fold expression change cutoff. In contrast, SAMS was significantly down-regulated at D6, with low expression values maintained two days after watering was re-established (Fig. 4). In terms of enzymes involved in ethylene biosynthesis, we were able to detect the expression of both ACS and ACO in mature nitrogen-fixing nodules. ACS, considered the rate-limiting step in the biosynthesis of ethylene in plants (Kende, 1993), showed a sharp decline in transcript

304 levels in samples from drought-stressed plants, which was only partially alleviated by
305 rewatering (Fig. 4).
306
307 **Absolute quantification of S-metabolism enzymes in *M. truncatula* root and nodule**
308 **tissue during drought and recovery**
309 To more accurately quantify the drought-induced changes at the protein level, we
310 designed stably-labeled peptides specific for each of the enzymes in the S metabolism
311 pathway described in the previous section. This targeted proteomic technique allows the
312 quantification of the levels of a protein in absolute terms, providing more reliable results
313 compared to traditional protein immunodetection-based methods (Wienkoop *et al.*
314 2008). Measurements were carried out both in nodule (Fig. 5) and in root tissue (Fig. 6)
315 from samples collected at different drought stages and after a two-day recovery period.
316 With the exception of SAMS, for which the peptide used for quantification targeted a
317 conserved region of the protein shared by other isoforms, peptide sequences were
318 selected so that measurements would specifically target one single protein (see
319 Supporting Information Table S2).
320 We were able to successfully detect and quantify all the enzymes tested in both
321 nodule and root protein extracts. Side by side comparison of protein levels shows
322 increased absolute protein content in nodule tissue, in some cases several hundred-fold
323 higher than in roots. For instance, the average content of MetS in control nodules was
324 664.8 ± 190.9 fmol µg protein^{-1}, while the content in roots was only 8.2 ± 3.3 fmol µg
325 protein^{-1}. These remarkable differences in protein levels highlight the active role of
326 protein biosynthesis and S metabolism of symbiotic root nodules.
327 In terms of effects of drought on the levels of these enzymes, there was a reduction
328 in the nodule content of MetS and SAMS, in agreement with previous results, a
329 decrease that was more obvious in roots for MetS (Fig. 6) and in nodules for SAMS

(Fig. 5). The levels of cystathionine γ-synthase and cystathionine β-lyase did not show significant changes during the drought experiment, which suggests a tight regulation of the levels of these enzymes in these tissues. Although the protein content of enzymes involved in ethylene biosynthesis was not as affected by drought as observed at the expression level, ACS showed a transient reduction in protein levels in roots three days after the onset of drought.

DISCUSSION

In this work we have explored the involvement of S metabolism, with a special focus on Met and ethylene biosynthesis, in the response of *M. truncatula* plants subjected to a progressive drought and a subsequent recovery treatment. Physiological characterization of the plants during the experimental time course shows a gradual inhibition of nitrogen fixation rates before any measurable effect in photosynthesis (Fig. 1). These results provide further support to the hypothesis that symbiotic nitrogen fixation is one of the first processes showing a response to water deficit (Durand *et al.* 1987; Djekoun & Planchon, 1991).

In order to test whether the decline in the content of enzymes involved in Met and SAM biosynthesis could be attributed to a limitation of S availability, the levels of total S and sulfate in roots and nodules were measured at different time points during the drought/recovery experiment. In nodules neither the total level of S nor the content of sulfate significantly changed, while in roots increased contents were actually observed during the water-deficit period (Fig. 2). Interestingly, rewatering provoked a rapid reduction of the levels of sulfate to control values in roots (Fig. 2d), suggesting that a mobilization of sulfate from roots to the aerial part of the plant occurs once water transport is re-established. Therefore, far from being limiting during the nutrient-deprivation period, S does accumulate in roots during drought, remaining at relatively

356 constant values in nodule tissue. Thus, the previously observed down-regulation of S
357 metabolic enzymes cannot be explained by a limitation in S availability for the plant.

358 Regarding the main thiols present in nodules, both GSH and its homologue hGSH
359 were detected in *M. truncatula* nodules at average control concentrations of 8.3 ± 0.7
360 and 4.1 ± 0.2 μmol per g dry weight, respectively (Fig. 3a and c), in line with previous
361 studies in this model legume (Frendo *et al.* 1999). Interestingly, we observed a drought-
362 induced decline in the levels of GSH, while the content of hGSH barely changed along
363 the time course. This differential response of GSH and hGSH to water deficit has also
364 been described in *M. sativa* nodules (Naya *et al.* 2007), as well as in other legumes upon
365 hormone treatment (Clemente *et al.* 2012). Taken together, these results provide further
366 evidence for a functional specialization of these thiols in legumes, although the specific
367 roles of GSH and hGSH in legume nodules are yet to be defined (Frendo *et al.* 2013). In
368 the present work, the decline in GSH can only be partially attributed to direct
369 antioxidant functions since the levels of the oxidized form, GSSG, remained relatively
370 constant during the experiment (Fig. 3). However, other possible explanations for the
371 observed drop in GSH content include its participation as a substrate for other
372 antioxidant enzymes (Buchanan & Balmer, 2005; Dalton *et al.* 2009), its involvement in
373 protein S-glutathionylation reactions (Dixon *et al.* 2005; Zaffagnini *et al.* 2012), as well
374 as early sulfur assimilation steps, among others (reviewed by Noctor *et al.* 2012).

375 Results presented in this work, along with previous reports in which a number of
376 proteins involved in S assimilation were identified in root plastids (Brunold & Suter,
377 1989; Hatzfeld *et al.* 2000; Phartiyal *et al.* 2006) and nodules (Larrainzar *et al.* 2007;
378 2009), suggest that roots are an important site for S assimilation in plants. This is, to our
379 knowledge, the first study centered on S metabolism in legume nodule and root tissue in
380 response to water-deficit conditions. We were able to detect the whole set of enzymes
381 assayed, both at the gene expression and protein levels, suggesting that legume nodules

are an active site for the *de novo* biosynthesis of Met, SAMS and, to a lesser extent, ethylene. Met biosynthesis, accumulation and catabolism are assumed to be under a high level of regulatory control (Hesse *et al.* 2004; Takahashi *et al.* 2011). In *Arabidopsis thaliana* and *M. sativa* several lines of evidence indicate that cystathionine γ-synthase controls the rate of Met synthesis (Inaba *et al.* 1994; Kim *et al.* 2002; Avraham *et al.* 2005). However, this regulation seems to be species-dependent, since the over-expression of cystathionine γ-synthase and other downstream enzymes does not lead to increase Met content in potato (Maimann *et al.* 2001; Nikiforova *et al.* 2002; Kreft *et al.* 2003). In the present work, results show that the expression of cystathionine γ-synthase and cystathionine β-lyase is largely unaffected by the stress. Even though a drought-induced reduction in the content of MetS and SAMS occurs in *M. truncatula*, the levels of S-containing amino acids, Met and Cys, do not show significant variations in nodules, with Met is partially accumulated in drought-stressed roots (Gil-Quintana *et al.* 2013a). This balance in the nodule content of Met and Cys is in sharp contrast to the drought-induced accumulation trend observed for most amino acids (Gil-Quintana *et al.* 2013a) and indicates a finely controlled homeostasis of S-containing amino acids in root nodules.

It is interesting to note that there was not a perfect correlation between the transcript level and protein content of MetS in nodules (Fig. 4 and 5). At the expression level, MetS showed a moderate decline at D6, but expression values were close to control during rewatering. At the protein level, however, a significant reduction in protein content was measured during recovery. These results suggest that additional regulatory mechanisms are operating at the translational and posttranslational level. Indeed, recent evidence suggests that MetS is targeted to S-nitrosylation, at least under *in vitro* conditions (Lindermayr *et al.* 2005) and S-glutathionylation (Dixon *et al.* 2005). These types of regulatory mechanisms also regulate the expression of related genes,

including the autoregulation of cystathionine γ-synthase mRNA (Chiba *et al.* 1999) and the microRNA-395-based regulation of several ATP sulfurylase isoforms and a sulfate transporter in *A. thaliana* (Jones-Rhoades & Bartel, 2004; Kawashima *et al.* 2009).

One of the most dramatic effects of drought in the gene expression data set was the down-regulation of ACS, one of the isoenzymes committed to the biosynthesis of the ethylene precursor, ACC. In legume plants the role of ethylene has mostly been studied during early symbiotic stages (Peters & Meeks, 1989; Heidstra *et al.* 1997; Penmetsa & Cook, 2000; Oldroyd *et al.* 2001; Penmetsa *et al.* 2008) but its role at later stages has not yet been analyzed. Results presented here show that ACS and ACO are expressed in *M. truncatula* roots and nitrogen-fixing nodules and that drought stress causes the specific down-regulation of one of the ACS isoforms. Interestingly, although ethylene has often been considered a stress response hormone and its production generally assumed to increase in response to environmental stimuli (Ecker, 1995; Kim *et al.* 2002; Wang *et al.* 2002), its involvement in drought responses remains a controversial issue. Early work, in which excised leaves were subjected to rapid drying, concluded that ethylene production increased under water deficit conditions (El-Beltagy & Hall, 1974; Apelbaum & Yang, 1981). However, subsequent studies invalidated these conclusions and demonstrated that, when whole plants were subjected to a gradual water deficit, ethylene emission actually declined (Morgan *et al.* 1990; Narayana *et al.* 1991). In agreement with this conclusion, results presented here suggest that ethylene production is reduced in *M. truncatula* root and nodule tissue at early drought stages. Under control conditions, both the levels of ethylene emission and the concentration of ACC in nodulated *M. truncatula* plants appear to fall bellow the technical detection limits (data not shown), thus hindering direct measurements of ethylene or its precursor. Thus, whether ethylene acts as a signaling mechanism to regulate nitrogen fixation during

433 drought stress remains an open question, which may be answered with the use of
434 mutants with altered ethylene production or perception pathways.

435 In conclusion, we have characterized the pathway from the biosynthesis of Met to
436 the production of ethylene in *M. truncatula* nodules, showing that this symbiotic organ
437 is an important site for S metabolism in plants. Both MetS and SAMS show a decline in
438 protein content in drought-stressed roots and nodules and at least the expression of one
439 of the ACS homologs is rapidly down-regulated. These results suggest that ethylene
440 production is reduced at early stress stages, with possible implications in nitrogen
441 fixation signaling. A summary of our current knowledge on the effects of drought on
442 carbon, nitrogen and now sulfur metabolism in nodulated legumes is schematized in
443 Fig. 7. Although the molecular mechanisms involved in the drought-induced decline of
444 nitrogen fixation rates are not clear yet, the present work sheds light on the involvement
445 of Met metabolism and ethylene biosynthesis in response to drought stress in nodulated
446 legumes and paves the way for future analysis on the role of ethylene regulating
447 nitrogen fixation at mature nodule stages. One possible question to address in future
448 work may be to determine whether the Met and ethylene biosynthesis reactions
449 described here occur throughout the whole nodule tissue or only at specific zones, given
450 the heterogeneity of indeterminate nodules at the functional and developmental level.
451

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FIGURE LEGENDS

Figure 1. Effect of drought stress and recovery on nodule water potential (a), photosynthesis (b) and apparent nitrogenase activity (ANA) as a measure of nitrogen fixation (c) in *M. truncatula* "Jemalong A17" plants. Control plants are represented as open squares, drought/recovery plants as closed squares. Values are the mean ± standard error (SE) of three biological replicates. An asterisk (*) represents significant differences with the corresponding control value at $P<0.05$; ** for $P\leq0.01$ (Student's t test). NDW: nodule dry weight.

Figure 2. Effect of drought and recovery in the content of total S (a and b) and sulfate (c and d) in *M. truncatula* nodules and roots, respectively. Control plants are represented as open squares, drought/recovery plants as closed squares. Values are the mean ± SE of three biological replicates. An asterisk (*) represents significant differences with the corresponding control value at $P<0.05$; ** for $P\leq0.01$.

Figure 3. Effect of drought stress and recovery on the content of low-molecular weight antioxidants in *M. truncatula* nodules. Levels of glutathione (GSH; a) and total pool (b); homoglutathione (hGSH; c) and total pool (d). Control samples are represented as open squares, drought/recovery samples as closed squares. Values are the mean ± SE of three biological replicates. An asterisk (*) represents significant differences with the corresponding control value at $P<0.05$; ** for $P\leq0.01$.

Fig. 4. Gene expression analysis of cystathionine γ-synthase (Medtr7g011230), cystathionine β-lyase (contig_8476_2), methionine synthase (MetS, Medtr7g086300),

S-adenosyl-L-methionine synthetase (SAMS, Gene Index ID: BT148742.1), ACC synthase (ACS, Medtr7g079080) and ACC oxidase (ACO, Medtr6g092620) in *M. truncatula* nodules subjected to drought and recovery. All ID numbers, except for SAMS, correspond to IMGAG Mt3.5 v4. Values represent normalized expression levels in respect to those of control plants according to the $2^{-\Delta\Delta Ct}$ method(Livak & Schmittgen, 2001). Bars represent the SE of three biological replicates measured in triplicate. An increase or decrease of at least 50% compared to control values is represented with an asterisk. Abbreviations used: C, average of control samples; D3, drought samples at day three; D6, drought samples at day six; DR, drought samples after two days of recovery. ACC, 1-aminocyclopropane-1-carboxylic acid; APS, adenosine 5'-phosphosulfate; GSH, glutathione; GSSG, glutathione disulfide; hGSH, homoglutathione; OAS(TL), *O*-acetylserine (thiol lyase).

Figure 5. Absolute quantification of the main enzymes involved the synthesis of Met and ethylene in *M. truncatula* nodules: cystathionine γ-synthase (Medtr7g011230), cystathionine β-lyase (contig_8476_2), methionine synthase (MetS, Medtr7g086300), S-adenosyl-L-methionine synthetase (SAMS, several isoforms), ACC synthase (ACS, Medtr7g079080) and ACC oxidase (ACO, Medtr6g092620). Values represent the average protein amount (expressed in fmol per µg of total protein) of five biological replicates. Bars represent SE. Significant differences from the corresponding control value at $P<0.05$ (Student's t-test) are represented with an asterisk. Abbreviations as in Fig. 4.

Figure 6. Absolute quantification of the main enzymes involved the synthesis of Met and ethylene in *M. truncatula* roots: cystathionine γ-synthase (Medtr7g011230), cystathionine β-lyase (IMGAG Mt3.5 v4 ID: contig_8476_2), Met synthase (MetS,

Medtr7g086300), S-adenosyl Met synthetase (SAMS, several isoforms), ACC synthase (ACS, Medtr7g079080) and ACC oxidase (ACO, Medtr6g092620). Values represent the average protein amount (expressed in fmol per µg of total protein) of three biological replicates. Bars represent SE. Significant differences with the corresponding control value at $P<0.05$ (Student's t-test) are represented with an asterisk. Abbreviations as in Fig. 4.

Figure 7. Schematic representation of the main pathways involved in carbon, nitrogen and sulfur metabolism in *M. truncatula* nodules and roots in response to drought stress. Sucrose (Suc) is the main carbon source transported to nodules, where it is metabolized through a series of reactions to yield malate, which used as a respiratory substrate for bacteroids to fuel the activity of the nitrogenase (Nase) complex. Besides the direct export of ammonium, an amino acid cycle has been shown for species such as pea (Lodwig *et al.* 2003). Reduced nitrogen enters the plant nitrogen assimilation pathway to generate asparagine (Asn) and glutamine (Gln), main nitrogen compounds exported to the rest of the plant. Drought stress (e.g. water potential values lower than -1.2 MPa for *M. truncatula*) provokes a rapid inhibition of Nase activity and a reduction in Suc catabolism, followed by a decline in the levels of nitrogen assimilation enzymes. Under water-deficit conditions, sulfate accumulates in roots but not in nodules, and this accumulation is rapidly reverted as soon as water is available. The levels of GSH in nodules decline during drought, but not those of the homolog thiol hGSH. Based on absolute quantification measurements, the content of Met synthase (MetS) and one of the isoforms of ACC synthase (ACS) is reduced in roots under water deficit conditions, while nodules show a decline in the content of S-adenosyl-Met synthetase (SAMS) and reduced expression of ACS. Background picture corresponds to a longitudinal cross section of a *M. truncatula* nodule attached to a root. Bar represents 100 µm.

SUPPORTING INFORMATION

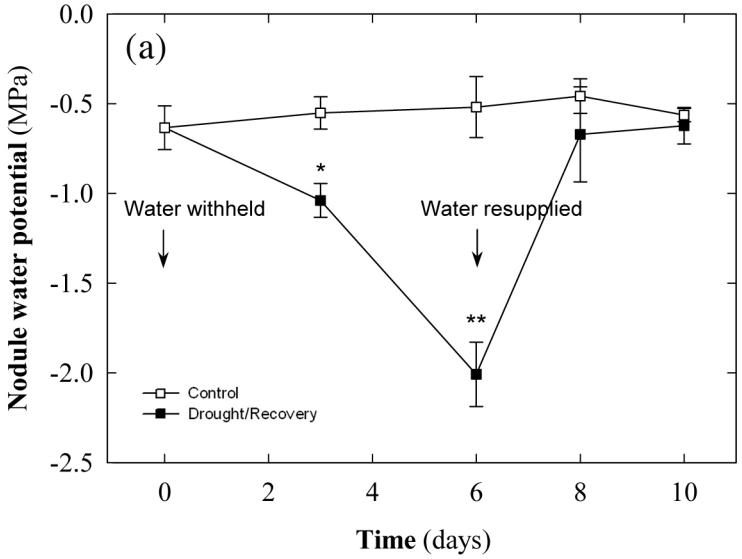
Additional Supporting Information may be found in the online version of this article:

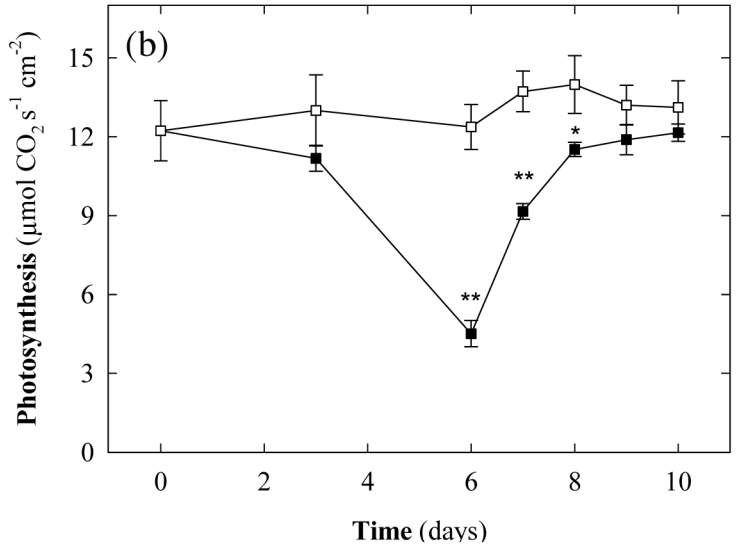
Figure S1. Effects of drought/recovery on plant and nodule dry weight, and nodule water content.

Table S1. Primers used for qRT-PCR expression analysis of enzymes involved in the biosynthesis of Met, SAM and ethylene.

Table S2. Peptides used for absolute protein quantification of enzymes involved in the biosynthesis of Met, SAM and ethylene.

Fig. 1


(a)
Nodule water potential (MPa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Water withheld
Water resupplied
*
**
Control
Drought/Recovery
0
2
4
6
8
10
Time (days)


(b)
Photosynthesis (μmol CO2 s-1 cm-2)
15
12
9
6
3
0
**
**
*
0
2
4
6
8
10
Time (days)

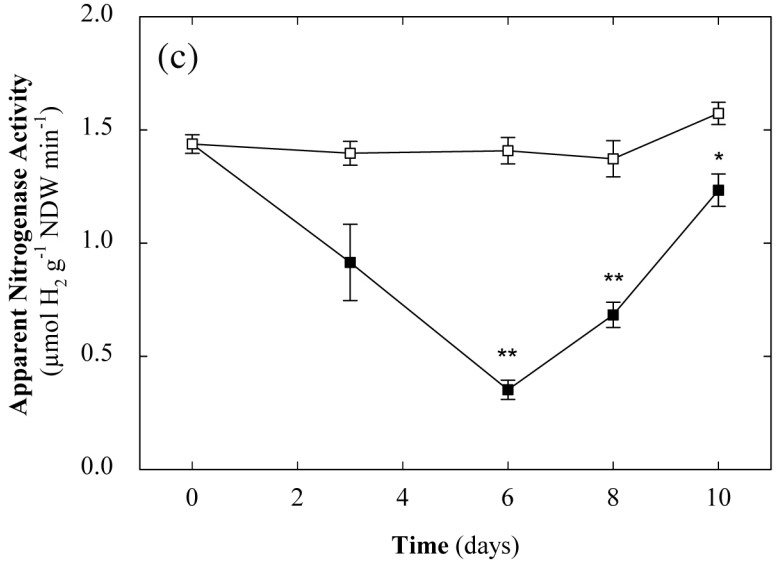

(c)
Apparent Nitrogenase Activity
(μmol H2 g-1 NDW min-1)
2.0
1.5
1.0
0.5
0.0
**
**
*
0
2
4
6
8
10
Time (days)

Fig. 2

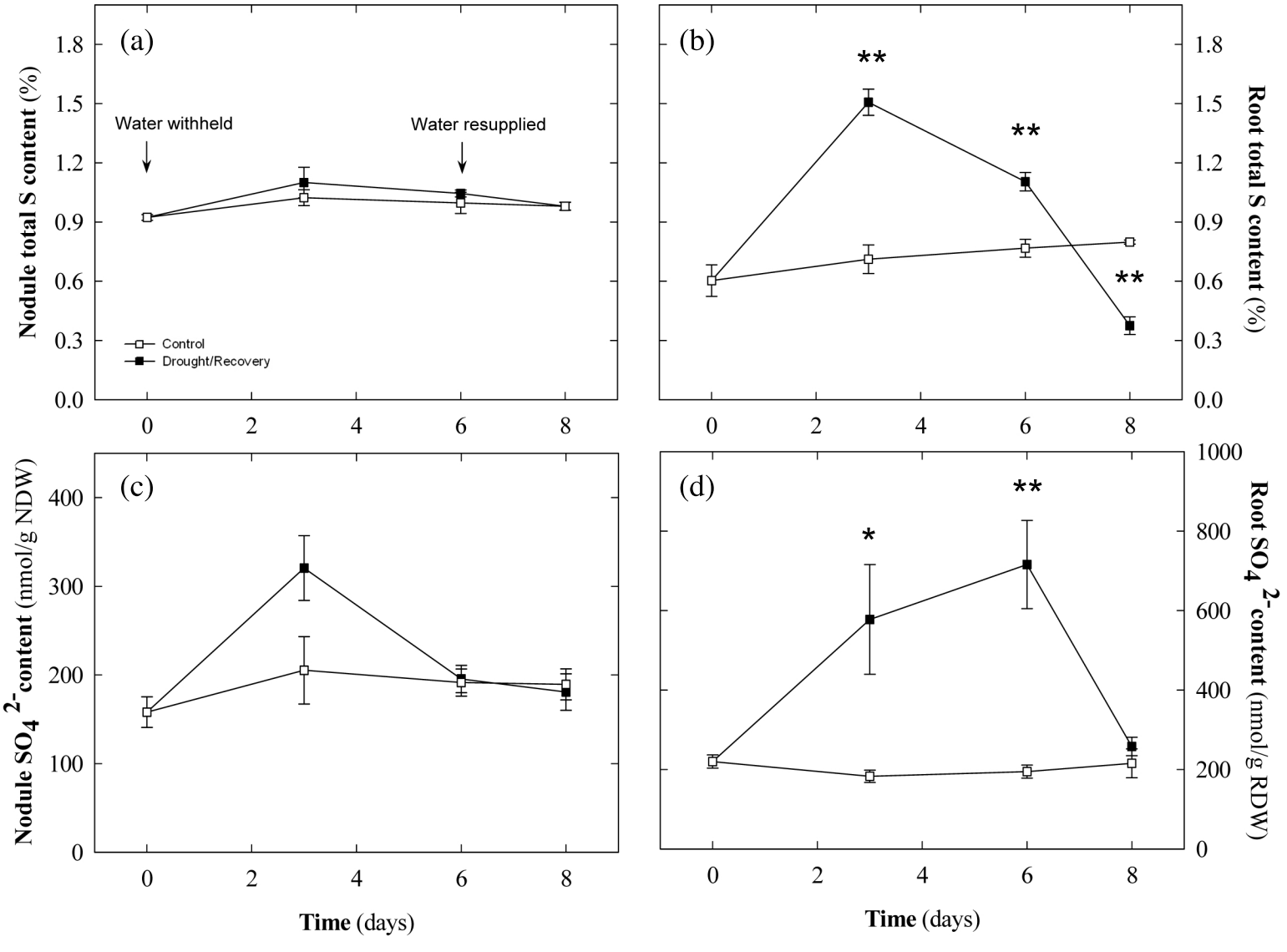
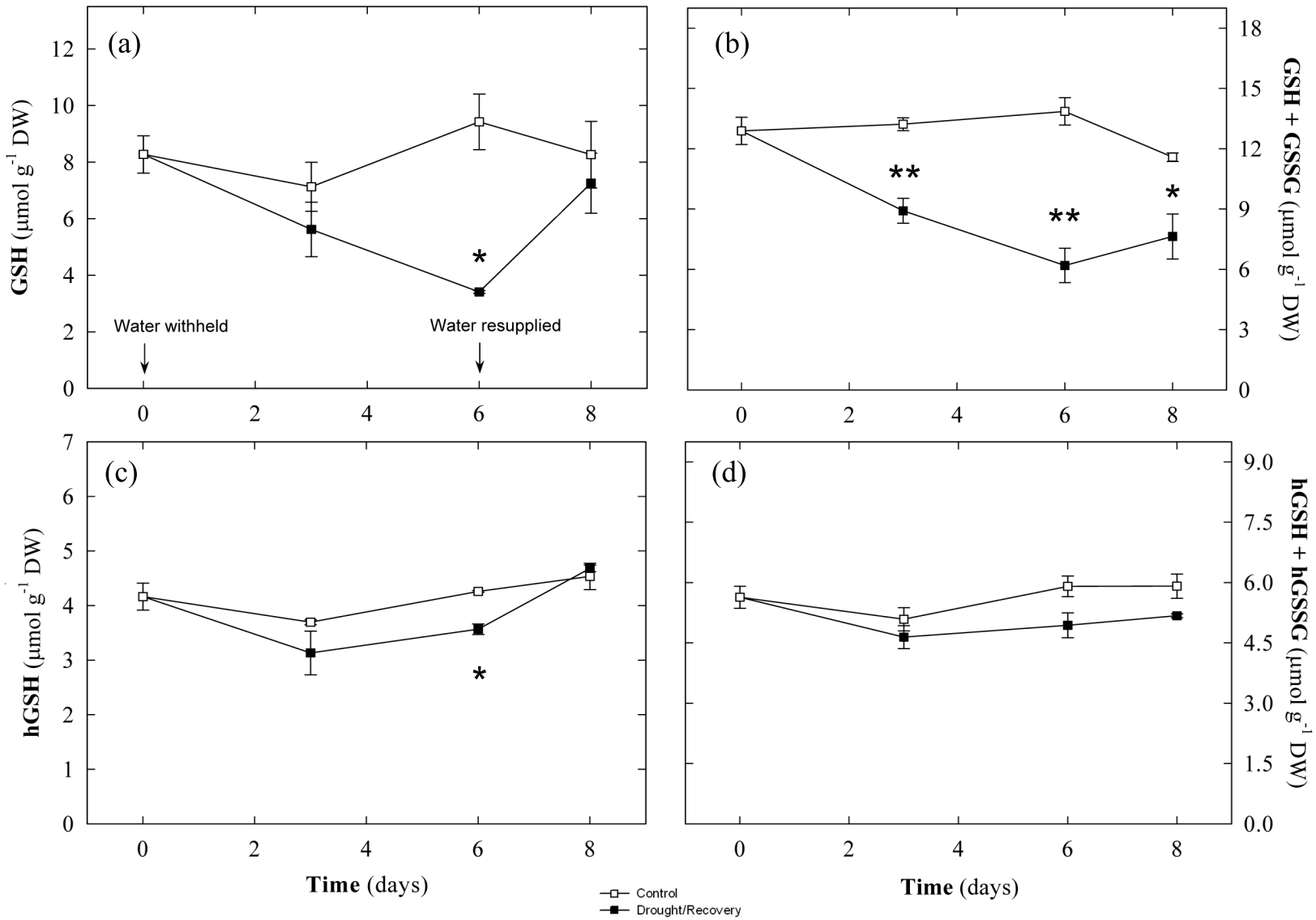
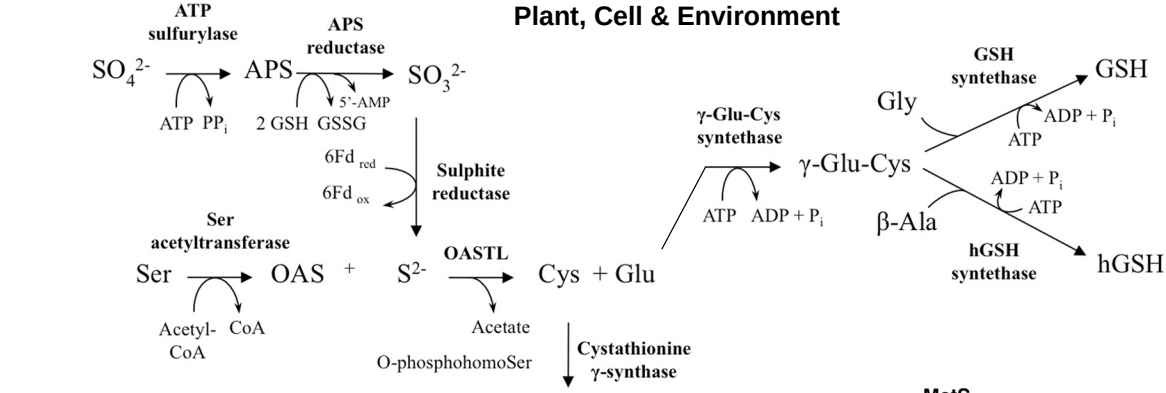
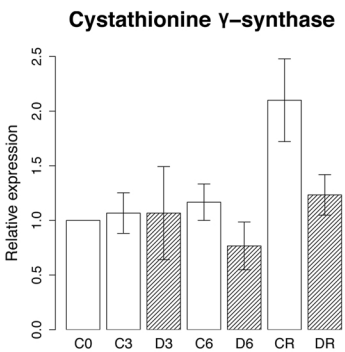
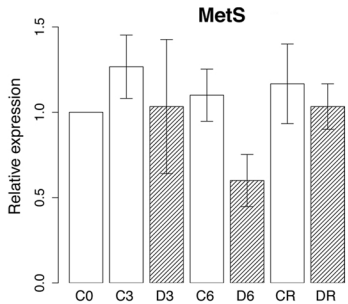

(a)
Water withheld
Water resupplied
Control
Drought/Recovery
Nodule total S content (%)
(b)
Root total S content (%)
(c)
Nodule SO4 2- content (nmol/g NDW)
(d)
Root SO4 2- content (nmol/g RDW)
Time (days)
Time (days)

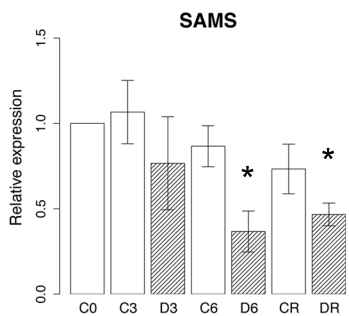
Fig. 3

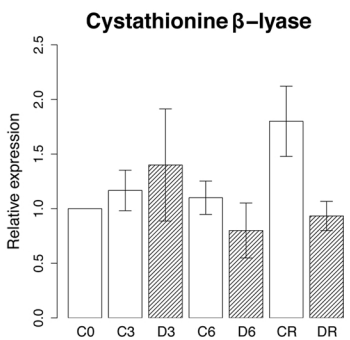

(a)
(b)
(c)
(d)
GSH (μmol g-1 DW)
GSH + GSSG (μmol g-1 DW)
hGSH (μmol g-1 DW)
hGSH + hGSSG (μmol g-1 DW)
Water withheld
Water resupplied
Time (days)
Time (days)
Control
Drought/Recovery

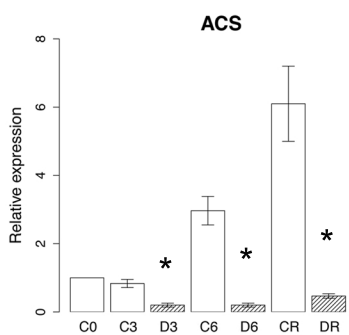

ATP sulfurylase
APS reductase
SO_4^{2-}
APS
SO_3^{2-}
ATP PP_i
2 GSH GSSG
5'-AMP
6Fd red
6Fd ox
Sulphite reductase
Ser acetyltransferase
Ser
OAS
S^{2-}
OASTL
Cys + Glu
Acetyl-CoA
CoA
Acetate
O-phosphohomoSer
γ-Glu-Cys syntethase
ATP ADP + P_i
γ-Glu-Cys
GSH syntethase
Gly
GSH
ADP + P_i
ATP
β-Ala
hGSH syntethase
hGSH
Cystathionine γ-synthase
cystathionine
H_2O
NH_4 + pyruvate
Cystathionine β-lyase
homoCys
THF
CH_2=THF
MetS
Met
ATP
PP_i + P_i
SAMS
SAM
ACS
ACC
ACO
C_2H_4
methylthioadenosine
O_2
CO_2 + HCN


Cystathionine γ–synthase
Relative expression
C0 C3 D3 C6 D6 CR DR


MetS
Relative expression
C0 C3 D3 C6 D6 CR DR


SAMS
Relative expression
C0 C3 D3 C6 D6 CR DR


Cystathionine β–lyase
Relative expression
C0 C3 D3 C6 D6 CR DR


ACS
Relative expression
C0 C3 D3 C6 D6 CR DR

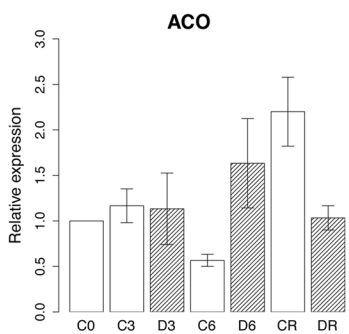
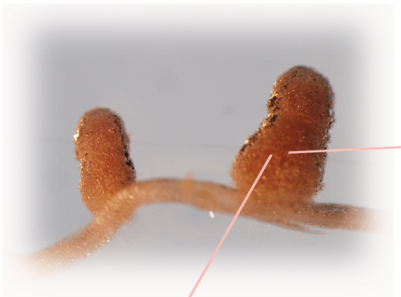
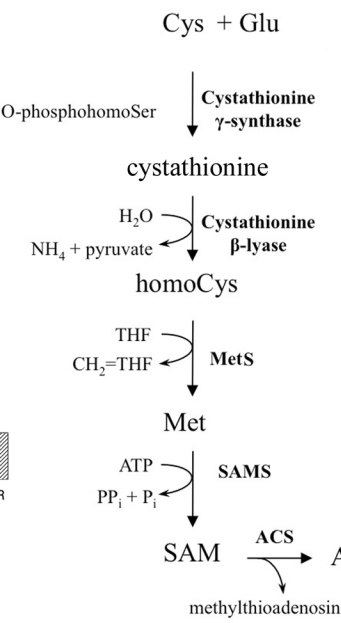
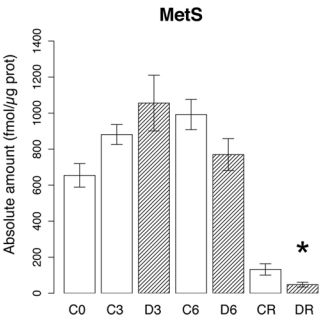
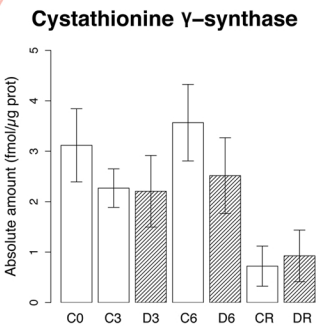

ACO
Relative expression
C0 C3 D3 C6 D6 CR DR

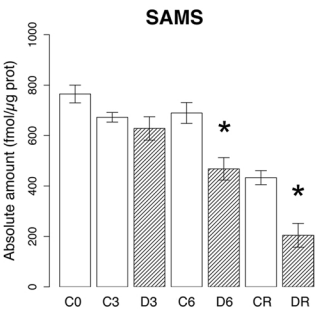
Fig. 5

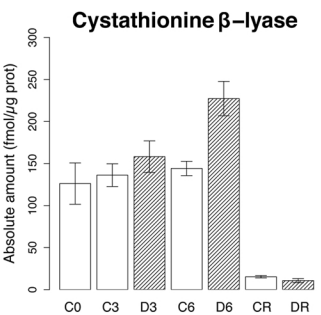


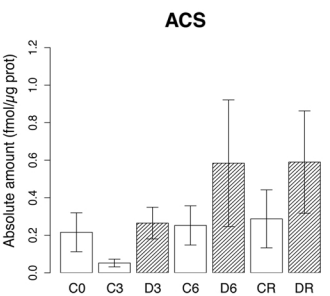

Cys + Glu
O-phosphohomoSer
Cystathionine γ-synthase
cystathionine
H2O
NH4 + pyruvate
Cystathionine β-lyase
homoCys
THF
CH2=THF
MetS
Met
ATP
PPi + Pi
SAMS
SAM
ACS
ACC
ACO
C2H4
methylthioadenosine
O2
CO2 + HCN


MetS
Absolute amount (fmol/μg prot)
0
200
400
600
800
1000
1200
1400
C0
C3
D3
C6
D6
CR
DR
*


Cystathionine γ–synthase
Absolute amount (fmol/μg prot)
0
1
2
3
4
5
C0
C3
D3
C6
D6
CR
DR


SAMS
Absolute amount (fmol/μg prot)
0
200
400
600
800
1000
C0
C3
D3
C6
D6
CR
DR
*
*


Cystathionine β–lyase
Absolute amount (fmol/μg prot)
0
50
100
150
200
250
300
C0
C3
D3
C6
D6
CR
DR


ACS
Absolute amount (fmol/μg prot)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
C0
C3
D3
C6
D6
CR
DR

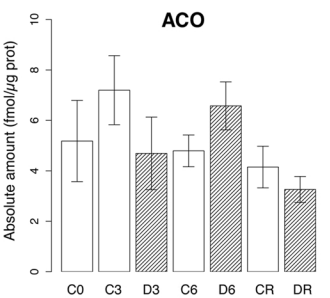
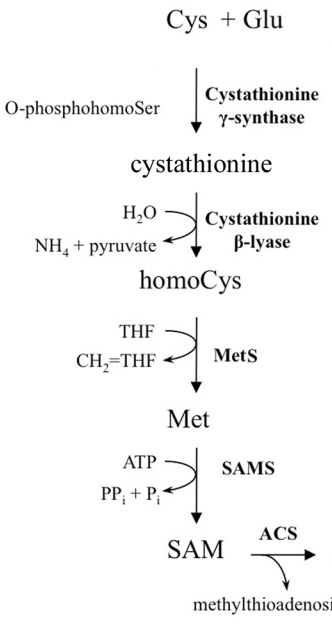
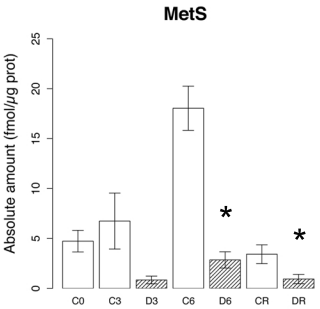
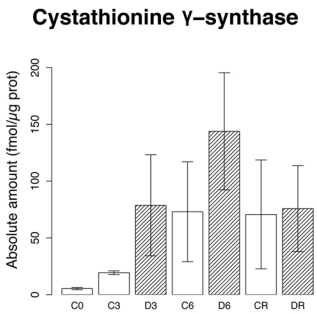
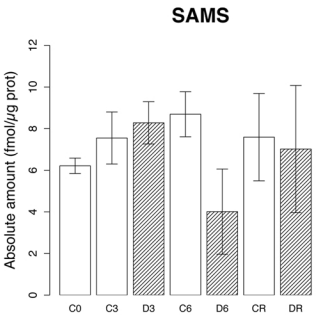

ACO
Absolute amount (fmol/μg prot)
0
2
4
6
8
10
C0
C3
D3
C6
D6
CR
DR

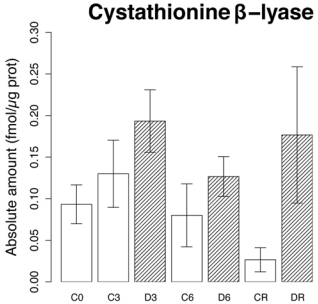
Fig. 6

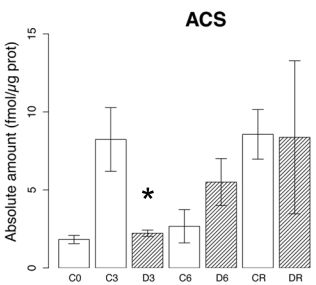

Cys + Glu
O-phosphohomoSer
Cystathionine γ-synthase
cystathionine
H2O
NH4 + pyruvate
Cystathionine β-lyase
homoCys
THF
CH2=THF
MetS
Met
ATP
PPi + Pi
SAMS
SAM
ACS
ACC
ACO
C2H4
methylthioadenosine
O2
CO2 + HCN


MetS
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR


Cystathionine γ–synthase
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR


SAMS
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR


Cystathionine β–lyase
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR


ACS
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR

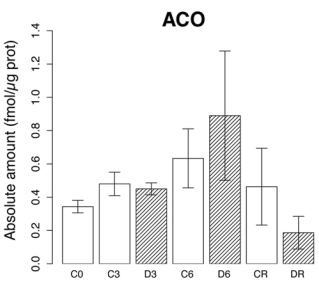
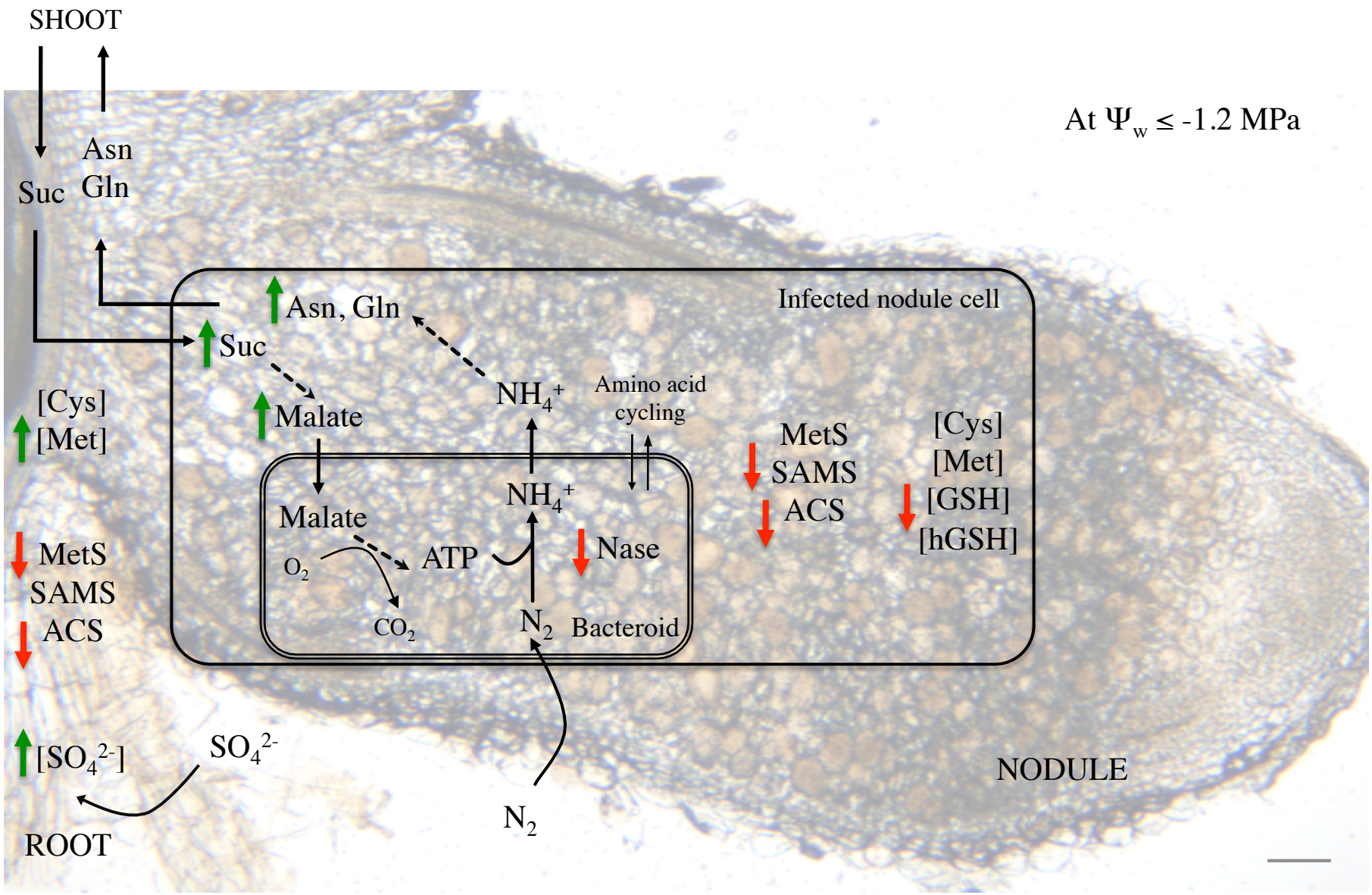

ACO
Absolute amount (fmol/µg prot)
C0 C3 D3 C6 D6 CR DR

Fig. 7


SHOOT
Asn
Gln
Suc
At $\Psi_w \leq$ -1.2 MPa
Asn, Gln
Infected nodule cell
Suc
NH_4^+
Amino acid cycling
[Cys]
[Met]
Malate
MetS
SAMS
ACS
[Cys]
[Met]
[GSH]
[hGSH]
Malate
NH_4^+
MetS
SAMS
ACS
O_2
ATP
Nase
CO_2
N_2
Bacteroid
$[SO_4^{2-}]$
SO_4^{2-}
NODULE
N_2
ROOT

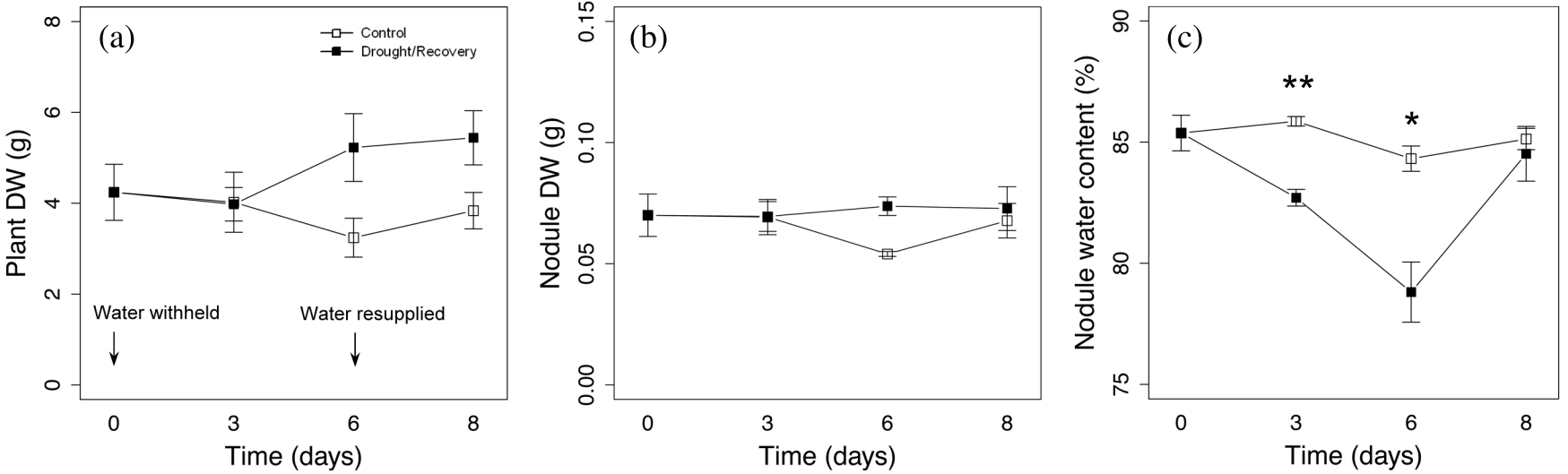


Fig. S1. Effect of drought stress and recovery on plant dry weight (DW, a), nodule DW (b) and nodule water content (c) in *M. truncatula* "Jemalong A17" plants. Control plants are represented as open squares, drought/recovery plants as closed squares. Values are the mean ± standard error (SE) of four biological replicates. An asterisk (*) represents significant differences with the corresponding control value at $P<0.05$; ** for $P\leq0.01$ (Student's t test).