Inhibition of Glutamine Synthetase by Phosphinothricin Leads to Transcriptome Reprogramming in Root Nodules of *Medicago truncatula*

Ana R. Seabra,¹ Patrícia A. Pereira,² Jörg D. Becker,² and Helena G. Carvalho¹

¹Instituto de Biologia Molecular e Celular da Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal;

²Instituto Gulbenkian de Ciência, Rua da Quinta Grande No. 6, 2780-156 Oeiras, Portugal

Submitted 23 December 2011. Accepted 7 March 2012.

Glutamine synthetase (GS) is a vital enzyme for the assimilation of ammonia into amino acids in higher plants. In legumes, GS plays a crucial role in the assimilation of the ammonium released by nitrogen-fixing bacteria in root nodules, constituting an important metabolic knob controlling the nitrogen (N) assimilatory pathways. To identify new regulators of nodule metabolism, we profiled the transcriptome of *Medicago truncatula* nodules impaired in N assimilation by specifically inhibiting GS activity using phosphinothricin (PPT). Global transcript expression of nodules collected before and after PPT addition (4, 8, and 24 h) was assessed using Affymetrix *M. truncatula* Gene-Chip arrays. Hundreds of genes were regulated at the three time points, illustrating the dramatic alterations in cell metabolism that are imposed on the nodules upon GS inhibition. The data indicate that GS inhibition triggers a fast plant defense response, induces premature nodule senescence, and promotes loss of root nodule identity. Consecutive metabolic changes were identified at the three time points analyzed. The results point to a fast repression of asparagine synthesis and of the glycolytic pathway and to the synthesis of glutamate via reactions alternative to the GS/GOGAT cycle. Several genes potentially involved in the molecular surveillance for internal organic N availability are identified and a number of transporters potentially important for nodule functioning are pinpointed. The data provided by this study contributes to the mapping of regulatory and metabolic networks involved in root nodule functioning and highlight candidate modulators for functional analysis.

The symbiotic interaction between legumes and soil bacteria of the genus *Rhizobium* involves complex signal exchange mechanisms that culminate with the formation of newly differentiated organs called root nodules, which provide a niche for bacterial nitrogen (N₂) fixation (Oldroyd et al. 2011). Inside the nodules, the ammonium produced by N₂ fixation is mainly released from the rhizobial bacteroids into the cytosol of the nodule-infected cells where it is assimilated into glutamine

(Gln) by plant glutamine synthetase (GS) (EC.6.3.1.2) (Carvalho and Cullimore 2003). In higher plants, GS is encoded by a multigene family with a complex pattern of expression which is influenced by developmental and environmental cues. The individual genes encode distinct GS isoenzymes, which are responsible for primary ammonium assimilation, reassimilation of ammonia resulting from photorespiration, and nitrogen (N) remobilization during developmental senescence (Lea and Milfin 2010). The model legume *Medicago truncatula* contains one of the smallest plant GS gene families, with only four expressed genes; *MtGS1a* and *MtGS1b* encode cytosolic polypeptides whereas *MtGS2a* and *MtGS2b* encode plastid-located enzymes (Carvalho and Cullimore 2003; Seabra et al. 2010). *MtGS1a* is highly expressed in the infected cells of root nodules, where it assimilates the ammonia derived from nitrogen fixation (Carvalho et al. 2000).

In addition to its fundamental role in ammonia assimilation and detoxification, GS potentially integrates a broad N-signaling network in plants by mediating amino acid synthesis. It has been suggested that the internal amino acids pool function as a signal to regulate N uptake and assimilation (Miller et al. 2008), and both Gln and glutamate (Glu) are considered important signaling molecules. It was shown that Gln is involved in the regulation of the expression of ammonium transporters in *Arabidopsis* and rice (Lima et al. 2010; Rawat et al. 1999; Sonoda et al. 2003), nitrate transporters in *Arabidopsis* (Nazoa et al., 2003), asparagine synthetase and GS in *Arabidopsis* (Lam et al. 1998; Oliveira and Coruzzi 1999), and nitrate reductase (NR) in tobacco leaves and barley roots (Fan et al. 2006; Vincentz et al. 1993). Glu was shown to induce the expression of both GS and Glu dehydrogenase in tobacco leaves (Masclaux-Daubresse et al. 2005) and the external application of Glu appears to elicit complex changes in the root architecture of *Arabidopsis* (Walch-Liu and Forde 2007). Interestingly, Glu also appears to function as a signal for the diurnal changes in the organic N status of the plant. The expression of the master clock control protein *cca1*, which modulates the expression of key N-assimilatory enzymes, appears to be regulated by Glu or a Glu-derived metabolite (Gutierrez et al. 2008).

Several large-scale transcriptome studies were devoted to the *M. truncatula*–*Sinorhizobium meliloti* symbiosis (Ampe et al. 2003; Barnett et al. 2004; El Yahyaoui et al. 2004; Fedorova et al. 2002; Godiard et al. 2007; Lohar et al. 2006; Manthey et al. 2004; Maunoury et al. 2010; Moreau et al. 2011; Starker et al. 2006; Tesfaye et al. 2006). Most of these studies were dedicated to the analysis of root nodule development; and, only recently, transcriptome approaches have focused on nodule functional aspects related to nitrogen fixation by comparing the

Nucleotide sequence data is available in the Gene Expression Omnibus database under accession number GSE34155.

Corresponding author: H. G. Carvalho; Telephone: +351 226074900; Fax: +351 22 6099157; E-mail: mhcarval@ibmc.up.pt

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables are published online.

transcript profiles of plant mutants with varying nitrogen-fixing phenotypes and nodules formed by different rhizobial mutant strains (Maunoury et al. 2010; Moreau et al. 2011; Starker et al. 2006; Tesfaye et al. 2006). The effects of local N starvation mediated by systemic signaling were also studied in *M. truncatula*, and included the response to low N₂-fixation conditions. Using the split-root system, it was shown that a localized repression of N₂ fixation in one part of the root does not lead to increased N₂ fixation in the other part (Ruffel et al. 2008) but, over the long term, the plants respond to the local N₂-fixation impairment by stimulating growth of preexisting nodules and formation of new nodules (Jeudy et al. 2010). Taken together, these results revealed that local and systemic

signals are integrated and demonstrated that the plant response to N starvation is highly dependent on the N source. However, the transcriptional changes occurring in root nodules specifically induced by impairment of N assimilation have never been studied. Considering the critical position of GS within root nodule metabolic pathways, the enzyme constitutes an important metabolic knob controlling N assimilation. Thus, the blocking of this critical step is expected to provide important insights into the regulation of root nodule metabolism and also to elucidate the mechanisms regulating GS activity.

Because GS is an essential enzyme for plant growth and development, it has been difficult, traditionally, to isolate mutants, especially for the cytosolic isoenzymes. Thus, the use of GS

Fig. 1. Histochemical localization of β -galactosidase activity in the nitrogen-fixation zone of phosphinothricin (PPT)-treated plants. Nodules formed by a *Sinorhizobium meliloti* strain carrying a constitutive hemA:LacZ gene were collected at **A**, 0; **B**, 4; **C**, 8; **D** and **E**, 24; and **F**, 48 h after PPT treatment. Nodules were sectioned (80 μ m thick), assayed for β -galactosidase activity and photographed. The blue precipitate indicate the location of β -galactosidase activity. Scale bars = 10 μ m.

inhibitors is a good alternative to address the transcriptional changes induced upon impairment of primary N assimilation. Phosphinothricine (PPT) is a structural analogue of Glu, which irreversibly inhibits GS activity (Lea and Ridley 1990). A successful approach to study the effects of organic N signals on the transcriptome of *Arabidopsis* seedlings was already accomplished by the strategic use of GS inhibitors (Gutierrez et al. 2008). In a previous study, we analyzed the metabolome changes induced by PPT in root nodules of *M. truncatula* (Barsch et al. 2006a), which has indicated consecutive metabolic changes imposed on the nodules upon GS inhibition. Here, we describe the transcriptional changes occurring in root nodules of *M. truncatula* upon GS inhibition by PPT. This transcriptome analysis, in combination with the metabolite profiling previously reported (Barsch et al. 2006a), provide new insights into the metabolic and developmental adjustments occurring in root nodules upon impairment of ammonia assimilation. Several genes potentially involved in the molecular surveillance of internal organic N availability and N assimilation during root nodule symbiosis are identified and important transporters involved in nitrogen metabolism are pinpointed.

RESULTS AND DISCUSSION

Selection of samples for transcript profiling and evaluation of the alterations induced by PPT in root nodules.

Because GS is an essential enzyme, the inhibition of its activity will inevitably lead to plant death. Thus, it is important to discriminate between changes caused by a direct effect of local GS inhibition and those induced by long-term nitrogen deprivation. In order to select samples representative of the plant's progression from a healthy to a stressed state for a transcriptome analysis, we carefully monitored the plant's health status and the morphologic alterations occurring in the nodules over a time course of 3 days following PPT application. No visible stress symptoms were observed before 24 h of continuous exposure to the herbicide. At 48 h, the symptoms worsened to leaf wilting, tumbled stems, and brownish roots and, after 56 h of continuous exposure to PPT, the leaves started to show symptoms of advanced chlorosis and necrosis. Root nodules gradually changed from typical pink (0, 4, and 8 h) to faintly pink (24 h) and, finally, to colorless with a slight green tone, characteristic of leghemoglobin heme cofactor degradation (48 h).

Morphologic alterations induced by GS inhibition in root nodules were further analyzed by optical microscopy of nodules formed by a rhizobial strain carrying a constitutive *hemA::LacZ* gene, allowing histochemical localization of β -galactosidase activity. No significant structural differences were detected between untreated and 4-h PPT-treated nodules (Fig. 1A and B) but, at 8 h, a substantial reduction in the plastid starch content was observed (Fig. 1C) and, at 24 h, starch was severely reduced and some cells showed symptoms of premature senescence such as plasma membrane loosening and a degraded cell content (Fig. 1D), and an abnormal proliferation of infection threads was detected in nodule fixation zone III (Fig. 1E). After 48 h of PPT treatment, numerous infection threads could be observed as well as a massive invasion and proliferation of saprophytic bacteria (Fig. 1F).

Senescence is a natural process involving N remobilization from protein and amino acid catabolism but a premature senescence of root nodules can be induced under certain stress conditions (González et al. 1998; Matamoros et al. 1999; Guerra et al. 2010). The stress-induced senescence progresses faster than developmental senescence and encompasses distinctive characteristics associated with oxidative stress and plant cell death (Puppo et al. 2005; Van de Velde et al. 2006). A consid-

erable reduction in the size of the nitrogen-fixing zone and the invasion of saprophytic bacteria has been observed in root nodules of *M. truncatula* after 3 and 5 days of continuous darkness, respectively (Guerra et al. 2010). In the present study, the overall inhibition of GS activity prematurely triggered senescence of *M. truncatula* root nodules, the first structural features of global nodule senescence were detected at 24 h of PPT treatment, and invasion of saprophytic bacteria was detected after 48 h. GS inhibition not only leads to Gln depletion and, subsequently, to nitrogen deprivation but also is likely to result in the toxic accumulation of ammonia. Furthermore, GS activity is tightly linked to N remobilization during senescence. This could explain why the stress-induced structural alterations observed in this study progressed more rapidly than those reported for dark-induced senescence (Guerra et al. 2010).

Evaluation of the effects of PPT on GS activity and on the expression of individual GS genes.

The inhibition of GS activity was monitored over a time course of PPT application in protein extracts from nodules, roots, and leaves (Fig. 2A). GS activity was completely lost in roots and nodules as early as at 8 h after PPT application and also severely reduced in the leaves. Since the herbicide was applied to the nutrient solution of the aeroponic container in which the plants were grown, PPT is likely to be primarily absorbed by roots and nodules and, subsequently, translocated to the leaves. Despite the effective GS inhibition, the polypeptide content of both GS1 and GS2 remained roughly unaltered (Fig. 2B), indicating that the proteins were not rapidly degraded. However, a lower molecular weight GS polypeptide was detected at 24 and 48 h after PPT treatment (Fig. 2B). This GS proteolysis fragment has been previously detected in root nodules following PPT treatment (Carvalho et al. 2003) and could not be detected in root or leaf extracts (Fig. 2B). Because this fragment is not recognized by a GS2-specific antibody (data not shown), it is likely to arise from a selective proteolysis of the cytosolic enzyme and probably reflects a posttranslational mechanism for the regulation of GS1, exclusive to root nodules. Even though the loss of enzymatic activity does not necessarily imply a decrease in protein stability, it is possible that the presence of PPT contributes to maintain protein stability, because it has been shown that the addition of GS inhibitors increases GS protein stability in vitro (Gill and Eisenberg 2001).

To evaluate whether PPT could differentially affect the expression of the individual GS genes, we followed the transcript levels of the three GS genes expressed in the nodules *MtGS1a*, *MtGS1b*, and *MtGS2a* by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) in samples collected at several time points (0, 4, 8, 24, and 48 h) following PPT application. The expression of both the nodule-enhanced cytosolic isoform *MtGS1a* and the plastid-located isoform *MtGS2a* was severely affected by PPT (Fig. 2C), whereas the expression of *MtGS1b* was maintained at relatively constant levels (Fig. 2C). Because the expression of *MtGS1a* is related to both active nitrogen fixation and root nodule developmental constraints and *MtGS1b* is related to N remobilization during the process of senescence (Carvalho et al. 2000; Stanford et al. 1993), the observed changes in GS gene expression reflect the transition from initial inhibition of nitrogen fixation to late senescence processes. Repression of *MtGS2a* expression during senescence and as a consequence of abiotic stress has been previously reported (De Michele et al. 2009; Larrainzar et al. 2009).

Interestingly, the reduced GS transcript levels do not directly correlate with the GS polypeptide content, which remains

largely unaltered over the time course of PPT treatment. Taken together, these results reflect the complexity of GS regulation in root nodules and point toward the existence of unknown molecular players acting at both the transcriptional and post-translational levels.

Inhibition of GS significantly alters the nodule transcriptome.

In order to identify overall transcriptional changes occurring in root nodules in response to organic N depletion, we profiled the *M. truncatula* nodule transcriptome before (0 h) and after (4, 8, and 24 h) inhibition of GS activity by PPT. Global transcript changes induced by GS inhibition were assessed using the Affymetrix *M. truncatula* GeneChip array. A clear separation between the profiles of the four different samples and the clustering of the three biological replicates of each independent sample is evidenced by principal component analysis (PCA) (Fig. 3). The transcriptome of the PPT-treated nodules (at 4, 8, and 24 h) was individually compared with untreated nodules (0 h) and approximately 20% of the probe sets present in the GeneChip array were found to be at least twofold differentially expressed. Statistical significance was assessed using one-way analysis of variance (ANOVA) with a false discovery rate of 0.05 (Partek Genomic Suite 6.5).

A global view of the transcriptional changes induced by PPT is displayed as a Venn diagram (Fig. 4) and the complete dataset has been deposited in the Gene Expression Omnibus (GEO; accession number GSE34155). More than 8,000 probe sets were differentially expressed following 8 and 24 h of PPT treatment and approximately 5,000 after 4 h. In all, 2,397 probe sets overlapped in the three analyses (Fig. 4) and we anticipate that they include genes related to alterations in nodule metabolism and sensing of N starvation. A relatively high number of probe sets (1,741) were specifically altered at 4 h after PPT treatment. Transcriptional changes occurring at this time point are expected to reflect responses to a local increase in ammonia or to low levels of Gln and are unlikely to be due to a plant systemic response. A larger number of differentially expressed probes (3,037) overlap exclusively at 8 and 24 h and probably reflect a general answer to a prolonged deficit of Gln and other nitrogen and carbon building blocks. A smaller number of probe sets (697) were commonly altered at 4 and 8 h only. Among the 2,509 probe sets that change only at 24 h after PPT treatment, the majority are downregulated when compared with untreated nodules, and a substantial number correspond to nodule-specific or nodule-enhanced genes as evaluated by crossing with the public available data at the *M. truncatula* gene atlas (Benedito et al. 2008). To validate the GeneChip results, we selected 10 genes for an analysis by qRT-PCR. In general, this analysis supported the trends observed in the expression pattern from the GeneChip analysis, with some discrepancies in the expression ratios, as usually observed when comparing expression ratios using different methods (Supplementary Table S1). The differentially expressed probe sets were distributed into 34 functional categories using the MapMan software (Thimm et al. 2004) (Supplementary Table S2), which will be discussed in the following sections.

GS inhibition leads to a fast transcriptional activation of defense responses and promotes nodule senescence and loss of root nodule identity.

The molecular mechanisms underlying the evasion of the plant defense responses by *Rhizobium* spp. leading to a successful symbiotic bacterial colonization in legumes are still poorly understood (Samac and Graham 2007) but the transcriptional changes detected by the present study clearly indicate that they are disturbed upon GS inhibition. An abnormal

proliferation of infection threads was observed after 24 h of PPT treatment (Fig. 1), indicating increased bacterial infections and noticeable transcript changes related to the activation of plant defense responses were also detected. Significant alterations in the expression of genes related to biotic stress occurred at 4 h of PPT treatment and included genes involved

Fig. 2. Evaluation of the effects of phosphinothricin (PPT) on glutamine synthetase (GS) activity and on the expression of individual GS genes. Total soluble proteins or total RNA were extracted from roots, leaves, and nodules at 0, 4, 8, 24, and 48 h after PPT treatment. **A**, Total GS activity in nodules, roots, and leaves. GS activity was normalized to that found before PPT treatment. **B**, Western blot analysis of GS polypeptides. An equal amount of proteins (10 μ g) was loaded on each lane. **C**, Quantification of *MtGS1a*, *MtGS1b*, and *MtGS2a* transcripts in root nodules by quantitative reverse-transcriptase polymerase chain reaction using equal amounts of cDNA (50 ng). Transcript abundance was normalized to that found before PPT treatment. Significance test: one-way analysis of variance with Bonferroni's multiple test correction; superscript a = $0.01 < P < 0.05$, b = $0.001 < P < 0.01$, and c = $P < 0.001$.

in cell wall biology, redox, protein degradation, and secondary metabolism (Table 1). It is noteworthy that the genes belonging to this functional category displayed the highest fold-increase at all time points analyzed, with some genes being highly upregulated (>100-fold change), which is the case of TC195994 encoding a plant leucine-rich repeat polygalacturonase-inhibiting protein. These observations suggest that the activation of plant defense responses is probably one of the first outcomes of GS inhibition in root nodules. In fact, growing evidence indicates that amino acid homeostasis may play a role in plant defense (Stuttman et al. 2011; Taler et al. 2004; van Damme et al. 2009) and Gln deficiency has been especially implicated in the activation of defense responses in *Arabidopsis thaliana* (Liu et al. 2010).

Parallel to the activation of the plant defense responses, the present transcriptome analyses revealed important changes, typical of stress-induced senescence (Guerra et al. 2010), occurring predominately after 8 h of PPT treatment. These changes included a general upregulation of genes encoding proteins involved in the degradation of macromolecules (nucleic acids, lipids, sugars, and proteins) and nutrient remobilization (transporters), and downregulation of genes encoding proteins involved in cell division (DNA synthesis and cell organization) and energy production (glycolysis and tricarboxylic acid [TCA] cycle). A number of genes encoding nodule-specific proteases were upregulated (Table 2). Considering that GS1 appears to be specifically cleaved in PPT-treated nodules (Fig. 2B), it would be interesting to investigate whether GS is a substrate of any of these proteases. Another good candidate for a functional analysis is the protease encoded by TC182502, which is generally highly expressed in flowers (according to

the *Medicago* Gene Expression Atlas [MtGEA]) and was strongly induced at 24 h after PPT treatment.

Interestingly, a general inhibition in the expression of genes associated with root nodule identity was observed at the three time points analyzed, with the number of repressed probes

Fig. 4. Venn diagram depicting the number of differentially expressed and overlapping probe sets in root nodules after incubation with phosphinothricin (PPT) for 4, 8, and 24 h in relation to untreated nodules.

Fig. 3. Principal component analysis (PCA) of transcript profiles of the three replicates that were used per sample treatment; phosphinothricin (PPT) at 0, 4, 8, and 24 h.

increasing over time, being significantly higher at 24 h. Among the downregulated genes are common nodulins such as leghemoglobin, nodule-specific cysteine and glycine-rich proteins, and nodule-specific calmodulin-like proteins (Table 3). Also interesting is the repression of genes potentially involved in nodule signal transduction pathways, such as transcripts encoding NPK1-like kinases and one encoding MtIRE kinase (TC172447). MtIRE relates with the maturation of infected cells during nodule development and appears to be involved in signal transduction cascades important for the expansion and

development of both plant cells and symbiosomes (Pislariu and Dickstein 2007).

Contrasting with the general repression in nodulin expression, two nodulin-like genes were significantly upregulated upon GS inhibition: early nodulin 12 (*ENOD12*, TC187336 and TC191278) and a gene annotated as *MtN9-like* (TC174513). The *M. truncatula ENOD12* encodes a hydroxyproline-rich protein related to the rhizobial infection process (Pichon et al. 1992; Scheres et al. 1990). *MtN9-like* is similar to *MtMMPL1* (TC175348), previously annotated as *MtN9*, and was downreg-

Table 1. Selected differentially expressed transcripts related to biotic stress

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
mtr.45188.1.s1_at	AJ498901	Putative defensin 3.1 precursor	2.0	7.9	4.5
mtr.28149.1.s1_at	BG584008	NtEIG-E80 protein	4.2	9.4	6.3
mtr.41905.1.s1_at	TC182086	NtEIG-E80 protein	2.6	4.0	6.0
mtr.43144.1.s1_at	TC176656	NtEIG-E80 protein	4.8	10.9	8.7
mtr.13957.1.s1_s_at ^b	TC188548	Stress kinase	...	...	-2.7
mtr.5165.1.s1_at ^b	AW690113	Stress kinase	1.4	...	-2.5
Cell wall					
mtr.15098.1.s1_at	Medtr1g100050.1	Polygalacturonase-like protein	31.2	82.7	16.2
mtr.43467.1.s1_at	TC195994	Polygalacturonase-inhibiting protein 2 precursor	136.9	310.8	228.1
mtr.23578.1.s1_s_at	TC182150	Polygalacturonase-inhibitor protein 8	12.2	49.4	22.3
mtr.40843.1.s1_at	TC174954	Polygalacturonase-inhibitor protein	...	3.0	2.2
mtr.23580.1.s1_at	TC174330	Polygalacturonase-inhibitor protein	...	7.9	8.7
mtr.23575.1.s1_x_at	TC179106	Leucine-rich repeat, plant specific	25.1	70.0	19.8
mtr.10454.1.s1_at	TC189836	Leucine-rich repeat, plant specific	...	9.3	7.2
mtr.38181.1.s1_at	TC176405	Expansin-like B1 precursor	8.2	96.8	103.7
mtr.6648.1.s1_s_at	BQ153446	Xyloglucan endotransglycosylase/hydrolase XTH-6	12.7	18.0	21.2
Redox					
mtr.12316.1.s1_at	TC178077	Probable glutathione S-transferase	11.3	46.4	32.7
mtr.12560.1.s1_at	TC175013	Probable glutathione S-transferase	36.1	126.8	88.2
mtr.37204.1.s1_at	TC193788	Glutathione S-transferase GST 18	14.9	10.0	3.3
mtr.6489.1.s1_at	TC183170	Glutathione S-transferase, C-terminal; thioredoxin-like	28.0	56.8	28.5
mtr.31347.1.s1_at	TC176616	Peroxidase 10 precursor	2.4	5.8	10.9
mtr.38635.1.s1_at	TC183203	Peroxidase 10 precursor	4.6	14.2	34.4
mtr.8653.1.s1_at	TC200298	Peroxidase 21 precursor	2.1	3.1	28.2
mtr.8654.1.s1_at	TC177579	Peroxidase 21 precursor	2.4	5.0	42.9
mtr.42692.1.s1_at	TC186498	Glutaredoxin-C11	11.3	13.4	19.5
Protein degradation					
mtr.8066.1.s1_at	BQ139568	clp Protease proteolytic subunit	13.3	11.8	2.4
mtr.10815.1.s1_at	TC182117	F-box family protein	33.7	207.0	72.5
mtr.9107.1.s1_at	TC179896	Uncharacterized protein At3g44326.1	10.532	45.0	10.6
Secondary metabolism					
mtr.17610.1.s1_at	TC173058	Chalcone synthase 2	10.9	9.3	4.9
mtr.20185.1.s1_x_at	TC179076	Chalcone synthase 2	23.5	56.4	20.1
mtr.20464.1.s1_x_at	TC176043	Chalcone synthase 2	15.7	48.6	18.0
mtr.40122.1.s1_s_at	TC178607	Chalcone synthase 4	24.9	168.1	30.5
mtr.49572.1.s1_x_at	TC179756	Chalcone synthase 9	12.3	21.0	8.3
mtr.8555.1.s1_at	TC185309	Chalcone-flavonone isomerase	18.6	9.9	3.5
mtr.10760.1.s1_at	TC180822	N-hydroxycinnamoyl/benzoyltransferase 6	20.2	33.3	14.6
mtr.49862.1.s1_at	TC173171	SAM dependent isoflavone 7-O-methyltransferase	12.6	18.0	9.0
mtr.37340.1.s1_at	TC183962	Probable mannitol dehydrogenase	9.3	18.5	15.7
mtr.8517.1.s1_at	TC180073	Probable mannitol dehydrogenase	10.2	92.4	158.0
mtr.40504.1.s1_at	TC174347	O-methyltransferase	21.6	47.3	31.9
mtr.20618.1.s1_at	TC172944	Transferase	24.5	42.7	41.8
mtr.33589.1.s1_at	BG454551	Hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyltransferase	10.2	25.8	11.9
Disease Resistance					
mtr.12193.1.s1_at	TC180906	TIR-NBS-LRR ^c type disease resistance protein	6.8	38.8	40.5
mtr.43189.1.s1_at	TC175301	Disease resistance-responsive family protein	52.7	77.0	15.5
mtr.43322.1.s1_at	TC175807	Trypsin protein inhibitor 2	5.6	148.1	20.3
mtr.50585.1.s1_at	TC181193	Calcium-binding EF-hand; Kunitz inhibitor ST1-like	6.5	11.3	12.1
Development					
mtr.12662.1.s1_at	TC179595	Patatin-like protein	2.7	22.0	3.9
mtr.11565.1.s1_at	TC179013	Patatin-like protein	5.2	26.4	6.6
mtr.8831.1.s1_at	TC190335	Patatin-like protein 1	19.1	20.0	14.1
mtr.32228.1.s1_at	AW685311	Patatin-like protein	2.0	2.90	3.4

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

^c TIR-NBS-LRR = Toll interleukin-1 receptor nucleotide-binding site leucine-rich repeat.

ulated at 8 and 24 h. MtMMPL1 was the first nodulin shown to belong to the large-matrix metalloendoproteinase (MMP) family and it appears to play a key role in bacterial release from the infection threads (Combiér et al. 2007). Thus, it is tempting to speculate that the proliferation of infection threads that was observed after 24 h of PPT treatment (Fig. 2E) is related to the significant changes in the expression of *MtIRE*, *ENOD12*, *MtN9-like*, and *MtMMPL1*.

Taken together, the present transcriptome analysis suggests that GS inhibition triggers a fast plant defense response, inhibits nodule growth or function, and induces premature nodule senescence. In the legume–rhizobia symbiosis, the plant exerts a tight control over the bacteroids, mainly imposed by the metabolic dependence of the microsymbiont on nutrients delivered by the plant cell (Oldroyd et al. 2011). Considering the time lag between the activation of plant defense responses (4 h)

Table 2. Selected differentially transcribed genes encoding proteases

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
mtr.43786.1.s1_at ^b	TC176928	Cysteine proteinase	1.4	2.5	2.0
mtr.43787.1.s1_at ^b	TC174778	Cysteine proteinase	...	2.2	2.2
mtr.24920.1.s1_at	TC175694	Wound-inducible carboxypeptidase	...	2.4	2.4
mtr.27783.1.s1_s_at	BE999360	Serine carboxypeptidase	...	3.9	2.4
mtr.44810.1.s1_at	TC202096	Cysteine protease 8	...	3.4	2.5
mtr.38411.1.s1_at	TC182502	Pre-pro-TPE4A protein precursor	...	2.7	15.1

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

Table 3. Selected differential transcribed genes related to root nodule identity

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
mtr.20845.1.s1_at	TC197903	MtN3 and saliva related transmembrane protein	–2.9	–14.2	–5.3
mtr.43552.1.s1_at	TC175348	MtMMPL1 (MtN9)	...	–2.9	–7.8
mtr.37328.1.s1_at	TC199253	MtN11	...	–7.2	–6.1
mtr.8912.1.s1_at	TC192818	MtN16	...	–4.9	–10.8
mtr.13992.1.s1_at	TC196390	MtN17 precursor	...	–5.1	–5.9
mtr.35863.1.s1_at	TC194315	MtN17 precursor	–1.8	–11.8	–4.6
mtr.12845.1.s1_at	TC174820	MtN21	...	–1.2	–4.0
mtr.41330.1.s1_at	TC191980	MtN25	–1.3	–1.7	–3.0
mtr.41813.1.s1_at	TC183769	MtN25	...	–1.1	–2.5
mtr.4672.1.s1_at	AL374752	MtN28 precursor	...	...	–4.3
mtr.38556.1.s1_at	TC187558	Nodule-specific cysteine-rich peptide 101	...	–1.2	–2.1
mtr.43624.1.s1_at	TC192476	Nodule-specific cysteine-rich peptide 144	...	...	–2.2
mtr.9428.1.s1_at	TC194580	Nodule-specific cysteine-rich peptide 146	2.4	...	–2.4
mtr.8724.1.s1_at	TC188203	Nodule-specific cysteine-rich peptide 165	–1.3	–1.6	–4.8
mtr.27142.1.s1_at	AW573795	Nodule-specific cysteine-rich peptide 290	...	–6.2	–47.6
mtr.3701.1.s1_at	BG580305	Nodule-specific cysteine-rich peptide 290	–1.2	–1.8	–2.8
mtr.7542.1.s1_at	TC199561	Nodule-specific cysteine-rich peptide 93	...	–1.5	–3.4
mtr.44665.1.s1_at	TC183607	Nodule-specific glycine-rich protein 2A	...	...	–2.6
mtr.50169.1.s1_at	TC175315	Nodule-specific glycine-rich protein 4	–1.4	–1.2	–4.3
mtr.44835.1.s1_at	TC192001	Nodule-specific glycine-rich protein 5A	...	–2.0	–4.9
mtr.10578.1.s1_at	TC195776	Calmodulin-like protein 1	–1.2	–1.2	–2.4
mtr.12956.1.s1_at	TC174472	Calmodulin-like protein 4	...	–2.0	–3.6
mtr.1587.1.s1_at	AW573662	Calmodulin-related	–1.5	–1.2	–3.3
mtr.37968.1.s1_at	TC180046	Calmodulin-like protein 3	...	–1.5	–7.5
mtr.38519.1.s1_at	TC185827	Calmodulin-like protein 5	...	–1.4	–2.1
mtr.40731.1.s1_at	TC174499	Calmodulin-like protein 2	...	–1.2	–2.4
mtr.43719.1.s1_at	TC177985	Calmodulin-like protein 6b	...	–1.5	–2.5
mtr.43465.1.s1_at	TC179415	Leghemoglobin 29	–1.1	–1.1	–2.5
mtr.38572.1.s1_at	TC177903	Leghemoglobin	...	–1.3	–3.8
mtr.51231.1.s1_x_at	TC177238	Leghemoglobin 2	–1.1	–1.1	–2.9
mtr.40141.1.s1_at	TC197420	Leghemoglobin 1	...	–1.1	–2.3
mtr.7223.1.s1_at	TC191342	DMI3 gene for calcium/calmodulin dependent protein kinase	...	–14.4	–12.0
mtr.12197.1.s1_at	TC193956	Leucine-rich repeat transmembrane protein kinase	–4.9	–8.5	–10.9
mtr.39419.1.s1_at	TC187093	Putative receptor protein kinase	–2.2	–5.2	–5.5
mtr.28099.1.s1_at	BG581960	Receptor protein kinase putative	–5.5	–5.1	–11.6
mtr.2208.1.s1_at	BG580126	C2 calcium/lipid-binding region (CaLB)	–1.6	–2.2	–2.4
mtr.39939.1.s1_at	TC182412	NPK1-like kinase	–1.4	–2.9	–8.4
mtr.49069.1.s1_at	TC187808	NPK1-like kinase	–1.9	–4.3	–11.6
mtr.38617.1.s1_at	TC172447	IRE-like protein	–1.6	–2.7	–5.2
mtr.899.1.s1_at	TC172467	U-box protein	–1.2	...	–2.5
mtr.13417.1.s1_at	TC182139	SINA2p	–1.4	–1.8	–3.5
mtr.3197.1.s1_at	Medtr5g091550.1	Ubiquitin carboxyl-terminal hydrolase	–3.3	–25.5	–11.4
mtr.43047.1.s1_at	TC187336	Early nodulin 12	...	3.1	2.2
mtr.43048.1.s1_at	TC191278	Early nodulin 12	...	...	12.3
mtr.18178.1.s1_at	TC174513	MtN9-like protein	13.7	13.5	5.0

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

and visible signs of abnormal infection (24 h), it appears that, when no metabolic compensation is attained, the plant activates another level of control involving a rapid induction of defense responses and nodule senescence. The activation of these processes is probably reversible at least until 24 h of PPT treatment, a time when the nodule identity appears to be severely compromised.

GS inhibition leads to consecutive changes in nitrogen metabolism.

As expected, the present study identified marked changes in the expression of several genes directly involved in nitrogen and amino acid metabolic pathways (Table 4). Major changes were detected in the pathways for the synthesis of asparagine (the major N-export compound in *M. truncatula*) and amino acid catabolism, with genes involved in Asn synthesis being repressed and many genes involved in amino acid catabolism being upregulated. Finally, an alternative pathway for Glu synthesis and subsequent production of the metabolite γ -amino butyrate (GABA) from Glu appears to be activated upon GS inhibition. The results will be discussed, taking into account the previous metabolome analysis of PPT-treated *M. truncatula* root nodules (Barsch et al. 2006a).

The metabolite profiling indicates that the primary effect of GS inhibition by PPT is metabolically reflected by a drastic decrease in Gln and Asn to almost undetectable levels over the

course of PPT treatment (Barsch et al. 2006a). However, Glu is maintained at relatively constant levels throughout the time course (Barsch et al. 2006a). Gln is a substrate for the enzymatic reactions catalyzed by NADH-GOGAT and asparagine synthetase (AS). Consistent with the reduction in Asn content, we detected a decrease in AS transcripts (AW684943, TC193976, TC200096, and TC179002) over the treatment, possibly reflecting the lack of Gln for Asn synthesis. However, NADH-GOGAT transcripts do not appear to be affected by GS inhibition, in spite of the lack of Gln, an essential substrate for Glu synthesis. Conversely, Glu dehydrogenase (GDH, TC191599) was upregulated after 8 h of PPT treatment, suggesting that this enzyme could contribute to Glu production, replacing inhibited GS. Although it is considered that GDH is only involved in Glu catabolism to form ammonia (Lea and Mifflin 2010), several reports suggested that GDH could operate in vivo in the direction of ammonium assimilation (Magalhaes 1991; Magalhaes et al. 1990; Melo-Oliveira et al. 1996; Yamaya and Oaks 1987; Yamaya et al. 1986). Studies in tobacco demonstrated that, in the presence of the GS inhibitor methionine-S-sulfoximine, GDH acquired increased in vivo aminating activity, evidenced by the incorporation of $^{15}\text{NH}_4$ into Glu (Skopelitis et al. 2006, 2007). Thus, it is conceivable that GDH could represent an alternative pathway for ammonia assimilation in PPT-treated nodules. Although less energetically favorable compared with the GS/GOGAT cycle, the assimilation of nitrogen-fixed ammonia by GDH could be

Table 4. Selected differentially expressed transcripts related to metabolism

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
Metabolism, nitrogen					
mtr.43550.1.s1_at	TC189738	Tyrosine aminotransferase	5.1	22.2	49.0
mtr.28325.1.s1_at	BG589023	Branched-chain α -keto acid decarboxylase	2.0	2.6	4.2
mtr.40481.1.s1_at	TC173473	Isovaleryl-CoA Dehydrogenase	2.2	4.8	5.6
mtr.32211.1.s1_at ^b	AW684943	Asparagine synthetase	...	-4.2	-6.1
mtr.7084.1.s1_at	TC193976	Asparagine synthetase, root (glutamine-hydrolyzing)	-2.1	-7.6	-2.1
mtr.25243.1.s1_at	TC200096	Asparagine synthetase, root (glutamine-hydrolyzing)	...	-2.2	-2.6
mtr.39335.1.s1_at	TC179002	Asparagine synthetase, type III	-3.4	-2.2	-3.0
mtr.43193.1.s1_at	TC191599	Glutamate dehydrogenase 1	1.6	2.2	2.6
mtr.43279.1.s1_at	TC188693	Proline dehydrogenase	3.2	5.6	7.8
mtr.919.1.s1_s_at	TC187778	Pyrroline-5-carboxylate reductase	-3.0	-1.9	-1.3
mtr.44607.1.s1_at	TC188400	Pyrroline-5-carboxylate synthetase 2	...	-2.2	-2.9
mtr.12906.1.s1_at	TC185281	Glutamate decarboxylase	5.9	22.7	5.0
mtr.52344.1.s1_at	TC172971	Aspartate aminotransferase, mitochondrial precursor	2.8	6.6	6.9
mtr.52346.1.s1_at	TC172996	Aspartate aminotransferase, mitochondrial precursor	1.8	2.8	2.7
mtr.31448.1.s1_at ^b	AJ848642	Nitrate reductase	3.0	2.2	-2.1
mtr.10480.1.s1_at	TC181750	Glutamine synthetase leaf isoenzyme, chloroplast precursor	...	-1.3	-2.4
Metabolism, carbon					
mtr.13813.1.s1_s_at	TC178491	Alkaline/neutral invertase	-3.8	-3.2	-2.2
mtr.2239.1.s1_at ^b	TC200940	Sucrose synthase	...	-3.4	...
mtr.45037.1.s1_at	TC189675	Sucrose synthase 2	...	-2.1	-1.7
mtr.35037.1.s1_at ^b	CX531367	Sucrose synthase	...	-3.3	-2.1
mtr.38643.1.s1_s_at ^b	TC172471	Sucrose-phosphate synthase	...	-2.3	-2.4
mtr.10532.1.s1_s_at ^b	TC172490	Phosphoenolpyruvate carboxylase	-4.7	-4.6	-4.8
mtr.23663.1.s1_at ^b	TC174339	Phosphoenolpyruvate carboxylase kinase	-1.5	-3.2	-4.4
mtr.8784.1.s1_s_at	CB891719	Pyruvate kinase	-2.8	...	...
mtr.754.1.s1_at	TC180986	Pyrophosphate-dependent phosphofructokinase	-2.2	-3.4	-2.1
mtr.49030.1.s1_at	TC172754	Pyrophosphate-dependent phosphofructo-1-kinase	...	-1.4	-2.1
mtr.43821.1.s1_at ^b	TC175608	Pyrophosphate-dependent phosphofructo-1-kinase	-1.2	-1.4	-2.9
mtr.43088.1.s1_at	TC178525	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase	-1.4	-2.2	-2.4
mtr.37562.1.s1_at	TC183421	Succinate dehydrogenase, mitochondrial precursor	-5.8	-4.4	-3.2
mtr.29250.1.s1_s_at	TC192517	Malate dehydrogenase precursor; mitochondria	-2.6	-2.4	-2.3
mtr.7569.1.s1_at	TC194668	Fructose-1,6-bisphosphatase, cytosolic	2.3	6.8	9.7
mtr.22592.1.s1_at	TC180413	Fructose-1,6-bisphosphatase, cytosolic	2.5	4.8	7.1
mtr.34416.1.s1_at	TC186614	Pyruvate, phosphate dikinase, chloroplast precursor	1.7	14.6	35.6
mtr.45269.1.s1_at	TC180431	NADP-dependent malic enzyme; chloroplast	1.6	1.9	2.7
mtr.8803.1.s1_at	TC177256	Malic enzyme; chloroplast	...	1.6	2.1
mtr.40239.1.s1_at	TC175091	Phosphoenolpyruvate carboxykinase	-1.6	-1.4	2.2

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

a temporary option to cope with the metabolome fluctuations induced by GS inhibition.

Alternatively, Glu could be derived from proline (Pro) catabolism, via the sequential action of Pro dehydrogenase and pyrroline-5-carboxylate dehydrogenase. PPT treatment stimulated the sustained expression of Pro dehydrogenase (TC188693) and, conversely, repressed the expression of transcripts related to Pro synthesis, pyrroline-5-carboxylate reductase (P5CR; TC187778), and pyrroline-5-carboxylate synthetase (P5CS; TC188400). P5CR was repressed earlier, at 4 h of PPT treatment, and P5CS was repressed later on, at 8 and 24 h of treatment. These results suggest that, upon GS inhibition, Glu could be produced from Pro catabolism. Pro, in turn, may result from catabolism of abundant nodule hydroxyproline-rich glycoproteins or by de novo synthesis from ornithine (Szabados and Savoure 2010).

The metabolome profile of PPT-treated nodules also revealed an increase in the amount of several amino acids at the later time points (24 and 48 h), possibly arising from protein degradation resulting from senescence (Barsch et al. 2006a). Consistently, the transcriptome analysis identified several genes related to amino acid catabolism upregulated, correlating with N-remobilization processes occurring during senescence. Because it has been suggested that the bacteroids become auxotroph for branched-chain amino acids (Prell et al. 2009), and the branched-chain amino acids valine, isoleucine, and leucine are among the amino acids showing the highest increase in the metabolome analyses, it is tempting to consider that, upon GS inhibition, these amino acids are no longer transported to the bacteroid and accumulate in the nodule. Consistent with this hypothesis, several genes related to branched-chain amino acid catabolism (for example, TC189738, BG589023, and TC173473) were found to be upregulated at the later time points of PPT treatment.

Another interesting finding is that the inhibition of GS activity leads to a sustained upregulation of a gene encoding Glu decarboxylase (GAD, TC185281), an enzyme responsible for the synthesis of GABA from Glu. It is well known that GABA is very abundant in root nodules but its physiological function has still

not been clarified (Miller 1991; Scharff et al. 2003; Streeter 1987; Sulieman and Schulze 2010; White et al. 2008). It was suggested that it could be involved in an amino acid cycle between the bacteroid and the plant, possibly playing a dual role as a metabolite and as a signal molecule. Even though GABA usually accumulates in response to stress situations (Crawford et al. 1994; Ramputh and Bown 1996; Wallace et al. 1984; Xing et al. 2007), the metabolome analysis indicated a decrease in GABA following PPT treatment of root nodules (Barsch et al. 2006a). This apparent contradiction suggests that GABA could be rapidly metabolized in response to GS inhibition.

With respect to alterations in nitrogen metabolic enzymes, it is also worth referring to the upregulation of two transcripts encoding mitochondrial aspartate aminotransferases (TC172971 and TC172996) at the three time points analyzed. Finally, it is worth mentioning a transcript coding for a nodule-specific NR (AJ848642), which was differentially expressed in response to PPT. The existence of nodule-specific NR is intriguing, because nitrate is not a source of nitrogen in root nodules; however, recently, it was proposed that a nitrate-NO respiration process in nodules could play a role in the maintenance of the energy status required for nitrogen fixation under oxygen-limiting conditions (Horchani et al. 2011).

Carbon and nitrogen metabolism are tightly linked and, thus, it is not surprising that the expression of a great number of genes related to carbon metabolism was considerably changed in response to GS inhibition (Table 4). The first steps of sucrose metabolism were found to be repressed, and a transcriptional deregulation of the glycolytic pathway and the TCA was observed, followed by the activation of gluconeogenesis. Although PPT was shown to affect the rate of photosynthesis (Wendler et al. 1990) and, thus, sucrose production is likely reduced in the PPT-treated plants, it is also conceivable that the decrease in organic N content induced upon GS inhibition is sensed by the plant, most probably as Asn, subsequently leading to a repression of photosynthate allocation to root nodules. Indeed, the synthesis of organic acids was found to be reduced in inefficient alfalfa root nodules, leading to a premature senescence (Barsch et al. 2006b).

Table 5. Selected differentially regulated transcripts related to starch metabolism

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
Starch degradation					
mtr.34260.1.s1_at	BQ141786	β-Amylase	2.4	1.6	−1.4
mtr.47857.1.s1_at	TC173806	β-Amylase	5.4	2.1	−4.4
mtr.41955.1.s1_at	TC194935	β-Amylase	−1.7	3.2	4.1
mtr.11170.1.s1_at	TC184048	β-Amylase	...	2.6	3.2
mtr.44431.1.s1_at	TC189925	β-Amylase	−3.3	−2.5	−2.2
mtr.44856.1.s1_at	TC174426	β-Amylase 1	−3.3	−1.8	−1.6
mtr.32662.1.s1_at	BE249138	β-Amylase 1	−6.6	−2.0	...
mtr.13958.1.s1_at	TC195261	β-Amylase	−10.8	−2.4	...
mtr.24415.1.s1_at	TC178239	α-Amylase	1.7	3.2	3.0
mtr.33111.1.s1_at	BF642292	α-Amylase, Plastid	−2.1	−1.9	−2.9
mtr.12907.1.s1_at	TC183382	α-Amylase, Plastid	...	−2.3	−2.8
mtr.13829.1.s1_at	TC199794	α-Amylase	...	−1.4	−2.2
mtr.31115.1.s1_at	AJ388863	α-Amylase, Plastid	...	−1.4	−2.4
mtr.11554.1.s1_at ^b	TC180717	α-Amylase 2	...	−1.7	−2.2
mtr.26355.1.s1_s_at ^b	Medtr2g014460.1	Glycogen/starch/α-glucan phosphorylase	−3.0	−4.7	−3.9
mtr.32476.1.s1_at	AW693214	α-1,4 Glucan phosphorylase L, chloroplast precursor	−7.1	−20.5	−23.0
mtr.36133.1.s1_at	AJ848698	α-1,4 Glucan phosphorylase L, chloroplast precursor	−8.9	−14.4	−13.2
Starch synthesis					
mtr.40706.1.s1_s_at	TC172751	Glucose-1-phosphate adenylyltransferase	−3.0	−16.3	−15.6
mtr.3071.1.s1_at	CB893818	Starch synthase, isoform V	−2.2	−2.6	−5.9
mtr.6780.1.s1_at	CX524404	Starch synthase-like protein	−2.6	−3.5	−6.4

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

As discussed in previous sections, PPT treatment resulted in a dramatic loss in nodule starch content (Fig. 2). Not surprisingly, genes encoding enzymes involved in starch synthesis were downregulated (Table 5), which in turn can be related to the decreased sucrose pool in PPT-treated nodules. In addition, the expression of some genes related to starch degradation was upregulated (Table 5).

The metabolic alterations induced by GS inhibition are reflected by changes in the expression of specific transporters.

Transport between the bacteroids and the plant cell is crucial for nodule functioning. The main nutrient exchange between the plant and the bacteroid through the peribacteroid membrane is fixed nitrogen to the plant and reduced carbon to the bacteroid (Peiter and Schubert 2003). Thus, transporters involved in nutrient sharing between the plant and the bacteroid as well as the transporters involved in the translocation of metabolites to and from other plant organs are essential for root nodule functioning. Upon GS inhibition, major changes in transport compounds will inevitably occur, as illustrated by the extensive metabolic changes described in the previous sections. Thus, the present transcriptome analysis provides a valuable tool to identify new transporters important for nodule functioning. It is noteworthy that the genome of *M. truncatula* encodes at least 3,830 transporters, from which only 50 are specifically expressed in root nodules (Benedito et al. 2010).

A number of transporters were found to be differentially regulated upon GS inhibition and are likely to be of critical importance for symbiotic nitrogen fixation (Table 6). Among the upregulated genes, we identified several genes encoding putative amino acid permeases, which are possibly related to alterations in the amino acid metabolism, including a transcript related to a Pro transporter (TC188303). Interestingly, two

transcripts encoding putative cationic amino acid transporters (TC202527 and AL375006), which generally show a nodule-enhanced expression (according to MtGEA), were downregulated.

The metabolic alterations induced in root nodules upon GS inhibition are also reflected by changes in several metabolite transporters associated with different organelles. A consistent repression of transcripts related to a chloroplast glutamate or malate translocator (TC182829, TC189528, and CX522228) was detected in response to GS inhibition. On the other hand, the expression of several genes encoding mitochondrial carriers was considerably upregulated (TC180166, Medtr8g086200.1, TC174183, and TC184453), with the expression of the mitochondrial phosphate transporter (TC184453) being particularly high at the three time points analyzed.

We also detected remarkable changes in the expression of transporters associated with vacuoles and peribacteroid membranes, from which a vacuolar citrate/H⁺ symporter (TC193835) showed the highest upregulation ratio. The expression of several genes encoding transporters belonging to the H⁺-dependent oligopeptide transporter family (POT/PTR), which, according to MtGEA, are normally induced during nodule development in *M. truncatula*, was consistently repressed (AW980440, Medtr7g092250.1, Medtr7g092230.1, and AL377677). It is noteworthy that a dicarboxylate transporter (AgDCAT1) related to the POT/PTR has been located on the symbiotic membrane of actinorrhizal nodules in alder (Jeong et al. 2004). Although the transporters identified in the present analysis may not be related to AgDCAT1, it would be interesting to explore their localization and biochemical functionality.

Upon GS inhibition, nitrogenase is subsequently inactivated and, thus, a considerable decrease in ammonium production and export from the bacteroids to the plant cell is expected to occur. Consistently, a number of aquaporins and other trans-

Table 6. Selected differentially regulated transcripts related to transport

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
mtr.17426.1.s1_at	TC185304	Amino acid transporter	3.6	6.5	4.7
mtr.41255.1.s1_at	TC173772	Amino acid permease AAP3	2.6	4.3	2.6
mtr.31888.1.s1_at	AL384207	Amino acid permease	1.6	2.2	2.7
mtr.7077.1.s1_at	TC174746	Amino acid carrier	1.5	4.8	2.6
mtr.44129.1.s1_at	TC188303	Betaine/proline transporter	2.9	5.1	2.9
mtr.42325.1.s1_at ^b	TC202527	Cationic amino acid transporter, plastid-located	-2.3	-6.4	-11.8
mtr.4676.1.s1_at ^b	AL375006	Cationic amino acid transporter, plastid-located	...	-2.5	-5.0
mtr.8920.1.s1_at	TC182829	Glutamate/malate translocator, plastid-located	-3.2	-3.8	-3.7
mtr.13956.1.s1_at	TC189528	Glutamate/malate translocator, plastid-located	-2.0	-3.0	-3.1
mtr.6743.1.s1_at	CX522228	2-oxoglutarate/malate translocator, plastid-located	-2.3	-3.7	-2.9
mtr.1281.1.s1_at	TC180166	Carnitine/acylcarnitine translocase, mitochondrial	2.4	4.8	7.9
mtr.19711.1.s1_at	Medtr8g086200.1	2-oxoglutarate/malate carrier protein, mitochondrial	2.8	3.6	5.1
mtr.22571.1.s1_at	TC174183	Dicarboxylate carrier protein, mitochondrial	...	3.2	2.8
mtr.41653.1.s1_at	TC184453	Phosphate transporter, mitochondrial	24.9	85.8	39.8
mtr.43425.1.s1_at	TC193835	Citrate/H ⁺ symporter, vacuolar	3.0	6.1	8.3
mtr.12578.1.s1_at	TC176111	Citrate/H ⁺ symporter, vacuolar	...	3.6	5.2
mtr.32523.1.s1_at ^b	AW694862	Proton-pumped oligopeptide transporter family (POT)	-2.6	-2.4	-7.0
mtr.18056.1.s1_at ^b	Medtr7g092250.1	Oligopeptide transporter superfamily (OPT)	-2.0	-5.6	-6.1
mtr.49182.1.s1_at ^b	Medtr7g092230.1	Oligopeptide transporter superfamily (OPT)	-1.6	-3.4	-4.9
mtr.31737.1.s1_at ^b	AL377677	Peptide transporter	...	-2.8	-5.9
mtr.36842.1.s1_s_at	CX531726	Nodulin 26-like protein	-2.1	-2.3	-2.3
mtr.2246.1.s1_at ^b	BG582951	Multifunctional aquaporin, Nodulin 26-like protein	1.3	-2.8	-5.2
mtr.37525.1.s1_at ^b	TC173643	Multifunctional aquaporin, Nodulin 26-like protein	...	-1.8	-3.1
mtr.24929.1.s1_at ^b	TC173346	NIP3	-2.6	-7.4	-2.2
mtr.36889.1.s1_s_at ^b	TC194213	NIP3	-2.4	-6.2	-1.9
mtr.19346.1.s1_s_at ^b	TC174264	NIP2	2.3	8.5	6.9
mtr.10556.1.s1_at	TC173899	Ammonium transporter	1.9	2.8	2.0
mtr.25576.1.s1_at	Medtr8g095040.1	Ammonium transporter 3 member 1	-2.1	-7.8	-11.8

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

porters related to ammonium transport showed altered expression. PPT treatment repressed the expression of the aquaporin nodulin 26-like intrinsic protein (NIP) (CX531726) at the three time points analyzed, and of two additional transcripts encoding multifunctional aquaporins, which show a nodule-enhanced expression (BG582951 and TC173643). The expression of NIP3 (TC194213 and TC173346) was also downregulated but the expression of NIP2 (TC174264) was consistently increased in response to PPT treatment. This contrasting alteration in two related NIP suggest a specific involvement in different transport processes. It has been shown that N compounds can differentially modulate the expression of some aquaporins; the maize aquaporin ZmPIP1;5b is strongly induced by nitrate (Gaspar et al. 2003) whereas the *Arabidopsis* transporter AtTIP2;1 is induced under long-term N starvation or short-term NH₄⁺ supply (Loque et al. 2005). Thus, it is believable that the expression of the *M. truncatula* NIP2 and NIP3 is differentially regulated in response to changes in the Glu/Gln ratio.

Strikingly, a high-affinity ammonium transporter related to *Lotus japonicus* AMT1.1 (TC173899) was upregulated at 8 and 24 h (D'Apuzzo et al. 2004). In *L. japonicus*, it has been shown that even though AMT1;1 is expressed in N₂-fixing nodules, the protein is located at the plasma-membrane and, therefore, is likely to be involved in the uptake of ammonia lost from infected cells to the apoplast prior to its assimilation (Rogato et al. 2008). The upregulation of a high-affinity ammonium transporter in response to PPT treatment suggests that the plant operates to increase recovery of free ammonium from the apoplast despite GS inhibition. Particularly interesting is the sustained PPT-induced downregulation of a transcript annotated as ammonium transporter 3 (Medtr8g095040.1). Because the molecular identity of the symbiotic ammonium transporter is currently unknown, it would be interesting to

explore the subcellular localization and biochemical functionality of this protein because it represents a good potential candidate.

Candidate genes potentially involved in nitrogen sensing and regulation.

Control of nutrient and energy homeostasis is crucial for plant survival, particularly under the energy-demanding process of root nodule symbiosis. Hence, the plants must possess sensing mechanisms to enable the coordination of nitrogen fixation and the control of carbon allocation to root nodules. It has been recently suggested that a large part of the regulators of active nitrogen fixation are shared with other metabolic pathways in the plant (Moreau et al. 2011). Although essential genes involved in the autoregulation of nodulation have already been identified (Krusell et al. 2002; Nishimura et al. 2002; Schnabel et al. 2005; Searle et al. 2003), the key regulators of nodule function remain to be identified. Previous studies have assigned some genes as putative nitrogen sensors or regulators. The current transcriptome analyses support those conjectures and pinpoint novel candidates (Table 7).

Regulation of gene expression is a complex mechanism in which transcription factors (TF) are key components. Specifically, at 4 h of PPT treatment, we detected increased expression of six TF: one HLH (TC180866), one WRKY (TC199439), one C2H2 (BG584080), two homeobox (TC178713 and TC180000), and one DOF (TC180225). TF showing a sustained increase in expression include a gene encoding a protein with a MADS-box domain (TC190669), one GRAS TF (TC179041), and two TF, homologues to AtbZIP63 (TC174709) and AtbZIP53 (TC184041). The upregulation of the bZIP TF in response to GS inhibition is particularly interesting, because they appear to be involved in nutrient signaling. The *A. thaliana* group S1 basic leucine zipper TF AtbZIP1 and AtbZIP53

Table 7. Selected differentially regulated transcripts related to nitrogen sensing and regulation

Target identification	Gene ID	Annotation	Fold change, phosphinothricin at ^a		
			4 h	8 h	24 h
Transcription factors					
mtr.48694.1.s1_at	TC180866	Helix-loop-helix DNA-binding	2.3	...	...
mtr.32745.1.s1_at	TC199439	WRKY4	2.2	1.7	...
mtr.28153.1.s1_at	BG584080	Zinc finger, C2H2-type	2.1	...	...
mtr.28788.1.s1_at	TC178713	Homeobox-leucine zipper protein 3	2.1	1.9	...
mtr.43645.1.s1_at	TC180000	Homeobox-leucine zipper protein	2.9	...	...
mtr.10824.1.s1_at	TC180225	C2C2(Zn) DOF zinc finger family	2.3	1.4	1.6
mtr.17688.1.s1_at	TC190669	MADS-box	4.0	2.6	2.3
mtr.22745.1.s1_at	TC179041	GRAS	7.2	11.5	19.0
mtr.26044.1.s1_at	TC197332	Homeodomain-related cca1	-8.8	-21.6	-3.0
mtr.42879.1.s1_at	TC174709	BZIP (AtbZIP63)	4.6	8.3	7.3
mtr.46539.1.s1_at	TC184041	BZIP (AtbZIP53)	1.2	2.6	2.6
mtr.4532.1.s1_at ^b	TC19629	Zinc finger, C2H2-type	-2.3	-3.0	-2.7
mtr.50932.1.s1_at ^b	TC195970	Homeodomain-like	-2.3	-9.2	-6.4
mtr.9880.1.s1_at ^b	TC188675	DHHC-type zinc finger family protein	-2.1	-2.2	-2.2
mtr.48656.1.s1_at	TC172416	ENBP1 protein	-2.1	-2.8	-2.0
mtr.43750.1.s1_at ^b	TC95981	MtHAP2.1	...	-2.3	-2.4
mtr.44789.1.s1_at	TC98097	MtNSP2	-1.7	-2.5	-4.0
mtr.14008.1.s1_at	TC182346	NIN-like protein 7, plant regulator RWP-RK	1.4	1.9	2.3
mtr.41967.1.s1_at	TC186859	NIN-like protein 1	-2.3	-3.4	-2.1
Putative nitrogen sensors and regulators					
mtr.30792.1.s1_at ^b	CA989716	ES43 like protein.	2.3	-1.7	-2.7
mtr.18794.1.s1_at	Medtr6g065170.1	Cell differentiation protein. Rcd1-like	2.3	...	1.7
mtr.7416.1.s1_at	TC175949	Uridyl transferases-like	2.1	-4.6	-4.9
mtr.42555.1.s1_at	TC190362	Glutamine synthetase I	...	1.5	2.9
mtr.5776.1.s1_at	BF648697	Nodulin/glutamate-ammonia ligase-like (FluG)	-2.0	...	1.9
mtr.9502.1.s1_at	TC193978	Nodulin 6	-2.3	-2.0	...
mtr.44342.1.s1_at ^b	TC179293	Nitrate transporter	-2.2	-4.0	-9.3

^a Ellipses (...) indicate values with no statistical significance ($P > 0.05$).

^b Probe sets found to be nodule specific or preferentially expressed in nodules according to the *Medicago truncatula* gene atlas database (Benedito et al. 2008).

are activated under low energy stress and it has been shown that they bind regulatory *cis* elements present in the promoters of asparagine synthetase and Pro dehydrogenase (Dietrich et al. 2011). The binding capacity of AtbZIP1 to the promoters appears to be affected by heterodimerization with a C-group AtbZIP as AtbZIP63 (Kang et al. 2010). Therefore, is it appealing to investigate whether these two TF are modulated by Gln and whether they are involved in the regulation of AS and PDH expression in *M. truncatula* root nodules. Also noteworthy is the continued decrease in expression of a homeodomain TF (TC197332) related to the circadian clock-associated gene (*CCA1*), because its expression was shown to be modulated by Glu or a Glu-derived metabolite in *Arabidopsis* and to subsequently regulate the expression of key N-assimilatory genes (Gutierrez et al. 2008).

Regarding downregulated TF, it is worth referring three putative TF which were repressed at the three time points analyzed (TC19629, TC195970, and TC188675) which, according to in silico analysis (based on the current MtGEA), correspond to genes with either nodule-specific or nodule-enhanced expression. These uncharacterized genes may constitute transcriptional regulators important for nodule function. Among the downregulated TF we also detected a transcript encoding ENBP1 (TC172416), whose *Vicia sativa* ortholog has been shown to act as a repressor of ENOD12b (Hansen et al. 1999). This result is consistent with the observed increased expression of *ENOD12* (Table 7).

One special feature of indeterminate nodules is the maintenance of a persistent meristem. The CCAAT-binding factor MtHAP2.1 is a key regulator of root nodule meristem function (Combier et al. 2006, 2008) and its expression was repressed after 8 h of PPT treatment. Interestingly, NODULATION SIGNALING PATHWAY 2 (NSP2) is also among the TF with decreased expression at 8 and 24 h of PPT treatment. Because NSP2 together with NSP1 are indispensable for Nod-factor signaling and nodule morphogenesis (Heckmann et al. 2006; Kalo et al. 2005; Smit et al. 2005), these findings support the notion that, at 8 h of PPT treatment, the symbiotic identity is compromised and that these two genes may be under the control of nitrogen-responsive elements.

Among the nodule-specific genes showing altered expression are also nodule inception (*Nin*)-like genes. NIN proteins are key components of nodule development (Geurts et al. 2005; Marsh et al. 2007; Schauser et al. 1999), but *Nin*-like genes are also present in non-legume plants and in algae genomes (Schauser et al. 2005). The proteins are characterized by a conserved RWP-RK DNA-binding domain, which appears to be involved in the regulation of genes that are controlled by nitrogen status. The chlamydomonas NIT2 protein is structurally related to NIN and its expression was found to be negatively regulated by ammonium (Camargo et al. 2007), and the *A. thaliana* NIN-like protein NLP7 was recently proposed as a new regulatory protein specific for nitrogen assimilation (Castaings et al. 2009). Our transcriptome analysis revealed an increased expression of a gene encoding a protein homologue to AtNLP7 (TC182346) after 8 h of PPT treatment. Conversely, a gene related to the *L. japonicus* NIN-like protein 1 was downregulated at the three time points analyzed (TC186859).

Also interesting is the upregulation of a gene encoding a protein with BAH and PHD domains (CA989716) only at 4 h. BAH and the PHD domains were suggested to mediate protein-protein interactions and are probably associated with chromatin remodeling processes crucial for transcriptome reprogramming. CA989716 is also specifically expressed in root nodules (according to MtGEA) and encodes a protein similar to the uncharacterized ES43 protein from barley (Speulman and Salamini 1995) and to two important *Arabidopsis* develop-

mental proteins, SHORT LIFE (Mussig et al. 2000) and SHL1 EARLY BOLTING IN SHORT DAYS (Pineiro et al. 2003). It is noteworthy that another gene encoding a required cell differentiation 1 (Rcd1)-like domain protein (Medtr6g065170.1) was also upregulated at 4 h of PPT treatment. Rcd1-like proteins were initially identified as essential factors for differentiation in fission yeast during nitrogen starvation (Okazaki et al. 1998) and it is reasonable to hypothesize that these uncharacterized protein may serve similar functions in plants.

Interestingly, a gene encoding a protein similar to the bacterial sensor protein GlnD (TC175949) was upregulated at 4 h of PPT treatment but later reduced at 8 h. The predicted protein contains a conserved regulatory ACT domain which, in bacteria, are amino-acid-binding domains present in metabolic enzymes or transcriptional activators. Proteins holding ACT domains have been described in *Arabidopsis* and rice (Hayakawa et al. 2006; Kudo et al. 2008). In bacteria, GlnD is a bifunctional uridylyltransferase/uridylyl-removing enzyme that either activates or inhibits PII proteins according to nitrogen availability. Bacterial PII proteins are sensors for 2-oxoglutarate and are known to modulate GS activity. However, in plants, PII-like proteins do not seem to be involved in the modulation of GS activity but are known to regulate plastidic N-acetyl glutamate kinase, an enzyme which catalyzes the key regulatory step in the pathway to arginine biosynthesis (Chen et al. 2006; Ferrario-Mery et al. 2006; Sugiyama et al. 2004).

Other attention-grabbing putative nitrogen sensors that showed altered expression upon GS inhibition are nodulin 6 and GSI-like proteins, which share homology with the N-terminal and C-terminal regions, respectively, of the *Aspergillus nidulans* FluG protein. The function of FluG is still not fully understood but it is known that it is implicated in important signaling pathways during fungal conidiogenesis (Lee and Adams 1994). *FluG*-like genes are present in plant genomes such as *Os10g0456500* in rice and *At3g53180* (NodGS) in *Arabidopsis*. In *M. truncatula*, individual genes encode the two domains of FluG-like proteins, and both are differentially expressed in response to GS inhibition. TC193978, a transcript similar to nodulin 6, was downregulated at 4 h of PPT treatment, and a GSI-like gene (TC190362) was upregulated at 24 h of PPT treatment. In root nodules of *M. truncatula*, the transcripts for nodulin 6 (*MtN6*) accumulate specifically ahead of growing infection threads, including in the infection zone of mature root nodules (Mathis et al. 1999), and the gene was found to respond to N signals in a transcriptome study using split-roots to compare N-sufficient with N-limited roots (Ruffel et al. 2008). Although GSI-like genes are widespread in higher plants, there is one single report in the literature (Mathis et al. 2000) and, because the protein is predicted not to have GS activity, its function remains unknown. Interestingly, in a recent work, flagellin was shown to increase the expression of the *Arabidopsis* NodGS gene (Doskočilová et al. 2011). Thus, it would be exciting to investigate whether nodulin 6 and GSI-like proteins function together as a two-component system important for plant-microbe interactions in *M. truncatula*.

Finally, it is worth mentioning a gene predicted to encode a putative nitrate transporter (TC179293) which was repressed in response to PPT treatment. Nitrate transporters have been previously described in root nodules but no concrete function was attributed to them. The *LATD/NIP* gene encodes a putative nitrate transporter in *M. truncatula*, and it was shown to be important for the normal development of root nodules (Yendrek et al. 2010). In *nip-1* mutant plants, the infection threads proliferate abnormally and fail to release bacteria into the plant cell cytoplasm (Veereshlingam et al. 2004). The expression of this gene was shown to be regulated by auxin, cytokinin, and abscisic acid (ABA) but not by nitrate (Yendrek et al. 2010).

Unlike *LATD/NIP*, the expression of TC179293 appears to be nodule specific, which is an interesting observation on its own; however, the finding that its expression is affected by GS inhibition, together with the altered expression of a nodule-specific NR, strongly suggests that it can constitute an important player in root nodule functioning. Apart from their primary function in transport, nitrate transporters may play an additional role related to nitrate sensing (Remans et al. 2006) and it is conceivable that this particular gene could fulfill a similar sensing-function.

Conclusion.

This work studies the effects of the impairment of ammonium assimilation on gene transcription in root nodules of *M. truncatula* over a time course. The present transcriptome analysis combined with a previous metabolome analysis of PPT-treated nodules (Barsch et al. 2006a) provides a comprehensive overview of the metabolic rearrangements occurring in root nodules in response to overall GS inhibition.

The results suggest a three-step response of the plant to GS inhibition. The first step relates to a local reduction in Gln and Asn content. The results point to a fast repression of Asn synthesis and of the glycolytic pathway which, in turn, impairs the synthesis of dicarboxylic acids, which are crucial to sustain bacteroid metabolism. On the other hand, the synthesis of Glu via reactions alternative to the GS/GOGAT cycle, the catabolism of amino acids, and the gluconeogenesis pathway appear to be activated upon GS inhibition. In a second step, the plant systemically perceives the organic-N deprivation and, to avoid ammonia accumulation and the unfruitful allocation of carbon resources to the nodules, the plant ceases the transport of sucrose to the nodules and triggers the activation of genes related to senescence. In the final step, the symbiotic identity seems to be severely compromised. The expression of nodule-specific genes is significantly downregulated and an abnormal proliferation of free bacteria is observed in the nitrogen fixation zone, suggesting that the bacteria continue to divide but are unable to infect nodule cells. Thus, GS inhibition appears to hinder the symbiotic relationship corroborating the dominant position of the plant over the microsymbiont.

Functional studies of the identified candidate genes are required to bring about a complete understanding of the molecular players involved in the nodule responses to impaired N assimilation. Nevertheless, the results presented here contribute to the construction of a solid basis toward the selection of genes potentially important for the regulation of the metabolic networks involved in root nodule functioning and provides important insights into how the genes governing root nodule symbiotic identity are influenced by N starvation.

MATERIALS AND METHODS

Plant growth conditions.

Seeds of *M. truncatula* Gaertn. 'Jemalong' were germinated on agar plates and 7-day-old seedlings were transferred to aeroponic containers and grown for 3 weeks on ammonium nitrate in the medium described by Lullien and associates (1987). The plants were grown under cycles of 16 h of light (22°C) and 8 h of darkness (19°C) and light intensity of 150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For nodule induction, plants were nitrogen starved for 7 days before inoculation with the *S. meliloti* strain Rm1021 pXLGD4 RCR 2011(GMI51). The nutrient solution was renewed every week. Nodulated plants (20 days postinoculation) were treated with a volume of the herbicide BASTA corresponding to 0.25 mM PPT, which was added to fresh nutrient solution. Plants were harvested just before addition of PPT and at 4, 8, 24, and 48 h after herbicide application, and

nodules, roots, and leaves were collected separately. All biological material was immediately frozen in liquid nitrogen and stored at -80°C .

Histochemical localization of β -galactosidase activity.

Histochemical staining of β -galactosidase activity was performed as described by Boivin and associates (1990). Root nodules collected from plants just before herbicide application and after the PPT treatment (4, 8, 24, and 48 h) were immediately fixed by vacuum infiltration in 1.25% (vol/vol) glutaraldehyde in 0.2 M sodium cacodylate, pH 7.2, and rinsed in 0.2 M sodium cacodylate, pH 7.2. Root nodules were subsequently incubated overnight at room temperature in staining solution: 0.08 % (wt/vol) 5-bromo-4-chloro-3-indolyl-galactosidase, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.2 M sodium cacodylate at pH 7.2. The material was rinsed in 0.2 M sodium cacodylate (pH 7.2) and cut into 80- μm sections using a vibratome (VT 1000; Leica, Ryswyk, The Netherlands). The sections were briefly cleared with NaOCl to improve the contrast between stained and nonreactive tissues. Samples were mounted between a slide and a coverslip and observed using an Olympus BX50 light microscope. Photographs were taken using an Olympus DP10 digital camera.

Protein extraction and Western blot analysis.

Plant material was homogenized at 0 to 4°C using a mortar and pestle as described before (Carvalho et al. 2003) and the extracts were assayed for GS activity by the transferase assay (Cullimore and Sims 1980). One unit of activity is equivalent to glutamyl hydroxamate at $1 \mu\text{mol min}^{-1}$ produced per total amount of protein (μg^{-1}) at 30°C . GS activity was expressed as a percentage of GS activity in each extract relative to the activity of the untreated samples. Soluble protein concentration was measured by the Coomassie dye-binding assay (Bio-Rad, Munich) using bovine serum albumen as a standard. For Western blot analyses, total soluble protein extracts (10 μg) were separated by 12.5% (wt/vol) sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.), and incubated with polyclonal antibody raised against GS (Cullimore et al. 1984). Immunodetection was performed using goat anti-rabbit peroxidase-conjugated antibody (Vector Laboratories, Burlingame, CA, U.S.A.) and ECL (GE Healthcare, Life Sciences, Piscataway, NJ, U.S.A.) detection system.

RNA extraction, target synthesis, and hybridization to Affymetrix GeneChips.

Total RNA was extracted from 100 mg of root nodules collected from PPT-treated plants (at 4, 8, and 24 h) and from plants collected before application of the herbicide (0 h), using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each sample consisted of RNA extracted from three independent biological replicates and each replicate comprised root nodules pooled from five independent plants. Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 bioanalyzer with a RNA 6000 nano assay (Agilent Technologies, Palo Alto, CA, U.S.A.).

RNA was processed for use on Affymetrix (Santa Clara, CA, U.S.A.) GeneChip Medicago Genome Arrays, according to the manufacturer's GeneChip 3' IVT Express kit user manual. Briefly, 100 ng of total RNA containing spiked-in Poly-A RNA controls was used in a reverse-transcription reaction (GeneChip 3' IVT Express Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in a 16-h in vitro transcription (IVT) reaction

to generate amplified (a)RNA (GeneChip 3' IVT express kit; Affymetrix). Size distribution of the aRNA and fragmented aRNA was assessed using an Agilent 2100 bioanalyzer with an RNA 6000 nano assay. Fragmented aRNA (12.5 µg) was used in a 250-µl hybridization cocktail containing added hybridization controls. Then, 200 µl of the mixture was hybridized on arrays for 16 h at 45°C. Standard post-hybridization wash and double-stain protocols (FS450_0001; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

GeneChip data analysis.

Scanned arrays were analyzed first with Affymetrix Expression Console software to obtain absent or present calls and to assure that all quality parameters were in the recommended range. Subsequent analysis was carried out with DNA-Chip Analyzer (dChip) 2010 (Cheng Li Lab, Harvard). Then, the 12 arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li and Wong 2001) available in DNA-Chip Analyzer 2010 (Cheng Li Lab, Harvard). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a perfect-match-only model (Li and Wong 2001). Subsequently, log₂ transformed expression data for the 50,963 probe sets on the array representing *M. truncatula* transcripts were imported into Partek Genomics Suite 6.5. Differential expression was determined using one-way ANOVA with a false discovery rate of 0.05.

Gene functional characterization.

The differentially expressed probe sets were divided into functional categories using the MapMan software (version 3.5.1). Functional characterization is assessed based on similarities with *Arabidopsis* proteins (Thimm et al. 2004). The software distributes the genes into 34 functional BINs (i.e., functional classes) and one BIN corresponding to unknown function. The annotation and sequence reference of the genes presented in Tables 1 to 7 are given according to the Medicago Gene Index Release 11.0 from March 23, 2011. The sequences were further blasted against the National Center for Biotechnology Information nucleotide and protein database and the *Medicago* genome assembly Mt3.5 release. Sequence comparisons were performed using the multalin software. The expression of the genes in Tables 1 to 7 were analyzed in silico using the MtGEA (Benedito et al. 2008).

qRT-PCR analysis.

Three biological replicates consisting of a pool of three independent plants, different from the ones used for the GeneChip analysis, were analyzed for each sample. Total RNA was extracted as described before using the RNeasy plant mini kit (Qiagen) followed by on-column DNase digestion. Total RNA (2.5 µg) was reverse transcribed using Superscript-RT III (Invitrogen, Life Science) and oligo(dT) according to the manufacturer's instructions. qRT-PCR was performed using an iCycler Thermal cycler (Bio-Rad) detection system with iQ SYBR Green Supermix (Bio-Rad). Two technical replicates were performed for each primer and sample combination in 20 µl of reaction mix including 50 ng of cDNA and 250 nM each primer. PCR conditions were as follows: initial denaturation (95°C for 3 min) followed by 40 cycles of amplification and quantification (95°C for 10 s, 53°C for 30 s, and 72°C for 30 s with a single fluorescence measurement) and melting curve generation (55 to 95°C with one fluorescence read every 0.5°C). Calculation of the cycle threshold (C_t) and primer efficiency was performed by the iQ5 optical system software (ver-

sion 2.0). The relative transcript abundance was calculated as fold change between C_t values of control (0 h) and PPT-treated samples (4, 8, and 24 h). Data were analyzed statistically using the one-way ANOVA with Bonferroni's correction method. Primers are listed in Supplementary Table S3 and were designed using Beacon Designer (PREMIER Biosoft, Palo Alto, CA, U.S.A.).

The entire dataset has been deposited in GEO with GEO accession number GSE34155 and can be accessed online.

ACKNOWLEDGMENTS

This work was supported by the Portuguese Foundation for Science and Technology projects PTDC/AGR-GPL/70592/2006 and PTDC/AGR-AAM/099577/2008.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

dChip software: www.dchip.org
 Medicago Gene Index:
compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago
 National Center for Biotechnology Information database:
www.ncbi.nlm.nih.gov/guide
Medicago truncatula HapMap project: medicagohapmap.org
 Multalin software: multalin.toulouse.inra.fr/multalin
 The Medicago truncatula Gene Expression Atlas (MtGEA) server:
mtgea.noble.org/v2
 Gene Expression Omnibus Series GSE34155 webpage:
www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zzsvfawacimouvc&acc=GSE34155