Glutamine Synthetase Is a Molecular Target of Nitric Oxide in Root Nodules of *Medicago truncatula* and Is Regulated by Tyrosine Nitration\(^1\)*\(^{[W]}\)\(^{[OA]}\)

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Nitric oxide (NO) is emerging as an important regulatory player in the *Rhizobium*-legume symbiosis, but its biological role in nodule functioning is still far from being understood. To unravel the signal transduction cascade and ultimately NO function, it is necessary to identify its molecular targets. This study provides evidence that glutamine synthetase (GS), a key enzyme for root nodule metabolism, is a molecular target of NO in root nodules of *Medicago truncatula*, being regulated by tyrosine (Tyr) nitration in relation to active nitrogen fixation. In vitro studies, using purified recombinant enzymes produced in *Escherichia coli*, demonstrated that the *M. truncatula* nodule GS isoenzyme (MGS1a) is subjected to NO-mediated inactivation through Tyr nitration and identified Tyr-167 as the regulatory nitration site crucial for enzyme inactivation. Using a sandwich enzyme-linked immunosorbent assay, it is shown that GS is nitrated in planta and that its nitration status changes in relation to active nitrogen fixation. In ineffective nodules and in nodules fed with nitrate, two conditions in which nitrogen fixation is impaired and GS activity is reduced, a significant increase in nodule GS nitration levels was observed. Furthermore, treatment of root nodules with the NO donor sodium nitroprusside resulted in increased in vivo GS nitration accompanied by a reduction in GS activity. Our results support a role of NO in the regulation of nitrogen metabolism in root nodules and places GS as an important player in the process. We propose that the NO-mediated GS posttranslational inactivation is related to metabolite channeling to boost the nodule antioxidant defenses in response to NO.

Nitric oxide (NO) is widely recognized as an endogenous signaling molecule involved in various physiological processes, from stress responses to normal plant growth and development, including the legume-rhizobia symbiosis (Besson-Bard et al., 2007; Neil et al., 2008; Wilson et al., 2008). This symbiotic interaction involves complex signal-exchange mechanisms that culminate with the formation of newly differentiated organs called root nodules, which provide a niche for bacterial nitrogen fixation (Oldroyd et al., 2011). An increasing number of reports on the occurrence of NO during the legume-rhizobia symbiosis suggest an important, but yet unknown, signaling role of this molecule for the symbiosis (Wang and Ruby, 2011). The identification of the molecular elements involved in the nodule response to NO will be essential to understand its role and mechanisms of action. In functional nodules of *Medicago truncatula*, NO production has been located in the bacteroid-containing cells of the nodule fixation zone (Baudouin et al., 2006), where the ammonium generated by bacterial nitrogenase activity is released from the rhizobial bacteroids into the cytosol of the infected plant cells and assimilated into the organic pools by plant glutamine synthetase (GS; EC 6.3.1.2); thus, the enzyme can be considered a potential candidate to respond to NO.

GS is an extremely complex and highly regulated enzyme that, in addition to nitrogen fixation, is involved in a number of essential metabolic pathways in the plant. GS catalyzes the ATP-dependent condensation of ammonium with Glu to yield Gln, which is then used for the biosynthesis of essentially all nitrogenous compounds (Lea and Miflin, 2011). The ammonium for GS activity may be derived from primary sources (nitrogen fixation, nitrate assimilation, and ammonium uptake) or from secondary metabolism such as photorespiration and amino acid catabolism; therefore, the enzyme plays essential roles both in primary nitrogen assimilation and in nitrogen recycling (Lea and Miflin, 2011). Consistent with the diversity of metabolic roles, GS exists in the plant as a number of isoenzymes that are located both in the cytosol (GS1) and in the plastids (GS2) and are encoded by a small family of genes. Each of the GS genes shows distinct patterns of expression in different organs of the plant and appears to participate in different metabolic processes. In *M. truncatula*, the GS

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gene family consists of four expressed genes, MtGS1a, MtGS1b, MtGS2a, and MtGS2b (Stanford et al., 1993; Carvalho et al., 2000a, 2000b; Melo et al., 2003; Seabra et al., 2010). MtGS2a and MtGS2b encode precursors to plastid-located isoenzymes, with MtGS2b being a second plastid-located isozyme unique to *M. truncatula* and closely related species and exclusively expressed in developing seeds (Seabra et al., 2010). The other three GS genes are expressed in root nodules, but MtGS1a is highly up regulated in the central infected cells, accounts for the production of over 90% of the total nodule GS activity, and encodes the isoenzyme responsible for the assimilation of the ammonia released by nitrogen fixation (Carvalho et al., 2000a).

The essentially of GS for plant life predicts the existence of several precise and tightly controlled mechanisms for the regulation of enzyme activity. The regulation of GS at the transcriptional level has been well studied, and it is generally accepted as the primary regulatory point, but a number of posttranslational regulatory controls were shown to be critical for the regulation of GS activity (Hoellzel et al., 1992; Temple et al., 1996, 1998; Ortega et al., 1999, 2001). In higher plants, GS activity is known to be modulated by oxidative turnover (Ortega et al., 1999; Palatnik et al., 1999; Ishida et al., 2002) and phosphorylation along with 14-3-3 interaction (Finnemann and Schoerring, 2000; Man and Kaiser, 2001; Riedel et al., 2001; Lima et al., 2006a, 2006b). In *Escherichia coli*, the regulation of GS activity involves cysc adenyllylation and nitration of Tyr residues (Berlet et al., 1996). Tyr nitration has also been implicated in the regulation of GS in animals (Gorg et al., 2007), being conceivably a similar mechanism for the regulation of the plant enzyme. Protein Tyr nitration is a NO-mediated posttranslational modification that consists on the addition of a nitro (NO₂) group to one of the equivalent ortho carbons of the aromatic ring of Tyr residues (Radi, 2004), which can alter the conformation and structure of proteins, the catalytic properties of enzymes, and their susceptibility to proteolysis (Abello et al., 2009). Protein Tyr nitration has been best studied in animal systems, and little is known regarding the functional effects of protein Tyr nitration in plants (Corpas et al., 2009), but recent proteomic studies revealed the existence of a high number of nitrated proteins in sunflower (*Helianthus annuus*; Chaki et al., 2009) and in Arabidopsis (*Arabidopsis thaliana*; Cecconi et al., 2009; Lozano-Juste et al., 2011), suggesting that Tyr nitration also represents an important NO-mediated regulatory mechanism in plants.

The formation of NO and its involvement in the legume-rhizobia symbiosis has been the subject of much research in the last few years, and it is believed that it plays an important, but yet unknown, signaling role in the symbiosis (Wang and Ruby, 2011). The model legume *M. truncatula* and its symbiotic partner *Sinorhizobium meliloti* provided the background for many of those studies. NO production has been located at the infection sites during the initial stages of nodule development of *M. truncatula* and seems to be required for the establishment of the symbiosis (Del Giudice et al., 2011), whereas in fully developed root nodules, it appears to be confined to the nodule fixation zone, suggesting an involvement of NO in root nodule metabolism (Baudouin et al., 2006; Horchani et al., 2011). Proposed sources of NO in root nodules include NO synthase-like proteins (Cueto et al., 1996; Baudouin et al., 2006; Leach et al., 2010) and nitrate reductase and electron transfer chains from both plants and bacteria (Kato et al., 2003; Mesa et al., 2004; Meakin et al., 2007; Gupta et al., 2011; Horchani et al., 2011).

NO appears to directly affect nodule metabolism by inhibiting nitrogenase activity. The involvement of NO in nitrogenase inactivation has been demonstrated in soybean (*Glycine max*) and *Lotus* after nitrate supply (Kanayama et al., 1990; Meakin et al., 2007; Kato et al., 2010). In *Lotus japonicus*, the artificial application of the NO donor sodium nitroprusside (SNP) decreased nitrogen fixation, whereas the application of a NO scavenger (2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) enhanced nitrogen fixation (Shimoda et al., 2009; Kato et al., 2010). In spite of the inhibitory effect of NO on nitrogenase, it appears that NO production is required for nodule development and functioning, and the plant antioxidant systems appear to be crucial to maintain nodule functioning (Pauly et al., 2006; Keyster et al., 2010; Leach et al., 2010; Sanchez et al., 2011). The levels of NO inside the nodule appear to be controlled by leghemoglobin, which is able to scavenge NO and in this way may protect nitrogenase from inactivation (Kanayama et al., 1990; Herold and Puppo, 2005; Meakin et al., 2007; Kato et al., 2010; Sanchez et al., 2011). Nitroso- and leghemoglobin complexes have been detected in nodules of soybean and *Lotus* (Kanayama et al., 1990; Mathieu et al., 1998; Meakin et al., 2007; Sanchez et al., 2010). This NO-scavenging function has also been attributed to nonsymbiotic hemoglobins in *L. japonicus*, which are induced upon symbiotic infection and accumulate in nitrogen-fixing nodules (Shimoda et al., 2009).

There is still much controversy and uncertainty around the possible roles of NO in the legume-rhizobia symbiosis, and the identification of its molecular targets is a major asset to start dissecting its mechanisms of signaling and action. In this study, we provide evidence that GS is a molecular target of NO in root nodules of *M. truncatula* and that its activity is posttranslationally regulated by Tyr nitration in relation to active nitrogen fixation. A model is proposed to integrate the posttranslational regulation of GS by Tyr nitration within the context of root nodule metabolism.

**RESULTS**

**In Vitro Inactivation of GS by NO**

The major protein posttranslational modifications induced by NO are nitrosylation of Cys and nitration...
of Tyr residues (Radi, 2004). To investigate whether nodule GS could be affected by any of these modifications, we started by evaluating in vitro the effects of two reactive nitrogen species producers on the M. truncatula nodule isoenzyme MtGS1a and the plastid-located isoenzyme MtGS2a. The plant proteins were produced in E. coli with a His tag, purified by Ni-affinity chromatography, and used to evaluate GS activity following incubation with increasing concentrations of either peroxynitrite (PN) or tetrynitromethane at pH 8 (TNM). GS activity was found to be inhibited by the two compounds in a dose-dependent manner for the two isoenzymes (Fig. 1), reaching total inactivation after incubation with 100 μM of either PN or TNM. To assess whether the inhibitory effect could be due to Tyr nitration, the treated enzymes were analyzed by western blotting using a specific anti-3-nitrotyrosine antibody. Immunoreactivity was found to increase with increasing PN or TNM concentration for both isoenzymes (Fig. 1).

To further investigate whether the cause of the NO-induced GS inactivation is nitration or other oxidative processes triggered by the reactive nitrogen species, we tested the effect of a number of reagents known to selectively modify Tyr, Cys, or Met residues on the activity of each GS isoenzyme. The widely used Tyr O-acetylation agent N-acetyl-imidazole was used to specifically modify Tyr residues, the alkylating sulfhydryl reagent iodocetamide and the S-nitrosylation reagent S-nitrosoglutathione (S-GSNO) were used as Cys modification agents, and hydrogen peroxide was used as a potent Met and Cys oxidant. The activity of MtGS1a was not significantly affected by Cys alkylation (Fig. 2A), nitrosylation (Fig. 2B), or oxidation (Fig. 2C). Conversely, the acetylation of Tyr residues by N-acetyl-imidazole clearly induced MtGS1a inactivation (Fig. 2D). These results indicate the existence of Tyr residue(s) that, upon modification, lead to MtGS1a inactivation. Interestingly, this effect appears to be specific for the cytosolic isoenzyme MtGS1a, as treatment of the plastid-located MtGS2a isoenzyme with Cys-modifying reagents results in enzyme inactivation (Fig. 2, A–C), whereas N-acetyl-imidazole has a minor inhibitory effect on this isoenzyme (Fig. 2D). Additional evidence that MtGS1a inhibition is a result of Tyr modifications is given by the finding that Cys alkylation, induced by incubation of the enzyme with iodoacetamide, does not prevent MtGS1a from being inactivated by either PN or TNM (Fig. 3). Taken together, these results suggest that NO-dependent inactivation of MtGS1a is due to Tyr nitration, whereas MtGS2a activity is inhibited by thiol residue nitrosylation.

The flavonoid epicatechin has been shown to selectively prevent the nitration of Tyr residues (Schroeder et al., 2001); therefore, we examined whether this flavonoid could protect the enzymes from inactivation. Both MtGS1a and MtGS2a were incubated with increasing concentrations of epicatechin before treatment with 50 μM PN (Fig. 4). Epicatechin was able to protect MtGS1a from inactivation (Fig. 4A) but not MtGS2 (Fig. 4B). Furthermore, the incubation of both GS isoenzymes with epicatechin prior to PN treatment resulted in decreased immunoreactivity against nitrotyrosine, confirming that this flavonoid does indeed prevent Tyr nitration. These results substantiate that MtGS1a inactivation is a consequence of Tyr nitration mediated by reactive nitrogen species. As for MtGS2a, PN and TNM induced Tyr nitration, but this modification does not seem to be the cause of enzyme inactivation, as epicatechin was able to prevent nitration of the protein but not the inactivation induced by the reactive nitrogen species producers (Fig. 4B).

Figure 1. Effects of PN and TNM on GS activity and nitration. Purified MtGS1a or MtGS2a was incubated with PN (A) or TNM at pH 8 (B) at increasing concentrations, assayed for GS transferase activity, and immunodetected with an anti-3-nitrotyrosine specific antibody (NO2-Tyr). Loading controls were obtained by immunoblotting the membranes with anti-GS1 or anti-GS2 antibody (GS1 or GS2, respectively). GS activity was normalized to that found in the absence of NO donor and is represented as means ± SD of at least three independent experiments, assayed in triplicate. The average initial GS activities were 0.36 and 0.1 μmol min⁻¹ μg⁻¹ protein for MtGS1a and MtGS2a, respectively.
Tyr nitration is generally considered an irreversible process, whereas S-nitrosylation is rapidly reversible (Besson-Bard et al., 2007). In order to examine whether the NO-induced GS inactivation could be reversed, we incubated the NO-treated enzymes with dithiothreitol (DTT) and looked for activity recovery. Following incubation with DTT, the activity of MtGS2a could be totally recovered while MtGS1a inactivation could not be reversed (Fig. 5), further strengthening the idea that the MtGS1a NO-inhibitory effect is due to Tyr nitration whereas MtGS2 inactivation is likely to result from Cys nitrosylation.

Identification of Tyr-167 as a Relevant Nitration Site

Once MtGS1a and MtGS2a appeared to be differentially regulated by NO-mediated modifications, either through Tyr nitration or Cys modification, respectively, we searched for Tyr residues present in MtGS1a but not in MtGS2a. An alignment of the amino acid sequences of the two proteins identified three Tyr residues on MtGS1a, Tyr-108, Tyr-167, and Tyr-263, that are substituted in MtGS2a (Fig. 6). From the analysis of the three-dimensional structure of MtGS1a (Seabra et al., 2009), it is predicted that only the residues Tyr-167 and Tyr-263 are placed within a local environment favorable to nitration (Abello et al., 2009); additionally, their modification is likely to affect GS activity. To investigate whether any of these residues could be a regulatory nitration site and account for the specific susceptibility of the mutated proteins to inactivation induced by TNM. The mutated recombinant proteins were found to be active, and their specific activities were similar to the nonmutated enzymes. However, the inhibitory effect of TNM was significantly reduced for the Tyr-167 mutated protein (MtGS1aY167F), whereas mutation of Tyr-263 did not appear to affect TNM-induced GS inhibition, with the protein showing a pattern of inhibition comparable with the nonmutated enzyme (Fig. 7A). These results demonstrate that...
Tyr-167 is a relevant site for the inhibition of MtGS1a activity by nitration. However, we cannot exclude the existence of additional Tyr residues important for the inactivation process or the contribution of other oxidative modifications induced by NO, as the mutated protein was still susceptible to inhibition by TNM, although to a much lower extent (20% inactivation). Pretreatment of the mutated enzyme with 20 μM epicatechin could not prevent this inactivation (Fig. 7B), suggesting that Tyr-167 is the only Tyr implicated in the regulation of MtGS1a activity by nitration and that the observed 20% reduction in GS activity results from oxidative modifications different from Tyr nitration. Western-blot analysis of the mutated proteins treated with TNM (Fig. 7C) showed a reduction in nitrotyrosine immunoreactivity of the two mutated enzymes (Tyr-167 and Tyr-263) relative to the non-mutated enzyme, indicating that the two residues are subjected to nitration, but only Tyr-167 appears to be critical for GS inactivation.

In Vivo GS Nitration in Effective versus Ineffective Root Nodules

To begin studying the physiological significance of GS Tyr nitration for root nodule functioning, we compared GS nitration in planta in situations where nitrogen fixation is impaired. For this purpose, a sandwich ELISA was developed as a tool to compare the amount of in vivo nitrated GS in nodules collected under different physiological conditions. This assay uses an anti-GS antibody as a capture antibody to immunoprecipitate GS from the other nodule proteins and a specific anti-3-nitrotyrosine antibody for detection and quantification of the relative amount of nitrated GS in the different nodule extracts. The relative quantification of GS nitration was based on standards using pure recombinant MtGS1a, which was previously subjected to in vitro nitration by incubation with 100 μM PN. Since the GS polypeptide content and GS activity change under different physiological conditions, the nodule protein extracts were also subjected to GS activity measurements and to immunoblot analysis using anti-GS antibodies. The cytosolic GS immunoreactive bands were quantified by densitometry and the values were plotted. The analyses were performed in at least four biological replicates, and for each biological sample GS nitration, GS quantity, and GS activity were compared.

In ineffective root nodules formed by the Rhizobium mutant FixJ, GS activity was significantly reduced (Fig. 8A), which is an expected result, since the Rhizobium mutant is unable to produce ammonium for GS assimilation, yet the GS polypeptide content was only slightly reduced (Fig. 8B). Interestingly, the amount of nitrated GS was found to be significantly higher in the
mutant nodules when compared with wild-type nodules (Fig. 8C). Thus, the reduction in GS activity in ineffective root nodules can be related with an increase in in vivo GS nitration, strongly suggesting a direct relationship between GS nitration and nitrogen fixation.

In Vivo GS Nitration in Roots and Root Nodules following Nitrate Application

Nitrate is known to induce NO production inside the nodule, leading to the inhibition of nitrogenase activity (Kanayama et al., 1990; Meakin et al., 2007; Kato et al., 2010). To evaluate the effect of nitrate application on GS nitration in roots and nodules of *M. truncatula*, nodulated plants (19 d after inoculation) were fed with nitrate for 2 d, and the levels of GS nitration were compared in nitrate-treated and non-treated plants and both in roots and root nodules. Following nitrate application, GS activity was found to be significantly reduced in root nodules (Fig. 9A), and this reduction was accompanied by a decrease in GS polypeptide content (Fig. 9B). The amount of cytosolic GS polypeptides (39 kD) was 50% reduced, and the plastid-located polypeptides (42 kD) were reduced to undetectable levels. Interestingly, in spite of the observed reduction in GS abundance, the amount of nitrated GS was found to be significantly increased in the nitrate-treated nodules (Fig. 9C), further suggesting a relationship between GS nitration and active nitrogen fixation.

In roots, nitrate induced an increase in root GS activity (Fig. 9A) as well as in the amount of cytosolic and plastid-located polypeptides (Fig. 9B), reflecting the fact that the roots start to assimilate nitrate. In opposition to what was observed in the nodules, the amount of nitrated GS in the root, which is mainly composed of the isoenzyme MtGS1b (Carvalho et al., 2000b), does not seem to be affected by the nitrate treatment (Fig. 9C). In spite of the higher production of NO, which is likely to occur following nitrate feeding, the root enzyme does not appear to be regulated by nitration. This result supports a specific regulation of the nodule isoenzyme MtGS1a by Tyr nitration.

In Vivo GS Nitration in Root Nodules Treated with SNP

To further evaluate whether GS nitration is the cause of GS inactivation in vivo, we applied the widely used NO donor SNP to root nodules of *M. truncatula* and followed GS activity, GS polypeptide content, and GS nitration in nodules treated with 1 and 10 mM SNP. The GS polypeptide content was not affected by the SNP treatment (Fig. 10B), but GS activity was found to be reduced (Fig. 10A). The reduction in GS activity can be directly correlated with an increase in the levels of nitrated GS evaluated by ELISA (Fig. 10C).

DISCUSSION

There is increasing evidence for a role of NO in the legume-rhizobia symbiosis, but the nature of this role is still far from being understood. Elucidation of mechanisms of action of NO in root nodules will inevitably require the identification of its molecular targets. This study represents a first step in that direction, as it...
provides evidence that the key metabolic enzyme GS is a target of NO in root nodules of *M. truncatula*, being posttranslationally regulated by Tyr nitration in relation to active nitrogen fixation. GS is abundantly present in root nodules and plays a pivotal role in the assimilation of the ammonium released by nitrogen fixation. We have previously shown that in *M. truncatula*, this function is fulfilled by the cytosolic isoenzyme MtGS1a, which is abundantly present in the infected cells of the nodule fixation zone (Carvalho et al., 2000a), coinciding with the major site of NO formation in this model species (Baudouin et al., 2006; Horchani et al., 2011). The enzyme is thus accessible to the oxidative effects induced by this reactive compound. NO can affect protein function and in this way may signal fundamental physiological processes, with S-nitrosylation of Cys residues and Tyr nitration being the major protein posttranslational modifications induced by NO (Besson-Bard et al., 2007). Therefore, we started by evaluating whether the *M. truncatula* nodule enzyme MtGS1a could be affected by either of these modifications. In vitro studies using purified recombinant proteins demonstrated that the nodule enzyme MtGS1a is subjected to Tyr nitration and that this modification provokes a loss of enzyme activity. To our knowledge, this is the first report on GS inactivation by Tyr nitration in plants, but previous studies reported an in vitro inhibition of the mammalian and bacterial GS activity by Tyr nitration (Berlet et al., 1996; Gorg et al., 2007). In Arabidopsis, the plastid-located GS (GS2) has been previously identified both as an S-nitrosylated protein (Lindermayr et al., 2005) and as a nitrated protein in more recent proteomic studies (Cecconi et al., 2009; Lozano-Juste et al., 2011), but the effect of either of these modifications on the activity of the enzyme was not assessed before. Our study demonstrates that the activity of the cytosolic enzyme is modulated by Tyr nitration and suggests that the plastid-located GS is susceptible to both nitration and S-nitrosylation, but the cause of GS2 inactivation appears to be S-nitrosylation. However, we cannot rule out the possibility that GS2 inactivation could result from other Cys oxidative processes triggered by reactive nitrogen species. The finding that two isoenzymes sharing a high degree of sequence homology and a remarkably conserved active site fold are differentially modified by NO strengthens the idea that the NO signaling effects are specific under different physiological contexts. In addition to a differential susceptibility of individual GS isoenzymes to NO, the differential localization of the isoenzymes in a specific plant tissue is likely to be implied in the NO-mediated regulation of GS activity.

Tyr nitration is considered a selective process, and typically, only one or two of the Tyr residues present in a protein become preferentially nitrated, depending on the structural environment (Abello et al., 2009). The two *M. truncatula* GS enzymes MtGS1a and MtGS2a are very similar in their amino acid sequences, so the fact that only MtGS1a is regulated by Tyr nitration is in accordance with the selectivity of the Tyr nitration process and prompted us to search for specific Tyr residues on MtGS1a not present in MtGS2a. This
search identified Tyr-108, Tyr-167, and Tyr-263. Previously characterized factors determining the selectivity of Tyr nitration in proteins include the exposure of the aromatic ring at the surface of the protein and the location of the Tyr residue on a loop and in the neighborhood of a negatively charged residue (Souza et al., 1999; Ischiropoulos 2003; Chaki et al., 2009). For the identification of the Tyr residue(s) potentially nitrated in MtGS1a, we analyzed the structural environment within the three-dimensional structure of the protein and identified Tyr-167 to be favorably placed for nitration (Seabra et al., 2009). The residue is located in a solvent-accessible loop, close to the enzyme active site and in close proximity to a basic residue (Lys-137). Tyr-167 establishes a hydrogen bond with Lys-137, which may be important to maintain a correct conformation of the active site and is likely to interfere with

Figure 8. Evaluation of GS nitration in effective and ineffective root nodules of *M. truncatula*. A, Total soluble proteins were extracted from root nodules formed by the wild type (Wt) and *fix* (*Fix2*) mutant strains of *S. meliloti*. GS activity was measured by the transferase reaction. B, The cytosolic GS (GS1) immunoreactive bands were quantified by densitometry and the values were plotted. A representative GS western blot of total soluble proteins (10 μg per lane) is shown. C, GS nitration was quantified by sandwich ELISA relative to GS1 polypeptide content. Values shown are means of four biological replicates ± so, assayed in duplicate.

Figure 9. Evaluation of GS nitration in roots and root nodules treated with nitrate. A, Total soluble proteins were extracted from roots and from 21-d-old root nodules collected from plants treated for 2 d with NH₄NO₃. GS activity was measured by the transferase reaction. B, The cytosolic GS (GS1) immunoreactive bands were quantified by densitometry and the values were plotted. A representative GS western blot of total soluble proteins (10 μg per lane) is shown. C, GS nitration was quantified by sandwich ELISA relative to GS1 polypeptide content. Values shown are means of three biological replicates ± so, assayed in duplicate.
the catalytic activity of the enzyme. Experimental evidence that Tyr-167 is a regulatory nitration site is given by the fact that a mutation of this residue to Phe results in a significantly reduced NO-mediated inhibitory effect. These results strongly suggest that Tyr-167 is the only Tyr implicated in the modulation of MtGS1a activity by nitration, but Tyr-263 constitutes an additional nitration target without apparent functional significance, as mutation of this residue to Phe does not affect the susceptibility of the enzyme to NO, in spite of its reduced nitrotyrosine immunoreactivity in response to NO treatment. The nitration sites have been appointed for some plant proteins in a recent proteomic approach, but the functional significance of nitration at a particular residue was not demonstrated (Lozano-Juste et al., 2011). Here, by site-directed mutagenesis, we present unequivocal evidence that Tyr-167 is the regulatory nitration site responsible for enzyme inactivation. To our knowledge, this is the first report that unambiguously identifies a nitration site in a plant enzyme and determines its functional significance.

Contrary to S-nitrosylation, Tyr nitration is generally considered an irreversible process; however, it has been reported that the inhibition of mammalian GS by Tyr nitration could be reversed by a putative denitrase existing in spleen extracts from lipopolysaccharide-treated mouse (Gorg et al., 2007). We tested whether the NO-induced inactivation of MtGS1a could be reversed by incubation of the in vitro nitrated protein with protein extracts from different organs of the plant and even from spleen extracts from lipopolysaccharide-treated mouse (data not shown). All our attempts failed to recover MtGS1a activity; thus, the inhibitory effect of NO on MtGS1a activity does not appear to be reversible. Conversely, the NO-mediated inactivation of MtGS2a could be readily reversed by incubation with reducing agents, further suggesting that the plastid-located enzyme is inactivated by S-nitrosylation.

Due to the interest in the physiological significance of GS nitration for root nodule functioning, we investigated whether GS was nitrated in vivo in root nodules and whether the levels of GS nitration could be related to lower levels of GS activity in vivo. Central to these studies was the development of a sandwich ELISA to quantify the amount of in vivo nitrated GS in root nodules under different physiological conditions. Using this method, the levels of in vivo GS nitration were compared in two situations where nitrogen fixation is impaired, GS activity is known to change, and NO is expected to be produced, namely, in nodules fed with nitrate and in effective versus ineffective nodules. Interestingly, a direct relationship could be established between reduced nodule GS activity, increased GS nitration, and reduced nitrogen fixation activity, strongly suggesting that GS is posttranslationally inactivated by NO-mediated nitration in response to lower nitrogen fixation rates. Further evidence for the effect of in vivo GS nitration on the reduction of GS activity is given by the finding that nodules treated with the NO donor SNP show reduced GS activity, which can be directly correlated with increased GS nitration. Since the GS polypeptide content was not altered by SNP treatment, the reduction in GS activity can be
directly attributed to the increased GS nitrination levels. Given that the ammonium released by nitrogen fixation is assimilated in the cytosol of the infected cells by GS, it seems reasonable that the enzyme activity is modulated in response to the cell requirements to shut down ammonia assimilation, if it is not being produced.

It is noteworthy that the NO-induced inactivation of GS by nitrination appears to be somehow specific in the root nodules. Following nitrate application, the root enzyme behaved in an opposite manner in relation to the nodule enzyme. Root GS activity increased following nitrate treatment, a process accompanied by de novo synthesis of the enzyme. However, in spite of the elevated GS polypeptide content, there were no detectable changes in the amount of nitrated GS in the root, further suggesting a specific regulation of the nodule enzyme by NO. As mentioned above, in *M. truncatula* roots, GS is mainly composed of a different cytosolic isoenzyme, MtGS1b, that is mainly located in the root cortex, whereas MtGS1a is confined to the root vascular tissues (Carvalho et al., 2000b). It is likely that both the formation of NO at the sites of expression of each individual isoenzyme and the differential susceptibility of the two isoenzymes to NO account for the differential regulation of GS in roots and root nodules.

Altogether, the results presented in this work indicate that GS is posttranslationally regulated by Tyr nitrination in root nodules of *M. truncatula* in relation to nitrogen fixation. A model for the regulation of the nodule enzyme is proposed in Figure II. According to this model, the inhibition of GS activity by Tyr nitrination would be directly related to NO-induced nitrogenase inhibition. In view of the fact that elevated levels of NO in root nodules lead to decreased production of ammonium for GS assimilation, the enzyme would be shut down by posttranslational inactivation through Tyr nitrination in response to the signal NO, the same signal that shuts down nitrogenase. On the other hand, it is proposed that GS inhibition is involved in the nodule antioxidant response to NO. Glu, a substrate for GS activity, is also the precursor for the synthesis of glutathione (GSH), which is known to be highly abundant in root nodules of several plant species and to play a major role in the antioxidant defense, participating in the ascorbate/GSH cycle (Matamoros et al., 1999, 2003; Becana et al., 2010). Upon NO-mediated GS inhibition, Glu could be channeled for the synthesis of GSH, contributing to the antioxidant defenses, and like this the NO signaling events would meet the nodule metabolic pathways to provide an adaptive response to the inhibition of symbiotic nitrogen fixation by reactive nitrogen species.

In conclusion, this study identifies GS as a target of NO in root nodules of *M. truncatula* and shows that the enzyme is in vivo posttranslationally regulated by Tyr nitrination in relation to nitrogen fixation. These findings are significant, as the identification of the molecular targets of NO is determinant for the assembly of the signal transduction cascade that will ultimately help to unravel NO function. This study also highlighted subtle differences between the regulatory effects of NO on different GS isoenzymes and under different physiological contexts, corroborating the idea that NO signaling is specific in determined physiological backgrounds. This specificity is probably governed by the compartmentalization and dynamics of NO production as well as the differential regulation of individual GS isoenzymes by NO. Particularly interesting is the clear relationship that was observed

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**Figure 11.** Proposed model to integrate the posttranslational regulation of GS by Tyr nitrination within the context of root nodule metabolism. Enzymes are as follows: nitrogenase (Nase), GS, Glu synthase (GO-GAT), γ-glutamyl-Cys synthetase (γ-ECS), and glutathione synthetase (GSHS). Dark gray arrows indicate down-regulation by NO, and white arrows indicate up-regulation by NO.
between reduced nitrogen fixation and the inactivation of nodule GS by Tyr nitration. This NO-mediated posttranslational inactivation, which is possibly related to metabolite channeling to the cell antioxidant defenses, provides a link between NO signaling and nitrogen metabolism in root nodules.

**MATERIALS AND METHODS**

**Materials**

PN was prepared as described by Radi et al. (1991). PN concentration was determined spectrophotometrically at 302 nm using a molar absorptivity of 1,670 M⁻¹ cm⁻¹.

**Plant Material and Growth Conditions**

Plants of *Medicago truncatula* 'Jemalong J2' were grown in apericnic conditions under 16-h-light (22°C)/8-h-dark (19°C) cycles and under a light intensity of 150 to 200 µmol m⁻² s⁻¹ in the growth medium supplemented with ammonium nitrate as described by Lullien et al. (1987). For nodule induction, the growth medium was replaced with fresh medium lacking a nitrogen source 3 d before inoculation with either the wild-type *Sinorhizobium meliloti* effective wild-type strain Rm1021 pXLC4D 1R2011 (CM1 1531) or the ineffective *fixJ S.* nodules were harvested at 21 d after infection. For the experiment with nitrate, nodulated plants (19 d after inoculation) were aeroponically grown for 2 d in a growth medium containing 10 mM NH₄NO₃ before harvesting. For the treatment with SNP, mature nodules of *M. truncatula* were infiltrated with a solution containing 1 or 10 mM SNP in phosphate buffer, pH 7.0, under vacuum for 10 min and incubated at room temperature for 3 h. All plant material was immediately frozen in liquid nitrogen and stored at -80°C.

**Protein Extraction from Plant Tissues**

Roots or root nodules of *M. truncatula* were homogenized at 4°C with a mortar and pestle with 2 volumes of extraction buffer (phosphate-buffered saline [PBS], pH 7.4, containing 0.05% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride), and the homogenates were centrifuged at 13,000g for 20 min at 4°C.

**Expression and Purification of Recombinant GS in Escherichia coli**

The *M. truncatula* GS isoenzymes MtGS1a and MtGS2a were produced in *E. coli* BL21-<b>CodonPlus (DE3)-RP* cells harboring previously described gene constructs *pET28a-MtGS1a* and *pET28a-MtGS2a* encoding N-terminally His-tagged fusion proteins (Lima et al., 2006b).

Expression was induced with isopropylthio-β-galactoside (final concentration, 1 mM) at midexponential growth (optical density at 600 nm = 0.5) and proceeded for 3 to 5 h at 37°C. The cells were harvested by centrifugation at 2,800g, resuspended in 0.2 M potassium phosphate, pH 7.4, 0.01 M magnesium sulfate, 0.005 M Glu, 0.5 M NaCl, and 0.02 M imidazole (buffer A), disrupted by sonication, and centrifuged (60 min, 36,700g, 4°C) to remove cell debris. The crude protein extract was filtered through a 0.5-µm protein-binding filter and loaded onto a 5-ml nickel Sepharose column (GE Healthcare) equilibrated with buffer A. Elution of the bound fusion protein was achieved with buffer A supplemented with 0.23 M imidazole. MtGS1a-containing fractions were pooled and dialyzed against 0.02 M potassium phosphate, pH 7.4, 0.005 M Glu, and 0.01 M magnesium sulfate and further purified by size-exclusion chromatography on a Sephacryl S-300 16/60 column (GE Healthcare) equilibrated in the same buffer. The fractions containing purified MtGS1a were pooled and concentrated on a centrifugal concentration device with a 10-kD molecular weight cutoff membrane.

**Site-Directed Mutagenesis**

The Quick-Change Site-Directed Mutagenesis Kit (Agilent/Stratagene) was used to mutate Tyr-167 and Tyr-263 to Phe in *M. truncatula* MtGS1a protein.
Quantification of Nitrated GS by ELISA

A microtiter plate (F8 Maxisorp Nunc-Immuno module; Nunc) was coated with the anti-GlnA antibody (Cullimore and Millin, 1984), diluted in PBS (50 μl well⁻¹), and incubated overnight at 4°C. After two washings with a washing buffer (PBS, pH 7.4, and 0.05% Tween 20), the plate was blocked with a blocking buffer (PBS, pH 7.4, and 3% bovine serum albumin; 100 μl well⁻¹) for 2 h at room temperature. The blocking buffer was removed, the plant extracts were diluted in extraction buffer to 10 μg in 50 μl, and the standard calibration solution was added to the plate (50 μl well⁻¹) and incubated for 4 h at room temperature. All experiments were performed in duplicate. After five washings, a diluted biotin-labeled monoclonal antibody against 3-nitrotyrosine (100 μl well⁻¹) was added to the plate and incubated for 2 h at room temperature. After washing, a NeutraAvidin-HRP-conjugate (Invitrogen) was added to the plate and incubated for 1 h at room temperature.

The plates were washed four times with wash buffer before adding 100 μl well⁻¹ 1 p-nitrophenyl phosphate substrate (Sigma Fast; Sigma-Aldrich). The plates were incubated in the dark for 30 min at room temperature after stopping the reaction by the addition of 100 μl well⁻¹ 0.25 M H₂SO₄. The absorbance was measured at 405 nm using a microplate reader (Multiscan Microplat Spec; Biotek). Standard curves were established by serial dilutions (1–5 ng) of in vitro nitrated GS per well. For this purpose, the purified enzyme (Microplat Spec; Biotek). Standard curves were established by serial dilutions (1–5 ng) of in vitro nitrated GS per well. For this purpose, the purified enzyme (Microplat Spec; Biotek). Standard curves were established by serial dilutions (1–5 ng) of in vitro nitrated GS per well. For this purpose, the purified enzyme.


