Nitric Oxide Detoxification by *Mesorhizobium loti* Affects Root Nodule Symbiosis with *Lotus japonicus*

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(Rceived May 14, 2021—Accepted July 28, 2021—Published online August 31, 2021)

Root nodule symbiosis between legumes and rhizobia involves nitric oxide (NO) regulation by both the host plant and symbiotic rhizobia. However, the mechanisms by which the rhizobial control of NO affects root nodule symbiosis in *Lotus japonicus* are unknown. Therefore, we herein investigated the effects of enhanced NO removal by *Mesorhizobium loti* on symbiosis with *L. japonicus*. The *hmp* gene, which in *Sinorhizobium meliloti* encodes a flavohemoglobin involved in NO detoxification, was introduced into *M. loti* to generate a transconjugant with enhanced NO removal. The symbiotic phenotype of the transconjugant with *L. japonicus* was examined. The transconjugant showed delayed infection and higher nitrogenase activity in mature nodules than the wild type, whereas nodule senescence was normal. This result is in contrast to previous findings showing that enhanced NO removal in *L. japonicus* by class 1 phytoglobin affected nodule senescence. To evaluate differences in NO detoxification between *M. loti* and *L. japonicus*, NO localization in nodules was investigated. The enhanced expression of class 1 phytoglobin in *L. japonicus* reduced the amount of NO not only in infected cells, but also in vascular bundles, whereas that of *hmp* in *M. loti* reduced the amount of NO in infected cells only. This difference suggests that NO detoxification by *M. loti* exerts different effects in symbiosis than that by *L. japonicus*.

**Key words:** hemoglobin, flavohemoglobin, nitric oxide, root nodule symbiosis

Nitric oxide (NO) is produced in plants as a biotic or abiotic response (Delledonne et al., 1998; Beligni and Lamattina, 2000; Dordas et al., 2003a, 2003b; Qiao and Fan, 2008; Simontacchi et al., 2015). Previous studies that focused on the production and detoxification of NO in plant–microbe interactions reported that NO was detectable in both pathogenic and symbiotic responses and was widely involved in these interactions (Romero-Puertas et al., 2004; Wendehenne et al., 2004; Delledonne, 2005; Baudouin et al., 2006; Pi et al., 2007; Leitner et al., 2009; Gaupels et al., 2011; Murakami et al., 2011). In root nodule symbiosis (RNS) between legumes and rhizobia, NO has been detected at various stages from infection to nodule senescence, indicating the importance of NO regulation for the establishment of RNS (Baudouin et al., 2006; Nagata et al., 2008; del Giudice et al., 2011; Cam et al., 2012; Hichri et al., 2015; 2016; Meilhoc et al., 2015; Fukudome et al., 2016). NO is controlled by hemoglobin derived from both the host plant and rhizobia (Meilhoc et al., 2010; Fukudome et al., 2016; 2019a; Berger et al., 2020; Larrazanzar et al., 2020; Salas et al., 2020). Host plants remove NO by plant hemoglobin, now generally known as phytoglobin, specifically by non-symbiotic class 1 phytoglobin (Phytogb1) (Hill et al., 2016; Becana et al., 2020; Larrazanzar et al., 2020). In *Lotus japonicus*, the low expression of the *LjGlb1-1* gene, which encodes Phytogb1, reduces infection efficiency and symbiotic nitrogen fixation (SNF) (Fukudome et al., 2016). SNF increases in hairy root cultures and transgenic lines that highly express *LjGlb1-1*, and nodule senescence is also delayed in these transgenic lines (Shimoda et al., 2009; Fukudome et al., 2019a). Similarly, the low expression of the Phytogb1-encoding *Glb1.1* gene in *Medicago truncatula* decreased SNF, while high expression increased it (Berger et al., 2020).

Symbiotic rhizobia remove NO by the flavohemoglobin Hmp, such as in *Sinorhizobium meliloti* (Meilhoc et al., 2010; del Giudice et al., 2011; Cam et al., 2012), or by a single-domain globin, such as Bgb of *Bradyrhizobium diazoefficiens* (Cabrera et al., 2011; 2016; Sánchez et al., 2011). In a strain of *S. meliloti* deficient in the *hmp* gene, infected nodules showed low SNF and early senescence (Cam et al., 2012; Blanquet et al., 2015), whereas in a strain that highly expresses *hmp*, nodules in the late stage of RNS showed higher SNF and delayed senescence than those infected with wild-type (WT) bacteria (Cam et al., 2012). The *hmp* and *hgb* genes both contribute to NO tolerance in rhizobia under free-living conditions (Meilhoc et al., 2010; Cabrera et al., 2011; 2016; Sánchez et al., 2011), and NO reduction by NorB and NorC in the denitrification pathway and NO metabolism by NnrS1 and NnrS2 have been reported (Cabrera et al., 2011; 2016; Meilhoc et al., 2013; Blanquet et al., 2015). Although NO functions as a signaling molecule in diverse physiological responses...
(Delledonne et al., 1998; Beligni and Lamattina, 2000; Pagnussat et al., 2002; He et al., 2004; Qiao and Fan, 2008; Mur et al., 2013), it also inhibits nitrogenase activity (Trinchant and Rigaud, 1982; Kato et al., 2010); therefore, these regulatory mechanisms are extremely complex, which makes it difficult to understand the full extent of NO regulation in each symbiosis.

NO regulation in RNS has generally been investigated using L. japonicus, M. truncatula, and Glycine max as host plants or in symbiosis between non-leguminous actinorhizal plants and Frankia (Sasakura et al., 2006; Niemann and Tisa, 2008; Coats et al., 2009). Specifically, the effects of deficient or enhanced NO regulation by symbiotic rhizobia have been examined in the RNS of M. truncatula infected with S. meliloti, but not L. japonicus. However, we previously reported that deficient or enhanced NO detoxification by LjGlb1-1 (NO detoxification derived from the host plant) affected the RNS of L. japonicus with Mesorhizobium loti (Shimoda et al., 2009; Fukudome et al., 2016; 2019a; 2019b). Since the developmental process and morphology of L. japonicus root nodules differ from those of M. truncatula (Hirsch, 1992; Larrianzar et al., 2020), the difference in symbiotic nodule organogenesis is often described as one of the factors causing variations in the role and control of NO; however, the underlying mechanisms remain unclear (del Giudice et al., 2011; Hichri et al., 2015; Fukudome et al., 2016). Although a more detailed understanding of the contributions that L. japonicus and M. loti make to the control of NO during RNS is needed in order to discuss differences and similarities in the roles and regulation of NO, research into this issue has been difficult. Some genes in the Mesorhizobium species are annotated as encoding flavohemoproteins (Larrianzar et al., 2020); however, the homology of these genes to hmp in L. japonicus is low and their NO-scavenging activity and NO responsiveness are unknown. Furthermore, genes known to be involved in NO metabolism, hgb and norBC, have not been identified in the genome of M. loti MAFF303099; therefore, the mechanisms underlying NO metabolism in M. loti, which is symbiotic with L. japonicus, have not yet been elucidated.

The present study investigated the effects of enhanced NO detoxification by symbiotic rhizobia in the RNS of L. japonicus–M. loti. A transconjugant of M. loti that ectopically expressed the hmp gene of S. meliloti was generated and its symbiotic phenotype was characterized based on comparisons of the infection process, resistance to NO, and nodular senescence in the Hmp-expressing strain with those of a WT strain. Unexpected results in senescence prompted us to investigate the localization of NO in nodules.

Materials and Methods

Growth conditions of plants

Plants of L. japonicus accession Gifu B-129 and its derivative lines were germinated and grown as previously described (Nagata et al., 2008). In brief, 5 d after germination, seedlings were transferred to 1.5% Fåhraeus agar plates (Fåhraeus, 1957) and inoculated with a cell suspension (10⁶ cells mL⁻¹ in water) of M. loti MAFF303099 (Kaneko et al., 2000) and its derivative strains. Plants were grown under controlled conditions with photo-synthetically active radiation of 150 μmol photons m⁻² s⁻¹ (16-h photoperiod) at 25°C for up to 8 weeks post-inoculation (wpi). The LjGlb1-1 overexpression line (Ox1) of L. japonicus was produced according to the method reported by Aoki et al. (2002) using a binary vector with the CaMV 35S promoter and cDNA of LjGlb1-1 that was constructed based on pG121-Hm. The Ox1 line was used in the same manner as that generated by Fukudome et al. (2019a; 2019b).

Gene editing and cloning of M. loti strains

Since M. loti MAFF303099 did not stably maintain the plasmid pBBR-hmp (Meilhoc et al., 2010) during RNS (data not shown), we integrated the hmp gene containing the promoter sequence into the genome of M. loti MAFF303099. Using pBBR-hmp as a template, the DNA region that codes from the hmp gene to the gentamicin resistance gene (hmp–Gm, approximately 2.7 kb) was amplified by PCR using the primer pair 5’-egcggcctgtgaaaccctgaag-3’ and 5’-ccatcttcctctccatcagc-3’. On the genome of M. loti MAFF303099, a region that does not affect growth or RNS and that ranges between approximately 1 kb upstream and downstream of the mfr8031 gene locus (mfr8031-UP and mfr8031-DW) was amplified by PCR. The primer pair of 5’-taccactcgctgctggctttc-3’ and 5’-ccatcttcctctccatcagc-3’ and that of 5’-egcggcctgtgaaaccctgaag-3’ and 5’-ccatcttcctctccatcagc-3’ were used for up- and downstream PCR, respectively. The DNA fragments of mfr8031-UP, hmp–Gm, and mfr8031-DW were linked by crossover PCR. The resulting fragment (mfr8031-UP/hmp–Gm/mfr8031-DW) was cloned into the suicide plasmid pK18mobbacB, which retains the SacB marker, with the aid of the restriction enzymes SceI and XbaI (both from Takara Bio). The resulting plasmid (pK18mobbacB/mfr8031-UP/hmp–Gm/mfr8031-DW) was transformed into Escherichia coli HST08 (Takara Bio) and then transferred into M. loti and DsRed-labeled M. loti (M. loti-DsRed, Maekawa et al., 2009) by tri-parental mating using helper E. coli MM294 (pRK2013). To obtain transconjugants derived from M. loti and M. loti-DsRed with the plasmid pK18mobbacB/mfr8031-UP/hmp–Gm/mfr8031-DW integrated into their genomes by homologous recombination, the resulting strains were spread on TY agar plates containing gentamycin (50 μg mL⁻¹) or kanamycin (50 μg mL⁻¹), respectively. Colonies were screened by sensitivity to 10% sucrose, and two sensitive clones in which the hmp gene was amplified by PCR were referred to as W315 (derived from M. loti) and RW45 (derived from M. loti-DsRed). To confirm that mfr8031 was replaced by homologous recombination with hmp–Gm, W315 was spread on TY agar medium containing 10% sucrose and gentamycin (50 μg mL⁻¹) and colonies were subjected to PCR. One of the clones was selected and referred to as transconjugant E109. The bacterial strains and plasmids used in the present study are listed in Table 1. To confirm hmp expression, we used the primer set 5’-ttacctgcaggcatagcctcggcgggt-3′ and 5′-cggcggccttgcctgcctggc-3′ for hmp, and the primer set 5′-ccatcttcctctccatcagc-3′ and 5′-taccactcgctgctggctttc-3′ for sigA as housekeeping genes. All bacterial strains were cultured in liquid HM medium (Cole and Elkan, 1973).

Bacterial growth conditions and the NO resistance assay

When the OD₆₀₀ of the culture reached between 0.4 to 0.5, cells were harvested and washed twice with HM medium. Cells were then suspended in HM medium to achieve an OD₆₀₀ equal to 0.2. The NO donor, sodium nitroprusside (SNP), was added to the bacterial suspension to final concentrations of 5, 25, 50, 100, 250, and 500 μM, and following by an incubation with shaking at 26°C for 12 h. OD₆₀₀ was measured using Miniphoto518R (Taisei) every 2 h during the incubation. As a control, potassium ferricyanide (PF) was used at the same concentrations as SNP.
Nitrogenase activity

The nitrogenase activity of nodules was assessed by measuring acetylene reduction activity (ARA) according to the method reported by Shimoda et al. (2009). Whole plants were placed in glass tubes containing wet filter paper. The tubes were filled with an acetylene and air mixture (C₂H₂:air = 1:9 [v/v]). After a 1-h incubation at 25°C, the amount of ethylene in the gas phase was evaluated using a GC-3A gas chromatograph (Shimadzu).

Endogenous NO production in and NO released from nodules

The endogenous production of NO in nodules at 4 and 6 wpi was monitored by fluorescence microscopy, as described by Shimoda et al. (2020). The probes were dissolved in phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM NaH₂PO₄, and 2 mM NaHPO₄ (pH 7.4). To detect NO inside cells, 5% agarose was mixed and the number of plants that formed nodules by 1 wpi was compared in the strains E109, W315, and RW45 (Supplementary Fig. S2). PF at 5 μM did not inhibit the growth of WT or E109.

Infection and nodulation by Hmp-expressing strains

The number of nodules that formed on L. japonicus from 1 to 6 wpi was compared in the strains E109, W315, and WT (Fig. 2a). Although no significant differences were observed in the number of nodules between 2 and 6 wpi, E109 and W315 induced fewer nodules than WT at 1 wpi and the number of plants that formed nodules by 1 wpi was also lower (Fig. 2b). The number of infection threads (ITs) formed by DsRed-labeled WT (M. loti-DsRed) and RW45 were counted at 10 d post-inoculation (dpi) (Fig. 2c). ITs were categorized into two groups, incipient or long ITs, according to the terminology of Malolepszy et al. (2015), except that we included elongating ITs in the long IT category. The numbers of long and incipient ITs were both significantly smaller in RW45 than in M. loti-DsRed. The fresh weights of plants and total nodules from 3 to 8 wpi did not significantly differ between E109 and WT (Supplementary Fig. S3).

Symbiotic phenotypes of mature nodules formed by an Hmp-expressing transconjugant

The symbiotic phenotype of mature nodules was compared at 3 and 4 wpi (Fig. 3). To assess nodule function, ARA was measured to evaluate nitrogenase activity, which was found to be significantly higher in mature nodules with E109 than in those with WT at both 3 and 4 wpi (Fig. 3a). To assess the ability of E109 to scavenge NO in nodules, the fluorescence intensities from the NO-specific probes DAF-FM DA and DAF-FM were measured (Fig. 3b, c, and d).
d). After labeling with DAF-FM DA to reveal endogenous NO levels in nodules, cells in mature nodules infected with E109 exhibited lower levels of intrinsic NO than those with WT (Fig. 3b). A comparison using ImageJ also supported significantly lower NO levels in E109 than in WT (Fig. 3c). NO released from nodules was revealed by labeling with DAF-FM, and the results obtained showed that NO-dependent fluorescence from released NO in mature nodules was lower in E109 than in WT (Fig. 3d).

Fig. 1. Effects of the hmp gene from Sinorhizobium meliloti on the growth of Mesorhizobium loti strains. (a) M. loti WT cells were cultured with or without various concentrations of SNP, as indicated. (b) M. loti WT (black symbols) or E109 cells (open symbols) were cultured with (5 μM or 25 μM) or without SNP. Cell growth was measured every 2 h after the addition of SNP. In (a) and (b), values are the mean of four biological replicates.

Symbiotic phenotype of senescent nodules in Hmp-expressing strains

The phenotypes of old nodules in plants infected with WT and E109 were compared at 6 and 8 wpi (Fig. 4). No significant differences were observed in overall nitrogenase activity per plant at 6 or 8 wpi (Fig. 4a). To examine nitrogenase activity specifically in old nodules, we measured ARA at 6 wpi in nodules that had appeared at 2 wpi and found that nitrogenase activity in these nodules did not significantly differ between E109 and WT (Supplementary Fig. S4a). Neither endogenous nor released NO in old nodules significantly differed between WT and E109 (Fig. 4b, c, and d). Normal and disintegrating infected cells were observed in old nodules, and the progression of aging in nodules
induced by WT and by E109 was similar (Fig. 4b). Furthermore, no significant differences were noted in the rate of greening in old nodules, indicating similar advances in senescence in nodules induced by WT or E109 (Supplementary Fig. S4b). In all experiments on late-stage symbiosis, no significant differences were observed between E109 and WT.

**Differences in effects of Hmp and phytoglobin on NO localization in nodules**

To investigate differences in the effects on nodules of NO detoxification by rhizobia with that by host plants, we compared these effects at 4 wpi on NO localization in the E109 *M. loti* strain, which highly expresses Hmp, with that in the Ox1 line of *L. japonicus*, which highly expresses class 1 phytoglobin (Fig. 5). DAF-FM DA labeling revealed that NO-specific fluorescence intensity was lower in cells infected with E109 than in those infected with WT (Fig. 5 left-hand panel). On the other hand, when nodules in infected cells of *L. japonicus* Gifu were compared with those of the Ox1 line, they showed lower NO-specific fluorescence intensity not only in infected cells, but also in vascular bundles (Fig. 5 right-hand panel).

**Discussion**

In the present study, the effects of enhanced NO-scavenging activity by rhizobia in RNS was investigated in *L. japonicus–M. loti* symbiosis. Enhanced NO detoxification...
fication, which was achieved by inserting the \textit{hmp} gene from \textit{S. meliloti} into \textit{M. loti}, delayed the infection of \textit{L. japonicus}. The nodules that formed after infection by the strain with \textit{hmp} expression (E109) exhibited enhanced nitro‐
genase activity, but not delayed senescence. Furthermore, NO localization differed when it was scavenged by rhizobia in nodules from when it was scavenged by the host plant.

Enhanced NO detoxification by \textit{M. loti} had a negative impact on the early stages of infection, suggesting that a certain amount of local NO is required for infection (Fig. 2a and b). The present results are consistent with previous findings; in the symbiosis between \textit{M. truncatula} and \textit{S. meliloti}, delayed infection was reported for high \textit{hmp} expression in \textit{S. meliloti} and for ectopic \textit{hmp} expression and high \textit{Glb1.1} expression in the hairy roots of \textit{M. truncatula} (del Giudice et al., 2011; Berger et al., 2020). Although the function of NO during IT progression remains unclear, it may be closely involved in the establishment of infection. Infection in \textit{L. japonicus} was not delayed in \textit{LjGlb1-1} lines or hairy roots with high expression, even though root NO levels were low (Shimoda et al., 2009; Fukudome et al., 2019a). The rate of infection of \textit{L. japonicus} by \textit{M. loti} may vary depending on whether NO decreases in the whole root or at the site of infection. Although these possibilities may be examined by observing the amount and localization of NO during the formation of ITs over time, an appropriate

Fig. 4. Nitrogenase activity and NO levels in senescent nodules. (a) Nitrogenase activity (estimated as ARA) was measured at 6 and 8 wpi (late stage of symbiosis). Values indicate the mean±SE (\textit{n}=40). (b) Fluorescence microscopy images were taken of senescent nodules incubated with DAF-FM DA. Scale bars, 100 μm. (c) The fluorescence intensity of each image taken of senescent nodules was quantified using ImageJ. Values indicate the mean±SE (\textit{n}=40). (d) NO released from senescent nodules was assessed by measuring fluorescence intensity at 6 wpi (4 weeks after nodulation). Fluorescence was quantified and expressed per min and per weight of fresh nodules. Values indicate the mean±SE (\textit{n}=12). In (c) and (d), the fluorescence intensity of E109 is expressed relative to that of WT, which was set at 1. In (a), (c), and (d), none of the values showed significant differences (the Student’s \textit{t}-test, \textit{P}<0.05).
method is not yet available. Investigations on the function of NO in the infection process will require novel methods for observing NO microscopically.

In *L. japonicus* and *M. truncatula*, the high expression of Phytogb1 in the host plant increased nitrogenase activity, while low expression decreased this activity (Shimoda et al., 2009; Fukudome et al., 2016; 2019a; Berger et al., 2020). Nitrogenase activity was also reduced in a hmp-deficient strain of *S. meliloti* (Meilhoc et al., 2010; Cam et al., 2012). The present results demonstrated that enhanced NO removal in nodules contributed to their high nitrogenase activity (Fig. 3) and are, thus, consistent with previous findings. Other studies on the nodules of *M. truncatula* and *Glycine max* reported that NO inhibited not only nitrogenase, but also the expression and activity of leghemoglobin and glutamine synthetase (Melo et al., 2011; Navascués et al., 2012; Berger et al., 2020), which are essential for symbiosis. Furthermore, NO repressed the expression of nifH and nifD in soybean symbiosis (Sánchez et al., 2010). The expression and activity of these symbiosis-related genes and molecules need to examined in nodules induced by Hmp-expressing *M. loti*.

We previously reported that the high expression of *LjGlb1-1* delayed nodule senescence in *L. japonicus* by enhancing NO scavenging activity (Fukudome et al., 2019a). Additionally, the deletion or high expression of the *hmp* gene in *S. meliloti* induced early or delayed senescence, respectively, in the nodules that formed in *M. truncatula* (Cam et al., 2012). These findings suggest that the regulation of NO concentrations in nodules delays the onset of nodule senescence. In contrast, in the present study, the ectopic expression of *hmp* in *M. loti* did not delay nodule senescence (Fig. 4 and Supplementary Fig. S4). We currently cannot provide any explanations for this discrepancy. We speculate that the excessive supply of photosynthetic products by host plants to mature nodules with high nitrogenase activity may have limited the nutrient supply to old nodules. NO in nodules has also been suggested to play a role in ATP regeneration via phytoglobin–NO respiration (Igamberdiev and Hill, 2004); therefore, we cannot exclude the possibility that the excessive removal of NO may negatively affect SNF. Limited information is currently available on how much NO is required to delay senescence in old nodules, and, thus, further studies are warranted.

For example, since the NO levels detected in the old nodules of WT and E109 were similar (Fig. 4b, c, and d), the rates of NO production and removal in old nodules need to be compared. The delay in nodule senescence in the Ox1 line, an overexpression line of *LjGlb1-1*, may be due to the less excessive accumulation of NO in vascular bundles. Vascular bundles of nodules are a major pathway connecting host and symbiotic organs and may function as a site for nutrient exchange and signaling (Vadez et al., 2000; King and Purcell, 2005; Sulieman et al., 2010; Sinclair and Nogueira, 2018; Livingston et al., 2019). *LjGlb1-1* mRNAs are mainly localized in the infected zone and in vascular bundles (Bustos-Sanmamed et al., 2011). In soybean, NO is localized in the nodule parenchyma (Calvo-Beguería et al., 2018). Further studies are needed on the effects on RNS of NO localized outside of infected cells.
In RNS, the NO regulatory system is complex because NO functions either positively or negatively depending on the growth stage and location (del Giudice et al., 2011; Hichri et al., 2015; Fukudome et al., 2016; Berger et al., 2020). The genes associated with NO metabolism in bacteria are not highly conserved, which suggests that each rhizobium strain may have established its own NO metabolic pathway. The present study shows the effects of enhanced NO detoxification by M. loti on RNS in L. japonicus, which broadens our knowledge on the role that NO regulation plays in the RNS of microsymbionts and their host plants.

Acknowledgements

The authors thank the National BioResource Project for providing seeds of L. japonicus B-129 Gifu and Dr. Claude Bruand for providing the plasmid pBBR-hmp. The authors also thank Dr. Panlada Tittabutr, Suranaree University of Technology, for her valuable comments and discussion.

Funding: The present study was funded by the JSPS KAKENHI Research Fellowship (JP18J11872 to Mitsutaka Fukudome) and was partially supported by the National Institute for Basic Biology (NIBB) Collaborative Research Program (16-305, 17-309, and 18-312 to Toshiki Uchiimi).

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