Expression of a Class 1 Hemoglobin Gene and Production of Nitric Oxide in Response to Symbiotic and Pathogenic Bacteria in *Lotus japonicus*

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Symbiotic nitrogen fixation by the collaboration between leguminous plants and rhizobia is an important system in the global nitrogen cycle, and some molecular aspects during the early stage of host-symbiont recognition have been revealed. To understand the responses of a host plant against various bacteria, we examined expression of hemoglobin (Hb) genes and production of nitric oxide (NO) in *Lotus japonicus* after inoculation with rhizobia or plant pathogens. When the symbiotic rhizobium *Mesorhizobium loti* was inoculated, expression of *LjHb1* and NO production were induced transiently in the roots at 4 h after inoculation. In contrast, inoculation with the nonsymbiotic rhizobia *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* induced neither expression of *LjHb1* nor NO production. When *L. japonicus* was inoculated with plant pathogens (*Ralstonia solanacearum* or *Pseudomonas syringae*), continuous NO production was observed in roots but induction of *LjHb1* did not occur. These results suggest that modulation of NO levels and expression of class 1 Hb are involved in the establishment of the symbiosis.

Leguminous plants form root nodules, in which symbiotic rhizobia fix atmospheric nitrogen and supply the fixation products to their host plants as a nitrogen source. The nodulation process of legume-rhizobium symbiosis consists of a series of events initiated by an exchange of specific signaling compounds between both partners.

Plant hemoglobin (Hb) was first reported as leghemoglobin (Lb) in the root nodules of *Glycine max* (Kubo 1939). Since their discovery, Hb have been identified in both leguminous and nonleguminous plants, and Hb are now believed to exist in all plants (Arredondo-Peter et al. 1998; Hill 1998). There are at least three distinct types of plant Hb: class 1, class 2, and truncated (Dordas et al. 2003a). All Lb belong to class 2. Lb of legumes and a class 2 Hb of *Casuarina glauca*, which establishes symbiosis with nitrogen-fixing *Frankia* bacteria, function as both an oxygen transporter to allow respiration of micro-symbionts in the nodules and an oxygen regulator to keep a

low oxygen concentration for nitrogenase activity of nitrogen-fixing bacteria in the symbiotic zone (Appleby 1992).

Although class 1 Hb have been identified in many plant species (Hunt et al. 2001), their functions are not fully understood. Class 1 Hb exhibit an extremely high affinity for oxygen (Arredondo-Peter et al. 1997; Trevaskis et al. 1997) and nitric oxide (NO) (Dordas et al. 2004; Perazzolli et al. 2004). In plants, NO is produced under various conditions and serves as a signal molecule that induces the plant defense system (Dordas et al. 2003b; Durner et al. 1998). Dordas and associates (2004) reported that NO was produced in maize cell-suspension cultures by nitrate reductase during hypoxia, and class 1 Hb had a significant function in regulating NO levels. In another study, transgenic alfalfa-root cultures overexpressing barley Hb showed lower levels of NO and a lower rate of cell disruption as compared with wild-type root under hypoxia (Dordas et al. 2003b). A class 1 Hb gene of *Arabidopsis thaliana*, *AHB1*, is strongly induced by hypoxia, sucrose treatment (Trevaskis et al. 1997), and nitrate addition (Wang et al. 2000). A transgenic line of *A. thaliana* overexpressing *AHB1* showed an increased survival rate under severe hypoxic conditions (Hunt et al. 2002). The overexpression of *GhHb1*, a class 1 Hb gene of cotton, in *Arabidopsis* increased disease tolerance (Qu et al. 2006). These reports indicate that class 1 Hb control various NO-regulated physiological processes of plants by modulating NO levels (Seregelyes et al. 2003, 2004).

In many plants, infection by pathogens was shown to induce the expression of pathogenesis-related genes, including the phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes (Mishina and Zeier 2007; Zabala et al. 2006). PAL and CHS are involved in the phenylpropanoid metabolic pathway leading to the synthesis of phytoalexins and other phenolic compounds in response to pathogen attack. When *G. max* was inoculated with the symbiotic rhizobia *Bradyrhizobium japonicum*, PAL and CHS were induced during the early stages of the interaction (Estabrook and Sengupta-Gopalan 1991). CHS of *Trifolium subterraneum* was induced with inoculation of *Rhizobium leguminosarum* bv. *trifolii*, but no induction of CHS was detected in uninoculated plants and with inoculation of the heterologous rhizobia *Sinorhizobium meliloti* (Lawson et al. 1994). The early stages of the interaction between plants and symbiotic and pathogenic bacteria have much in common (Baron and Zambryski 1995); the reactions against symbiotic rhizobia resemble plant defense response to pathogens. However, it is

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unclear how rhizobia cancel the induction of the plant defense system to establish the symbiosis.

Two class 1 Hb genes and three class 2 Hb (Lb) genes exist in the genome of *Lotus japonicus* (Uchiumi et al. 2002). The Lb genes of *L. japonicus* (*LjLb*) are expressed exclusively in root nodules. *LjHb1*, one of the class 1 Hb genes of *L. japonicus*, is strongly induced under both hypoxic and cold stresses, and the gene is induced as well by treatment with the NO donor *S*-nitroso-*N*-acetyl-D, L-penicillamine, and inoculation with the symbiotic rhizobium *Mesorhizobium loti* MAFF303099 (Shimoda et al. 2005). Although NO generation was induced by *M. loti* MAFF303099, the intensity was less than that under cold and hypoxia conditions. Observations using fluorescence microscopy revealed the induction of *LjHb1* expression corresponded to the generation of NO. These results suggest that *LjHb1* and NO have roles in stress adaptation and during the early stage of legume-rhizobium symbiosis (Shimoda et al. 2005).

Fig. 1. *Lotus japonicus* inoculated with symbiotic rhizobia and plant pathogens. **A** through **D**, Whole *Lotus japonicus* plants, **E** and **F**, leaves, and **G** through **J**, root tips, and **K**, nodules inoculated by symbiotic rhizobia and plant pathogens at 28 days after inoculation with *Mesorhizobium loti* MAFF303099 (**A**, **E**, **G**, and **K**), *Ralstonia solanacearum* (**B**, **F**, and **J**), *Pseudomonas syringae* pv. *pisi* MAFF730032 (**C** and **H**), and *P. syringae* pv. *glycinea* MAFF302690 (**D** and **I**). **L**, Leaves at 5 days after infiltration with *R. solanacearum* MAFF730135 and **M**, with *M. loti* MAFF303099. White bars = 1 cm (**A** through **D**); black bars = 1 mm (**E** through **K**). In **A**, arrowheads indicate nodules; in **M**, the arrow indicates the site where *M. loti* MAFF303099 was injected.

To understand the responses of leguminous plants against microbes in the rhizosphere, including rhizobia and pathogens, in the present study, we investigated the expression of *LjHb1* and production of NO in *L. japonicus*. *L. japonicus* plants were inoculated with symbiotic or nonsymbiotic rhizobial strains or plant pathogens. The timecourse of the expression level of *LjHb1* was estimated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) at various time intervals up to 24 h after bacterial inoculation. NO production in *L. japonicus* was observed using the fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM DA) as a NO-specific detector. Our findings will improve understanding of the functions of class 1 Hb and NO in the plant recognition system for symbiotic and pathogenic microbes.

RESULTS

Responses of *L. japonicus* induced by plant pathogens.

L. japonicus plants inoculated with the symbiotic rhizobium *M. loti* MAFF303099 grew well, with the formation of nitrogen-fixing nodules (Fig. 1A and K). Their leaves were green (Fig. 1E) and no pigmentation was observed in the root tips (Fig. 1G). In contrast, when *L. japonicus* was inoculated with the plant pathogen *R. solanacearum* MAFF730135, etiolation of leaves, brownish pigmentation of the elongation zone of the root tip, and inhibition of plant growth were observed by 28 days after inoculation (Fig. 1B, F, and J). Similarly, at 28 days after inoculation with the plant pathogen *P. syringae* pv. *pisi* MAFF730032, brownish pigmentation of the meristematic zone of the root tip, inhibition of primary root elongation, and curling of shoots were observed (Fig. 1C and H). When *L. japonicus* was inoculated with the plant pathogen *P. syringae* pv. *glycinea* MAFF302690, brownish pigmentation of the meristematic zone of the root tip was observed but shoots grew like those of uninoculated plants (Fig. 1D and I). It is known that infiltration of these plant pathogens induce characteristic responses on the leaf of *L. japonicus* (for *R. solanacearum* MAFF730135, H. Kaku and H. Ochiai, unpublished data; for *P. syringae* pvs. *pisi* MAFF730032 and *glycinea* MAFF302690, T. Nakagawa and K. Takeuchi, unpublished data). We performed infiltration experiments according to their findings. When *R. solanacearum* MAFF730135 was infiltrated into leaves of *L. japonicus*, the leaves wilted and dropped but the symptom was restricted to the infiltrated leaf (Fig. 1L). Infiltration of

Fig. 2. Expression of hemoglobin genes in *Lotus japonicus* by inoculation with rhizobia. *L. japonicus* was inoculated with symbiotic rhizobium *Mesorhizobium loti* MAFF303099 (black bars), or nonsymbiotic rhizobia *Sinorhizobium meliloti* 1021 (gray bars) or *Bradyrhizobium japonicum* USDA110 (hatched bars). Control plants were treated with sterilized water (white bars). Total RNAs were isolated from whole plants at the indicated times after inoculation. Relative amounts of transcripts, as normalized against control plants, are shown. Data represent the average of three independent experiments with standard error.

P. syringae pv. *pisii* MAFF730032 caused similar symptoms as those of *R. solanacearum* MAFF730135 (data not shown). Infiltration of *P. syringae* pv. *glycinea* MAFF302690 (data not shown) and *M. loti* MAFF303099 (Fig. 1M) caused no obvious disease symptoms. These results indicate that the characteristic responses observed by inoculation of roots or infiltration of leaves of *L. japonicus* depend on the plant pathogens.

Expression of plant Hb genes in response to rhizobia and plant pathogens.

To examine the responses of *LjHb1* and *LjLb* to rhizobia, the roots of *L. japonicus* plants were inoculated with the symbiotic rhizobial strain *M. loti* MAFF303099 or the nonsymbiotic rhizobial strains *S. meliloti* 1021 or *B. japonicum* USDA110. Total RNA was prepared from a whole plant, and the expression levels of *LjHb1* and *LjLb* were estimated by quantitative RT-PCR.

Inoculation with *M. loti* MAFF303099 induced transient expression of *LjHb1*; the expression of *LjHb1* increased at 4 h after inoculation and decreased to its basal level at 10 h (Fig. 2). At 4 h after inoculation, the expression level of *LjHb1* in plants inoculated with *M. loti* was enhanced approximately three times higher than that of control plants. However, inoculation with the nonsymbiotic rhizobia *S. meliloti* 1021 and *B. japonicum* USDA110 did not induce the expression of *LjHb1* (Fig. 2). *LjLb* (the three class 2 Hb genes of *L. japonicus*) did not respond to inoculation with rhizobial strains (Fig. 2).

To further investigate how the induction of class 1 Hb genes by symbiotic rhizobia generally occurs in leguminous plants, the expression of *Mhb1* (class 1 Hb of *Medicago sativa*) and *MsLb* (Lb of *Medicago sativa*) were also analyzed. When roots of *Medicago sativa* were inoculated with the symbiotic rhizobium *S. meliloti* 1021, the expression of *Mhb1* was enhanced transiently, similar to the case of *L. japonicus* with *M. loti*

Fig. 3. Expression of the *Lotus japonicus* Hb1 gene in roots and shoots. *L. japonicus* plants were inoculated with *Mesorhizobium loti* MAFF303099 (black bars), *Sinorhizobium meliloti* 1021 (gray bars), or *Bradyrhizobium japonicum* USDA110 (hatched bars). Control plants were treated with sterilized water (white bars). Total RNAs were isolated from roots and shoots at the indicated times after inoculation. Relative amounts of transcripts normalized by control plants are shown. Data represent the average of three independent experiments with standard error.

Fig. 4. Expression of hemoglobin genes by inoculation with plant pathogens. *Lotus japonicus* was inoculated with *Ralstonia solanacearum* (hatched bars), *Pseudomonas syringae* pv. *pisii* (gray bars), or *P. syringae* pv. *glycinea* (black bars). Control plants were treated with sterilized water (white bars). Relative amounts of transcripts, as normalized against control plants, are shown. Time indicates the sampling time after inoculation with plant pathogens. Data represent the average of three independent experiments with standard error.

MAFF303099. The expression of *Mhb1* increased at 4 h after inoculation and decreased to the basal levels at 10 h. The expression level of *MsLb* in infected plants did not change (Supplementary Fig. 1). The inoculation with the nonsymbiotic rhizobium *M. loti* MAFF303099 induced neither *Mhb1* nor *MsLb* (data not shown). These data clearly indicate that only symbiotic rhizobia induce transient expression of class I Hb genes in their host legumes.

The location of *LjHb1* expression was examined by quantitative RT-PCR. When *M. loti* MAFF303099 was inoculated,

the expression of *LjHb1* was enhanced in roots to about five times the level of uninfected root at 4 h after inoculation (Fig. 3), whereas *LjHb1* was expressed at basal levels in the shoot. In the case of inoculation with *S. meliloti* 1021 or *B. japonicum* USDA110, the expression level of *LjHb1* did not change either in shoot or root (Fig. 3). These results indicate that the expression of *LjHb1* was enhanced transiently only in the roots by inoculation with symbiotic rhizobia.

The responses of *LjHb1* and *LjLb* to the plant pathogens were also examined. When roots of *L. japonicus* were inocu-

Fig. 5. Nitric oxide (NO) production by inoculation with symbiotic and nonsymbiotic rhizobia. A through D, *Mesorhizobium loti* MAFF303099 and E through H, *Sinorhizobium meliloti* 1021 were used to inoculate *Lotus japonicus* (A, B, E, F, I, and J) and *Medicago sativa* (C, D, G, H, K, and L). I through L, As a control, sterilized distilled water was applied instead of rhizobia. Images of roots were taken at 4 h after inoculation by fluorescence microscopy, using 4-amino-5-methylamino-2',7'-difluorescein diacetate as a NO detector (B, D, F, H, J, and L) and Bright-field imaging (A, C, E, G, I, and K).

Fig. 6. Nitric oxide (NO) production at various times after rhizobial inoculation. Roots of *Lotus japonicus* and *Medicago sativa* at A through D, 0 h, E through H, 4 h, I through L, 10 h, and M through P, 24 h after inoculation with *Mesorhizobium loti* MAFF303099 (A, B, E, F, I, J, M, and N) and *Sinorhizobium meliloti* 1021 (C, D, G, H, K, L, O, and P). NO production was observed by fluorescence microscopy, using 4-amino-5-methylamino-2',7'-difluorescein diacetate (B, D, F, H, J, L, N, P), and Bright-field imaging (A, C, E, G, I, K, M, and O).

lated with *R. solanacearum* MAFF730135, *P. syringae* pv. *lisi* MAFF730032, or *P. syringae* pv. *glycinea* MAFF302690, expression of neither *LjHb1* nor *LjLb* was induced (Fig. 4).

NO production by inoculation with rhizobia and plant pathogens.

In accordance with the expression of *LjHb1*, NO was detected in the roots of *L. japonicus* by 4 h after *M. loti* inoculation (Shimoda et al. 2005). To assess the specificity of NO production, cross-inoculation tests were performed, and the roots were observed using the dye DAF-FM DA as a NO-specific detector.

The distinct fluorescence was detected when the roots of *L. japonicus* (Fig. 5A and B), *Medicago sativa* (Fig. 5G and H), and *Trifolium repens* (Supplementary Fig. 2) were inoculated with their symbiotic rhizobia *M. loti* MAFF303099, *S. meliloti* 1021, and *Rhizobium leguminosarum* bv. *trifolii* 4S, respectively. In contrast, the fluorescence was not observed when those plants were inoculated with nonsymbiotic rhizobia (Fig. 5C through F). The distinct fluorescence as a response to symbiotic rhizobia was transiently observed at 4 h after inoculation (Fig. 6E through H) and, then, the intensity decreased to its basal level at 10 h (Fig. 6I through L). These results suggest that symbiotic rhizobia specifically induce transient NO production in the roots of its host plant in a pattern similar to that of *LjHb1* expression.

When *L. japonicus* was inoculated with *R. solanacearum* MAFF730135, *P. syringae* pv. *lisi* MAFF730032, or *P. syringae* pv. *glycinea* MAFF302690, strong fluorescence was detected at 4 h after inoculation (Fig. 7A through F). Furthermore, the fluorescence was detectable until at least 24 h after

inoculation (Fig. 7G through R). These observation suggests that the plant pathogens induce continuous NO production in the root of *L. japonicus*.

Effect of NO scavenger on the induction of *LjHb1*.

To make clear whether the induction of *LjHb1* is mediated by NO, the expression of *LjHb1* and NO production was examined, using *L. japonicus* plants inoculated by *M. loti* MAFF303099 with or without a NO scavenger, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (c-PTIO). At 3 h after inoculation, the plants were placed for 1 h on filter paper soaked with 1 mM c-PTIO and the expression of *LjHb1* was determined by quantitative RT-PCR. In inoculated plants treated with c-PTIO, the expression of *LjHb1* was strongly repressed (Fig. 8A). The roots were observed by fluorescent microscope using DAF-FM DA. The fluorescence intensity of roots treated with c-PTIO was very low compared with that of roots without c-PTIO (Fig. 8B). The very deep color of c-PTIO solution sometimes masks the green fluorescence. However, c-PTIO did not mask the fluorescence of roots expressing the green fluorescent protein gene when we observed by the method used in this study. Considering the results of the other combination of host-microsymbiont (Fig. 5), these results suggest that the inoculation of rhizobia induces NO production and expression of class 1 Hb genes of host plants.

Quantification of NO produced by bacterial inoculation.

NO production in the roots inoculated with bacteria was detected by the fluorescent dye DAF-FM, and relative fluorescent unit (RFU) values were estimated. When *L. japonicus* was inoculated with *M. loti* MAFF303099, the RFU value increased

Fig. 7. Nitric oxide (NO) production by inoculation with plant pathogens. Roots of *Lotus japonicus* at A through F, 4 h, G through L, 10 h, and M through R, 24 h after inoculation with *Ralstonia solanacearum* (A, D, G, J, M, and P), *Pseudomonas syringae* pv. *lisi* (B, E, H, K, N, and Q), or *P. syringae* pv. *glycinea* (C, F, I, L, O, and R). NO production was observed by fluorescence microscopy, using 4-amino-5-methylamino-2',7'-difluorescein diacetate (D through F, J through L, and P through R) and Bright-field imaging (A through C, G through I, and M through O).

at 4 h after inoculation and decreased to the basal level at 10 h (Fig. 9). In contrast, inoculation with *R. solanacearum* MAFF730135 induced continuous NO production until at least 24 h after inoculation (Fig. 9). Likewise, inoculation with *P. syringae* pv. *pisi* MAFF730032 and *P. syringae* pv. *glycinea* MAFF302690 also induced continuous NO production (Fig. 9).

DISCUSSION

We analyzed the responses of *L. japonicus* against the plant pathogens *R. solanacearum* and *P. syringae* pvs. *pisi* and *glycinea*. The effects of these bacterial strains will be helpful in understanding the responses of the model legume *L. japonicus* against various microorganisms. *R. solanacearum* is the causal agent of bacterial wilt and attacks more than 200 plant species, including legumes. *R. solanacearum* GMI1000 induces typical symptoms on *Medicago truncatula* A17, namely, chlorosis on cotyledons and the first leaf, and inhibition of development of secondary roots and leaves (Vailleau et al. 2007). *P. syringae* causes disease symptoms ranging from leaf spots to stem cankers on many plant species (Nomura et al. 2005). *P. syringae* enters the host tissues (usually leaves) through wounds and natural openings, such as stomata, and the pathogen multiplies to high population numbers in the intercellular spaces (Katagiri et al. 2002). Although none of the plant pathogens we used (i.e., *R. solanacearum* MAFF730135, *P. syringae* pv. *pisi* MAFF730032, and *P. syringae* pv. *glycinea* MAFF302690) are pathogens of *L. japonicus* in nature, the characteristic responses were observed on *L. japonicus* after inoculation of roots or infiltration of leaves (Table 1).

In inoculated root tissues, NO was produced transiently against the symbiotic rhizobium *M. loti* but continuously against all plant pathogens we used (Figs. 6 and 7). In plants, NO is involved in root growth, leaf expansion, photomorpho-

Fig. 9. Quantification of nitric oxide produced in roots. Roots of *Lotus japonicus* were inoculated with *Mesorhizobium loti* MAFF303099 (black bars), *Ralstonia solanacearum* MAFF730135 (gray bars), *Pseudomonas syringae* pv. *pisi* (hatched bars), and *P. syringae* pv. *glycinea* (dotted bars). Roots of *L. japonicus* treated with sterilized water were used as control (white bars). Relative fluorescent units (RFU) per 10 mg of roots at 515 nm normalized against control plants are shown. The data represent the average of three independent experiments with standard error.

Fig. 8. Effect of nitric oxide (NO) scavenger on *Lotus japonicus* Hb1 (*LjHb1*) gene expression. **A**, The expression of *LjHb1* was examined using *Lotus japonicus* plants inoculated with *Mesorhizobium loti* MAFF303099 with or without NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazole-3-oxide-1-oxyl (c-PTIO) by quantitative reverse transcription-polymerase chain reaction. Control plants were treated with sterilized water (SDW). Relative amounts of transcripts normalized against control plants are shown. Data represent the average of three independent experiments with standard error. **B**, Detection of fluorescence, using 4-amino-5-methylamino-2',7'-difluorescein diacetate in roots treated with 1 mM c-PTIO.

genesis senescence (Beligni and Lamattina 2000; Neill et al. 2003), and stomatal closure (Desikan et al. 2004). The production of NO and reactive oxygen species (ROS) is induced by pathogen attack (Hancock et al. 2002). NO is one of the key molecules in plant defense against pathogenic bacteria, and it induces the expression of defense genes and the accumulation of antimicrobial compounds such as phytoalexin (Delledonne 2005; Polverari et al. 2003; Romero-Puertas et al. 2004).

Various defense reactions might occur during the process of legume-rhizobium symbiotic interactions. For instance, *Medicago sativa* responds to infection with *S. meliloti* by the production of superoxide and hydrogen peroxide (Santos et al. 2001). Our previous study indicated that *M. loti* induces NO production in the roots of *L. japonicus* during the first hours of infection (Shimoda et al. 2005). Transcriptome analysis at the early nodulation stage showed an induction of putative defense response genes at 1 h after inoculation with rhizobia (Lohar et al. 2006). Either NO, ROS, or both may trigger plant defense responses against symbiotic rhizobia; that is, like pathogenic microorganisms, symbiotic rhizobia also appear to induce genes involved in the defense system of host plants. Thus, to establish a successful symbiosis, rhizobia must cancel or avoid the host defense responses (Mithöfer 2002).

Class 1 Hb affect various physiological processes through their association with NO. For instance, *Mhb1*, a class 1 Hb of *Medicago sativa*, interferes with NO functions during pathogenic responses. Overexpression of *Mhb1* in tobacco plants resulted in fewer lesions in leaves when they were challenged with incompatible bacteria or tobacco necrosis virus, as compared with control plants (Seregélyes et al. 2003). Thus, in this study we focused on NO production and the expression of plant class 1 Hb genes as indicators of plant responses against various bacteria in the rhizosphere.

We reported previously that NO was produced rapidly in *L. japonicus* by inoculation with *M. loti* MAFF303099 (Shimoda et al. 2005). In the present study, NO was specifically and transiently induced by symbiotic *M. loti*. Considering the function of NO in the plant defense system, initially *L. japonicus* roots seem to recognize *M. loti* as a biotic stress or a pathogen attack rather than as a symbiont. Lohar and associates (2006) also reported a strong suppression of putative defense response genes in *Medicago truncatula* after inoculation with symbiotic rhizobia, particularly at 48 h. This suggests that the defense response of the host plant is aborted through an unknown mechanism.

It has been reported that *LjHb1* is induced by NO and that c-PTIO represses the induction, suggesting that NO is an actual inducer of *LjHb1* in *L. japonicus* (Shimoda et al. 2005). When a host legume interacts with its symbiotic rhizobia, NO was produced accompanied by the expression of a class 1 Hb

gene (Figs. 3 and 5). We propose that NO in roots is produced initially as a defense against the inoculation of symbiotic rhizobia and that class 1 Hb then modulates the NO levels. Furthermore, class 1 Hb serves as a NO scavenger only in the roots at the site of inoculation with symbiotic rhizobia. It is unclear whether the transient NO production is essential for initiation of the symbiosis; however, once NO is produced by the host plant, it is essential to reduce the NO level in order for the symbiotic rhizobia to colonize on the root. Because NO itself functions as an inducer of class 1 Hb genes (Shimoda et al. 2005), the transient expression of these genes may be induced by NO. When the NO level is low enough to be fully removed by class 1 Hb, the defense response of the host plant will be cancelled. Another possibility is that the symbiotic rhizobia actively induce class 1 Hb genes to reduce the level of NO. If class 1 Hb serves as a NO scavenger in the roots, it might be involved in the first step of symbiosis. We cannot elucidate why nonsymbiotic rhizobia do not induce the production of NO and the gene expression of class 1 Hb. Further research is needed to clarify whether rhizobia possess some factor (e.g., Nod factor) that induces class 1 Hb genes as well as to identify the rhizobial components that induce NO production.

In the interaction between *L. japonicus* and the plant pathogens we used in the present study, NO was produced continuously without expression of the class 1 Hb gene *LjHb1*, despite the fact that NO itself is an inducer of *LjHb1*. Some but not all plant pathogens, at least the plant pathogens used in the present study, may be able to repress the expression of class 1 Hb and induce high levels of NO. Although we need further testing, it might be one of the common characteristics of plant pathogens. High NO levels can either be protective, by activating defense responses and suppressing the growth of pathogenic bacteria, or toxic for the plant cells (Beligni and Lamattina 1999). However, continuous production of NO by inoculation with a plant pathogen may cause damage to roots of *L. japonicus*, thus promoting the growth of pathogenic bacteria.

The infiltration of *P. syringae* pv. *glycinea* MAFF302690 in the leaves did not induce any obvious symptoms in *L. japonicus*, although inoculation of the roots with this strain induced continuous NO production. This suggests that *L. japonicus* recognizes *P. syringae* pv. *glycinea* MAFF302690 as a putative pathogen. In contrast, *P. syringae* pv. *pisi* MAFF730032 induced hypersensitive reaction-like symptoms in the leaves. Tada and associates (2004) reported that alteration of the ratio of NO and ROS may induce a variant set of defense responses. The NO/ROS ratio may be different between *P. syringae* pvs. *glycinea* and *pisi*, such that the hypersensitive reaction-like symptoms will be induced only by the latter. Infiltration by *P. syringae* pv. *glycinea* into other ecotypes and *L. japonicus* mutants such as *har1*, which is hyperinfected by mycorrhizal fungi and root-parasitic nematodes, will provide clues to under-

Table 1. Bacterial strains

Bacterial strain	Host plant (isolation source)	Response of <i>Lotus japonicus</i>	
		Inoculation to roots	Infiltration to leaves
<i>Mesorhizobium loti</i> MAFF303099	<i>L. japonicus</i> (root nodule)	Formation of nitrogen-fixing nodules	No symptom
<i>Sinorhizobium meliloti</i> 1021	<i>Medicago sativa</i> (root nodule)	No change	N.t. ^a
<i>Bradyrhizobium japonicum</i> USDA110	<i>Glycine max</i> (root nodule)	No change	N.t.
<i>Ralstonia solanacearum</i> MAFF730135	<i>Solanum tuberosum</i> (stem)	Etiolation of leaves, brownish pigmentation of root tip, inhibition of plant growth	Withering and death of the infiltrated leaf ^b
<i>Pseudomonas syringae</i> pv. <i>pisi</i> MAFF730032	<i>Pisum sativum</i> (stem)	Brownish pigmentation of root tip, inhibition of primary root elongation	Withering and death of the infiltrated leaf ^c
<i>P. syringae</i> pv. <i>glycinea</i> MAFF302690	<i>Glycine max</i> (leaf)	Brownish pigmentation of root tip	No symptom ^c

^a N.t. = not tested.

^b Kaku et al., unpublished data.

^c Nakagawa et al., unpublished data.

standing the different responses of *L. japonicus* against the two *P. syringae* pathovars.

In plant-microbe interactions, the function of NO and class 1 Hb is not completely understood. Recently, Baudouin associates (2006) and Pii and associates (2007) reported NO in the root nodules of *Medicago truncatula* and *Medicago sativa*, and we detected enhanced expression of class 1 Hb genes in the root nodules of *Medicago sativa* (data not shown) and *L. japonicus* (Uchiumi et al. 2002). Sasakura and associates (2006) suggested that class 1 Hb contributes to the symbiotic nitrogen fixation between *Frankia* spp. and the actinorhizal plant *Alnus firma*. Based on the present study, class 1 Hb appears to be involved in controlling the plant defense response during the initial stage of plant-microbe interactions. The modulation of NO by class 1 Hb may be one of the critical steps for successful symbiotic nitrogen fixation.

MATERIALS AND METHODS

Plant materials and bacterial strains.

L. japonicus MG-20 Miyakojima (Kawaguchi 2000) was used throughout the experiments. *M. loti* MAFF303099 (Kaneko et al. 2000) was used as a symbiotic partner of *L. japonicus*. *S. meliloti* 1021 (Meade et al. 1982) and *B. japonicum* USDA110 (Johnson and Means 1964) were used as non-symbiotic partners of *L. japonicus*. *R. solanacearum* MAFF730135 (isolated from potato stem), *P. syringae* pv. *pisi* MAFF730032 (isolated from *Pisum sativum* stem), and *P. syringae* pv. *glycinea* MAFF302690 (isolated from *G. max* leaves) were used as plant pathogens. The relevant characteristics of bacterial strains are listed in Table 1.

Inoculation of bacteria to plants.

L. japonicus seeds were scarified with sulfuric acid for 10 min and were then rinsed with sterilized distilled water. Seeds were surface-sterilized with a 2% sodium hypochlorite solution containing 0.02% Tween 20 and were germinated on agar plates at 25°C in the dark. After 3 days, the seedlings were transferred to 1.5% Fåhræus agar plates (Fåhræus 1957) and were grown under a 16-h-light and 8-h-dark cycle at 25°C for 10 days.

Rhizobia were grown at 28°C to the stationary phase in yeast-mannitol liquid medium (Keele et al. 1969). *R. solanacearum* MAFF730135 was grown at 28°C to the stationary phase in peptone-sucrose liquid medium (Tsuchiya et al. 1982). *P. syringae* pvs. *pisi* MAFF730032 and *glycinea* MAFF302690 were grown at 28°C to the stationary phase in Luria-Bertani medium containing 10 mM MgCl₂. Each bacterial culture was centrifuged and washed with sterilized distilled water; bacterial cells were then suspended in sterilized water at a concentration of 1×10^7 cells per milliliter. The bacterial suspension was directly applied to the roots of 14-day-old plants growing on plates or infiltrated into the leaves using a disposable syringe.

Isolation of total RNA from *L. japonicus*.

Whole plants or tissues were quickly frozen in liquid nitrogen and were stored at -80°C until further use. Total RNA was prepared using the Plant Total RNA Extraction miniprep system (Viogene, Sunnyvale, CA, U.S.A.). DNase treatment was performed using deoxyribonuclease RT-Grade (Wako, Osaka, Japan). RNA was then precipitated by ethanol in the presence of ethachinmate (Wako) as a carrier and was resuspended in RNase-free water.

Analysis of expression of Hb genes by real-time RT-PCR.

To quantify the relative amount of transcripts derived from each Hb gene, real-time RT-PCR was performed in a 25-μl

reaction mixture containing 12.5 μl of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.), 100 ng of total RNA, 10 U of RNase inhibitor, 12.5 U of Superscript II-RT (Invitrogen Japan, Tokyo), and 0.2 μM of each primer. The enzymatic reactions were performed with a 7300 Real-Time PCR system (Applied Biosystems). A reverse transcription reaction was performed at 48°C for 30 min, followed by heating at 95°C for 10 min. Target genes were then amplified by 40 cycles of 95°C for 15 s and 60°C for 1 min. Sequences of primers for real-time RT-PCR were as follows (forward and reverse): 5'-CCTTTGGAGGAGAACCCCAA-3' and 5'-GAG CTGCTGATTCAAGTCATG-3' for *LjHb1*; 5'-TCTGGRC CYAMGCAYAGTC-3' and 5'-CRTCRGWTGTCAGTSCAAA A-3' for *LjLb*; and 5'-AGAGGGTTTAAAGATCAAAT-3' and 5'-ATGTCAATTCATCACGTTTT-3' for initiation factor 4A3 gene (*LjeIF-4A*). Primers were designed with the Primer Express software (Applied Biosystems). *LjeIF-4A* of *L. japonicus* was used for normalization of the results of real-time RT-PCR.

Detection of NO production by fluorescence microscopy.

A stock solution of DAF-FM DA (5 mM in dimethylsulfoxide) was diluted 500-fold in water before use. Throughout the experiments, 14-day-old plants were used. Plants that generated strong autofluorescence were not used for further experiments. The plants inoculated with *M. loti* MAFF303099, *R. solanacearum* MAFF730135, *P. syringae* pv. *pisi* MAFF730032, or *P. syringae* pv. *glycinea* MAFF302690 were divided into four groups. The plants in one of the four groups were observed immediately (0 h) and those in the other three groups were incubated for 4, 10, or 24 h. After incubation, the plants to be observed were placed for 30 min on filter paper soaked with the DAF-FM DA solution, and the fluorescence images of the roots were then observed under a microscope. Images were captured by a Leica DMLB microscope equipped with a Leica DC2000 digital camera (Leica Microsystems, Wetzlar, Germany). For detection of DAF-FM fluorescence, filter sets of 470 (excitation) and 525 nm (emission) were used.

Quantitation of NO by fluorescence spectrophotometer.

A stock solution of DAF-FM (7 mM in dimethylsulfoxide) was diluted 1,000-fold in water before use. Roots of *L. japonicus* (14 days after germination) were inoculated with *M. loti* MAFF303099, *R. solanacearum* MAFF730135, *P. syringae* pv. *pisi* MAFF730032, or *P. syringae* pv. *glycinea* MAFF302690 and were incubated for 3.5 h. Roots were cut off and soaked in DAF-FM solution for 30 min. The RFU of the DAF-FM solution were measured using a NanoDrop ND-3300 fluorospectrometer (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.). Wavelengths for excitation and emission were 470 and 515 nm, respectively.

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