**Stably-Transformed *Lotus japonicus* Plants Overexpressing
Phytoglobulin LjGlb1-1 Show Decreased Nitric Oxide Levels in
Roots and Nodules as well as Delayed Nodule Senescence**

Running head: M. Fukudome *et al.* / Overexpression of LjGlb1-1 improves symbiosis

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2',7'-difluorescein (diacetate); Glb, phytoglobin; NO, nitric oxide; PAS, Periodic Acid-Schiff staining; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; WT, wild-type.

1 Table; 5 Figures; 3 Figures in colour (print).

Supplementary data: 6 Figures.

The class 1 phytoglobin, LjGlb1-1, is expressed in various tissues of the model legume *Lotus japonicus*, where it may play multiple functions by interacting with nitric oxide (NO). One of such functions is the onset of a proper symbiosis with *Mesorhizobium loti* resulting in the formation of actively N₂-fixing nodules. Stable overexpression lines (Ox1 and Ox2) of LjGlb1-1 were generated and phenotyped. Both Ox lines showed reduced NO levels in roots and enhanced nitrogenase activity in mature and senescent nodules relative to the wild-type (WT). Physiological and cytological observations indicated that overexpression of LjGlb1-1 delayed nodule senescence. The application to WT nodules of the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) or the phytohormones abscisic acid (ABA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) repressed nitrogenase activity, induced the expression of three senescence-associated genes, and caused cytological changes evidencing nodule senescence. These effects were almost completely reverted by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Our results reveal that overexpression of LjGlb1-1 improves the activity of mature nodules and delays nodule senescence in the *L. japonicus*-*M. loti* symbiosis. These beneficial effects are probably mediated by the participation of LjGlb1-1 in controlling the concentration of NO that may be produced downstream in the phytohormone signaling pathway in nodules.

Keywords: Hemoglobin, *Lotus japonicus*, *Mesorhizobium loti*, nitric oxide, nitrogen fixation, symbiosis.

Introduction

Symbiotic hemoglobins are present in nodules of legumes and actinorhizal plants, where they provide the endosymbionts with a steady and low oxygen concentration compatible with bacteroid respiration and nitrogenase activity (Appleby 1984). In contrast, nonsymbiotic hemoglobins, now termed phytoglobins (Glbs), are ubiquitous in plant tissues where they are found at submicromolar concentrations (Trevaskis et al. 1997, Watts et al. 2001, Smaghe et al. 2009). Phytoglobins are classified into three types based on phylogenetic analyses and

1 biochemical properties (Smagghe et al. 2009). Class 1 Glbs have an extremely high affinity
2 for O₂ and at least in some cases may function by modulating nitric oxide (NO) levels and
3 preserving cellular energy during hypoxia (Hill 2012). Class 2 Glbs are considered as the
4 evolutionary precursors of leghemoglobins (Lbs) and, like them, they show a moderate O₂
5 affinity. The functions of these globins remain unclear but they are able to scavenge NO *in*
6 *vivo* (Hebelstrup and Jensen 2008) and are preferentially expressed in developing organs,
7 suggesting a role in tissues with high metabolic demand (Vigeolas et al. 2011, Elhiti et al.
8 2013). Class 3 or truncated Glbs have relatively low O₂ affinities and their functions are
9 unknown. Several of them are induced in nodules and mycorrhizal roots (Vieweg et al. 2005)
10 and might interact also with NO (Sanz-Luque et al. 2015).

11 The expression of class 1 Glbs is induced in response to stressful factors known to trigger
12 NO production, such as low temperature, hypoxia, and high osmotic conditions (Igamberdiev
13 and Hill 2004, Shimoda et al. 2005, Bustos-Sanmamed et al. 2011). Overexpression of class 1
14 Glbs confers tolerance to hypoxic stress by decreasing the level of NO accumulation in
15 *Arabidopsis thaliana* (Hunt et al. 2002), alfalfa (Dordas et al. 2003), and maize (Mira et al.
16 2016). Conversely, mutant plants of *A. thaliana* deficient in its single class 1 Glb (AtGlb1,
17 At2g16060) are more sensitive to pathogen infection (Mur et al. 2012). Many phytohormones
18 trigger NO production and affect Glb gene expression in plant tissues (Hill 2012). In
19 particular, treatment of *Lotus japonicus* plants with the immediate ethylene precursor
20 1-aminocyclopropane-1-carboxylic acid (ACC) or with abscisic acid (ABA) induces the
21 expression of a class 1 Glb gene, *LjGlb1-1* (Lj3g3v3338170), in nodules (Bustos-Sanmamed
22 et al. 2011). It is thus tempting to speculate that Glbs participate in the cross-talk between NO
23 and phytohormones by modulating NO levels. Some functions of class 1 Glbs may rest on
24 their ability to modulate NO concentration through its NO dioxygenase activity, which
25 converts NO into nitrate with the concomitant oxidation of the oxyferrous Glb to ferric Glb
26 (Igamberdiev and Hill 2004, Hebelstrup et al. 2013). However, the NO dioxygenase activity
27 is shared by all classes of Glbs because this reaction is a property of the hemes and, hence, the
28 specificity of this mechanism of action needs to be reassessed (Smagghe et al. 2008).

29 Notably, NO is produced during the rhizobia-legume interaction and is required for the
30 onset of symbiosis (del Giudice et al. 2011, Hichri et al. 2015). On the other hand, NO is a

1 direct inhibitor of nitrogenase (Trinchant and Rigaud 1982, Kato et al. 2010). Consistent with
2 this, treatment of plants with NO scavengers enhances nitrogenase activity (Sasakura et al.
3 2006, Shimoda et al. 2009). There is good evidence that NO concentration in nodules is low
4 under physiological conditions and only increases when the mechanisms responsible for its
5 homeostasis fail, as occurs in senescent nodules and in nodules elicited by rhizobia defective
6 in flavohemoglobin, nitrite reductase, or NO reductase (Sánchez et al. 2010, Horchani et al.
7 2011, Meilhoc et al. 2013, Calvo-Begueria et al. 2018). All these observations indicate that
8 NO homeostasis is essential at several stages of symbiosis. Inoculation of roots with
9 compatible rhizobia induces concomitantly NO generation and LjGlb1-1 expression (Shimoda
10 et al. 2005, Nagata et al. 2008). The use of mutant lines of LjGlb1-1 revealed that the NO
11 scavenging activity of LjGlb1-1 is required for normal infection and nodule formation
12 (Fukudome et al. 2016). The genome of *L. japonicus* contains a second functional class 1 Glb
13 gene, termed *LjGlb1-2* (Lj3g3v3338180), but it is not involved in the NO-dependent response
14 during the onset of symbiosis (Fukudome et al. 2016).

15 In the first part of the present study, we established and characterized stable transgenic
16 lines overexpressing LjGlb1-1 (Ox). In contrast to the transient hairy root transformation
17 system (Shimoda et al. 2009), stably-transformed plants allowed us to determine the
18 symbiotic performance of the Ox lines in mature and senescent nodules. We conclude that the
19 Ox lines outperform the WT line, showing higher nodule activity and delayed nodule
20 senescence because of their low NO levels. In the second part of our study, we performed
21 complementary experiments with NO donors and phytohormones known to elicit NO
22 production, and showed that NO was involved in the detrimental effect of phytohormones on
23 nodule activity and structure.

24 25 26 **Results**

27 28 **Nodulated Ox plants show better symbiotic performance**

29 Two transgenic lines of *L. japonicus* bearing the overexpression construct of *LjGlb1-1* under
30 the control of the constitutive CaMV 35S promoter were generated and designated as Ox1

1 and Ox2. Both lines showed enhanced expression of *LjGlb1-1* in seedlings five days after
2 germination and in nodules five weeks post-inoculation (wpi) compared with the wild-type
3 (WT) and with the *LjGlb1-1* knockout mutant 96642 (Table 1). The expression of the Ox1
4 line doubled the expression of the Ox2 line, both in whole plants and in nodules (Table 1).
5 We monitored the plant length and the nodule numbers in WT, Ox1, Ox2, and 96642 plants
6 grown on nitrogen-free Fåhræus medium; however, because the Ox1 and Ox2 lines behaved
7 similarly we show only the Ox1 data for simplicity (Supplementary Fig. S1A, B). The two
8 parameters did not differ between the WT and Ox1 lines, but the growth of the 96642 line
9 was delayed. The plant length was also measured in plants grown on Fåhræus medium
10 containing 1.5 mM NH_4NO_3 (Supplementary Fig. S1C). In this case, the growth of both Ox1
11 and 96642 plants was delayed relative to the WT plants. There were no morphological
12 differences among the three lines, except for the size.

13 Nitrogenase activity (Fig. 1A) and nodule numbers (Fig. 1B) were measured in plants of
14 the four lines at 4 wpi (mature nodules). Nitrogenase was estimated as acetylene reduction
15 activity (ARA) and expressed per fresh weight of nodules. The two Ox lines exhibited
16 significantly greater ARA per nodule fresh weight than the WT plants but similar nodule
17 number, whereas the 96642 plants had lower nitrogenase activity and fewer nodules. The
18 average weight of nodules was similar at 4 wpi and only tended to increase slightly in the Ox1
19 line at 8 wpi (senescent nodules) (Fig. 1C). Thus, the enhanced ARA of Ox plants was not
20 due to a greater number or size of nodules, but to their better symbiotic performance. Control
21 plants transformed with the empty vector did not show differences on ARA or nodule
22 numbers with respect to the WT (Supplementary Fig. S2).

24 **Roots of Ox lines produce less NO after infection by *Mesorhizobium loti***

25 As part of the phenotyping of the Ox lines, we compared the endogenous production of NO in
26 the roots of WT and Ox plants in response to *M. loti* infection by using the cell permeable
27 fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM DA). This
28 compound is deacetylated by intracellular esterases to DAF-FM, which in turn reacts with
29 endogenous NO (actually, with the NO oxidation product N_2O_3) forming a highly fluorescent
30 triazole. Therefore, fluorescence marks the presence of NO provided that adequate controls

are used in parallel. We applied DAF-FM DA to the roots 3 h after inoculation with *M. loti* and the roots were incubated for 1 h (Fig. 1D). Previous studies from our laboratory using DAF-FM DA have shown that intracellular NO production in WT roots is maximal 4 h after inoculation with *M. loti* (Nagata et al. 2008), and that the roots of 96642 plants produce greater NO levels than those of WT plants (Fukudome et al. 2016). We used the same method to estimate NO production in the roots of the Ox plants including the 96642 line as a control. The fluorescence intensity was observed with a confocal microscope (Fig. 1D). To complement this experiment, we also employed an indirect method based on the cell non-permeable probe DAF-FM. Intact roots were immersed into a solution of the probe and, after appropriate incubation, the fluorescence intensity of the solution was measured with a fluorometer. The reading provided an estimate of the NO released by the intact roots and, indirectly, of the NO being accumulated inside them (Fig. 1E). The results using the two methods were identical: fluorescence intensity was lower in the roots of the Ox plants and, as expected, higher in the roots of the 96642 plants (Fig. 1D, E).

Ox lines also outperform during nodule senescence

Nodule senescence was also compared in plants of the WT, Ox, and 96642 lines. To this end, we conducted experiments with plants at 3-5 wpi (mature nodules) and at 6-8 wpi (senescent nodules). In preliminary tests, we noticed that the WT nodules had already turned to green at 6 wpi, whereas the corresponding Ox nodules remained pale pink (Supplementary Fig. S3). In contrast, no obvious difference was seen in the color of nodules of line 96642 with respect to the WT (data not shown). Because formation of green pigments from Lb is a well-known hallmark of nodule senescence (Lehtovaara and Pertilä 1978, Navascués et al. 2012), this observation strongly suggested that senescence was delayed in the Ox nodules.

To further support this hypothesis, we measured ARA at 8 wpi (senescent nodules). As occurred at 4 wpi (mature nodules), the two Ox lines outperformed the WT plants, whereas the 96642 plants behaved similarly (Fig. 2A). A plot of the ratios of ARA at 8 wpi to ARA at 4 wpi also evidenced that the Ox lines retained higher nodule activity during senescence than did the WT and 96642 lines (Supplementary Fig. S4). Because NO is efficiently scavenged in roots of Ox lines by LjGlb1-1 (Fig. 1D, E), we also investigated whether the Ox nodules have

1 lower NO levels than the WT nodules. For this purpose, we used nodules at 6 wpi (senescent
2 nodules) and measured the release of NO using the DAF-FM probe. The fluorescence signal,
3 expressed as relative fluorescence units (RFUs) per nodule fresh weight, was clearly reduced
4 in the Ox nodules whereas it was higher in the nodules of the 96642 plants (Fig. 2B).

5 Two additional experiments were carried out to demonstrate that the Ox nodules have
6 delayed senescence: (1) expression analysis of Lb, a marker of the nodule physiological state,
7 and of some senescence-associated genes; and (2) light microscopic examination of nodules.
8 For the first experiment, total RNA was isolated from mature and senescent nodules and
9 mRNA levels were quantified by qRT-PCR (Fig. 2C). We performed a multiple comparison
10 of the means of the four lines and of the two developmental stages, but also considered
11 threshold values for the relative mRNA levels (R). These threshold values were $R > 2$ for gene
12 up-regulation and $R < 0.5$ for gene down-regulation (with respect to a value of $R = 1$ given to
13 controls) and are routinely used in transcriptomic studies of *L. japonicus* (Ott et al. 2009). To
14 quantify Lb expression, we used a pair of primers that allows the joint quantification of
15 mRNA levels of Lb1 and Lb2, two Lb isoproteins with almost identical sequences that are
16 highly expressed in *L. japonicus* nodules (Uchiumi et al. 2002, Ott et al. 2005). We found that
17 two sets of primers previously reported to specifically amplify *Lb1* and *Lb2* (Ott et al. 2005)
18 recognize both genes. Figure 2C shows that the expression of *Lb1/2* is higher in both the
19 mature and senescent nodules of the Ox lines than in the respective nodules of the WT and
20 96642 plants, strongly supporting that the nodules overexpressing *LjGlb1-1* retain higher
21 metabolic activity during senescence. We also quantified the expression of three genes
22 generally used as senescence markers: heat shock protein (Lj4g3v0473190), osmotin
23 precursor (Lj2g3v2017460), and cysteine protease Cyp2 (Lj1g3v4047250) (Fujie et al. 2009,
24 Chungopast et al. 2014). As could be anticipated, these genes were up-regulated in senescing
25 nodules relative to mature nodules for all four lines (Fig. 2C). Notably, the senescent nodules
26 of the Ox1 and Ox2 plants had much lower expression levels of the three marker genes than
27 the nodules of the other plants, further confirming that the Ox nodules show delayed
28 senescence (Fig. 2C).

29 In the second experiment, we examined microscopically nodule sections after staining with
30 toluidine blue (Fig. 3A) and Periodic Acid-Schiff (PAS) reagent (Fig. 3B). The infected zone

of WT nodules contained large infected cells uniformly packed with bacteroids and smaller uninfected cells that were almost completely vacuolated at 4 wpi. The infected cells contained one or two large vacuoles and agglutination of bacteroids was apparent at 6 wpi. The infected cells in the central area exhibited disintegration and were almost empty at 8 wpi. The vacuoles in the infected cells of the Ox lines increased in size and number at 8 wpi compared with 4 wpi (Fig. 3A). In addition, PAS staining revealed that a great number of large starch granules accumulated in the uninfected cells as well as in the inner cortex of the nodules of WT at 6 wpi (Fig. 3B). Compared with the WT, the starch granules of the Ox lines were smaller and fewer. In contrast, toluidine blue and PAS stainings revealed that the nodules of the WT and 96642 plants underwent similar extent of disintegration of infected cells and accumulation of starch granules (Fig. 3B).

Phytohormones induce NO production in nodules as well as nodule senescence

The second part of our study was focused on the effects of ABA and ethylene on nodule functioning because both phytohormones are known to inhibit nodulation (Suzuki et al. 2004, Lohar et al. 2009), cross-talk with NO in plant tissues (Neill et al. 2002, Hill 2012, Mur et al. 2012), and induce *LjGlb1-1* (Bustos-Sanmamed et al. 2011). First, we confirmed by qRT-PCR that the exogenous addition of an NO donor (SNAP; *S*-nitroso-*N*-acetyl-DL-penicillamine), ABA, or ACC increased the *LjGlb1-1* mRNA level. Furthermore, we observed that the induction of the gene was blocked when each compound was applied together with an NO scavenger [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, cPTIO] (Supplementary Fig. S5). Then, we quantified the NO released from nodules using DAF-FM. Nodules attached to the roots were treated with 100 μ M ABA or 100 μ M ACC for 24 h. They were then detached and incubated with the probe and the fluorescence of the solution was measured. Under these conditions, we found that ABA, but not ACC, doubled the NO level of the control (Supplementary Fig. S6).

We also examined the effects of the two phytohormones on the nodule functional state using as parameters ARA per nodule fresh weight, ARA per plant, and *Lb1/2* expression (Fig. 4). In this experiment, we included SNAP as a positive control for NO production as well as co-treatments with cPTIO to verify that the effects were genuinely due to NO. Our data reveal

1 a consistent inhibition of ARA by all three compounds (SNAP, ABA, and ACC) and a
2 reversal of this inhibition by cPTIO (Fig. 4A, B). Likewise, we investigated whether NO and
3 the phytohormones induce the expression of *Lb1/2* and the senescence marker genes in the
4 WT nodules (Fig. 4C). Nodules at 4 wpi were treated for 72 h with SNAP, ABA, or ACC, in
5 combination or not with cPTIO, and gene expression was analyzed by qRT-PCR. Figure 4C
6 shows that the *Lb1/2* mRNA levels did not significantly change upon the supply of plants
7 with SNAP, ABA, or ACC, and that the combined application of cPTIO increased gene
8 expression only slightly when considering cut-off values for up-regulation ($R>2$) and
9 down-regulation ($R<0.5$). The heat-shock protein transcript was slightly induced by SNAP but
10 not by the phytohormones, whereas the osmotin precursor and cysteine protease transcripts
11 were induced by the three compounds. With a few exceptions, the induction of the genes by
12 SNAP, ABA, and ACC was partially suppressed by cPTIO; however, the combined treatment
13 of SNAP with cPTIO enhanced the expression of cysteine protease (Fig. 4C).

14 Finally, the nodules of WT plants were treated with SNAP, ABA, or ACC for 72 h, and
15 were examined by light microscopy after PAS staining (Fig. 5). In nodules treated with SNAP
16 or ACC, we observed an increased number of vacuoles in the infected cells as well as spread
17 starch granules, features characteristic of nodule senescence. Also, we found that nodules
18 treated with ABA had a higher number of vacuoles in the infected cells, but similar
19 distribution of starch granules to the control nodules. Most of the changes in the nodule
20 tissues caused by SNAP, ABA, and ACC were suppressed by cPTIO; however, the combined
21 treatment of ACC and cPTIO resulted in an increase of the vacuole number (Fig. 5).

24 Discussion

26 In this work, we have generated stably-transformed lines, Ox1 and Ox2, of *L. japonicus* that
27 overexpress the phytoglobin LjGlb1-1 under the control of a constitutive promoter. These
28 plants allowed us to study the effects of LjGlb1-1 on nodule development and senescence,
29 which was not possible using plants with transient overexpression (Shimoda et al. 2009). First,
30 we show here that the roots and nodules of the Ox lines have lower NO levels and greater

1 nitrogenase activities than the WT plants. The accumulation of NO in nodules decreases N₂
2 fixation rates and induces premature senescence (Shimoda et al. 2009, Cam et al. 2012,
3 Fukudome et al. 2016). These effects are somewhat expected as NO is a potent inhibitor of
4 nitrogenase (Trinchant and Rigaud 1982, Kato et al. 2010) and interferes with Lb function
5 (Hichri et al. 2005, Sánchez et al. 2010, Navascués et al. 2012, Calvo-Begueria et al. 2018).
6 The lower NO level in the nodules of the Ox lines may thus contribute to enhance the
7 nitrogenase activity. Because the number of nodules and the weight per nodule were similar
8 in the Ox and WT plants, the increased activity should be ascribed to a better efficiency of the
9 Ox nodule tissue to fix N₂ and not to a larger weight of nodules per plant. It also suggests that
10 the mechanism for autoregulation of nodulation (Kosslak and Bohlool 1984) remains intact in
11 the Ox plants.

12 The enhanced symbiotic performance of the Ox plants is also reflected by the observation
13 that their nodules retain the red color at 6 wpi, when the WT nodules had turned green, which
14 is indicative of heme degradation (Lehtovaara and Perttilä 1978, Navascués et al. 2012).
15 Because the NO level is low even in the senescent Ox nodules, the oxidative reactions of Lb
16 heme in these nodules may be slower than in the senescent WT nodules. An additional
17 finding supporting the outperformance of the Ox lines is that the WT senescent nodules at 6
18 wpi and 8 wpi showed aggregation of bacteroids and disruption of infected cells, typical
19 features of senescence (Hossain et al. 2006), whereas the infected cells of the Ox nodules of
20 the same age did not display significant changes. Additionally, the number of starch granules
21 in the Ox nodules at 6 wpi was lower than in the corresponding WT nodules, probably
22 because the capacity of the latter to consume carbon metabolites declines due to deterioration
23 of nodule function. All these observations evidence that the overexpression of LjGlb1-1
24 extends the nodule's lifespan.

25 In the second part of our study, we analyzed the effects of ABA and ethylene on the
26 performance of the *L. japonicus*-*M. loti* symbiosis. These two phytohormones are known to
27 inhibit N₂ fixation and nodulation (González et al. 2001, Ferguson and Mathesius 2003, and
28 references therein) and to induce nodule senescence (Ligero et al. 1991, Suzuki et al. 2004,
29 Lohar et al. 2009). We observed that ABA and ACC, as occurs for SNAP, decreased
30 nitrogenase activity and induced three senescence marker genes. These effects were

1 suppressed by the NO scavenger cPTIO. Also, the two phytohormones induced histological
2 changes in the infected cells of mature nodules at 4 wpi, such as an increase in the number
3 and size of vacuoles and in starch deposition, resembling nodule senescence. These changes
4 in the infected cells were also suppressed by cPTIO. Based on all these observations, we
5 propose that ABA and ACC induce nodule senescence and that, at least in part, they do so by
6 increasing NO levels. However, SNAP together with cPTIO enhanced the expression of
7 cysteine protease and ABA did not induce the accumulation of starch grains in the nodule
8 tissue, pointing out differences in the nodule senescence induced by SNAP, ABA, and ACC.
9 In fact, the latter two observations suggest that additional mechanisms, independent of NO
10 signaling, are involved, which is not unexpected given the complexity of the nodule
11 senescence process at the metabolic and cellular levels (Dupont et al. 2012).

12 In conclusion, our results indicate that the overexpression of LjGlb1-1 improves the
13 efficiency of the symbiosis by increasing nodule activity without affecting the number or
14 weight of nodules and by delaying nodule senescence. This effect may be mediated by a
15 reduction in the NO levels inside the nodules. Our study also reveals that the phytohormones
16 ABA and ethylene trigger nodule senescence as evidenced by the decrease in nitrogenase
17 activity, the induction of senescence marker genes, and the deterioration of infected cells. The
18 two phytohormones induce NO accumulation and the senescence-associated changes are
19 suppressed by the NO scavenger cPTIO. This indicates that, at least partially, the mechanism
20 of senescence induction entails an increase in NO levels.

23 **Materials and Methods**

25 **Biological materials and growth conditions**

26 Plants of *Lotus japonicus* accession Gifu B-129 and their derivative lines were germinated and grown
27 as described (Nagata et al. 2008). In brief, five days after germination seedlings were transferred to
28 1.5% Fåhræus agar plates (Fåhræus 1957) and were inoculated with a cell suspension (10^7 cells ml⁻¹
29 in water) of *Mesorhizobium loti* strain MAFF303099 (Kaneko et al. 2000). For experiments with
30 non-nodulated plants, the Fåhræus medium was supplemented with 1.5 mM NH₄NO₃. The plants

were grown under controlled conditions with a photosynthetically active radiation of 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (16-h photoperiod) at 25°C for 4, 6, and 8 wpi. The knockout mutant line 30096642 (abbreviated as 96642) bearing the retrotransposon *LORE1* inserted in the 5'-untranslated region was obtained from the *LORE1* collection (Fukai et al. 2012, Urbański et al. 2012, Małolepszy et al. 2016). The binary vector with the cauliflower mosaic virus 35S (CaMV 35S) promoter and cDNA of *LjGlb1-1* was constructed based on pIG121-Hm, and the hemoglobin-ectopic overexpression lines of *L. japonicus* were produced according to Aoki et al. (2002).

Nitrogenase activity

Nitrogenase activity of nodules at 4 wpi was determined as ARA according to Shimoda et al. (2009). To estimate ARA at 8 wpi, visible nodules formed at 2 wpi were marked and 6 weeks later the unmarked nodules were removed from the roots. The ARA of the whole plant was then measured and ascribed to nodules at 8 wpi.

Endogenous NO production in roots and NO released from nodules

The endogenous production of NO in the roots of 5-day-old seedlings was monitored by fluorescence microscopy as described by Nagata et al. (2008). Seedlings were inoculated with *M. loti* and incubated for 3 h. The seedlings were then soaked for 1 h with 20 μM of the cell permeant probe DAF-FM DA (GORYO Chemical, Japan) in distilled water. Confocal images were captured using an A1si-90i microscope and epifluorescence images using an Eclipse 90i microscope (Nikon, Japan).

The NO released from roots and nodules was assessed using the cell non-permeant probe DAF-FM. The roots were incubated with *M. loti* for 4 h, followed by 3 min with 7 μM of DAF-FM, and NO in the solution was measured as RFUs. The NO released from nodules was determined in detached nodules that were immediately soaked in 7 μM DAF-FM for 10 min. The RFUs of the DAF-FM solution were measured using an e-spect2 (Malcom, Japan) fluorometer with excitation at 495 nm and emission at 519 nm.

Treatment of nodules with NO donor, NO scavenger, and phytohormones

The nodules on the roots at 4 wpi were sandwiched between two 7-mm square papers and impregnated with 40 μl of solutions containing 20 mM SNAP, 20 mM cPTIO, 100 μM ABA, or 100 μM ACC for

24 h. To investigate the effect of these compounds on the expression of senescence-associated genes and on the nodule tissue, the nodules were treated for 72 h with 20 µl of each solution at 24 h intervals.

Expression analysis of *Lb* and senescence-related genes by qRT-PCR

Total RNA was extracted from ~50 mg of nodules with the RNeasy plant mini kit (Qiagen). qRT-PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems) and One Step SYBR Prime Script RT-PCR kit (Takara, Japan). Primers for *LjGlb1-1* (5'-CCTTTGGAGGAGAACCCCAA-3' and 5'-GAGCTGCTGATTACACAAGTCA-3'), *Ljb1/2* (5'-GTGGTTAAAGAAGCACTGCT-3' and 5'-TTAATTGCAGCTGCGAGTCC-3'), *heat shock protein* (Lj4g3v0473190; 5'-CAGTGGGAAA TTCCAGAGGA-3' and 5'-AGTGAGAACCCCATCTCCA-3'), *osmotin precursor* (Lj2g3v2017460; 5'-GGACAGGTGCCATGATTCTT-3' and 5'-GAAAGTGCTGGT GGGATCAT-3'), *cysteine protease* LjCyp2 (Lj1g3v4047250; 5'-GGAGAACA ATGGGGTGAAGA-3' and 5'-GCCACACAAACCCAATACTG-3') and *LjeIF-4A* (Lj6g3v1382260; 5'-TGGAAGCTTCGAAGAGATGG-3' and 5'-GTGCCAGATTGAGCCTGAG-3') were used with a PCR program consisting of an initial denaturation and *Taq* polymerase activation step of 10 s at 95°C, followed by 40 cycles of 10 s at 95°C and 31s at 60°C, and a final melting curve stage. The reverse transcription step was 5 min at 42° C. Primer specificity and the absence of contaminating genomic DNA were verified, respectively, with amplicon dissociation curves and with PCR analysis of RNA samples prior to reverse transcription. Expression levels were normalized using *LjeIF-4A* as the internal reference gene. All genes used in this study are deposited in the *Lotus japonicus* Genome Sequencing Project (<http://www.kazusa.or.jp/lotus/>).

Light microscopy

Nodules were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C overnight. Fixed samples were dehydrated through a graded ethanol series, embedded in JB4 resin (Polysciences, Warrington, USA), and sectioned (3 µm thick). Nodule sections were stained with 0.5% (w/v) toluidine blue and starch granules were stained with the PAS reagent (Muto Pure Chemicals, Japan) according to the manufacturer's instructions.

Funding

This work was supported by Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects (Japan) and NIBB Collaborative Research Program (grants 16-305 and 17-309, Japan) to T.U., and by the Ministry of Economy and Competitiveness (grant AGL2017-53717-R, Spain) to M.B.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

The authors thank the National BioResource Project for providing seeds of *L. japonicus* B-129 Gifu.

Supplementary Data

Supplementary data are available at PCP online.

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Figure Legends

Fig. 1 Nitrogenase activity, number and weight of nodules, and NO production in roots of the WT, Ox, and 96642 lines. (A) Nitrogenase activity (estimated as ARA) was measured at 4 wpi (mature nodules) and expressed as ethylene produced per hour and nodule fresh weight. (B) The number of nodules per plant was counted at 4 wpi. (C) The average weight of nodules was calculated at 4 wpi. (D) Fluorescence imaging of NO production in roots 4 h after infection with *M. loti*. Scale bars, 100 μ m. (E) NO released from roots 4 h after infection with *M. loti*. Fluorescence was quantified and expressed per root fresh weight. For panels A-C and E, values are means $\pm$ SE of nine biological replicates. Means denoted by the same letter do not significantly differ at $P<0.05$ based on the Duncan's multiple range test.

Fig. 2 Effect of senescence on nodule activity, NO release from nodules, and expression of *Lb* and senescence-associated genes in nodules of WT, Ox1, Ox2, and 96642 plants. (A) Nitrogenase activity, estimated as ARA, was measured at 8 wpi (senescent nodules) and expressed as ethylene produced per hour and nodule fresh weight. Values are means $\pm$ SE of 18 biological replicates. (B) Quantification of NO released from nodules. Fluorescence was expressed per nodule fresh weight. Values are means $\pm$ SE of nine biological replicates. (C) Expression of *Lb* and senescence-associated genes in mature and senescent nodules. Steady-state mRNA levels (R) of senescent nodules were expressed relative to those of WT mature nodules, which were set at $R=1$. Values are means $\pm$ SE of three biological replicates. Asterisks indicate up-regulation ($R>2$) or down-regulation ($R<0.5$) compared with WT mature nodules. For panels A, B, and C, mean values denoted by the same letter do not significantly differ at $P<0.05$ based on the Duncan's multiple range test.

Fig. 3 Microscopic observation of nodules of WT and Ox1 plants during senescence. (A) Sections of nodules at 4, 6, and 8 wpi were stained with toluidine blue to visualize the general features of infected cells. Note that at 6 and 8 wpi the structural integrity of infected cells, including vacuoles, is better preserved in the Ox nodules than in the WT nodules. (B) Sections of nodules at 6 wpi were stained with PAS to show distribution of starch granules. Note that the nodules of the WT and 96642 plants underwent similar extent of disintegration of infected cells and of accumulation of starch granules. Scale bars, 20 μ m.

Fig 4 Effect of NO donor (SNAP) and phytohormones (ABA, ACC) on nitrogenase activity and on the expression of *Lb* and senescence-associated genes. Nodules at 4 wpi were treated with SNAP, ABA, or ACC, in combination or not with cPTIO. (A, B) Nitrogenase was measured as ARA and expressed either per hour and nodule fresh weight (A) or per plant (B). Values are means $\pm$ SE of nine biological replicates. Means denoted by the same letter do not significantly differ based on the Duncan's multiple range test ($P<0.05$). (C) Steady-state mRNA levels (*R*) of *Lb* and senescence-associated genes were expressed relative to the controls treated with distilled water, which were given a value of $R=1$. Asterisks indicate up-regulation ($R>2$) or down-regulation ($R<0.5$) compared with WT mature nodules. For panels A, B, and C, mean values denoted by the same letter do not significantly differ at $P<0.05$ based on the Duncan's multiple range test.

Fig. 5 Structure of nodules treated with NO donor (SNAP) or phytohormones (ABA and ACC). Nodules of 4 wpi were treated for 72 h with these compounds, in combination or not with cPTIO, and then were sectioned and stained with PAS. Microphotographs are representative of sections from nine nodules of different plants. Note that most of the changes in the nodule tissues caused by SNAP, ABA, and ACC were suppressed by cPTIO. Scale bars, 20 μ m.

Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Table 1 Relative expression level of *LjGlb1-1*.

	WT	Ox1	Ox2	96642
Whole plant	1	13.17 ± 1.67	5.41 ± 0.81	0.03 ± 0.00
Nodule	1	10.39 ± 1.95	4.12 ± 0.66	0.01 ± 0.00

Data are means ± SE of nine biological replicates.

Fig. S1 Plant growth and nodule numbers of the WT, Ox1, and 96642 lines. (A and B) Seedlings were inoculated with *M. loti* and grown on nitrogen-free Fåhræus medium. (C) Seedlings were grown on Fåhræus medium containing 1.5 mM NH_4NO_3 . For all panels, values are means $\pm$ SE of nine biological replicates. Means denoted by "a" significantly differ between WT (O) and Ox1 (■), and means denoted by "b" significantly differ between WT (O) and 96642 (▲), based on the Student's *t*-test ($P < 0.05$).

Fig. S2 Nitrogenase activity and nodule number of the empty vector control line. (A) Values of nitrogenase activity (ARA per nodule fresh weight) and (B) number of nodules were measured at 4 wpi and are means $\pm$ SE of 17 biological replicates. There were no statistically significant differences based on the Student's *t*-test ($P < 0.05$). EV, plants transformed with the empty vector pIG121-Hm.

Fig. S3 Senescent nodules (6 wpi) of the two Ox lines retain more Lb than do those of the WT line. Yellow arrows indicate the greenish color of WT nodules and the pink color of the Ox nodules. Images are representative of 18 nodules per line.

Fig. S4 The relative ARA of senescent nodules (ratio of ARA at 8 wpi to ARA at 4 wpi, in percent) was determined for the WT, Ox1, Ox2, and 96642 lines. Values are means $\pm$ SE of measurements from three independent experiments conducted in triplicate. Means denoted by the same letter do not significantly differ at $P < 0.05$ based on the Duncan's multiple range test.

Fig. S5 Induction of *LjGlb1-1* in nodules by NO and phytohormones. Total RNA was isolated after 24 h of treatment with SNAP, ABA, or ACC in the absence or presence of cPTIO. Steady-state mRNA levels (R) are expressed relative to the controls treated with distilled water, which were given a value of $R=1$. Values are means $\pm$ SE of three biological replicates. Means denoted by the same letter do not significantly differ at $P<0.05$ based on the Duncan's multiple range test.

Fig. S6 Effect of phytohormones on NO release from nodules. Nodules attached to the roots were treated with 100 μ M ABA or 100 μ M ACC for 24 h. Then, three or four nodules from two plants were detached and immersed in a solution of 7 μ M DAF-FM in distilled water. After 10 min, the fluorescence of the solution was measured and expressed as relative fluorescence units (RFUs) per nodule fresh weight. Control nodules were incubated with distilled water instead of ABA or ACC. Values are means $\pm$ SE of three biological replicates. Means denoted by the same letter do not significantly differ at $P < 0.05$ based on the Duncan's multiple range test.