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

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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 N_2-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-DL-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.

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; Lb, leghemoglobin; NO, nitric oxide; PAS, Periodic Acid-Schiff staining; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; WT, wild-type.

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, non-symbiotic 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, Smagghe et al. 2009). Glbs are classified into three types based on phylogenetic analyses and biochemical properties (Smagghe et al. 2009). Class 1 Glbs have an extremely high affinity for O_2 and at least in some cases may function by modulating nitric oxide (NO) levels and preserving cellular energy during hypoxia (Hill 2012). Class 2 Glbs are considered as the evolutionary precursors of leghemoglobins (Lbs) and, like them, they show a moderate O_2 affinity. The functions of these globins remain unclear but they are able to scavenge NO in vivo (Hebelstrup and Jensen 2008) and are preferentially expressed in developing organs, suggesting a role in tissues with high metabolic demand (Vigeolas et al. 2011, Elhiti et al. 2013). Class 3 or truncated Glbs have relatively low O_2 affinities and their functions are unknown. Several of them are induced in nodules and mycorrhizal roots (Vieweg et al. 2005) and might interact also with NO (Sanz-Luque et al. 2015).

The expression of class 1 Glbs is induced in response to stressful factors known to trigger NO production, such as low temperature, hypoxia and high osmotic conditions (Igamberdiev and Hill 2004, Shimoda et al. 2005, Bustos-Sanmamed et al. 2011). Overexpression of class 1 Glbs confers tolerance to hypoxic stress by decreasing the level of NO accumulation in *Arabidopsis thaliana* (Hunt et al. 2002), alfalfa (Dordas et al. 2003) and maize (Mira et al. 2016). Conversely, mutant plants of *A.thaliana* deficient in its single class 1 Glb (AtGlb1, At2g16060) are more sensitive to pathogen infection (Mur et al. 2012). Many phytohormones trigger NO production and affect Glb gene expression in plant tissues (Hill 2012). In particular, treatment of *Lotus japonicus* plants with the immediate

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ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or with abscisic acid (ABA) induces the expression of a class 1 Glb gene, *LjGlb1-1* (Lj3g3v3338170), in nodules (Bustos-Sanmamed et al. 2011). It is thus tempting to speculate that Glbs participate in the cross-talk between NO and phytohormones by modulating NO levels. Some functions of class 1 Glbs may rest on their ability to modulate NO concentration through its NO dioxygenase activity, which converts NO into nitrate with the concomitant oxidation of the oxyferrous Glb to ferric Glb (Igamberdiev and Hill 2004, Hebelstrup et al. 2013). However, the NO dioxygenase activity is shared by all classes of Glbs because this reaction is a property of the hemes and, hence, the specificity of this mechanism of action needs to be reassessed (Smagghe et al. 2008).

Notably, NO is produced during the rhizobia-legume interaction and is required for the onset of symbiosis (del Giudice et al. 2011, Hichri et al. 2015). On the other hand, NO is a direct inhibitor of nitrogenase (Trinchant and Rigaud 1982, Kato et al. 2010). Consistent with this, treatment of plants with NO scavengers enhances nitrogenase activity (Sasakura et al. 2006, Shimoda et al. 2009). There is good evidence that NO concentration in nodules is low under physiological conditions and only increases when the mechanisms responsible for its homeostasis fail, as occurs in senescent nodules and in nodules elicited by rhizobia defective in flavohemoglobin, nitrite reductase or NO reductase (Sánchez et al. 2010, Horchani et al. 2011, Meilhoc et al. 2013, Calvo-Begueria et al. 2018). All these observations indicate that NO homeostasis is essential at several stages of symbiosis. Inoculation of roots with compatible rhizobia induces concomitantly NO generation and LjGlb1-1 expression (Shimoda et al. 2005, Nagata et al. 2008). The use of mutant lines of LjGlb1-1 revealed that the NO scavenging activity of LjGlb1-1 is required for normal infection and nodule formation (Fukudome et al. 2016). The genome of *L.japonicus* contains a second functional class 1 Glb gene, termed *LjGlb1-2* (Lj3g3v3338180), but it is not involved in the NO-dependent response during the onset of symbiosis (Fukudome et al. 2016).

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

Results

Nodulated Ox plants show better symbiotic performance

Two transgenic lines of *L.japonicus* bearing the overexpression construct of *LjGlb1-1* under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter were generated and designated as Ox1 and Ox2. Both lines showed enhanced expression of *LjGlb1-1* in seedlings 5 d after germination and in nodules 5 weeks post-inoculation (wpi) compared with the wild-type (WT) and with the *LjGlb1-1* knockout mutant 30096642 (abbreviated as 96642) (**Table 1**). The expression of the Ox1 line doubled the expression of the Ox2 line, both in whole plants and in nodules (**Table 1**). We monitored the plant length and the nodule numbers in WT, Ox1, Ox2 and 96642 plants grown on nitrogen-free Fåhraeus medium; however, because the Ox1 and Ox2 lines behaved similarly we show only the Ox1 data for simplicity (Supplementary Fig. S1A, B). The two parameters did not differ between the WT and Ox1 lines, but the growth of the 96642 line was delayed. The plant length was also measured in plants grown on Fåhraeus medium containing 1.5 mM NH_4NO_3 (Supplementary Fig. S1C). In this case, the growth of both Ox1 and 96642 plants was delayed relative to the WT plants. There were no morphological differences among the three lines, except for the size.

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

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

As part of the phenotyping of the Ox lines, we compared the endogenous production of NO in the roots of WT and Ox plants in response to *Mesorhizobium loti* infection by using the cell permeable fluorescent dye 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA). This compound is deacetylated by intracellular esterases to DAF-FM, which in turn reacts with endogenous NO (actually, with the NO oxidation product N_2O_3) forming a highly fluorescent 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

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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.

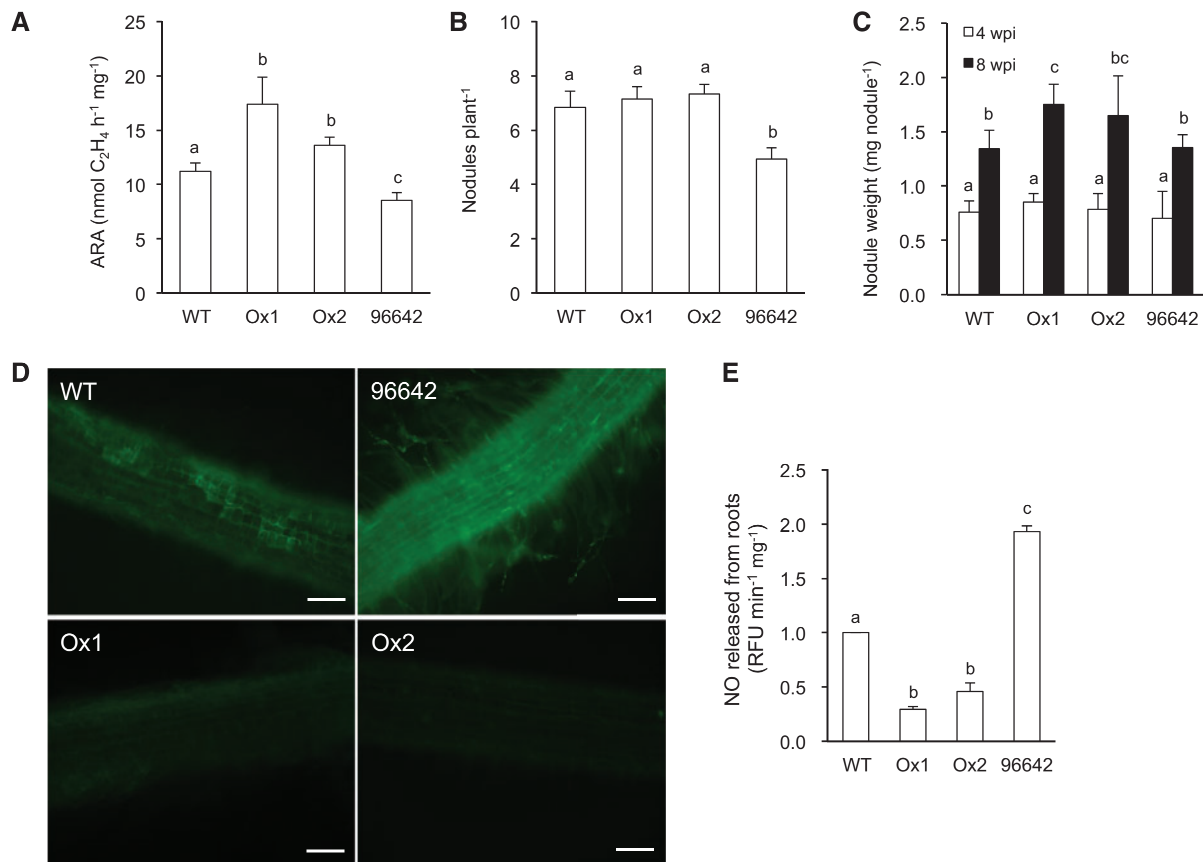

Fig. 1 Nitrogenase activity, number and weight of nodules, and NO production in roots of the WT, Ox and 96642 lines. (A) Nitrogenase activity (estimated as ARA) was measured at 4 wpi (mature nodules) and expressed as ethylene produced per hour and nodule fresh weight. (B) The number of nodules per plant 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 µm. (E) NO released from roots 4 h after infection with *M.loti*. Fluorescence was quantified and expressed per root fresh weight. For (A–C, E), values are means ± 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.

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 Perttilä 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

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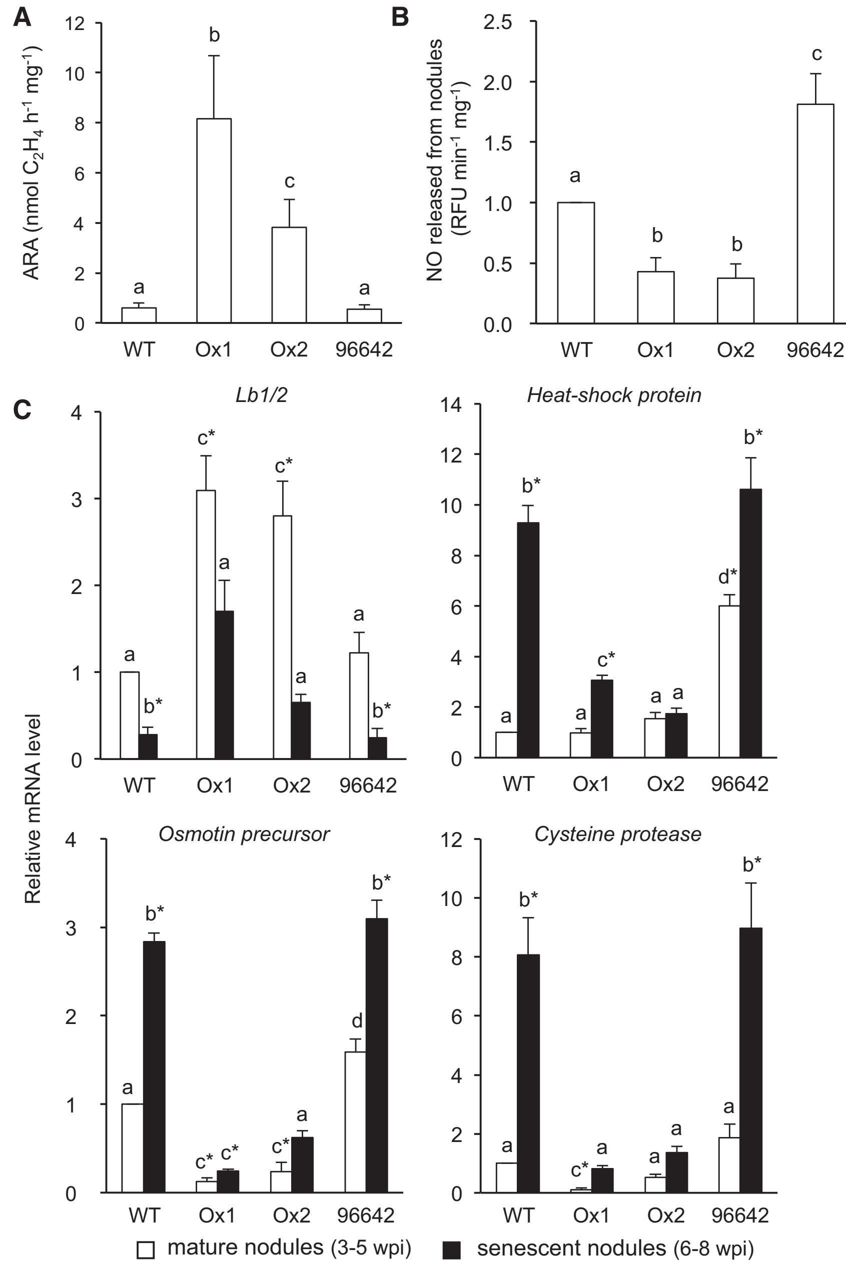

Fig. 2 Effect of senescence on nodule activity, NO release from nodules, and expression of *Lb* and senescence-associated genes in nodules of WT, Ox1, Ox2 and 96642 plants. (A) Nitrogenase activity, estimated as ARA, was measured at 8 wpi (senescent nodules) and expressed as ethylene produced per hour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

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

Two additional experiments were carried out to demonstrate that the Ox nodules have delayed senescence: (i) expression analysis of Lb, a marker of the nodule physiological state, and of some senescence-associated genes; and (ii) light microscopic examination of nodules. For the first experiment, total RNA was

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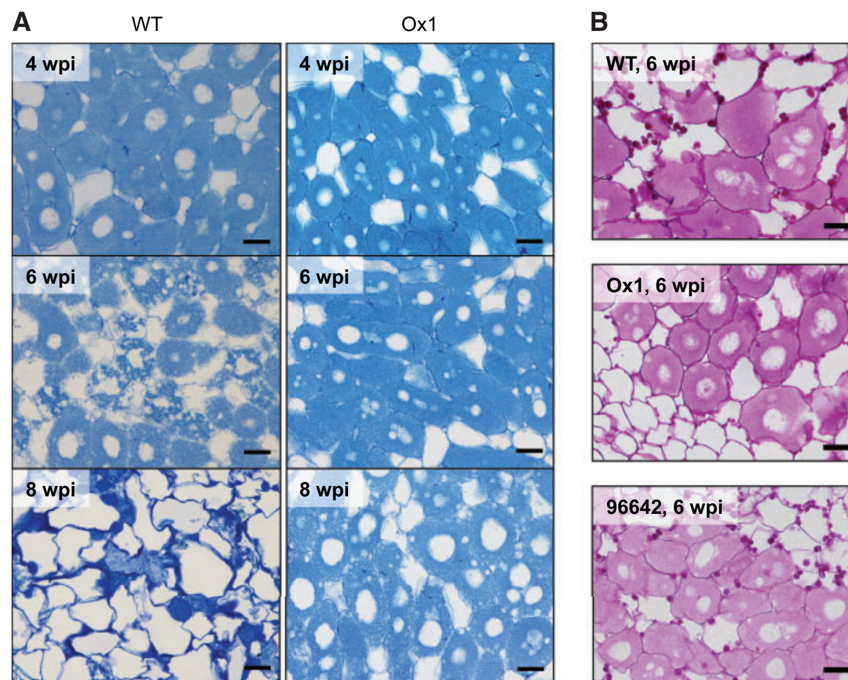

Fig. 3 Microscopic observation of nodules of WT and Ox1 plants during senescence. (A) Sections of nodules at 4, 6 and 8 wpi were stained with toluidine blue to visualize the general features of infected cells. Note that at 6 wpi and 8 wpi the structural integrity of infected cells, including vacuoles, 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 μm.

isolated from mature and senescent nodules and mRNA levels were quantified by qRT-PCR (Fig. 2C). We performed a multiple comparison of the means of the four lines and of the two developmental stages, but also considered threshold values for the relative mRNA levels (R). These threshold values were $R > 2$ for gene up-regulation and $R < 0.5$ for gene down-regulation (with respect to a value of $R = 1$ given to controls) and they are routinely used in transcriptomic studies of *L.japonicus* (Ott et al. 2009). To quantify Lb expression, we used a pair of primers that allows the joint quantification of mRNA levels of LjLb1 and LjLb2, two Lb isoproteins with almost identical sequences that are highly expressed in *L.japonicus* nodules (Uchiumi et al. 2002, Ott et al. 2005). We found that two sets of primers previously reported to specifically amplify *LjLb1* and *LjLb2* (Ott et al. 2005) recognize both genes. Fig. 2C shows that the expression of *LjLb1/2* is higher in both the mature and senescent nodules of the Ox lines than in the respective nodules of the WT and 96642 plants, strongly supporting that the nodules overexpressing *LjGlb1-1* retain higher metabolic activity during senescence. We also quantified the expression of three genes generally used as senescence markers: *heat shock protein* (Lj4g3v0473190), *osmotin precursor* (Lj2g3v2017460) and *cysteine protease Cyp2* (Lj1g3v4047250) (Fujie et al. 2009, Chungopast et al. 2014). As could be anticipated, these genes were up-regulated in senescing nodules relative to mature nodules for all four lines (Fig. 2C). Notably, the senescent nodules of the Ox1 and Ox2 plants had much lower expression levels of the three marker genes than the nodules of the other plants, further confirming that the Ox nodules show delayed senescence (Fig. 2C).

In the second experiment, we examined microscopically nodule sections after staining with 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 [*S*-nitroso-

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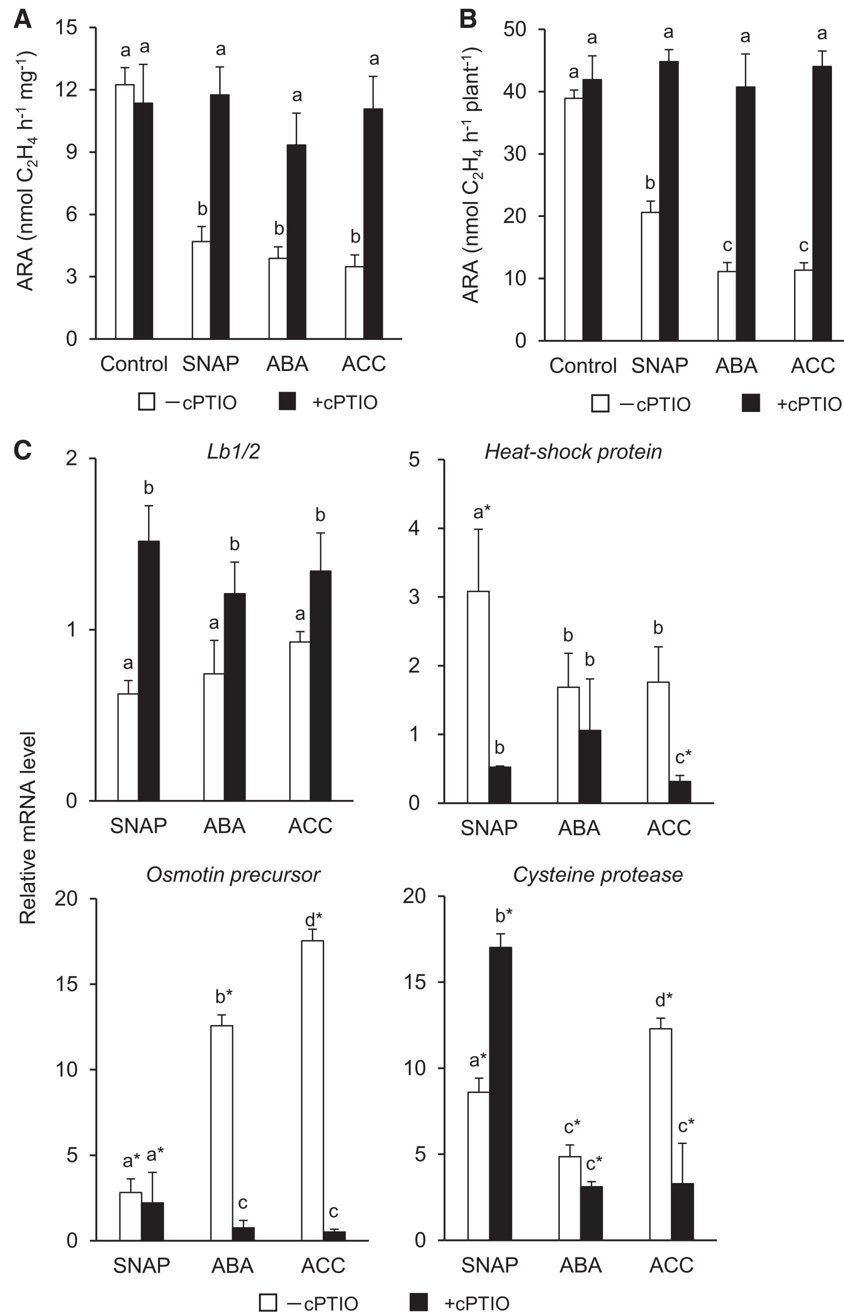

Fig. 4 Effect of NO donor (SNAP) and phytohormones (ABA, ACC) on nitrogenase activity and on the expression of *Lb* and senescence-associated genes. Nodules at 4 wpi were treated with SNAP, ABA or ACC, in combination or not with cPTIO. (A, B) Nitrogenase was measured as ARA and expressed either per hour and nodule fresh weight (A) or per plant (B). Values are means ± 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 (A–C), mean values denoted by the same letter do not significantly differ at $P < 0.05$ based on the Duncan's multiple range test.

N-acetyl-DL-penicillamine (SNAP)], 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 µM ABA or 100 µM 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 phytohomones on the nodule functional state using as parameters ARA per nodule fresh

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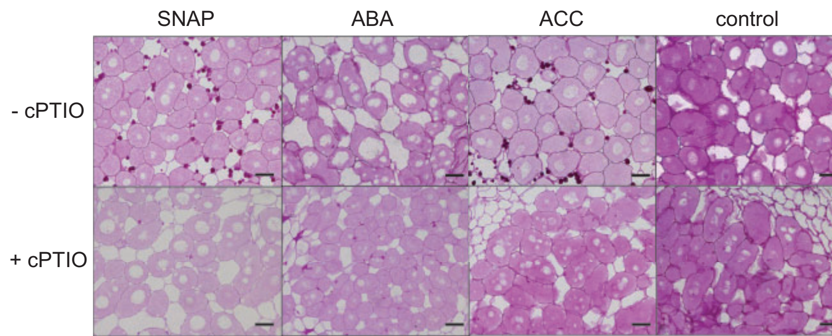

Fig. 5 Structure of nodules treated with NO donor (SNAP) or phytohormones (ABA and ACC). Nodules of 4 wpi were treated for 72 h with these compounds, in combination or not with cPTIO, and then were sectioned and stained with PAS. Microphotographs are representative of sections from nine nodules of different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

weight, ARA per plant and *LjLb1/2* 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 a consistent inhibition of ARA by all three compounds (SNAP, ABA and ACC) and a reversal of this inhibition by cPTIO (**Fig. 4A, B**). Likewise, we investigated whether NO and the phytohormones induce the expression of *LjLb1/2* and the senescence marker genes in the WT nodules (**Fig. 4C**). Nodules at 4 wpi were treated for 72 h with SNAP, ABA or ACC, in combination or not with cPTIO, and gene expression was analyzed by qRT-PCR. **Fig. 4C** shows that the *LjLb1/2* mRNA levels did not significantly change upon the supply of plants with SNAP, ABA or ACC, and that the combined application of cPTIO increased gene expression only slightly when considering cut-off values for up-regulation ($R > 2$) and down-regulation ($R < 0.5$). The heat shock protein transcript was slightly induced by SNAP but not by the phytohormones, whereas the osmotin precursor and cysteine protease transcripts were induced by the three compounds. With a few exceptions, the induction of the genes by SNAP, ABA and ACC was partially suppressed by cPTIO; however, the combined treatment of SNAP with cPTIO enhanced the expression of cysteine protease (**Fig. 4C**).

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

Discussion

In this work, we have generated stably transformed lines, Ox1 and Ox2, of *L.japonicus* that overexpress *LjGlb1-1* under the control of a constitutive promoter. These plants allowed us to study the effects of LjGlb1-1 on nodule development and senescence, which was not possible using plants with transient overexpression (Shimoda et al. 2009). First, we show here that the roots and nodules of the Ox lines have lower NO levels and greater nitrogenase activities than the WT plants. The accumulation of NO in nodules decreases N_2 fixation rates and induces premature senescence (Shimoda et al. 2009, Cam et al. 2012, Fukudome et al. 2016). These effects are somewhat expected as NO is a potent inhibitor of nitrogenase (Trinchant and Rigaud 1982, Kato et al. 2010) and interferes with Lb function (Sánchez et al. 2010, Navascués et al. 2012, Hichri et al. 2015, Calvo-Begueria et al. 2018). The lower NO level in the nodules of the Ox lines may thus contribute to enhance the nitrogenase activity. Because the number of nodules and the weight per nodule were similar in the Ox and WT plants, the increased activity should be ascribed to a better efficiency of the Ox nodule tissue to fix N_2 and not to a larger weight of nodules per plant. It also suggests that the mechanism for autoregulation of nodulation (Kosslak and Bohlool 1984) remains intact in the Ox plants.

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

In the second part of our study, we analyzed the effects of ABA and ethylene on the performance of the *L.japonicus–M.loti* symbiosis. These two phytohormones are known to inhibit N_2 fixation and nodulation (González et al. 2001, Ferguson and

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Mathesius 2003) and to induce nodule senescence (Ligero et al. 1991, Suzuki et al. 2004, Lohar et al. 2009). We observed that ABA and ACC, as occurs for SNAP, decreased nitrogenase activity and induced three senescence marker genes. These effects were suppressed by the NO scavenger cPTIO. Also, the two phytohormones induced histological changes in the infected cells of mature nodules at 4 wpi, such as an increase in the number and size of vacuoles and in starch deposition, resembling nodule senescence. These changes in the infected cells were also suppressed by cPTIO. Based on all these observations, we propose that ABA and ACC induce nodule senescence and that, at least in part, they do so by increasing NO levels. However, SNAP together with cPTIO enhanced the expression of cysteine protease and ABA did not induce the accumulation of starch grains in the nodule tissue, pointing out differences in the nodule senescence induced by SNAP, ABA and ACC. In fact, the latter two observations suggest that additional mechanisms, independent of NO signaling, are involved, which is not unexpected given the complexity of the nodule senescence process at the metabolic and cellular levels (Dupont et al. 2012).

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

Materials and Methods

Biological materials and growth conditions

Plants of *L.japonicus* accession Gifu B-129 and their derivative lines were germinated and grown as described (Nagata et al. 2008). In brief, 5 d after germination seedlings were transferred to 1.5% Fåhraeus agar plates (Fåhraeus 1957) and were inoculated with a cell suspension (10^7 cells ml^{-1} in water) of *M.loti* strain MAFF303099 (Kaneko et al. 2000). For experiments with non-nodulated plants, the Fåhraeus medium was supplemented with 1.5 mM NH_4NO_3. The plants were grown under controlled conditions with a photosynthetically active radiation of 150 µmol photons m^{-2} s^{-1} (16-h photoperiod) at 25°C for 4, 6 and 8 wpi. The knockout mutant line 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 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 approximately 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′-GAGCTGCTGATTCACAAGTCA-3′), *LjLb1/2* (5′-GTGGTTAAAGAAGC ACTGCT-3′ and 5′-TTAATTGCAGCTGCGAGTCC-3′), *heat shock protein* (Lj4g3v0473190; 5′-CAGTGGGAAA TTCCAGAGGA-3′ and 5′-AGTGAGAACC CCATTCTCCA-3′), *osmotin precursor* (Lj2g3v2017460; 5′-GGACAGGTGCCAT GATTCTT-3′ and 5′-GAAAGTGCTGGT GGGATCAT-3′), *cysteine protease* LjCyp2 (Lj1g3v4047250; 5′-GGAGAACA ATGGGGTGAAGA-3′ and 5′-GCCAC ACAAACCCAATACTG-3′) and *LjeIF-4A* (Lj6g3v1382260; 5′-TGGAAGCTTCGA AGAGATGG-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 31 s 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 *L.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.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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