RESEARCH LETTER

Glutathione produced by *Rhizobium tropici* is important to prevent early senescence in common bean nodules

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Abstract

In this paper, we examine the importance of glutathione in symbiosis, using a glutathione biosynthetic *gshB* mutant derived from *Rhizobium tropici* CIAT899, a common bean (*Phaseolus vulgaris*) endosymbiont. Plants infected with the mutant strain presented a delayed nodulation phenotype and a reduction in the dry weight of aerial part of plants, suggesting diminished nitrogen-fixation activity. In addition, bacterial *gshB* expression was assayed in wild-type infected nodules, during the different steps of nodulation, and found to increase in mature and early senescent nodules. Conspicuously, nodules induced by *gshB* mutant bacteria presented an early senescent pattern, which was associated with increased levels of superoxide accumulation. These results provide a direct evidence of the role of bacterial glutathione in protecting nodules from reactive oxygen species, which may determine nodule senescence.

Introduction

Rhizobia are soil bacteria that are able to fix atmospheric nitrogen in symbiosis with leguminous plants in specific plant root organs named nodules. The establishment of the symbiosis requires extensive recognition and signaling by both partners (Oldroyd & Downie, 2008). Nod factors, which are lipochitosaccharide-based signal molecules, are secreted by *Rhizobium*, inducing nodule formation in legumes. Nod factor receptors have been described in the model legumes *Lotus japonicus* and *Medicago truncatula*, and different components of the Nod factor signaling cascade have been identified (Geurts *et al.*, 2005).

Infection of the roots by rhizobia elicits a hypersensitive reaction, similar to that observed in plant pathogen response. Hence, detectable superoxide and hydrogen peroxide levels are produced in very early steps of nodule formation (Santos *et al.*, 2000; D'Haeze & Holsters, 2002). It is not clear whether this finding forms part of a defensive 'oxidative burst' that bacteria have to escape or inhibit in order to infect the plant roots. While accepting infection by the bacterial partner, the plant keeps reactive oxygen species (ROS) low, which is indeed necessary for the proper functioning of the rhizobial nitrogenase enzyme complex. Finally, during nodule senescence, a decrease in nitrogen-fixation levels and an increase in ROS occur. Antioxidant defenses diminish, leghemoglobin is oxidized to characteristic green pigments and autolytic processes are enhanced. Moreover, oxidative damage of lipids, DNA and proteins is

observed (Evans *et al.*, 1999; Matamoros *et al.*, 1999, 2003). In fast-growing annual legumes, nodules are relatively short lived compared with their parent roots (Puppo *et al.*, 2005). Nevertheless, the intricate control mechanisms that determine the maintenance of symbiosis, as well as nodule senescence, are still poorly understood (Barsch *et al.*, 2006). Different mechanisms of protection from ROS have been described in nodules, which include enzymes like superoxide dismutases, catalases and peroxidases, and small molecules like ascorbate and glutathione (GSH; γ-glutamylcysteinylglycine) (Dalton *et al.*, 1986; Matamoros *et al.*, 2003).

Different lines of evidence demonstrate that GSH produced by both the plant and the bacteria play an important role in the establishment and maintenance of symbiosis (Moran *et al.*, 2000; Matamoros *et al.*, 2003; Frendo *et al.*, 2005; Groten *et al.*, 2005; Harrison *et al.*, 2005). We have reported previously that a mutant derivative of *Rhizobium tropici* CIAT899 in the *gshB* gene encoding for GSH synthetase, the second enzyme in the GSH biosynthetic pathway, is significantly affected in its ability to compete for nodule occupancy during the process of bean nodulation. The growth of this mutant was found to be sensitive to environmental stresses, including acidity and oxidative stress (Riccillo *et al.*, 2000). Also, *gshB* expression was found to be induced under acid stress conditions (Muglia *et al.*, 2007).

In this work, we further characterize the *R. tropici gshB* mutant in its symbiotic association with common bean. We present evidence indicating that glutathione is important in the protection of nodules against early senescence.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1.

Table 1. Strains and plasmids used

Strain	Relevant characteristics	References
E. coli DH5α	*supE44*Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
E. coli S17-1	*thi pro hsd*R$^-$ *hsd*M$^+$ *recA* carrying a RP4 2-Tc::Mu integrated in the chromosome	Simon *et al.* (1983)
R. tropici strains		
CIAT899	Sm, wild-type strain	Martínez-Romero *et al.* (1991)
CIAT899-13T2	Sm, Nm, *gshB*::Tn*5-luxAB* mutant strain	Riccillo *et al.* (2000)
Plasmids		
pJP2	Tc, promoter-probe vector containing a promoterless *uidA* (GUS) as reporter; replicative in *Rhizobium*	Prell *et al.* (2002)
pCR 2.1-TOPO	Ap, cloning vector with 3′-T overhangs for cloning	Invitrogen Life Technologies
pCM152G	Tc, pJP2 derivative, containing a 152-bp fragment from upstream *gshB* gene forming a transcriptional fusion with *gusA*	This work

Ap, ampicillin resistance; Km, kanamycin resistance; Gm, gentamicin resistance; Nm, neomycin resistance; Sm, streptomycin resistance; Tc, tetracycline resistance.

Construction of *PgshB-gus* fusion

A PCR fragment of 152 bp containing the *gshB* promoter (*PgshB*) was amplified using primers F53 and R3 and cloned in vector plasmid pCR 2.1-TOPO (Muglia *et al.*, 2007). The fragment was then excised as XhoI and cloned in vector pJP2, generating plasmid pCM152G. The orientation of the insert was checked by sequencing analysis. Plasmid pCM152G was introduced by conjugative mating into strains CIAT899 and CIAT899-13T2.

Plant nodulation assay

The host plant used in this study was *Phaseolus vulgaris* cv. Negro Jamapa (common bean). Seeds were surface sterilized and germinated as described (Riccillo *et al.*, 2000). Seedlings were transferred into pots with sterile vermiculite and cultured in a growth chamber maintained at 80% relative humidity, 28 °C, and with a 16-h daylight period. One-week-old plantlets were inoculated using a rhizobial suspension of about 10^8 rhizobia mL^{-1} of Fahraeus's medium (Vincent, 1970). Plants were harvested 2, 3 and 4 weeks after inoculation. Shoot dry weight was determined in plant material after incubation at 60 °C.

Staining for Gus activity

Histochemical analysis of GUS activity in nodules was performed according to Wilson *et al.* (1995).

Quantitative real-time reverse transcriptase PCR (qRT-PCR)

RNA for reverse transcription was extracted from roots and nodules induced by strains CIAT899 or CIAT899-13T2 at 5, 14 and 28 days postinoculation (d.p.i.) Nodules were

harvested and immediately frozen in liquid nitrogen for later processing. TRIZOL reagent (Invitrogen LifeTechnology, Buenos Aires) was used for RNA purification according to the instructions provided by the manufacturer. Reverse transcription was performed using Superscript II reverse transcriptase and specific primers for 1 h at 42 °C. qRT-PCR was performed using the Platinum SYBR Green qPCR Supermix kit (Invitrogen). Primers gshbFrt and gshbRrt and the PCR conditions used were as described in Muglia *et al.* (2007). Relative transcript abundance was calculated on the basis of a standard curve. 16S rRNA gene was chosen as a reference for ratio normalization. Every reaction was performed three times. Results were analyzed using the Student *t*-test ($P < 0.05$).

In situ H_2O_2 and O_2^- detection

For the detection of H_2O_2, 5 mg mL^{-1} 3,3′-diaminobenzidine-HCl (DAB; Sigma) in dimethyl sulfoxide (DMSO), diluted 1 : 10 with 10 mM sodium phosphate buffer (pH 7.8), was used. Nitrobluetetrazolium (NBT, Sigma) (0.1%) was used to detect superoxide.

Nodules were vacuum infiltrated as described by Groten *et al.* (2006).

Light microscopy

The nodules were harvested at different days postinoculation and were fixed in 50 mM potassium phosphate buffer (pH 7.2) containing 2% paraformaldehyde and 2.5% glutaraldehyde. Thin sections were prepared and used for optical microscopic observations after staining with toluidine blue in 0.5% borate buffer.

Results and discussion

Glutathione deficiency in *R. tropici* affects symbiosis with common beans

To characterize the importance of bacteroid GSH for *R. tropici*-common bean symbiosis, we assayed the pattern of nodulation induced by the glutathione-deficient *R. tropici gshB* mutant in plant inoculation experiments. The number of nodules per plant at 14 d.p.i. was higher in plants inoculated with the *gshB* mutant strain (100 ± 30) compared with the wild-type strain (30 ± 10). The size of the nodules formed by strain CIAT899-13T2 was about half of those induced by the wild-type strain (data not shown). In addition, the effectiveness of nodules induced by the GSH mutant was evaluated by determination of the dry weight of the aerial part of plants harvested 14 and 28 d.p.i. Accumulation of dry matter has been accepted to be an indirect indicator of the effectiveness of nitrogen-fixing activity (Vincent, 1970). The dry matter of plants at 14 d.p.i. with the wild-type strain was found to be 30% higher than those inoculated with the mutant strain. Fourteen days later, this difference increased up to 50% (Fig. 1). In addition, the size of the leaves of plants inoculated with the mutant strain was significantly smaller than those of plants inoculated with the wild-type strain. Flowering and pod filling were observed in plants inoculated with wild-type strain CIAT899, and not in plants inoculated with the *gshB* mutant strain. These results demonstrate that plant development was affected in plants inoculated with the *gshB* mutant, most likely due to nitrogen deficiency.

Microscopic analysis of nodule sections was performed. Nodules that were formed by the wild-type and mutant strains were found to be structurally similar to each other at 14 d.p.i. Infected cells and other noninfected and smaller cells were observed in the central part of the nodule. This central tissue was surrounded by the peripheral tissue, including the nodule parenchyma and the nodule cortex separated by the nodule endodermis. The infected cells strongly stained with toluidine blue, and their nuclei were found to be predominantly localized in the cell center (Fig. 2a–d). At 28 d.p.i., nodules induced by the wild-type strain showed some symptoms of senescence, with large cells invaded by bacteroids and localized next to uninfected cells, which were rich in starch granules (Fig. 2e and f). On the contrary, the structural organization of nodules induced by the *gshB*-deficient strain displayed changes, and a range of phenotypes was observed. Some nodules appeared to be completely devoid of bacteroids, brownish green in color and very fragile, whereas other nodules presented a few conserved layers of cells beneath the cortex, which contained very few bacteroids that presented signs of bacterial degradation. Their central zone was filled with starch granules and devoid of bacteroids, and many cells seemed empty (Fig. 2g and h). This microscopic pattern appeared to be very similar to that described for nodule senescence (Cermola *et al.*, 2000).

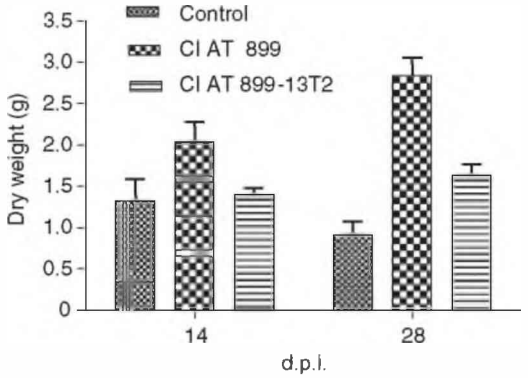

Fig. 1. Symbiotic phenotype of *gshB* mutant. Dry weight of plants inoculated with wild-type *Rhizobium tropici* and the *gshB* mutant strain, harvested at 14 and 28 d.p.i.

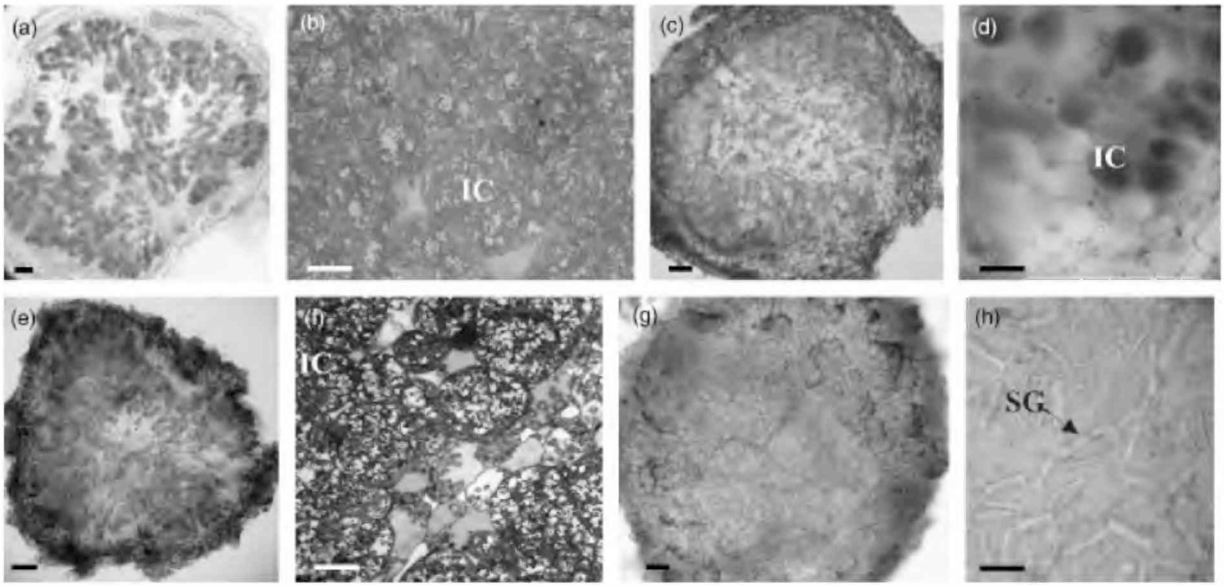

Fig. 2. Light micrographs of nodule structure of plants inoculated by wild-type and *gshB* strains, respectively. (a–h) Comparisons of nodule structures of plants inoculated with the wild-type (a and e) and *gshB* mutant strains (c and g) at 15 d.p.i. (a and c) and 28 d.p.i. (e and g). Scale bar = 500 μm. Infected cells of wild-type nodules at 14 d.p.i. (b) and 28 d.p.i. (f), and of *gshB*-induced nodules at 14 d.p.i. (d) and 28 d.p.i. (h). Scale bar = 20 μm. IC, infected cells; SG, starch granules.

From these results, we concluded that the bacterial GSH has an important impact on the senescence pattern of common bean nodules, which resembles that described for alfalfa nodules induced by *Sinorhizobium meliloti gshB* mutants (Harrison *et al.*, 2005).

Activation of rhizobial *gshB* during the late stages of nodulation

The expression of the *gshB* gene during the symbiotic interaction between common beans and *R. tropici* was examined using reporter genes. The gene fusion between the *R. tropici gshB* promoter sequence and the reporter gene *uidA* (GUS) carried on plasmid pCM152G was transferred into the wild-type strain *R. tropici* CIAT899 and the *gshB* mutant CIAT899-13T2 strain. The resulting transconjungants were used for plant inoculation, and *gshB* expression during the interaction with the host common beans was assayed. Transcriptional activity *in planta* was monitored by X-Glc staining of nodule sections and the results are shown in Fig. 3. Plants inoculated with the wild-type CIAT899 strain, carrying the *PgshB–gus* fusion, showed no detectable Gus activity in either infection threads or nodule primordia (Fig. 3a). Young nodules, at 5 d.p.i., appeared to be completely white after staining for Gus activity (Fig. 3b). Typical blue color evidencing GUS activity became detectable in the infected zone of nodules 6 d.p.i. (data not shown). Staining was found to be stronger in mature nodules (Fig. 3c and d). At 28 d.p.i., GUS activity reached the highest levels (Fig. 3e). At this stage, wild-type nodules presented early signs of senescence, such as greenish coloring, most probably due to oxidation of leghemoglobin. These results indicated that *gshB* expression increased in senescent nodules.

To confirm the data obtained using gene fusions, we performed qRT-PCR assays. *gshB* transcripts in extracts of nodules from the wild-type strain, harvested at 5, 14 and 28 d.p.i., were quantified. *gshB* transcripts were undetectable on samples harvested at 5 d.p.i. In contrast, at 14 d.p.i. *gshB* transcripts became detectable and levels increased about 20% at 28 d.p.i. (0.15 ± 0.025 vs. 0.21 ± 0.02 *gshB*/16S signal ratio), although this difference was not statistically significant ($P < 0.05$). The profile observed is consistent with the results obtained from the histological observations.

From these results, we conclude that there is a close relationship between *gshB* expression and nitrogen fixation. Levels of GSH and nitrogen fixation, in different legumes, have been described previously to correlate each other and to decrease in natural or induced senescence (Dalton *et al.*, 1986; Evans *et al.*, 1999; Matamoros *et al.*, 1999; Moran *et al.*, 2000; Groten *et al.*, 2005). We have also found that *gshB* expression increases at late stages of nodule development, suggesting that it may respond to senescence-induced signals. As nodules age, antioxidant defenses diminish, and a controlled degenerative process occurs that involves proteolysis and degradation of leghemoglobin, and thus the loss of nitrogen-fixation activity (Puppo *et al.*, 2005; Groten *et al.*, 2006). Because our data have shown that *gshB*-defective rhizobia induce nodules featuring earlier senescence, we therefore concluded that bacterial

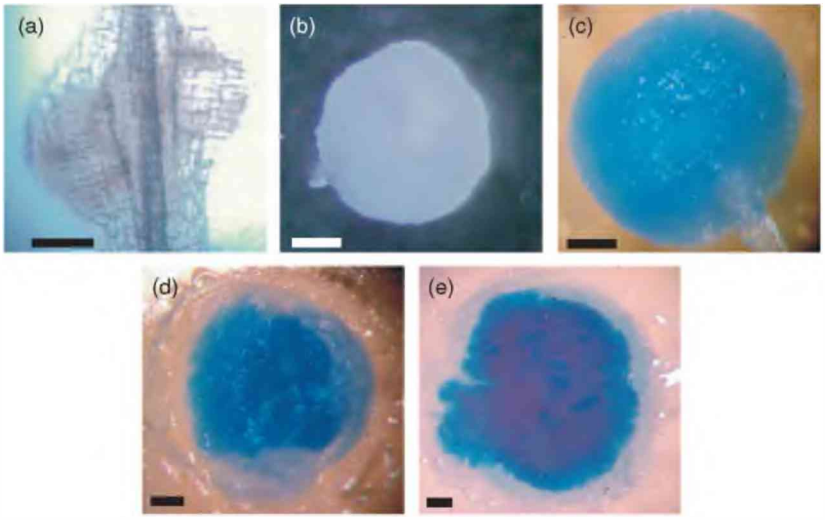

Fig. 3. *gusA* expression driven by the *gshB* promoter in common bean roots infected with wild-type strain *Rhizobium tropici* CIAT899 carrying plasmid pCM152G. Nodules were incubated with the substrates 5-bromo-4-chloro-3-indolyl β-D-glucuronide and potassium ferricyanide, and cleared with sodium hypochlorite. (a, b and c) β-Glucuronidase (GUS) activity in a 3-day-old nodule primordium, 5 and 10 d.p.i. nodules, respectively. (d and e) Hand cut sections of nodules at 21 and 28 d.p.i., respectively. Blue colour evidences GUS activity. (a) Scale bar = 100 μm; (b–e) Scale bar = 500 μm.

glutathione is important not only for efficient nitrogen fixation but also to keep nodules functional in time. Recently, Loscos *et al.* (2008) have found that common bean γ-glutamylcysteine synthetase enzyme is downregulated during senescence in nodules, at least in part, at the translational level, while homoglutathione synthetase levels remain nearly constant. We propose that by increasing GSH synthesis at late stages in the life of the nodule, bacteroids respond to ROS in order to maintain the redox homeostasis, albeit GSH is just one among the other components involved in preventing nodules from early senescence. For example, ascorbate has been indicated to have a regulatory role in nodule development as an antioxidant (Groten *et al.*, 2006). Furthermore, Rubio *et al.* (2007) have recently described that in *Mesorhozobium loti*, the antioxidant enzyme, Mn superoxide dismutase (MnSOD), presents maximum levels and enzymatic activity in old nodules. These bacteroid antioxidant responses are probably overwhelmed by the increase in ROS, and nitrogen fixation can no longer be maintained, triggering the process of senescence.

Another environmental factor that may induce an increase in bacteroid *gshB* levels is acidity. We have reported previously that in free-living *R. tropici* cells, *gshB* expression is activated by acidic conditions (Muglia *et al.*, 2007). Taking into consideration the acid pH of the peribacteroid space of nitrogen fixing pea and bean nodules (Szafran & Haaker, 1995; Krylova *et al.*, 2007), it is also possible that the acidic environment may also be involved in *gshB* activation.

O_2^- accumulates in common bean nodules in glutathione-deficient *R. tropici*

GSH has been reported to be involved in the antioxidant plant response through the ascorbate/glutathione cycle, and to act as a redox buffer of the plant cell (Noctor & Foyer, 1998; Noctor *et al.*, 2007). To determine whether bacterial GSH deficiency could affect the level and the spatial pattern of oxidant species within the nodule, we used *in situ* assays to reveal accumulation of hydrogen peroxide and superoxide, respectively. Nodules were assayed for H_2O_2 using diaminobenzoic acid (DAB), which relies on the presence of tissue peroxidases. H_2O_2 was largely detected in the meristem and invasion zones of young nodules (data not shown), whereas in mature nodules, H_2O_2 was detected in the cortex. No differences were observed in nodules formed by the wild-type strain or by the *gshB* mutant strain (Fig. 4a and b).

Accumulation of superoxide in nodule was revealed by blue formazan production resulting from the chemical reaction between NBT and O_2^-. In 3 d.p.i. roots, O_2^- was detected in nodule primordia formed by the wild-type strain (data not shown). In young nodules (14 d.p.i.) induced by the wild-type strain, O_2^- could be detected in the nodule cortex, in a few cell layers beneath the cortex and in a low number of cells following a divergent pattern inside the nodule parenchyma (Fig. 4c and d). Staining became less intense as nodules aged, such that 28 d.p.i. early senescent nodules showed staining only in the cortex and in a few cells of the parenchyma (Fig. 4e and f). In 35 d.p.i. nodules, staining was only observed in the cortex (Fig. 4g). This pattern of superoxide distribution that we observed in common bean nodules is similar to that described for pea nodules (Groten *et al.*, 2005).

The distribution of superoxides found in nodule primordial (not shown) and in young nodules induced by CIAT899-13T2 (14 d.p.i.) was similar to that of wild-type nodules (Fig. 4h). At 21 d.p.i., about half of the nodules induced by the *gshB* mutant were similar to the wild-type nodules (data not shown), whereas the parenchyma of other nodules were found to be strongly stained with NBT (Fig. 4i). In these nodules, the pattern of distribution of the blue deposition differed from

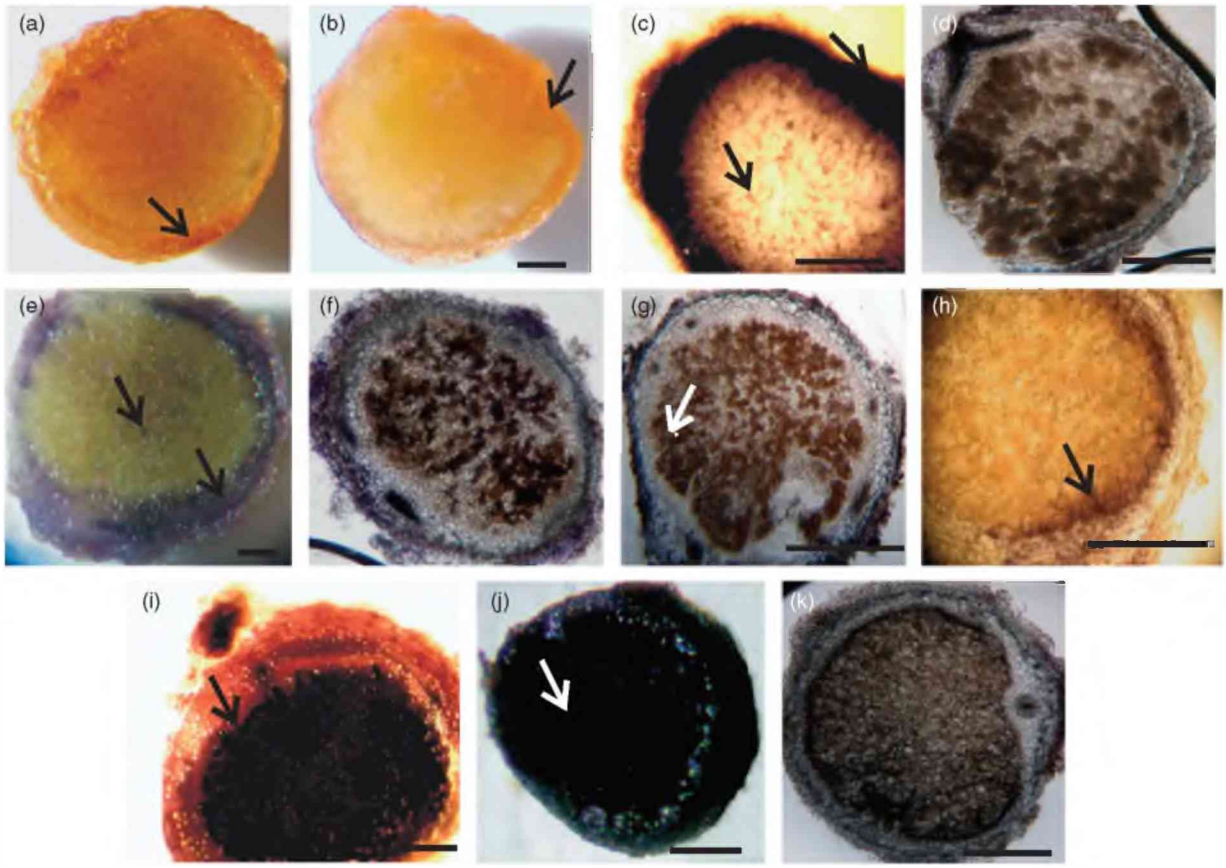

Fig. 4. Histochemical staining of H_2O_2 and O_2^- in common bean nodules induced by wild-type and *gshB* mutant strains. (a and b) sections of wild-type and mutant nodules 21 d.p.i., respectively, after treatment with DAB. Brown precipitate is indicated by arrows. Superoxide detection in wild-type nodules at 14 d.p.i. (c and d), 28 d.p.i. (e and f) and 35 (g) d.p.i. and superoxide detection in mutant nodules at 14 d.p.i. (h), 21 d.p.i. (i), 28 d.p.i. (j) and 35 d.p.i. (k) Arrows indicate blue formazan precipitate. Scale bars = 500 μm. Nodules were manually sectioned in (a)–(c), (e), (i) and (j), and ultra-thin sliced in (d)–(g) and (k).

that of the wild-type nodules, being more intense in the nodule parenchyma. Accumulation of superoxides increased as the nodules aged, at 28 and 35 d.p.i. All mutant nodules appeared completely dark blue after NBT staining (Fig. 4j and k). These results demonstrate different patterns of superoxide accumulation in nodules induced by wild-type and the GSH-deficient strains. ROS accumulated during the senescence stage and therefore suggested that nodules are under oxidative stress; however, this process was detectable earlier in the *gsh*B mutant-induced nodules.

ROS have been proposed to play a key role in symbiosis (Puppo *et al.*, 2005). In soybean nodules, H_2O_2 and lipid peroxides have been found to increase during senescence (Evans *et al.*, 1999; Puppo *et al.*, 2005). Similar symptoms have been observed in aging lupine nodules (Evans *et al.*, 1999) and also in stress-induced bean nodule senescence (Escudero *et al.*, 1996; Gogorcena *et al.*, 1997). To our knowledge, detection of superoxide accumulation in senescence nodules has not been reported. This is not unexpected, because this highly toxic anion is rapidly converted to hydrogen peroxide by superoxide dismutases of plant and bacteroid cells (Rubio *et al.*, 2004, 2007). The superoxide accumulation observed in *gshB* senescent nodules suggests that antioxidant mechanisms dependent on bacterial GSH are impaired in this mutant. Transcription of *sodCc* has been found to be activated by thiols such as GSH in *Nicotiana* (Hérouart *et al.*, 1993). Although there are no data regarding the occurrence of a similar mechanism to be functional in common bean, it could be assumed to determine a diminished superoxide dismutase activity in GSH-deficient nodules, and therefore superoxide accumulation. The increase in highly toxic superoxides may well trigger the early aging phenotype we have described in this work.

Conclusion

We believe that our results contribute to the current knowledge on nodule development and the role of bacterial

GSH in the lifetime of a nodule. Many questions remain to be answered as to the mechanisms involved in the protection exerted by glutathione from premature nodule senescence, limiting superoxide accumulation. Nevertheless, the relationship between bacterial glutathione and senescence and accumulation of ROS in nodule is shown. Further studies should be pursued in this direction.

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