Immunolocalization of antioxidant enzymes in high-pressure frozen root and stem nodules of *Sesbania rostrata*

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*Dedicated to the memory of our dear friend and colleague John Witty (18 November 1944 to 24 February 2009).

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Summary

- The activities and localizations of superoxide dismutases (SODs) were compared in root and stem nodules of the semi-aquatic legume *Sesbania rostrata* using gel-activity assays and immunogold labelling, respectively. Nodules were fixed by high-pressure freezing and dehydrated by freeze substitution.
- Stem nodules showed more total and specific SOD activities than root nodules due to the presence of chloroplastic CuZnSOD. Most of the total SOD activity of stem and root nodules was due to 'cytosolic' CuZnSOD, localized in the cytoplasm and chromatin, and to MnSOD in the bacteroids and in the mitochondria of vascular tissue. FeSOD was present in nodule plastids and in leaf chloroplasts and was found to be associated with chromatin.
- Superoxide production was detected histochemically in the vascular bundles and in the infected tissue of stem and root nodules, whereas peroxide accumulation was observed in the cortical cell walls and intercellular spaces, as well as within the infection threads of both nodule types.
- These data suggest a role of CuZnSOD and FeSOD in protecting nuclear DNA from reactive oxygen species and/or in modulating gene activity. The enhanced levels of CuZnSOD, MnSOD and superoxide production in vascular bundle cells are consistent with a role of CuZnSOD and superoxide in the lignification of xylem vessels, but also suggest additional functions in coping with superoxide production by the high respiratory activity of parenchyma cells.

Keywords: antioxidants, chromatin, nitrogen fixation, photosynthesis, reactive oxygen species, *Sesbania rostrata*, superoxide dismutases, vascular tissue.

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Introduction

Some (semi)tropical legumes, such as those belonging to the genus *Sesbania*, have the unique ability to form N₂-fixing nodules both on the roots and stems (Becker & George, 1995; James *et al.*, 2001; Den Herder *et al.*, 2006). The mechanisms of stem and root nodulation in these legumes have been studied in detail using as a model the symbiosis between *S. rostrata* and *Azorhizobium caulinodans* ORS571 (Den Herder *et al.*, 2006). Stem nodules develop by intercellular entry of the bacteria through fissures that are formed because of the protrusion of adventitious roots (“lateral root base invasion”). Root nodules are formed by the same mechanism when the plants are grown in flooded soils, or by intracellular entry of the bacteria via the hairs located near the root tip (“root hair curl invasion”) when the plants are grown in aerated, non-aquatic soils (Den Herder *et al.*, 2006). Formation of root nodules by lateral root base invasion involves reactive oxygen species (ROS), ethylene and gibberellins (D’Haeze *et al.*, 2003; Den Herder *et al.*, 2006).

Stem nodules can be formed in the aerial part, above the water level, or on the flooded stem. The aerial and some submerged stem nodules contain chloroplasts in the cortical cells that surround the infected zone (James *et al.*, 1996). These chloroplasts are photosynthetically competent and evolve O₂ (James *et al.*, 1998). Despite the O₂-sensitivity of nitrogenase, stem nodules have in general higher rates of N₂ fixation than root nodules, suggesting that special adaptations exist in stem nodules to deal with the additional burden of O₂ and ROS derived therefrom, such as the superoxide radicals and H₂O₂. Experiments with O₂-specific microelectrodes have shown that, whereas the O₂ concentration in the outer cortex increase from 1% to 23% upon illumination of nodules, the O₂ concentration in the inner cortex and infected zone remain below 0.00025% (James *et al.*, 1998). These data may reflect the operation of an O₂ diffusion barrier and/or of enhanced respiratory activity in the mid-cortex of stem nodules, as has been also proposed for the root nodules of crop legumes (Witty *et al.*, 1986; Dalton *et al.*, 1998).

An important aspect of O₂ metabolism in nodules is the production of ROS, which are either potentially damaging or signaling molecules, depending on their concentration and

chemical composition. For this reason, steady-state ROS concentrations need to be kept under tight control and this is mainly achieved by antioxidant metabolites and enzymes. The latter include three classes of superoxide dismutases (SODs) that catalyze the dismutation of superoxide radicals to H_2O_2 but that differ in their metal cofactors and subcellular localizations (for reviews see Bowler *et al.*, 1994; Alscher *et al.*, 2002). Thus, the CuZnSODs are typically localized in the cytosol and chloroplasts, the MnSODs in the mitochondria and peroxisomes, and the FeSODs in the chloroplasts and cytosol (Sevilla *et al.*, 1980; Bridges & Salin, 1981; Kanematsu & Asada, 1990). The three classes of SODs are present in nodules (Puppo *et al.*, 1982; Rubio *et al.*, 2007). The H_2O_2 produced by SODs and other enzymatic and nonenzymatic reactions that take place in nodules is eliminated by cytosolic ascorbate peroxidase (APXc) and other peroxidases, and by peroxisomal catalase (Dalton *et al.*, 1993; Matamoros *et al.*, 2003). However, there is scant information about ROS and antioxidant defenses in mature nodules of *S. rostrata*. An early study reported the presence of a CuZnSOD in the chloroplasts of stem nodules and of MnSOD and FeSOD in the bacteroids from stem and root nodules, and the authors proposed that SOD and nitrogenase activities are positively correlated (Puppo *et al.*, 1986). We have conducted a detailed immunolocalization study of some important enzymes involved in ROS scavenging in fully developed stem and root nodules of *S. rostrata*. Moreover, the sites of superoxide radical and H_2O_2 production have been localized in nodules with histochemical techniques. Our results point out additional functions of SODs in the nuclei of nodule and leaf cells, as well as in the metabolic activity of vascular bundle cells.

Materials and Methods

Plant material

Plants of *Sesbania rostrata* were inoculated with *Azorhizobium caulinodans* ORS571 and grown in pots in the greenhouse for 30 d according to James *et al.* (1996). Stem nodules, root nodules, and leaves were immediately frozen in liquid N_2 for later analysis. At the age of harvest, nodules were actively fixing N_2 and contained abundant leghemoglobin.

Total SOD activity and isoform composition

SODs were extracted at 4°C with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1% (w/v) polyvinylpyrrolidone-10 and 0.1% (v/v) Triton X-100. Total SOD activity was determined spectrophotometrically at 25°C by the ferric cytochrome *c* method (Rubio *et al.*, 2004). One unit of SOD activity was defined as the amount of the enzyme required to inhibit the reduction of ferric cytochrome *c* by 50%. The SOD isoforms were resolved on 15% native gels and identified by differential inhibition of SOD activity with 3 mM KCN or 5 mM H₂O₂, followed by gel staining by the NBT method (Rubio *et al.*, 2004). Specific activities of SOD isoforms were calculated by applying the relative proportions determined by densitometry of native gels using the ImageJ software (National Institutes of Health, Bethesda) to the total SOD activities.

Immunoblot analyses of antioxidant enzymes

Proteins were extracted from plant material at 4°C with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and a complete protease inhibitor cocktail (Roche, Penzberg, Germany), and were quantified by the Bradford microassay (Bio-Rad, Hercules, CA, USA). Proteins were resolved in 12.5% SDS-gels and transferred onto polyvinylidene fluoride membranes (Pall Corporation-Gelman) using a transfer buffer consisting of 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. Membranes were stained with Ponceau to verify equal loading of the lanes and the efficiency of the protein transfer.

Immunoblot analyses of SODs and other antioxidant enzymes were performed following standard protocols using rabbit polyclonal antibodies (dilutions used and references are given in parentheses) raised against CuZnSODp and CuZnSODc from spinach (1:3000; Kanematsu & Asada, 1990), MnSOD from rice (1:1000; Kanazawa *et al.*, 2000), FeSOD from cowpea (1:1000; Moran *et al.*, 2003), APXc from soybean (1:3000; Dalton *et al.*, 1998) and catalase from pumpkin (1:5000; Yamaguchi & Nishimura, 1984). For all the enzymes, the secondary antibody was a goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA), which was used at a dilution of 1:20000. Immunoreactive proteins were visualized using a highly-sensitive chemiluminescent reagent for peroxidase detection (SuperSignal West Pico; Pierce, Rockford, IL, USA).

Immunogold localization of antioxidant enzymes

Discs (1 mm in diameter) were punched out of leaves and nodule slices (200 μm thickness) of *S. rostrata*. Both the leaf and nodule discs were then immediately high-pressure frozen (HPF) using an EM-PACT (Leica) according to Studer *et al.* (2001). Initial samples of HPF nodules were then freeze-substituted using an EM-AFS freeze-substitution unit (Leica) in anhydrous acetone containing 0.1% glutaraldehyde, 0.2% OsO_4 , and 0.25% uranyl acetate at -90°C , -65°C and -45°C over a period of 68.5 h. After replacing the substitution fluid with pure anhydrous acetone, the samples were slowly (over a period of 4 h) brought up to room temperature (25°C) before being infiltrated and embedded within Durcupan epoxy resin (Sigma-Aldrich). Further additional samples were freeze-substituted in the same acetone solution, but omitting OsO_4 , and these samples were embedded in Lowicryl HM23 resin (Polysciences; Warrington, PA, USA) at -45°C under UV light according to Moran *et al.* (2003). Ultrathin sections were taken from osmicated, epoxy resin-embedded samples using a Leica Ultracut E microtome. These were collected on copper grids coated in pioloform and carbon before being stained, viewed, and photographed under a JEOL 1200 EX transmission electron microscope according to Rubio *et al.* (2004). Ultrathin sections from samples without OsO_4 and embedded in Lowicryl HM23 were collected on nickel grids coated in pioloform and carbon, and immunogold labelled using the same polyclonal antibodies described previously for immunoblots, as well as an antibody against the iron-protein of nitrogenase (a kind gift of Paul Ludden; Madison, WI, USA), according to James *et al.* (1996). Sections were first incubated for 1 h on a blocking/diluting buffer containing 1% (v/v) Tween 20, 1% (w/v) BSA and 1% (v/v) normal goat serum (Sigma-Aldrich) in Tris-buffered saline containing 0.5 g L^{-1} polyethylene glycol-20 K and 14 mM sodium azide, then for 1 h in a 1:50 dilution (in buffer) of the primary antibody. After washing, the grids were incubated in a 1:100 dilution of goat anti-rabbit antibodies conjugated to 15-nm gold particles (GE Healthcare; Little Chalfont, UK) for 30 min. As negative controls, serial sections were immunogold labelled with nonimmune serum (diluted 1:50) substituted for the primary antibodies. The immunogold labelled sections were stained with 2% (w/v) aqueous uranyl acetate for 10 min before viewing and photographing.

Localization of superoxide radical and H₂O₂ production

Superoxide generation in fresh root and stem nodules was detected using a solution of 0.25 mM NBT (Sigma-Aldrich) and 2 mM sodium *N,N*-diethyldithiocarbamate in 50 mM potassium phosphate buffer (pH 7.8) for 20 min (Ogawa *et al.*, 1996). Superoxide radicals give rise to a blue colouration, owing to the production and precipitation of formazan, in the presence of NBT when CuZnSOD is inhibited by diethyldithiocarbamate. Sections that had been pre-infused with TMP (Acros Organics, Geel, Belgium) were used as an additional negative control. For the *in situ* detection of H₂O₂ in root and stem nodules, the cerium chloride method (Bestwick *et al.*, 1997) was followed with some modifications (Rubio *et al.*, 2004). Briefly, fresh nodule slices were immediately perfused in 10 mM cerium chloride (Sigma-Aldrich) in 50 mM MOPS (pH 7.2) for 1 h prior to fixation in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate. Nodules with and without cerium chloride were post-fixed for 1 h in 1% (w/v) OsO₄ in 0.1 M cacodylate, dehydrated in an ethanol series at room temperature, and finally embedded in Agar 100 epoxy resin (Agar Aids) at 55°C for 48 h. Ultrathin sections were stained with lead citrate for 5 min, followed by 2% aqueous uranyl acetate for 10 min, and H₂O₂ was localized as electron-dense precipitates of cerium perhydroxides (Bestwick *et al.*, 1997). Nodule slices treated with catalase prior to perfusion with cerium chloride were used as negative controls (Rubio *et al.*, 2004).

Results

Total SOD activity and isoform composition in nodules and leaves of *S. rostrata*

The first aim of this work was to compare the isoform composition and activities of SODs in root and stem nodules of *S. rostrata* (Fig. 1). Leaf extracts were also included in this part of the study to confirm the identity of the isoforms. The total SOD activities [units per gram of fresh weight (FW)] and the specific SOD activities (units per milligram of protein) of root nodules, stem nodules and leaves were in the range of those reported for other legumes (Rubio *et al.*, 2004). However, the SOD activity in leaves was significantly greater than in nodules when expressed on a FW basis but lower when expressed as specific activity (Fig. 1a).

Gel activity staining, in the absence or presence of the inhibitors KCN and H₂O₂, was used to individualize and identify the SOD isoforms in the extracts (Fig. 1b). Root nodules, stem nodules and leaves contained the three classes of SODs, but there were tissue-dependent differences in the levels of the corresponding activities and proteins. In root and stem nodules, the major activity bands corresponded to mitochondrial MnSOD (upper MnSOD band), cytosolic CuZnSOD (CuZnSODc) and bacteroid MnSOD (lower MnSOD band). A faint band of plastidic CuZnSOD (CuZnSODp) was also detectable in stem nodules but not in root nodules. In the leaf extracts, in addition to the two major activity bands of mitochondrial MnSOD and CuZnSODc, there was an intense band of CuZnSODp activity and a faint band of FeSOD activity, which could not be detected in nodules. The lower MnSOD activity band was absent, further confirming its bacterial origin. The activities of each SOD isoform in root nodules, stem nodules and leaves were also determined by scanning the activity gels for each individual extract and then applying the resulting proportions of the isoforms to the total SOD activity determined in the same extract (Fig. 1c). This analysis showed that, in terms of specific activity, the leaves have lower mitochondrial MnSOD activity and greater CuZnSODp activity, whereas the stem and root nodules have greater CuZnSODc activity. Also, FeSOD was only measurable in leaf extracts, with a specific activity of 0.4 units mg⁻¹ of protein, which accounts for approximately 2.5% of the total SOD activity in the leaves (Fig. 1c).

Immunoblot analyses of SODs and other antioxidant enzymes of nodules

The second aim of this work was to investigate the localization of antioxidant enzymes in root and stem nodules at the organelle, cell, and tissue levels by electron microscopy. As a prerequisite, immunoblot analyses were performed for the SOD isoforms (Fig. 2a) using polyclonal antibodies raised against the CuZnSODc and CuZnSODp of spinach (*Spinacia oleracea*), MnSOD of rice (*Oryza sativa*) and FeSOD of cowpea (*Vigna unguiculata*). The CuZnSODc antibody recognized a single immunoreactive band of 19 kDa in the two types of nodule extracts, whereas the CuZnSODp antibody recognized a protein of 23 kDa and also a lower immunoreactive band of 19 kDa in both types of nodules. These observations, together

with the information provided by controls using leaf extracts (which exhibit an intense signal for CuZnSODp) and by immunoblots of native gels similar to that of Figure 1b (which allows a clear separation of the two CuZnSOD isoforms), indicate that the CuZnSODp antibody is not absolutely isoform-specific and that the immunoreactive bands of 19 and 23 kDa correspond to the CuZnSODc and CuZnSODp proteins, respectively. The signal intensity of the CuZnSODp protein band on the immunoblots correlated with the abundance of chloroplasts or plastids in the three plant organs tested, being highest in leaves (not shown), weaker in stem nodules, and barely detectable in root nodules (Fig. 2a). On the other hand, the MnSOD antibody recognized an immunoreactive protein of 25 kDa in the two types of nodule extracts, which correspond to the bacteroid and mitochondrial MnSODs, as these enzymes cannot be distinguished by the antibody and have similar subunit size (Rubio *et al.*, 2007). Finally, the FeSOD antibody recognized a protein of 27 kDa in root and stem nodules which is similar in size to that reported for nodules of cowpea (Moran *et al.*, 2003) and *Lotus japonicus* (Rubio *et al.*, 2007). The detection of FeSOD proteins in immunoblots of nodule extracts but not in the corresponding enzyme activity assays (Fig. 1c) can be attributed to intrinsic differences in the sensitivity of both methods.

Other important enzymes involved in H₂O₂ scavenging in plants were examined on immunoblots of nodule extracts (Fig. 2b) as a previous step to immunolocalization studies. In both root and stem nodules, the antibodies raised against APXc of soybean (*Glycine max*) and catalase of pumpkin (*Cucurbita* sp.) recognize single immunoreactive protein bands with corresponding apparent molecular masses of 32 and 54 kDa, as predicted (Yamaguchi & Nishimura, 1984; Dalton *et al.*, 1993).

Immunogold localization of antioxidant enzymes in nodules

Initial attempts to immunogold label SODs in root and stem nodule samples that had been chemically fixed and embedded in LR White resin according to James *et al.* (1996) were unsuccessful, most likely because the antigens were either removed during the dehydration process prior to the resin infiltration, or else they were damaged by the drastic effects of the

glutaraldehyde fixation and/or the resin-embedding procedures (Studer *et al.*, 1992; 2001). Therefore, in order to overcome these problems, fresh, unfixed root and stem nodule samples were subjected to high-pressure freezing (HPF), dehydrated by freeze substitution at -90°C, and embedded in low temperature resin at -45°C. In the first instance, however, it was necessary to check that nodules prepared in this way retained the typical ultrastructure of *S. rostrata* stem and root nodules. For this purpose, high-pressure frozen samples that had been subsequently embedded in an epoxy resin according to Studer *et al.* (1992) were examined and compared to high-pressure frozen nodules embedded in the low-temperature Lowicryl HM23 resin (Fig. 3). A close examination of the epoxy resin-embedded samples revealed that unfixed, high-pressure frozen *A. caulinodans* bacteroids in root (Fig. 3a) and stem (Fig. 3b) nodules were similar to those shown in earlier studies using chemical fixation (Tsien *et al.*, 1983; Duhoux, 1984). A similar result was obtained with *S. rostrata* nodules that had been embedded in Lowicryl HM23 (Fig. 3c,d). The latter samples also showed that the nodules were expressing nitrogenase proteins at the time of harvest (Fig. 3c), and that negative control sections incubated in nonimmune serum had negligible cross-reactions with immunogold particles (Fig. 3d). However, perhaps the most interesting observation was that, regardless of the resin used for embedding the samples, the symbiosome membranes were not closely adpressed against the bacteroids, which differ from previous observations of high-pressure frozen soybean nodules (Studer *et al.*, 1992). Also, it is interesting to note the similarly large amounts of poly- β -hydroxybutyrate granules (gray inclusions) in the bacteroids of both types of nodules (Fig. 3c,d), which suggests some O₂ limitation (Mandon *et al.*, 1998). Indeed, the high content of poly- β -hydroxybutyrate in free-living and symbiotic *A. caulinodans* has already been described and there is indirect evidence that the infected zone may be more O₂ deficient in *S. rostrata* nodules than in other legume nodules (Bergersen *et al.*, 1986; James *et al.*, 1998).

The four SOD isoforms examined in this study showed distinct localization patterns in nodules. CuZnSODc was sparsely localized in the cytoplasm of infected cells in both root and stem nodules, and also in the vascular bundle cells (not shown). The nucleate vascular bundle cells include phloem companion cells as well as xylem and phloem parenchyma cells. These

cannot be easily distinguished in transverse sections but all of them showed similar labelling intensity in the cytoplasm, and will therefore be referred to henceforth as (nucleate) vascular bundle cells. However, the most prominent sites of CuZnSODc localization were, by far (>90%), the nuclei (Supporting Information Table S1). These included the nuclei of infected cells (Fig. 4a), uninfected interstitial cells (not shown), cortical cells (Fig. 4b) and vascular bundle cells (Fig. 4c). The localization of CuZnSODc was highly specific, with negative control sections showing negligible labelling (Fig. 4d, Table S1). This pattern of localization was also observed for leaf cells (Fig. 4e). In all cells and tissues, the labelling was specifically localized to electron-dense regions in the nuclei, particularly at their peripheries. These regions were probably chromatin, and the labelling usually had a ring-shaped pattern, especially when the chromatin strands were sectioned transversely (eg. Fig. 4e). Nucleoli were not labelled. In the case of the nodule vascular tissue, as the cells are smaller than those in the other nodule tissues (compared to the infected cells and to the highly vacuolate cortical cells; James *et al.*, 1996, 1998), the nuclei occupy a much larger proportion of the cell volume, thus meaning that CuZnSODc is probably more concentrated within them.

CuZnSODp was localized in the chloroplasts of stem nodules (Fig. 4f) and leaves, and, albeit at a much lower level, in the plastids of root nodules (not shown). Labelling of MnSOD was observed on the bacteroids (Fig. 5a), but was not generally seen on mitochondria, except on those within the vascular parenchyma (Fig. 5b). Labelling with the FeSOD antibody was found on the chloroplasts of leaves and stem nodules (Fig. 5c), on the plastids of root nodules and on the cytoplasm (not shown) and nuclei of cells of all three plant organs examined. Within the nuclei, FeSOD labelling was found to be closely associated to chromatin (Fig. 5d), as occurred for CuZnSODc, but at lower levels. Consistent with the immunoblot data (Fig. 2), there were no obvious differences between stem and root nodules in either the localization or expression level of APXc and catalase. APXc was localized in the cytoplasm of infected cells surrounding the bacteroids (Fig. 5e), but the protein was also detected within the cortical cells and vascular bundle cells, although at a reduced level compared to the infected cells. Catalase localization was confined to the peroxisomes of infected and uninfected cells in both nodule types (Fig. 5f).

In situ detection of ROS production

The substrate (superoxide) and product (H_2O_2) of SODs were localized using optical and electron microscopy, respectively. The detection of superoxide radical production was based on the superoxide-dependent reduction of nitroblue tetrazolium (NBT) to formazan, which precipitates in tissues. Formation of purple-coloured spots of formazan, marking sites of superoxide production, was observed in fresh sections of stem (Fig. 6a) and root (Fig. 6b,c) nodules infiltrated with NBT and diethyldithiocarbamate, an inhibitor of CuZnSOD (Ogawa *et al.*, 1996). Similar results were obtained when sections were infiltrated with NBT and sodium azide as an inhibitor of CuZnSOD (E.K. James, unpublished). As an additional control, some root nodule sections (Fig. 6d) were incubated with 2,2,6,6-tetramethylpiperidinoxy (TMP), a scavenger of superoxide radicals (Romero-Puertas *et al.*, 2004). This control treatment was also useful to distinguish NBT reduction by superoxide radicals and by respiratory dehydrogenases, which are often studied with this procedure (Dalton *et al.*, 1998). The production of superoxide was particularly concentrated within the vascular tissue, as evidenced by deposition of formazan in the vascular bundles in the cortices in both nodule types (Fig. 6a,b), and also by its deposition in the vascular traces entering nodules from the subtending root (Fig. 6c) or stem. Some staining was also observed in the infected zone (Fig. 6a-c), but it was not as reliable because the infected zone contains abundant leghemoglobin, which can produce, or react with, superoxide radicals (Puppo *et al.*, 1982). In fact, control sections pretreated with TMP still show some dark-blue staining in the infected zone (Fig. 6d).

Production of H_2O_2 was detected in nodules with a cytochemical technique based on the oxidation of this ROS by cerium chloride to form cerium perhydroxides, which form dense precipitates (Bestwick *et al.*, 1997). Root and stem nodule samples were perfused with cerium chloride and then chemically fixed and prepared for electron microscopy. Sections of mature N_2 -fixing root and stem nodules prepared in this way had electron dense precipitates of cerium perhydroxide within the cell walls of the remaining infection threads in the invasion

zone as well as in nearby intercellular spaces (Fig. 7a). Thus, H_2O_2 was associated with the infection threads of developing nodules, and also with some of those within mature nodules (Fig. 7b), although most of the infection threads in the latter did not have H_2O_2 associated with them (Fig. 7c). H_2O_2 was also localized in the apoplast (cell walls and intercellular spaces) of the inner cortex and infected tissue of both types of nodules (Fig. 7a,b), but it was particularly observed within the walls of stem nodule cortical cells that contained chloroplasts (Fig. 7d), and somewhat less so within the walls of root nodule cortical cells. It was not commonly observed within the vascular tissue (Fig. 7e), except occasionally as a very slight precipitation of cerium perhydroxides on the lignified secondary thickening of the xylem vessels. Sections of nodules that had been perfused with catalase prior to the cerium chloride treatment showed no precipitation of cerium perhydroxides (Fig. 7f).

Discussion

The root and stem nodules used in this study were harvested at the approximate age for which nitrogenase and SOD activities are maximal (Puppo *et al.*, 1986). We found that the SOD specific activities in both nodule types are similar (35-40 units mg^{-1} protein) and twice as much as the corresponding activity in leaves. However, the total activities in nodules expressed per gram of FW were only 50-70% of the activity in leaves due to the greater content of total protein in the leaves (*c.* 60 mg protein per g FW) compared to nodules (*c.* 20 mg protein per g FW). The specific activities of CuZnSODc and MnSOD isoforms were similar in both nodule types, whereas CuZnSODp activity was detectable only in stem nodules and FeSOD activity only in leaves. The CuZnSODp isoform is probably responsible for the higher total SOD activity per FW found in stem nodules. In fact, CuZnSODp was localized in the stem nodule chloroplasts but also, less expectedly, in the root nodule plastids. This finding explains the faint immunoreactive protein band detected in root nodule extracts on western blots and suggests that CuZnSODp plays an additional role in nonphotosynthetic tissues. In stem nodules, CuZnSODp is involved in the dismutation of superoxide radicals

generated in the cortical chloroplasts through the Mehler reaction in photosystem I. The substrate for this reaction, molecular O₂, would be supplied at high concentrations by photosystem II (James *et al.*, 1998).

In terms of specific activity, CuZnSODc accounted for 50-60% of the total SOD activity in root nodules, stem nodules and leaves. An important finding of our study is the abundance of CuZnSODc (>90% of total labelling) in the nuclei of infected and uninfected cells of root and stem nodules, as well as in the cytoplasm of infected and vascular bundle cells. Ogawa *et al.* (1996) reported that, in spinach leaf cells, the so-called 'cytosolic' CuZnSOD is localized not only in the cytosol but also in the apoplast and nuclei. In our study, the HPF technique allowed for a much more definitive localization of this enzyme on the chromatin of leaf and nodule (infected and uninfected) cells. This suggests that CuZnSODc has the same functions in the nuclei of cells from leaves, root nodules and stem nodules. However, in contrast to the highly oxidizing hydroxyl radicals, superoxide or H₂O₂ are not sufficiently reactive on their own to cause damage to DNA or proteins (Halliwell & Gutteridge, 2007), and hence CuZnSODc may be protecting the nuclei by preventing formation of hydroxyl radicals through Fenton reactions, and/or the enzyme may have additional functions. Virtually nothing is known about ROS production in the nuclei of plant cells, although it has been recently shown that isolated nuclei of tobacco (*Nicotiana tabacum*) suspension cells can generate H₂O₂ in response to calcium addition (Ashtamker *et al.*, 2007). The finding of CuZnSODc and FeSOD, but not MnSOD, in nuclei is strong indirect evidence that ROS are generated under physiological conditions and suggests specific roles for the SOD isoforms in the nuclei. Conceivably, SOD activity in nuclei could modulate the levels of superoxide and H₂O₂, and these ROS may affect redox-sensitive transcription factors (Laloi *et al.*, 2004). This hypothesis is supported by the finding of a peroxiredoxin (PER1) in the nuclei of barley embryo and aleurone cells (Stacy *et al.*, 1999). Peroxiredoxins use H₂O₂ or other peroxides as substrates and have antioxidant and regulatory roles (Dietz, 2003).

Previous studies of nodules using the antibody against CuZnSODc have shown that this enzyme is abundant in young and meristematic tissues in nodules of alfalfa (*Medicago sativa*), pea (*Pisum sativum*) and *L. japonicus*, and that it is particularly associated with

infection threads and meristem cell walls (Rubio *et al.*, 2004; 2007). However, we could not determine if this also holds true for the infection threads of mature *S. rostrata* nodules, as meristem activity and bacterial invasion cease early in the development of these nodules (Den Herder *et al.*, 2006). This objective is certainly worth pursuing in future studies, especially as some of the infection threads that we observed were associated with the generation of H₂O₂, in agreement with previous observations in developing nodules of *S. rostrata* (D'Haeze *et al.*, 2003).

We have immunolocalized FeSOD in the chloroplasts, nonphotosynthetic plastids and cytoplasm, which is consistent with our previous study with *L. japonicus* nodules (Rubio *et al.*, 2007). However, the FeSOD protein was also detected in close association with chromatin in the three plant organs examined, although it was not so abundant as CuZnSODc. The similar locations of FeSOD and CuZnSODc in the chromatin of nodule cells led us to suggest that the two enzymes play analogous roles but possibly at different stages of nodule development. Thus, in a previous study with *L. japonicus* (Rubio *et al.*, 2007), we found that the CuZnSODc protein level is greater in the early stages of symbiosis and that FeSOD progressively replaces it with advancing age.

The antibody used in our study to localize MnSOD was raised against the enzyme purified from rice (Kanazawa *et al.*, 2000) but was unable to distinguish between the bacterial and plant isoforms. Thus, we detected MnSOD in the bacteroids and mitochondria from root and stem nodules. The MnSOD activity of bacteroids is essential for the onset of symbiosis (Santos *et al.*, 2000). Moreover, our histochemical study confirms that superoxide radicals are generated in the N₂-fixing tissue, probably associated to the respiration of bacteroids, which contain high affinity terminal oxidases coupled to nitrogenase activity (Bergersen *et al.*, 1986). A novel finding of this work is that MnSOD was localized specifically in the mitochondria of the vascular bundle cells. In a previous ultrastructural study of legume nodules using this antibody, we showed that MnSOD is present in the mitochondria of infected and uninfected cells of alfalfa (as well as in bacteroids and bacteria within infection threads), but did not find a preferential location in any particular cell type (Rubio *et al.*, 2004). This suggests that vascular bundle cells in *S. rostrata* nodules may have particularly

high rates of respiration and hence of ROS formation.

APXc and catalase are abundant hemoproteins of nodules which catalyze, respectively, the reduction of H_2O_2 by ascorbate and the decomposition of H_2O_2 to water and O_2 . APXc was localized in the infected cells of stem and root nodules of *S. rostrata*, as occurs in soybean and alfalfa nodules (Dalton *et al.*, 1993; 1998), whereas intense labelling for catalase was found in the peroxisomes of infected and uninfected cells in both types of nodules.

Indeed, an important finding of this study is the relatively high concentrations of the antioxidant enzymes CuZnSODc and MnSOD in vascular bundle cells. To our knowledge, the presence of antioxidants in the vascular tissue has been investigated only in two plant systems. First, in spinach leaves and hypocotyls, CuZnSODc was associated with the sites of superoxide and H_2O_2 production and of lignin deposition, suggesting that CuZnSODc activity is involved in lignification (Ogawa *et al.*, 1996; 1997). Second, in cucumber (*Cucumis sativus*) and pumpkin plants, the proteins and/or activities of CuZnSODc, monodehydroascorbate reductase, peroxidase and glutathione reductase were detected in the phloem sap (Alosi *et al.*, 1988; Walz *et al.*, 2002). Our co-localization of CuZnSODc and superoxide production in the vascular bundle cells of root and stem nodules supports a role of this enzyme in lignification. However, because mitochondrial MnSOD, but not FeSOD, is also particularly abundant in the vascular tissue, CuZnSODc and MnSOD may perform additional functions. We propose that the enhanced levels of these two SOD isoforms are related to the high respiratory activity of the vascular bundle cells and that mitochondrial respiration is a source of the superoxide radicals detected histochemically. The elevated respiration rates would be explained by intensive energy consuming processes such as the active transport of ions or metabolites.

Another major site of superoxide formation was the chloroplastic-containing cortex of the stem nodules, as well as in the infected zone. This localization pattern in *S. rostrata* nodules contrasts with that seen in the indeterminate nodules of alfalfa (Santos *et al.*, 2001; Rubio *et al.*, 2004) or pea (Groten *et al.*, 2005), in which ROS were found to be generated in the meristem and invasion zone. The mature nodules of *S. rostrata* lack an active meristem and invasion zone (Fernández-López *et al.*, 1998; Den Herder *et al.*, 2006) and hence we were

unable to confirm that superoxide and SOD expression are associated with meristematic activity and bacterial invasion in this species. However, the localization of H₂O₂ within and close to infection threads reported by D’Haeze *et al.* (2003) and in the remnants of infection threads found in the present study strongly suggests this possibility.

Finally, it is also important to note that this is the first time that the HPF technique has been used to study *S. rostrata* nodules. Our results show that the structure of nodules processed by HPF, treated with OsO₄, and embedded in conventional epoxy resin is similar to that of nodules prepared by conventional chemical fixation (Tsien *et al.*, 1983, Duhoux, 1984). In sharp contrast, in nodules of soybean, lupin (*Lupinus albus*) or common bean (*Phaseolus vulgaris*) prepared by HPF, the symbiosome membranes are tightly wrapped around the bacteroids (Studer *et al.*, 1992; de Felipe *et al.*, 1997), whereas in chemically fixed nodules the bacteroids are separated from the symbiosome membrane by a substantial “gap” (eg. Dalton *et al.*, 1993). In the case of soybean nodules, Studer *et al.* (1992) suggested that only symbiosomes in unfixed nodules prepared by HPF faithfully reflect their natural state, and that the close wrapping of the bacteroids would allow for their much closer proximity to the leghemoglobin-containing cytoplasm. We conclude that the gap between the bacteroids and the symbiosome membrane seen in our study is a genuine feature of the *S. rostrata* root and stem nodule structure. It is likely that, because the leghemoglobins of *S. rostrata* have very high affinities for O₂ compared to those of soybean (Bergersen *et al.*, 1986), the gap between the bacteroids and the symbiosome membrane may actually be required to prevent nitrogenase from being inactivated by the high O₂ flux supplied by *S. rostrata* leghemoglobins.

Conclusions

This study on the localization of SODs, other antioxidants, and ROS in *S. rostrata* nodules led us to a number of findings. First, the relatively high SOD activity of stem nodules is due to CuZnSODp, which helps to prevent photosynthetically-generated ROS from damaging the

N₂-fixing cells. Second, by far the greatest proportion of total SOD activity (>60%) in both nodule types is ascribable to CuZnSODc, and much of this enzyme appears to be associated with chromatin in the nuclei of infected and uninfected host cells and also of leaf cells, suggesting that CuZnSODc in nodules and leaves may be involved in protecting DNA from ROS or in modulating gene activity. Third, high levels of CuZnSODc and mitochondrial MnSOD were found in vascular bundle cells, in agreement with the histochemical localization of superoxide radicals. This is consistent with a role of superoxide and CuZnSODc in the lignification of xylem vessels, but also points out additional functions of these antioxidant enzymes in the vascular bundles, such as the scavenging of the high ROS levels produced during respiration in vascular bundle cells. Finally, superoxide generated within the photosynthetic tissue of stem nodules is scavenged by CuZnSODp in the chloroplasts and by CuZnSODc in the cytosol and nuclei. Because there was no excess production of H₂O₂ in these cells, this ROS may be efficiently removed by APXc and catalase. On the other hand, the accumulation of H₂O₂ in the apoplast suggests that some excess H₂O₂ is exported (or leaks?) from the cells. This H₂O₂ may be involved in the rapid H₂O₂-mediated cross-linking of the matrix glycoprotein that is located in the cell walls and intercellular spaces of the photosynthetic cortex of stem nodules and that is probably involved in the operation of the O₂ diffusion barrier (James *et al.*, 1996).

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Legends for Figures

Fig. 1. Total SOD activity and activities of SOD isoforms in root nodules (RN), stem nodules (SN), and leaves (L) of *S. rostrata*. (a) Total SOD activity expressed in units per gram of FW and in units per milligram of protein. Values are means $\pm$ SE of six replicates from plants grown independently. (b) Identification of SOD isoforms on native gels. The method is based on the reduction of NBT to blue formazan by the superoxide radicals generated photochemically. SOD activity inhibits this reaction and SOD isoforms are visualized as achromatic bands on a blue background. Each lane was loaded with 50 μ g of protein. (c) Activity of mitochondrial MnSOD (MnSODm), bacteroid MnSOD (MnSODb), CuZnSODc, CuZnSODp, and FeSOD. The activity of the FeSOD isoform in leaves was <3% of total. Values are means $\pm$ SE of six replicates from plants grown independently.

Fig. 2. Immunoblot analysis of antioxidant enzymes in root nodules (RN) and stem nodules (SN) of *S. rostrata*. (a) Blot of SOD isoforms. Lanes were loaded with 50 μ g (CuZnSODp and FeSOD) or 60 μ g (CuZnSODc and MnSOD) of protein. (b) Blot of catalase and APXc. Lanes were loaded with 40 μ g (catalase) or 50 μ g (APXc) of protein. Approximate molecular masses (kDa) are indicated on the left.

Fig. 3. Transmission electron micrographs of symbiosomes in *S. rostrata* root (a) and stem (b-d) nodules that had been high pressure frozen, freeze-substituted and embedded either in epoxy resin at 60°C (a, b) or in Lowicryl HM23 at -45°C (c, d). Note that the symbiosome membrane (arrows) is usually not closely adpressed against the bacteroids in any of the micrographs. The samples embedded in Lowicryl HM23 were immunogold labelled either with an antibody against the nitrogenase iron-protein (NifH protein) (large arrow) (c) or with nonimmune serum (d). These demonstrate, respectively, that the bacteroids were actively expressing the enzyme at the time of harvest (30 d after inoculation) and also that there was

negligible non-specific labelling. Note that bacteroids contain numerous granules of poly- β -hydroxybutyrate (gray inclusions in c,d). b, bacteroid. Bars= 2 μ m (a), 1 μ m (b), 500 nm (c, d).

Fig. 4. Transmission electron micrographs of sections of high-pressure frozen *S. rostrata* stem nodules (a-d, f) and leaves (e) immunogold labelled with an antibody raised against CuZnSODc (a-c, e), treated with nonimmune serum (d), or immunogold labelled with an antibody raised against CuZnSODp (f). In both root and stem nodules, CuZnSODc was almost exclusively localized (arrows) on electron-dense regions within the nuclei of all cells examined, including infected cells (a), cortical cells (b) and vascular parenchyma cells (c). Little or no labelling was seen in bacteroids, cytoplasm, mitochondria, plastids or vacuoles. Also, labelling was not seen on sections that had been treated with nonimmune serum substituted for the primary antibody, including the electron-dense regions in the nuclei (arrows in d). CuZnSODc was also localized in the nuclei of leaf cells (e), but was not present in chloroplasts. CuZnSODp was localized exclusively in chloroplasts (f) and plastids. b, bacteroid; c, cytoplasm; ch, chloroplast; m, mitochondrion; n, nucleus; p, plastid; v, vacuole. Bars= 500 nm (a, b, e), 1 μ m (c, d, f).

Fig. 5. Transmission electron micrographs of sections of high-pressure frozen *S. rostrata* stem (a-e) and root (f) nodules immunogold labelled with an antibody raised against MnSOD (a, b), FeSOD (c, d), APXc (e) and catalase (f). MnSOD was localized in bacteroids within infected cells (a) and also in mitochondria of parenchyma and phloem cells in the vascular bundles (b). FeSOD was localized in chloroplasts of stem nodules (c) and in electron-dense regions of nuclei (arrows) of all cell types in both stem (d) and root nodules. Immunolabelling with the APXc antibody was localized in the cytoplasm of infected cells (e), and that with the catalase antibody was found exclusively in peroxisomes (arrows). b, bacteroid; c, cytoplasm; ch, chloroplast; m, mitochondrion; n, nucleus; v, vacuole. Bars=500 nm (a-c), 1 μ m (d-f).

Fig. 6. Localization of superoxide in freshly-cut sections of stem (a) and root (b-d) nodules after treatment with NBT in the presence of diethyldithiocarbamate. Production of superoxide radicals was marked as purple-colored deposits of formazan in the vascular tissue (arrows) in the photosynthetic, chloroplast-containing cortices of stem nodules (a) and in the non-photosynthetic cortices of root nodules (b). Formazan was also seen in vascular tissue in the base of nodules where they were connected to the subtending root (c). The deposition of formazan in the vascular tissue was greatly reduced (arrows) in sections that were perfused with TMP prior to treatment with NBT (d). The infected tissue is indicated by an asterisk in each section. ch, chloroplast. Bars= 100 μ m (a-d).

Fig. 7. Localization of H_2O_2 in stem nodules as precipitates of electron-dense cerium perhydroxides after treatment with cerium chloride. Dark staining due to cerium perhydroxides was seen associated with infection threads (large arrows) and nearby cell walls and intercellular spaces (small arrows) in the invasion zone (a). This localization was also occasionally observed within the infected zone (b), but in most cases the remaining infection threads in the infected cells were not stained with cerium (c). Cerium deposits were frequently observed within the cell walls in the cortices of both stem and root nodules; in stem nodules these deposits were often close to chloroplasts (d). Little or no cerium staining was observed in the vascular bundles (e), and no cerium deposits were seen in sections that had been treated with catalase prior to the cerium chloride staining (f). b, bacteroid; ch, chloroplast; cw, cell wall; is, intercellular space; it, infection thread; x, xylem; xp, xylem parenchyma. Bars= 1 μ m (a-c, e, f), 2 μ m (d).

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Relative abundance of CuZnSODc protein in stem and root nodules of *Sesbania rostrata*

Fig. 1

Fig. 2

(a)

(b)

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Table S1 Relative abundance of CuZnSODc protein in stem and root nodules of *Sesbania rostrata*

Number of gold particles on organelles in cells from high-pressure frozen stem and root nodules after immunogold labeling with an antibody against CuZnSODc. Gold particles in sections with nonimmune serum for all three types of cells examined were <2.5 in the cytoplasm, nuclei, vacuoles and other cellular compartments. Values are means $\pm$ SE of 10 measurements, each corresponding to a different cell. n/a, not applicable.

Cell type	Cytoplasm	Nuclei	Vacuoles	Mitochondria and other organelles
Infected cells	21 $\pm$ 8	482 $\pm$ 27	n/a	8 $\pm$ 3
Cortical cells	10 $\pm$ 3	447 $\pm$ 29	19 $\pm$ 4	13 $\pm$ 4
Vascular bundle cells	7 $\pm$ 2	395 $\pm$ 39	n/a	10 $\pm$ 6