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Oxidative Stress in Nitrogen-Fixing Legume Nodules : Some Biochemical and Molecular Biology Aspects

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Summary

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Leghemoglobin, the oxygen-carrying hemoprotein present in great amounts in legume nodules, can react with hydrogen peroxide to form phenoxyl radicals. These radicals are quenched by at least two processes : the first one involves an intramolecular heme-protein cross-link and the second route results in the formation of intermolecular cross-links and hence dimeric forms of the protein. They can also interact with peribacteroid membrane fractions, leading to the generation of additional lipid-derived radicals. This transfer of damage may be of great biological significance during the nodule senescence process. Glutathione, which is present at high concentration in nodules, most probably exerts a very important protective role, as it can react with these radicals. Thus, glutathione synthesis is studied in *Medicago truncatula* : partial cDNAs corresponding to the two enzymes involved in this synthesis are cloned and show high homology with their *Arabidopsis* counterparts.

Introduction

The damaging effects of reactive oxygen species (ROS) on biomolecules and their involvement in degenerative processes in living cells are now well documented. In this framework, there is increasing evidence that ROS play an

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important role in the *Rhizobium* - Legume symbiosis, at least during the senescence period. Legumes and their symbiotic bacteria (collectively referred to as rhizobia) make the largest contribution to global biological nitrogen fixation. Rhizobia elicit on their hosts, in a specific manner, the formation of specialized organs, the nodules, in which they reduce molecular nitrogen into ammonia. In many cases, the mechanism of root infection consists of an entry through root hairs : rhizobia elicit root-hair curling and the development of a tubular structure, the infection thread, which grows through the root-hair cell and into the root cortex where it ramifies. Concomitantly, particular cortical cells divide to form a nodule primordium ; the development of primordia gives rise to nodules that are genuine organs (DENARIE & al. 1992). Bacteria multiply in infection threads and are then released into plant cells where they differentiate into nitrogen-fixing bacteroids. An important feature of this symbiosis lies in the presence of the peribacteroid membrane, derived from the root cell plasma membrane, which effectively excludes the bacteroids from the host cell cytoplasm (BREWIN & al. 1985).

Results and Discussion

Nodules have a high potential to produce damaging ROS, due to the strong reducing conditions required for nitrogen fixation and the action of several proteins, including ferredoxin, uricase, hydrogenase and leghemoglobin (Lb). This hemoprotein, present in great amounts in legume nodules where it ensures an adequate flux of oxygen to the bacteroids to support their respiration, is subject to an autoxidation process, generating superoxide anion and hydrogen peroxide (PUPPO & al. 1981). In the course of a further reaction between ferric Lb and hydrogen peroxide, globin derived radicals have been detected, as a result of the transfer of the second oxidising equivalent from the peroxide into the surrounding globin. An important site for this species is a tyrosine residue near the heme edge, and this intermediate has been identified as a sterically constrained phenoxyl radical (DAVIES & PUPPO 1992). These radicals are quenched by at least two processes. The first one involves an intramolecular heme-protein cross-link : this leads to the formation of a green compound with spectral characteristics differing markedly from those of ferryl and ferric Lbs. This green compound shows a much less intense Soret band than the untreated ferric Lb and the addition of ascorbate or dithionite results in the progressive disappearance of the two absorption peaks of this compound. The pyridine hemochromogen spectrum of the heme from the green compound provides strong evidence for changes to the heme. This is confirmed by a completely modified EPR spectrum : the obtained signals indicate that the heme group is both damaged and distorted. In order to assess possible heme binding to the protein, the ratio of the Soret band absorbance to that at 280 nm was measured before and after heme extraction by acidic butanone for both native Lb and the green compound. The results clearly suggest that the heme group was, at least partly, covalently bound to the protein (MOREAU & al. 1995). The

contribution of this process to the greening of senescing nodules can be proposed : this could be in agreement with the extraction of green, modified Lbs from 50-day-old soybean nodules (JUN & al. 1994). Furthermore, EPR signals obtained with these greening senescing nodules are very similar to those obtained with pink functioning nodules treated with exogenous hydrogen peroxide.

The second quenching route results in the formation of intermolecular cross-links and hence dimeric forms of the protein. When Western blots were performed using anti-Lb antibodies, two bands were revealed, indicating that the epitopes responsible for binding were still available in the dimer. ApoLb does not undergo dimerisation in the presence of hydrogen peroxide, clearly indicating that the presence of heme was necessary for cross-linking. An HPLC technique was used to purify sufficient amounts of the dimer : dityrosine was not detected in the purified and hydrolysed dimer samples, strongly suggesting that phenoxyl radicals may not be the only species found in these systems (MOREAU & al. 1995).

In EPR spin trapping studies, evidence has been obtained for the generation of a number of further radical species in addition to the previously detected phenoxyl radical. These radicals are transient (and hence reactive) in nature and are detected as the corresponding spin adducts. Analysis of the signals observed with a range of spin traps suggests that there are at least two further radical sites in the protein, and that these species are carbon-centred radicals ; some of these are tertiary species. At least some of these radicals are believed to be at surface exposed sites as they can be trapped with large, charged spin traps, and show considerable freedom of motion (MOREAU & al. 1996). These radicals may be key intermediates in the formation of the protein dimers described above. Further direct (two-way stopped flow) and spin trapping studies have shown that some of these radical species can interact with peribacteroid membrane fractions. This is manifested as a decrease in the concentration of the protein-derived radicals, and the generation of additional lipid-derived radicals (MOREAU & al. 1996). This transfer of damage from the protein to the neighbouring membrane may be of considerable biological significance, as the destruction of this membrane is one of the earliest observable events in root nodule senescence (PLADYS & RIGAUD 1985) and is associated with the loss of nitrogen-fixing activity. These processes, which can contribute to the formation of an oxidative stress in nodules, are summarized in Fig. 1.

One of the most important protective mechanisms against hydrogen peroxide in living cells involves glutathione (GSH) and in plant chloroplasts an ascorbate-GSH cycle has been shown to be operative (FOYER & HALLIWELL 1976). A similar protective system is present in functioning root nodules, where GSH concentration is high. Furthermore, it has been shown that the ascorbate-GSH cycle can adjust to varying physiological conditions in nodules and that there is a key link between nitrogen fixation and the detoxification process (DALTON & al. 1991). GSH most probably exerts also a direct protective role in vivo (see Fig. 1), as it can react with Lb radicals (PUPPO & al. 1993) and avoid, in functioning nodules, the

transfer of damage described above. Thus, a strategy to analyze the synthesis and the function of GSH in *Medicago truncatula* - which is a host model for studies on *Rhizobium* - Legume symbiosis - has been defined.

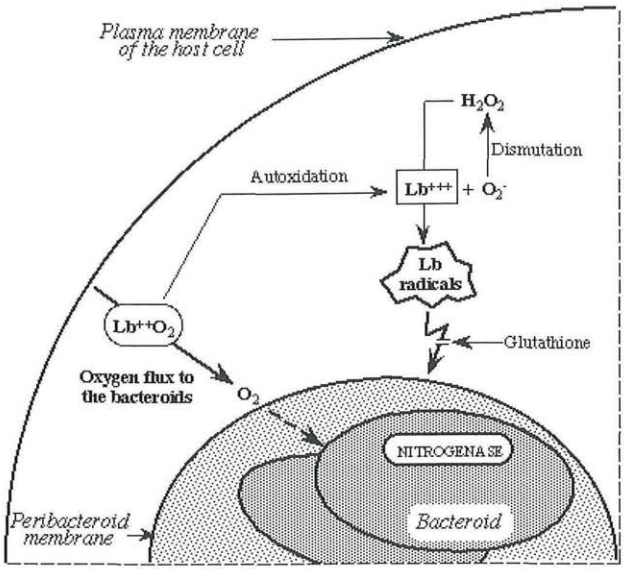

Fig. 1. General scheme summarizing the damaging effects of leghemoglobin during nodule senescence.

The synthesis of glutathione is a two stage process involving two enzymes, γ-glutamylcysteine synthetase (γ-ECS ; EC 6.3.2.2) and glutathione synthetase (GSHS ; EC 6.3.2.3). To obtain *M. truncatula* molecular probes corresponding to both enzymes, a cDNA library constructed from mRNAs of 4-day-old nodules (not already nitrogen fixing nodules) in lambda ZAP II vector (Stratagene) (GAMAS & al. 1996) was screened with *Arabidopsis* cDNAs corresponding to both γ-ECS and GSHS (MAY & LEAVER 1994, RAWLINS & al. 1995). The cDNAs fragments were radiolabelled with ^{32}P by random priming (Prime-a-gene labelling system, Promega) and used as a heterologous probe in the screening. About 400000 plaques of a *Medicago truncatula* cDNA library were screened, by transfer in duplicates onto Hybond-N (Amersham) and hybridisation with the radioactive probe was performed at 55°C in 6xSSC (20XSSC is 3M NaCl/0.3M sodium citrate, pH 7.0), 1 % SDS, 5xDenhart's solution (100xDenhart's solution is 2 % bovine serum albumine, 2 % Ficoll and 2 % polyvinylpyrrolidone) and denaturated salmon sperm DNA at 100 µg per ml. Filters were washed twice in 6xSSC/0.1% SDS at

55°C for 10 min and twice in 2xSSC/0.1% SDS at 55°C for 10 min. and exposed to Biomax film (Kodak) for 2 days.

Six putative positive clones were obtained using the *Arabidopsis* γ-ECS as an heterologous probe. The nucleotide sequence of the clone (pECS3) containing the longest insert (1300-bp) was determined and computer-aided analysis of the pECS3 insert amino acid sequence revealed that it is a partial cDNA with 90 % identity with γ-ECS from *Arabidopsis*. Partial sequences of the 5 other clones were also determined and one was found to be identical to the corresponding region of the pECS3 insert. These results show that these two clones belong to a single class and show very high homology with γ-ECS from *Arabidopsis* ; this suggests that the partial cDNAs correspond to γ-ECS in *M. truncatula*.

Sixteen positive clones were obtained using the *Arabidopsis* cDNA corresponding to GSHS as a probe. Digestion profiles of the different clones showed two different families named GSHS1 and GSHS2. The nucleotide sequence of the longest insert corresponding to each family was determined. Computer sequence analyses show that both GSH1 and GSH2 are partial cDNAs which have 70% identity with their *Arabidopsis* counterpart. These results indicate that two different truncated cDNAs showing high homology with *Arabidopsis* GSHS are cloned, suggesting that two different GSHSs are expressed in *M. truncatula*.

To determine the profile of expression of the genes corresponding to γ-ECS, GSH1 and GSH2, a Northern blot of total RNA isolated from leaves and roots of *Medicago truncatula* and *Medicago sativa* 8 days old plantlets was performed. Equal expression was observed in roots and in leaves of both plants for γ-ECS. Higher expression is found in leaves than in roots for GSHS1 and the reverse situation is observed for GSHS2 gene expression. These results suggest that γ-ECS and GSHS genes are very homologous in *M. sativa* and *M. truncatula* since the hybridisation signals are similar in both plants. The differential expression observed between GSHS1 and GSHS2 could reflect a different role for these two enzymes in *Medicago* plants.

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