Metabolism of Reactive Oxygen Species Is Attenuated in Leghemoglobin-Deficient Nodules of *Lotus japonicus*

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Leghemoglobins together with high rates of respiration are believed to be major sources of reactive oxygen species (ROS) in root nodules of leguminous plants. High capacities of antioxidative systems apparently protect this organ from oxidative damage. Using leghemoglobin-RNA interference (LbRNAi) lines of *Lotus japonicus*, we found that loss of leghemoglobin results in significantly lower H₂O₂ levels in nodules. Transcript levels and catalytic activities of ascorbate-glutathione cycle enzymes involved in H₂O₂ detoxification as well as concentrations of reduced ascorbate were also altered in LbRNAi nodules. Thus, symbiotic leghemoglobins contribute significantly to ROS generation in functional nodules.

Symbiotic nitrogen fixation (SNF) by rhizobia in legume root nodules is an energy-intensive process that requires O₂ for respiration to generate ATP and reducing equivalents for the reduction of N₂ to NH₃ (Marchal and Vanderleyden 2000). Paradoxically, the nitrogenase enzyme that catalyzes this reaction is inactivated by O₂ not only in rhizobia but also in other nitrogen-fixing prokaryotes. Resolution of this paradox is achieved in a variety of different ways in various organisms, including temporal and spatial separation of nitrogenase activity from photosynthesis in the coccoid cyanobacterium *Gloeocapsa* and in *Anabaena cylindrica*, respectively (Robson and Postgate 1980). In legume root nodules, bacteroid nitrogenase is protected from O₂ by reducing intracellular O₂ concentration from approximately 250 µM (the equilibrium concentration of O₂ in water) to a steady-state level of less than 50 nM in rhizobia-containing plant cells (Appleby 1984; Bergersen 1996; Hunt and Layzell 1993). This is achieved, in part, by limiting the rate of O₂ influx through the outer, uninfected cell layers of the nodule and by maintaining high rates of respiration in mitochondria and bacteroids in the infected cells of the central tissue (Bergersen 1996; Bergersen and Goodchil 1973; Denison and Layzell 1991; Sheehy et al. 1985; Tjepkema and Yocum 1974; Webb and Sheehy 1991; Wittenberg et al. 1972, 1974; Witty and Minchin

1998). Maintenance of high respiration rates in nodule tissue requires a third factor, namely the O₂-binding protein leghemoglobin (Lb), which, when present at millimolar concentrations in the cytoplasm as it is in infected nodule cells (Appleby 1984; Hargrove et al. 1997; Wittenberg et al. 1972), delivers O₂ efficiently to mitochondria and bacteroids for respiration while buffering free O₂ in the nanomolar range (Appleby 1984; Dakora 1995). The essential role of Lb in this ‘oxygen-juggling act’ for SNF was demonstrated recently when it was shown that an absence of Lb in RNA-interference (RNAi) lines of *Lotus japonicus* was accompanied by an increase in free O₂ and a loss of nitrogenase and nitrogen fixation in nodules (Ott et al. 2005). The importance of Lb in SNF in legumes is also underlined by the facts that the symbiotic clade of plant hemoglobins has expanded greatly in this family (Bogusz et al. 1988; Trevaskis et al. 1997), Lb genes are expressed in a nodule-specific manner (Barker et al. 1988; Davidowitz et al. 1994; Gallusci et al. 1991; Ott et al. 2005; Sato et al. 2001; Stougaard et al. 1987; Uchiumi et al. 2002), and Lb genes are among the most highly expressed of all plant genes in nodules (Colebatch et al. 2002, 2004; El Yahyaoui et al. 2004; Fedorova et al. 2002; Journet et al. 2002; Kouchi et al. 2004).

Despite the apparent necessity of Lb for SNF, this family of heme-containing proteins is believed to contribute to general oxidative stress in nodules (Becana and Klucas 1992a and b; Becana and Rodriguez-Barrueco 1989; Becana et al. 1998, 2000; Fridovich 1976; Moreau et al. 1996; Puppo and Halliwell 1988a; Puppo et al. 1981; Schneider and Schlegel 1981). Ferro-Lb (Lb²⁺), which is the active, oxygen-binding form, undergoes spontaneous autooxidation to inactive ferric-Lb³⁺ (Becana et al. 2000). Concomitantly, heme-bound oxygen molecules are reduced and released as superoxide anions (O₂^{•−}). Active Lb²⁺ can be regenerated from Lb³⁺ by the action of ferric-Lb reductase in nodules (Ji et al. 1992). Plant cells are well-equipped to deal with partially reduced forms of O₂, which include O₂^{•−}, hydrogen peroxide (H₂O₂), and the hydroxyl radical (HO•). These molecules are also known as reactive oxygen species (ROS) because of the oxidative damage they cause to various cellular components. The enzymatic armory possessed by plant cells to deal with ROS includes superoxide dismutase, catalase, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). The sequential action of APX, MDAR, DHAR, and GR constitutes the ascorbate-glutathione cycle, which is believed to be the main pathway for ROS detoxification in plant tissues (Mittler 2002; Noctor and Foyer 1998), including nodules (Dalton et al. 1986; Matamoros et al. 2003). The antioxidant ascorbate is a key substrate for the ascorbate-glutathione cycle and is present at millimolar concentrations in functional root nodules (Dalton et al. 1986; Gogorcena et al. 1995; Matamoros et al. 1999).

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Despite the belief that Lb are a major source of ROS in nodules, this has never been tested directly in planta. The existence of two independent LbRNAi lines of *Lotus japonicus* that completely lack Lb in nodules enabled us to test this idea for the first time. As both LbRNAi lines exhibited identical phenotypes with respect to nodule development and function as well as plant growth and development (Ott et al. 2005), we chose to focus on one representative line in the present study. Here, we present data on the concentrations of H₂O₂ and ascorbate and the levels of transcript, protein, and activity of enzymes of the ascorbate-glutathione cycle throughout development of wild-type and LbRNAi nodules, which support the notion that Lb are a significant source of ROS in nodules.

RESULTS

H₂O₂ and ascorbate concentrations are reduced in LbRNAi nodules.

Concentrations of H₂O₂ and reduced ascorbate were measured photometrically in developing nodules. While no difference in H₂O₂ concentration between wild-type and LbRNAi nodules was detected 2 weeks postinoculation (wpi), significantly less H₂O₂ was found in LbRNAi nodules 3 and 4 weeks after inoculation. Concentrations of H₂O₂ were approximately 40 and 60% lower in LbRNAi nodules 3 and 4 wpi, respectively (Fig. 1A). No changes in H₂O₂ concentrations were found over time in wild-type nodules.

Total concentrations of reduced ascorbate were significantly induced in LbRNAi nodules, with increases of 36% at 2 wpi and 22% at 4 wpi (Fig. 1B). Overall ascorbate concentrations did not change over time in both genotypes.

Expression of ascorbate-glutathione cycle genes is altered in LbRNAi nodules.

As significant changes in H₂O₂ and ascorbate concentrations were found in LbRNAi nodules relative to the wild type (Fig. 1), genes encoding enzymes involved in the ascorbate-glutathione cycle in *Lotus japonicus* were identified using the Gene Index Project for further studies. Genes were chosen based on their representation by expressed sequence tags from nodules. Thus, sequences of the two most abundant genes encoding ascorbate peroxidases in nodules represented by tentative consensus sequences TC7951/TC7952 (*LjAPX1*) and TC9623 (former TC779; *LjAPX2*) were selected for further analyses. The latter was previously found, by cDNA-array analysis, to be induced during nodule development (Colebatch et al. 2004). Genes encoding other enzymes of the ascorbate-

glutathione cycle were also chosen for expression analysis, including a dehydroascorbate reductase (*LjDHAR1* encoded by TC17380), three monodehydroascorbate reductases (*LjMDAR1*, *LjMDAR2*, and *LjMDAR3* corresponding to TC12910, TC19086, and TC15657, respectively), and three glutathione reductases (*LjGR1*, *LjGR12*, and *LjGR13* corresponding to TC19332, TC11942, and TC16174, respectively).

Expression of nine genes, *LjAPX1* and *LjAPX2*, *LjDHAR1*, *LjMDAR1* through *LjMDAR3*, and *LjGR1* through *LjGR3*, was measured 2, 3, and 4 wpi, using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Transcript levels were normalized to *LjUBI10*, a constitutively expressed ubiquitin gene used previously for this purpose (Colebatch et al. 2002). Expression of cytosolic *LjAPX1* was significantly lower in LbRNAi nodules than in wild-type nodules at 4 wpi but not at earlier developmental stages (Fig. 2). *LjAPX2* expression was significantly lower in LbRNAi nodules than in wild-type nodules at 3 wpi but not at other stages (Fig. 2). In contrast, transcript levels for *LjMDAR1* and *LjMDAR2* were unaltered in LbRNAi nodules as compared with the wild type (Fig. 2), while significantly more *LjMDAR3* transcripts were detected in LbRNAi nodules than in those of the wild type at 3 and 4 wpi (Fig. 2). Significant reductions in transcript levels were also found for *LjDHAR1* in LbRNAi nodules 2 and 3 wpi, as compared with the wild-type (Fig. 2), while no significant differences between LbRNAi and wild-type nodules were found with respect to transcript levels of the three *LjGR* genes (Fig. 2).

Changes in ascorbate-glutathione cycle enzyme activities in LbRNAi nodules.

As enzymes involved in ROS detoxification were previously found to be posttranscriptionally regulated (Contour-Ansel et al. 2006; Madhusudhan et al. 2003; Mittler et al. 1998; Shigeoka et al. 2002; Sunkar et al. 2006), we extended our analysis of ROS metabolism in nodules to measurements of enzyme activity. Analysis of the same biological material used for gene-expression analysis revealed significant reductions in APX activity in nodules of LbRNAi plants as compared with those of wild type, with 30 and 40% reduction at 3 and 4 wpi, respectively. No significant difference in APX activity was found between the two genotypes at 2 wpi (Fig. 3).

MDAR activity was significantly higher in LbRNAi nodules than in the wild type, i.e., 68, 43, and 38% higher at 2, 3, and 4 wpi, respectively (Fig. 3). A similar pattern was observed for DHAR activity, which was 69, 53, and 46% higher in LbRNAi nodules than in wild-type nodules at 2, 3, and 4 wpi, respectively (Fig. 3).

Fig. 1. Total ascorbate and hydrogen peroxide (H₂O₂) concentrations in nodules of wild-type and leghemoglobin-RNA interference (LbRNAi) plants. Nodules were harvested at different timepoints. **A**, Levels of H₂O₂ and **B**, total ascorbate concentrations were measured on the same set of samples. Black bars represent wild-type and gray bars LbRNAi nodules. Error bars indicate variation between three independent biological replicates. Significant differences obtained by student *t*-test (*P* < 0.05) are marked by an asterisk.

Fig. 2. Expression of genes encoding enzymes of the ascorbate-glutathione cycle. Expression level of two ascorbate peroxidases, *LjAPX1* and *LjAPX2*, three monodehydroascorbate dehydrogenases *LjMDHAR1*, *LjMDHAR2*, *LjMDHAR3*, one dehydroascorbate reductase *LjDHAR1*, and three glutathione reductases *LjGR1*, *LjGR2*, and *LjGR3* were measured using quantitative reverse transcriptase-polymerase chain reaction. Data were normalized against expression levels of ubiquitin10. Black bars represent data from wild-type and gray bars from leghemoglobin-RNA interference nodules. Error bars indicate standard deviation between three independent biological replicates. Significant differences obtained by student *t*-test ($P < 0.05$) are marked by an asterisk.

GR enzyme activity was significantly lower (between 30 and 40% lower) in LbRNAi nodules than in the wild-type nodules between 2 and 4 wpi (Fig. 3).

Regulation of cytosolic APX in LbRNAi nodules.

To test whether the lower APX activities in LbRNAi nodules as compared with the wild type were due to either changes in protein amount or protein *Vmax*, we performed parallel Western blot and in-gel activity assays. Using total protein extracted

from *Lotus* nodules and a polyclonal antibody raised against a cytosolic soybean ascorbate peroxidase (Dalton et al. 1993), a single band was detected via Western blotting at 28 kDa, in line with previous results from soybean (Caldwell et al. 1998; Dalton et al. 1987). No difference in the amount of APX protein in LbRNAi and wild-type nodules was detected 2, 3, and 4 wpi (Fig. 4A), indicating that the slightly lower levels of LjAPX1 and LjAPX2 transcripts in LbRNAi nodules (Fig. 2) did not affect protein amount. In contrast, in-gel activity stain-

Fig. 3. Enzymatic activities of (APX), monodehydroascorbate dehydrogenase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) in nodules. Enzyme activities of APX, MDHAR, DHAR, and GR were measured in nodules of wild-type (black bars) and transgenic leghemoglobin-RNA interference nodules (gray bars). Error bars indicate standard deviation between three independent biological replicates. Significant differences obtained by student *t*-test ($P < 0.05$) are marked by an asterisk.

Fig. 4. Detection of ascorbate peroxidase (APX) protein and activity in *Lotus* root nodule extracts. Total proteins were extracted from nodules of wild-type (wt) and mutant leghemoglobin-RNA interference (LbRNAi) plants at 2, 3, and 4 weeks postinoculation. **A**, Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were blotted onto membrane, and were detected with a specific antibody for APX. **B**, Native protein gel electrophoresis and enzymatic activities of APX were visualized by ascorbate-dependent reduction of nitroblue tetrazolium. Result shown is representative of three independent experiments that all yielded the same result.

ing revealed a single active protein band with substantially lower activity in LbRNAi than in wild-type nodules (Fig. 4B), supporting results from in vitro APX assays (Fig. 3).

DISCUSSION

Autooxidation of leghemoglobins and high rates of respiration have been proposed as major sources of ROS in legume root nodules (Becana and Klucas 1992a and b; Becana and Rodriguez-Barrueco 1989; Becana et al. 1998, 2000; Fridovich 1976; Moreau et al. 1996; Puppo and Halliwell 1988a; Puppo et al. 1981; Schneider and Schlegel 1981). In this study, we assessed the contribution of leghemoglobins to ROS generation in *Lotus japonicus* nodules by comparing H₂O₂ and ascorbate levels and ascorbate-glutathione cycle enzyme transcript, protein, and activity levels in a LbRNAi line devoid of leghemoglobin with those of wild-type plants. The data presented here show for the first time that leghemoglobins are a significant source of ROS in wild-type, nitrogen-fixing legume nodules.

Ascorbate is a key antioxidant in plant ROS metabolism (Noctor and Foyer 1998). Nodules of various legumes were shown to contain either high concentrations of ascorbate (in the millimolar range), elevated activities of antioxidant defense enzymes, or both compared with uninoculated roots (Bashor and Dalton 1999; Dalton et al. 1986; Desbrosses et al. 2005; Gogorcena et al. 1995; Matamoros et al. 1999, 2006). Ascorbate concentrations were found to be about 10-fold higher in nodules than in roots of soybean (Bashor and Dalton 1999), although ascorbate levels of nodules and roots were more similar in nodules of alfalfa, pea, bean, and *Lotus japonicus* (Matamoros et al. 2006). Concomitant with increases in ascorbate concentration during nodule development, enzymatic activities of APX, DHAR, and MDAR were at least twofold higher in nodules than in roots of soybean and common bean (Dalton et al. 1991, 1996; Jebara et al. 2005). Greater differences in APX activity have been reported in alfalfa, pea, bean, and *Lotus japonicus*, for which activities were found to be between 12- and 38-fold higher in nodules compared with roots (Matamoros et al. 2006).

To address the role of leghemoglobins in ROS generation in *Lotus* nodules, we took advantage of a LbRNAi line that completely lacks these proteins in nodules (Ott et al. 2005). We focused our attention on mature, nitrogen-fixing nodules harvested from roots 2, 3, and 4 weeks after inoculation with rhizobia, which typically contain high levels of leghemoglobins (Ott et al. 2005). Concentrations of H₂O₂ and ascorbate remained fairly constant in wild-type nodules between 2 and 4 wpi at approximately 1.2 and 40 μ mol per milligram of fresh weight, respectively (Fig. 1). Likewise, transcript levels and enzyme activities of APX, MDAR, DHAR, and GR were relatively constant in wild-type nodules between 2 and 4 wpi (Figs. 2 and 3), as was the amount of APX protein (Fig. 4). Loss of leghemoglobin from the RNAi line led to a significant decrease in H₂O₂ levels and a significant increase in reduced ascorbate levels in nodules (Fig. 1), consistent with the conclusion that leghemoglobins contribute significantly to ROS production in nodules. Differences in redox homeostasis between wild-type and LbRNAi nodules were partially reflected in changes in the level of transcript for specific enzymes of the ascorbate-glutathione cycle; significantly lower levels of *LjAPX1*, *LjAPX2*, and *LjGR1* transcripts were found in LbRNAi nodules than in the wild type, while the reverse (i.e., significantly higher levels) was true for *LjMDAR* (Fig. 2). Differences in redox homeostasis between wild-type and LbRNAi nodules were even more apparent at the level of enzymatic activity. LbRNAi nodules had significantly more MDAR and DHAR activity and significantly less APX and GR activity than wild-

type nodules (Fig. 3). Taken together with results of transcript analysis (Fig. 2), the data point to significant posttranscriptional regulation of ascorbate-glutathione cycle genes in response to changes in ROS status. DHAR activity was significantly higher in LbRNAi nodules than in the wild type, despite a drop in *LjDHAR* gene expression, while GR activity was significantly lower in LbRNAi nodules despite no difference between LbRNAi and wild-type nodules in *LjGR* gene expression (Figs. 2 and 3). This points towards posttranscriptional regulation of both proteins. Indeed, cytoplasmatic GR activities in pea were found to be regulated posttranscriptionally, as elevated levels in *PsGR1* transcripts were not reflected in higher enzyme activity (Vanacker et al. 2006). No such data have been published before for nodular *DHAR* genes.

It remains to be determined whether changes in the activity of the ascorbate-glutathione cycle enzymes in *Lotus* nodules in response to altered ROS load (Figs. 1, 2, and 3) result from changes in protein concentration rather than protein-specific activity, at least for MDAR, DHAR, and GR. In the case of APX, however, it is clear from the results of Western blot analysis and in-gel activity measurements (Fig. 4) that the decline in APX activity in LbRNAi nodules resulted from posttranslational regulation of APX. Posttranscriptional regulation of APX has been shown in *Arabidopsis* (Panchuk et al. 2002), *Raphanus sativus* (Lopez et al. 1996), tobacco (Madhusudhan et al. 2003; Mittler et al. 1998), and pea (Mittler and Zilinskas 1994).

While the work reported here is the first to probe the contribution of leghemoglobins to ROS generation in planta and the results are consistent with the idea that autooxidation of leghemoglobins is a major source of ROS in nodules (Becana and Klucas 1992b; Becana and Rodriguez-Barrueco 1989; Becana et al. 2000; Moreau et al. 1996; Puppo and Halliwell 1988b; Puppo et al. 1981), we cannot exclude the possibility that at least part of the decline in H₂O₂ in LbRNAi nodules and the concomitant changes in ascorbate-glutathione cycle activity resulted from indirect effects associated with the loss of leghemoglobins (e.g., the lack of demand for reducing equivalents required for Lb reduction). We have shown previously that despite the higher concentrations of free oxygen in LbRNAi nodules, respiration appeared to be compromised in these nodules, as judged by a two- to threefold decrease in the ATP/ADP ratio in these nodules compared with the wild type (Ott et al. 2005). If this is indeed the case, then reduced respiration could account for part of the reduction in ROS in LbRNAi nodules. Unfortunately, given the intimate connection between leghemoglobins and respiration in nodules, it may never be possible to separate the two entirely to determine the relative contribution of each to ROS generation. Nonetheless, it is clear from the results presented here that leghemoglobins in nodules, which are necessary for symbiotic nitrogen fixation, lead to ROS generation and an ameliorating response from the ascorbate-glutathione cycle.

MATERIALS AND METHODS

Biological material.

Seeds of *Lotus japonicus* ecotype GIFU (B-129) and its leghemoglobin-deficient LbRNAi line (Ott et al. 2005) were sterilized as described previously and were cultivated in quartz sand (Colebatch et al. 2004) under greenhouse conditions (16-h-light and 8-h-dark cycle, 22°C). Plants were inoculated after one week of growth with a 1:50 dilution of a 3-day-old liquid culture of *Mesorhizobium loti* NZP2235. Nodules were harvested weekly after inoculation and were stored at -70°C. Frozen nodules were homogenized using a Retch mixer mill prior to protein or RNA extraction.

Ascorbate and H₂O₂ measurements.

Ascorbate concentration was measured photometrically, according to a method based on the reduction of dipyrindyl (Kampfenkel et al. 1995). Nodules (10 mg) were homogenized and extracted in 100 µl of 6% trichloroacetic acid (TCA). Supernatant (25 µl) was mixed with 25 µl of 0.4 M sodium-phosphate buffer, pH 7.4, after centrifugation and 100 µl of staining reagent (fresh mixture 11:4 of solution A [4.6% TCA, 15.3% H₃PO₄]/0.6% FeCl₃ and solution B [4% 2,2-dipyridyl in 70% ethanol]) was added. Supernatant (100 µl) was transferred to a 96-well plate after incubation at 42°C for 45 min. The extinction coefficient of the red complex was measured at 525 nm.

H₂O₂ concentration in nodules was measured fluorimetrically, using the Amplex red H₂O₂ and peroxidase assay kit (Invitrogen, Karlsruhe, Germany). The samples were transferred in black 96-well plates and were excited at 530 nm. Emission was detected at 590 nm and intensity was correlated to H₂O₂ concentration using a calibration curve.

qRT-PCR.

Total RNA was isolated after homogenization of 10 mg of nodules per replicate. Extraction buffer (1 ml per sample; 200 mM, pH 9.0, Na-borate, 30 mM EGTA, 1% sodium dodecyl sulfate [SDS], 1% Na-deoxycholate, 10 mM dithiothreitol [DTT], 100 mM β-mercaptoethanol) was heated to 95°C and was mixed with the frozen sample. Then, 2% polyvinylpyrrolidone (PVPP), 0.15 mg proteinase K, and 78 µl KCl (2 M) were added and the mixture was incubated with slow rotation for approximately 45 min at 37°C. The reaction was stopped on ice for approximately 30 min before the debris was spun down. Nucleic acids were purified via aqueous phenol (pH 4.5)/chloroform (1:1) extraction using pure chloroform for the first and last extraction step. Nucleic acid precipitation was performed on ice for 45 min using 0.5 vol of isopropanol. After resuspending the pellet with diethylpyrocarbonate (DEPC)-treated water, 1 vol of LiCl (4 M) was added and the mixture was incubated at 4°C overnight. RNA was cleaned using 2 M LiCl and 70% ethanol, respectively. Resuspended RNA (1 µl) was run on a 1% agarose gel, and the concentration measured with a NanoDrop spectrometer (NanoDrop, Wilmington, DE, U.S.A.) at 260 nm.

DNA was removed using Turbo RNase-free DNase (Ambion, Applied Biosystems, Darmstadt, Germany). qRT-PCR was performed to check the success of the digestion using a primer binding in one of the introns of the *LjHAR1* gene (AJ495844) [fwd: 5'-CCTGAAATGCCTATTCGTTGAG-3', rev: 3'-CACAGCTTCTTCTGCATGCG-5']. Dnase-treated RNA (0.5 µg) was used for single-stranded cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's protocol.

Samples were diluted 1:8 with DEPC-treated water and were amplified using primers targeting the 3' end (fwd: 5'-CGGTTCACCTGGAAGATGATGTGG-3', rev: 3'-GATACTTGACCTGTTGTCGCA-5') and the 5' end (fwd: 5'-AAGGATCGGGC GTTTGG-3', rev: 3'-AGCAACAAGTTCAACATCGTCTCT-5') of a gene encoding a glyceraldehyde 3-phosphate dehydrogenase (TC14070), to ascertain the quality of the cDNA synthesis. Solely, cDNA with a 3':5' ratio above 0.98 was used for the experiments.

For expression analysis, replicates were amplified via RT-PCR, using gene-specific primers and normalized against ubiquitin10 (TC 14061) [fwd: 5'-TTCACCTGTGCTCCGTC TTC-3', rev: 3'-AACAACAGCACACAGACAATCC-5']. Each PCR reaction was carried out in a total volume of 10 µl, containing 1 µl of diluted RNA, 5 µl of 2× SYBR *LjGreen* PCR MasterMix (Applied Biosystems), and 4 µl of primer mix (0.5 µM each) using a 96- or 386-well Real-Time RT-PCR ma-

chine (Applied Biosystems). All RT-PCR steps and calculations of relative transcript levels were performed as described by Czechowski and associates (2004). Primer sequences used for qRT-PCR were as follows.

LjAPX1 (TC 7951):

fwd, 5'-CCAACCCCTCACATCTTCGACA-3'
rev, 3'-ACCTTCCTTCTCACCGGTCAA-5'

LjAPX2 (TC9623):

fwd, 5'-GGATGGCATGATGCTGGTACTT-3'
rev, 3'-AGCATTAGCTCCACCCCTCTGT-5'

LjMDAR1 (TC12910):

fwd, 5'-CAAACAACCTCTCTCAGCCGAG-3'
rev, 3'-TGTCTGAAAGCGACGGAAGTG-5']

LjMDAR2 (TC19086):

fwd, 5'-GCCAACTTCAAAACGCAACC-3'
rev, 3'-TTGCAAGGATCCCCGATTG-5'

LjMDAR3 (TC15657):

fwd, 5'-ACATGTTGACCATGCTCGCA-3'
rev, 3'-ACTGTTTTCCCATCCTCAGCTG-5'

LjDHAR (TC17380):

fwd, 5'-CATTGGTTGCTGAGTTGAGTGC-3'
rev, 3'-CCCAGCAACATATGGACCATG-5']

LjGR1 (TC19332):

fwd, 5'-CGTGGTTGTGTTCCTCCAAAAAGA-3'
rev, 3'-GCATCCTGAAGTTCACCACCAT-5'

LjGR2 (TC11942):

fwd, 5'-AAGGCACAGTTTGACAGCACG-3'
rev, 3'-CCGCATGGTCACAAATCTTCT-5']

LjGR3 (TC16174):

fwd, 5'-TACCAACTCCCAAACGAGGAGC-3'
rev, 3'-TCCACGTCTTCGTCCGTTGTT-5'

Enzymatic activities.

Nodules (20 mg) were homogenized and mixed with 1% PVPP and 600 µl of extraction buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM benzamidine, 1 mM ε-amino-capronic acid, 4 µM leupeptine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM DTT, 5 mM ascorbate) for 10 min at 4°C. Raw extracts (0.5 ml) were cleaned from proteins (MW < 5 kDa) after centrifugation using NAP-5 columns (Amersham Pharmacia Biotech, Braunschweig, Germany). Enzyme kinetics were measured photometrically in UV-permeable 96 Costar well plates (Vitaris, Baar, Germany) directly after elution in a 96-well Synergy HT microplate reader (Bio-Tek, Winooski, VT, U.S.A.).

APX activity was determined as described by Nakano and Edwards (1987), via the decline of ascorbate absorption at 290 nm.

MDAR activity was measured via the decrease of NADH⁺ H⁺ at 340 nm due to oxidation. Ascorbate oxidase (1.7 U) (Sigma, Deisenhofen, Germany) was used for this coupled-enzyme reaction, which was performed according to Borraccino and associates (1981).

DHAR activity was detected by measuring the increase of ascorbate extinction at 265 nm, following a protocol published by Hossain and Asada (1984).

GR activity was assayed as described by Foyer and Halliwell (1976), following the decrease in NADPH at 340 nm.

Native polyacrylamide gel electrophoresis (PAGE) and APX activity stain.

Homogenized nodules (20 mg) were mixed in the cold with 100 µl of extraction buffer (0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 5 mM ascorbate, 1 mM benzamidine, 1 mM ε-amino-capronic acid, 4 µM leupeptine, 1 mM PMSF, 1 mM DTT). Protein content was determined after centrifugation using Bradford reagent (Sigma).

Discontinuous polyacrylamide gels (1 mm; collecting gel, 5%; separating gel, 10%) without SDS were prepared using a Bio-Rad gel-casting system, following the manufacturer's instructions. Polymerized gels were run in the Mini Protean 3 system (Bio-Rad Laboratories, München, Germany) and were equilibrated in running buffer (25 mM Tris base, 200 mM glycine, 2 mM ascorbate, pH 9.1) for 30 min at 60 V. Protein extract (20 µg per well) was applied and electrophoresis was performed at 80 V for 15 min, 100 V for 20 min, and for 1 h at 120 V.

APX activity was visualized by negative bands on a purple-blue gel due to inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium (NBT; Roche, Mannheim, Germany), according to the method of Mittler and Zilinskas (1993).

Western blot hybridization.

Nodules (15 mg) were homogenized, were mixed with extraction buffer (10 mM Tris, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM DTT), and were centrifuged. Protein content of the supernatant was determined photometrically after using Bradford reagent. Protein extract (10 µg) was denatured by adding 0.25 vol loading buffer (40% glycerol, 20% β-mercaptoethanol, 400 mM Tris/HCl, pH 6.8, 7% SDS, 0.1% bromophenolblue) and was incubated at 95°C for 10 min. Proteins were separated by SDS-PAGE on a precasted 10% Bis-Tris gel (Invitrogen) according to the manufacturer's instructions. Proteins were transferred to a Protran nitrocellulose membrane (Whatman/Schleicher & Schuell, Einbeck, Germany) by using the XCell II blot module (Invitrogen), following the protocol provided by the manufacturer. Successful transfer was controlled by Red Ponceau staining (Sigma) of the membrane.

The membrane was blocked in phosphatesaline (PBS) buffer, 2% milk powder, 3% bovine serum albumin before being incubated overnight at 4°C with APX antibody. The membrane was incubated for 2 h with the secondary antibody (anti-rabbit immunoglobulin G alkaline phosphatase) after washing in PBS buffer containing 0.1% Tween 20 and 0.5% Tween 20, 1 M NaCl for 10 min, respectively. Washing steps were repeated and the membrane was incubated in 100 mM Tris base (pH 9.0) before NBT/5-bromo-4-chloro-3-indolyl phosphate staining.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Gene Index Project: compbio.dfci.harvard.edu/tgi/plant.html