Aldehyde Oxidase (AO) in the Root Nodules of *Lupinus albus* and *Medicago truncatula*: Identification of AO in Meristematic and Infection Zones

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Phytohormones are involved in the organogenesis of legume root nodules. The source of the auxin indole-3-acetic acid (IAA) in nodules has not been clearly determined. We studied the enzyme aldehyde oxidase (AO; EC 1.2.3.1), that catalyzes the last step of IAA biosynthesis in plants, in the nodules of *Lupinus albus* and *Medicago truncatula*. Primordia and young lupin nodules and mature *M. truncatula* nodules showed AO activity bands after native polyacrylamide gel electrophoresis. Gel activity analyses using indole-3-aldehyde as substrate indicated that the nodules of white lupin and *M. truncatula* have the capability to synthesize IAA via the indole-3-pyruvic acid pathway. Immunolocalization and in situ hybridization experiments revealed that AO is preferentially expressed in the meristematic and the invasion zones in *Medicago* nodules and in the lateral meristematic zone of *Lupinus* nodules. High IAA immunolabeling was also detected in the meristematic and invasion zones. Low expression levels and no AO activity were detected in lupin Fix⁻ nodules that displayed restricted growth and early senescence. We propose that local synthesis of IAA in the root nodule meristem and modulation of AO expression and activity are involved in regulation of nodule development.

Additional keywords: ABA, *Bradyrhizobium*, immunofluorescence, *Mesorhizobium loti*.

The association of leguminous plants and bacteria from the Rhizobiaceae family results in a symbiosis that allows the conversion of atmospheric nitrogen to ammonia in a new specific organ, the root nodule. Nodule development requires integrated processes, both in the host cell and in the microsymbiont (Stougaard 2000). In the early stages of infection, the rhizobial nodulation signal molecules known as Nod factors are induced in response to the secretion of flavonoids by the plant, representing the primary morphogenetic signal for nodulation (Long 1996). Phytohormones are considered to be secondary signals in the developmental program of the root nodule (Fang and Hirsch 1998; Hirsch 1992). Coregulation of several *enod* genes by Nod factors and plant hormones suggests that Nod factors and phytohormones may act synergistically or via the convergence of their signaling pathways during nodule development (Schultze and Kondorosi 1998). Flavonoids are poten-

tial candidates for endogenous inhibitors of auxin transport in the early stages of nodule initiation (Hirsch 1992). It has been shown that flavonoids induced in white clover cells undergoing nodule organogenesis can regulate peroxidase-mediated auxin breakdown (Mathesius 2001). Reduction in auxin transport and imbalance in phytohormonal levels cause the stimulation of root cell divisions and subsequent formation of root nodule primordia. Rhizobia themselves or Nod factors lead to local inhibition of acropetal auxin transport (Boot et al. 1999; Hirsch et al. 1989; Mathesius et al. 1998). Studies of auxin import carrier *AUX1*-like genes in *Medicago truncatula* indicate that auxin is required in early nodule development (de Billy et al. 2001).

Nodule formation requires high auxin levels to initiate the process of cell division and the establishment of the primordium. Expression of the auxin-responsive GH3 gene is high in dividing cells of primordia but decreases during later stages of development and differentiation (Mathesius et al. 1998).

Phytohormones exert a temporal and local control on the cell cycle and affect the transcription of cell-cycle genes or the activity of the cyclin-dependent kinases during nodule development. It has been shown that mitotic B-type cyclins Cyc1 and Cyc4 are induced by auxin and cytokinin in the meristematic tissue of yellow lupin (Jelenska et al. 2000). In root nodules of *M. truncatula*, cyclin A2, implicated in cell cycle activation, is up-regulated by auxins (Roudier et al. 2003). Auxins can also be involved in the control of nodule number (Varma et al. 2003).

The regulation of the early steps of nodule organogenesis by auxins, cytokinins, and ethylene has been studied extensively (Cooper and Long 1994; Ferguson and Mathesius 2003; Heidstra et al. 1997; Lohar et al. 2004; Mathesius et al. 1998, 2000; Oldroyd 2001). Information concerning the action of auxins has been obtained in experiments that involve the application of exogenous phytohormones or the synthetic auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) to legume roots. The observation that NPA elicits the formation of nodulelike structures and the expression of early nodulin genes (Hirsch 1992; Hirsch et al. 1989) and the fact that rhizobia (via the Nod factors) produce an accumulation of IAA (Mathesius et al. 1998) and a local reduction of auxin transport capacity prior to nodule primordium development (Boot et al. 1999) suggest a role for auxin in the earliest stages of nodule formation. As early as 1992, the study of gene expression or protein patterns relative to the hormone biosynthetic pathways was proposed as the best way to analyze the role of plant hormones

in nodulation (Hirsch 1992). However, to date, little is known about the auxin biosynthesis pathways and the factors that regulate auxin levels in vivo during root nodule development.

It has been shown that plants can synthesize indole-3-acetic acid (IAA) from tryptophan (Trp), and both Trp-dependent and Trp-independent pathways have been proposed for IAA synthesis from Trp precursors (Bartel 1997). The Trp-dependent indole-3-pyruvic acid pathway includes aldehyde oxidase (AO), which converts indole-3-acetaldehyde to IAA (Bartel 1997; Normanly and Bartel 1999).

The study of AO in maize (Koshiba et al. 1996; Sekimoto et al. 1997) and in the auxin-overproducing *Arabidopsis superroot 1* (*sur 1*) mutant (Seo et al. 1998) confirms its role in IAA biosynthesis. In *Arabidopsis*, one AO isoform, AO α , is able to oxidize indole-3 aldehyde as well as indole-3-acetaldehyde, a precursor of IAA (Akaba et al. 1999) and shows a higher substrate preference for these aldehydes than the other AO isoforms from *Arabidopsis* seedlings. This activity was significantly higher in *Arabidopsis superroot 1* than in wild-type *Arabidopsis* plants (Sekimoto et al. 1997; Seo et al. 1998). AO is also involved in abscisic acid (ABA) biosynthesis (Seo and Koshiba 2002). *AAO3* gene product catalyzes the final step in ABA synthesis in leaves (Seo et al. 2000b) and seeds (González-Guzmán et al. 2004). AO isoforms that can efficiently oxidize abscisic aldehyde have been described in *Arabidopsis* rosette leaves (Seo et al. 2000a), in barley roots (Omarov et al. 2003),

and in leaves and roots of pea plants (Zdunek-Zastocka et al. 2004).

Little information is available regarding AO localization in plants. The spatial distribution of this enzyme has been studied mainly with activity gels, showing that the enzyme is abundant in the apical region of maize coleoptiles and in the roots of *Arabidopsis* seedlings (Koshiba et al. 1996; Sekimoto et al. 1997; Seo et al. 1998). Most information about AO comes from studies on nonleguminous plants or nonnodulated legumes (Miyata et al. 1981; Zdunek and Lips 2001; Zdunek-Zastocka et al. 2004).

In the present work, we have studied the presence of AO in primordia and nodules of *Lupinus albus* and in nodules of *Medicago truncatula*, which represent two legume nodule types with different structure and developmental patterns. A potential role for AO in legume nodule organogenesis is discussed.

RESULTS

AO isoforms.

To analyze the presence of plant and bacterial AO isoforms in legume nodules, cytosol extracts from *L. albus* and *M. truncatula* nodules, bacteroids, inoculated and noninoculated roots of *L. albus*, and free-living bacteria were subjected to native polyacrylamide gel electrophoresis (PAGE) and were analyzed for AO activity. Figure 1 shows the results when indole-3-aldehyde (IAld) (an analog of indole-3-acetaldehyde) and heptaldehyde were used as substrates.

The analysis of the cytosol of lupin nodules induced by *Bradyrhizobium* sp. (*Lupinus*) (hereafter referred to as "wt nodules") showed two strongly stained bands, AOI and AOII, as well as a very faint band, AOIII, when IAld was used (Fig. 1A). No differences were found in the AO band pattern between primordia and mature nodules of lupin. *Mesorhizobium loti* is an incompatible strain that induces the formation of early-senescent Fix⁻ nodules (González-Sama et al. 2004). No active AO bands were detected in *Mesorhizobium loti*-induced Fix⁻ lupin nodules (Fig. 1A). No AO bands were observed in the cytosolic protein extracts of lupin wt nodules when heptaldehyde was used as a substrate (Fig. 1D).

Bacteroids were isolated from lupin nodules in order to test for AO activity in the microsymbiont and to confirm that nodule extracts were free of bacterial AO activity. The AO zymogram obtained from *Bradyrhizobium* bacteroids (Fig. 1C and D) was different from the pattern described above for cytosol extracts from nitrogen-fixing lupin nodules. Four bands, BAOI, BAOII, and a double BAOIV band, with clear differences in their electrophoretic mobility, were developed with IAld. The band pattern obtained with heptaldehyde as a substrate (Fig. 1D) included new active bands (BAOIII, BAOV). The intensity of the bands in free-living *Bradyrhizobium* extracts differed from that observed in bacteroid extracts (Fig. 1C and D), thus suggesting that the affinity of the AO isoenzymes for the substrates might be related to the symbiotic stage of the bacteria. No AO activity bands were detected in protein extracts from inoculated and noninoculated roots of *L. albus* collected five days after inoculation, with any of the substrates used (data not shown).

Active AO that might be involved in IAA biosynthesis were also found in the cytosol of *M. truncatula* nodule cells. Nodule extracts exhibited three AO activity bands, two of which had similar electrophoretic mobility to lupin nodule AOs, while the third band showed faster mobility and stronger IAld staining (Fig. 1A). Heptaldehyde was not oxidized by AO from *M. truncatula* nodules (data not shown).

Anti-AO antibodies raised against *A. thaliana* AAO1 and AAO2 were used to detect antigens in *L. albus* and *M. trunca-*

Fig. 1. Zymograms and immunoblot after native polyacrylamide gel electrophoresis of protein extracts from nodules of *Lupinus albus* and *Medicago truncatula* and *Bradyrhizobium* sp. (*Lupinus*) cell suspensions. **A**, and **C**, Aldehyde oxidase (AO) activity gels with indole-3-aldehyde as a substrate. **B**, Immunoblot with AAO1 antibody. **D**, AO activity gel with heptaldehyde as a substrate. Lane 1, *L. albus* wild-type (wt) nodule (21 days old); lane 2, *L. albus* wt nodule primordium (16 days old); lane 3, *L. albus* Fix⁻ nodule (16 days old); lane 4, *M. truncatula* nodule (21 days old); lane 5, bacteroids isolated from lupin wt nodules; and lane 6, free-living *Bradyrhizobium* cells.

tula nodules. Both antibodies cross-reacted similarly with nodule AO. Figure 1B shows the results when AAO1 antibody was used. Immunoblot analysis after native PAGE revealed several immunoreactive bands, some of which corresponded to AO activity bands. Additional bands could be inactive isoforms or related polypeptides immunolabeled by the antibodies, as previously described in *Arabidopsis* (Akaba et al. 1999). It is noteworthy that the extract from *Mesorhizobium loti*-elicited lupin nodules showed an intensely immunolabeled inactive band that was not observed in nitrogen-fixing lupin nodules. Immunoblot after sodium dodecyl sulfate-PAGE revealed that both antibodies immunorecognized polypeptides of approximately 140 and 145 kDa in protein extracts of lupin wt nodules and *M. truncatula* nodules (data not shown). In *A. thaliana*, polypeptides of 150 and 145 kDa have been found (Akaba et al. 1999). Polypeptides ranging from 140 to 160 kDa have been identified as subunits of AO isoforms in several plant species (Omarov et al. 2003; Zdnunek-Zastocka et al. 2004).

Localization of AO, ABA, and IAA in root nodules.

Nodules of *M. truncatula* and *L. albus* represent two different types of nodule structure and development, indeterminate and lupinoid, respectively. The alfalfa nodule has a persistent apical meristem (zone I) active for six to eight weeks and a clear zonation of the infected zone (Vasse et al. 1990). Infection takes place in zone II, in which bacteria are released from infection threads into the host cell cytoplasm. In zone III, infected cells grow and become host to developing bacteroids with high nitrogen-fixing activity. The senescence zone IV is located in the basal part of the nodule (Vasse et al. 1990).

The nodule of white lupin does not have the characteristic zonation of the elongated indeterminate nodules as does the *Medicago* nodule. The primordial white lupin nodule (7 to 10 days old) consists mainly of meristematic cells, situated in the central part, surrounded by several layers of cortical cells. In young nitrogen-fixing nodules (21 days old), the central infection zone is surrounded by the nodule cortex that contains the peripheral vascular elements. Several layers of young cells with high proliferative activity are situated in the periphery of infected tissue (Fig. 2A and B). In root nodules of yellow lupin,

these cell layers were referred to as the lateral meristematic zone (LMZ) (Jelenska et al. 2000). The central infected tissue surrounded by the LMZ contains mature infected cells with nitrogen-fixing bacteroids.

We studied *L. albus* nodules elicited by two different strains, *Bradyrhizobium* sp. (*Lupinus*) ISLU16 and *Mesorhizobium loti* NZP2037. Inoculation with *Bradyrhizobium* strain ISLU16 leads to the formation of active nitrogen-fixing nodules (Fig. 2A). *Mesorhizobium loti* NZP2037 is able to nodulate *L. albus* roots (González-Sama et al. 2004), but the resulting symbiosis is not effective, and small, white Fix⁻ nodules are formed (Fig. 2C and D). The meristematic activity of Fix⁻ nodules seized within 10 days, and 16-day-old nodules did not have a LMZ. The microsymbiont underwent premature senescence and complete lysis within three weeks (Fig. 2D).

AO immunolocalization with either AAO1 or AAO2 antibodies gave similar spatial patterns. In *M. truncatula* nodules, the strongest fluorescent signal was observed in the apical part, in the cytosol of meristematic and zone II cells (Fig. 3A, B, C, and D). Signal was also detected in the pericycle cells of the vascular bundles (data not shown). Mature infected cells displayed low levels of immunofluorescence, showing a gradient over the interzone II/III (Fig. 3A and D).

In lupin wt nodules induced by *Bradyrhizobium* strain ISLU16, the protein was relatively overexpressed in the LMZ (Fig. 3E, F, and G). In young nodules, fluorescence was located in a circle around the infected zone, which corresponds to the dividing infected and noninfected young cells. In mature lupin wt nodules (30 days, Fig. 3I) and in the central part of *M. truncatula* nodules (zone III, Fig. 3A and D), the signal for AO protein was uniformly present over the cytosol of nodule cells with a slightly higher intensity than the background fluorescence (Fig. 3J). In 10-day-old lupin nodules elicited by *Mesorhizobium loti*, in which both the infected cells and the noninfected cells surrounding them were still actively dividing (Fig. 2C), immunolabeling was detected in the dividing cell (not shown). In 16-day-old lupin Fix⁻ nodules elicited by *Mesorhizobium loti*, the pattern of AO immunolocalization was different from that described for young wt nodules. Fix⁻ nodules did not have the LMZ, and the level of fluorescence was low, similar to that observed in 30-day-old mature wt nodules (data not shown).

Fig. 2. Light micrographs showing the structure of a *Lupinus albus* root nodule after staining with methylene blue. **A**, A 21-day-old lupin nodule elicited by *Bradyrhizobium* sp. (*Lupinus*) showing the central infected cells (I) and the lateral meristematic zone (LMZ). Bar = 100 μ m. **B**, Magnification of the previous image showing LMZ dividing cells (arrows). Bar = 16 μ m. **C**, A 10-day-old nodule elicited by *Mesorhizobium loti* showing the central zone containing infected cells (I). Bar = 50 μ m. **D**, A 21-day-old nodule elicited by *M. loti*. Note the infected zone consisting of cells undergoing premature senescence. Bar = 100 μ m.

To confirm the immunofluorescence pattern, we performed analyses with purified antibodies. AAO1 antibody was eluted from a Western blot membrane (discussed below) and was employed to localize AO in *M. truncatula* nodules. The pattern was identical to that described above with a strong fluorescent signal in the apical part of the nodule (data not shown).

We analyzed the presence of two putative products of AO activity, ABA and IAA, in nodule tissues. The final step of

ABA biosynthesis is catalyzed by AO, and one AO isoform has been associated mainly with ABA synthesis in root-tip dividing cells and in lateral root primordia of *A. thaliana* (Koiwai et al. 2004; Seo et al. 2000a). In order to clarify the topology of ABA localization, we performed ABA immunolocalization using anti-ABA antibodies. ABA localization did not show any specific site of overexpression. The low level of immunofluorescent signal was uniformly present over all tissues of *Lu-*

Fig. 3. Localization and expression of aldehyde oxidase (AO) and localization of abscisic acid in nodules of *Medicago truncatula* and *Lupinus albus*. **A** through **D**, **K**, and **L**, Sections of *M. truncatula* nodules. **E** through **J**, **M**, and **N**, Sections of *L. albus* nodules. **A**, and **E**, Fluorescence microscopy with AAO2 and AAO1 antibodies, respectively. **B** through **D**, **F**, **G** and **I**, Laser confocal microscopy with AAO1 antibody. **A**, Micrograph showing immunolocalization of AO. Note the strong fluorescent signal in the meristem (M) and zone II. **B**, Detail of the meristematic zone, a metaphase can be observed (arrow). Nuclei contrasted with SYTOX are visualized in green. Bar = 50 μ m. **C**, Infected zone II in which bacteria are being released from the infection thread (it) into the cell cytosol. Cytosol shows AO immunolabeling in red, and green bacteria are contrasted with SYTOX. Bar = 10 μ m. **D**, Micrograph showing AO localization in zone II, interzone II/III, and zone III. Bar = 10 μ m. **E**, Strong fluorescent signal in the cells of the lateral meristematic zone (LMZ). Bar = 100 μ m. **F**, Detail of infected cells of the LMZ showing strong immunolabeling. Bar = 10 μ m. **G**, Localization of AO in the lateral part of a 16-day-old wild-type (wt) primordium showing strong AO immunostaining in the cytosol of several infected cell layers. Bar = 50 μ m. **H**, Lateral part of a 16-day-old wt primordium showing infected cells labeled with anti-abscisic acid antiserum. Bar = 16 μ m. **I**, Lateral part of a 30-day-old wt nodule with low immunolabeling in mature infected cells. Bar = 50 μ m. **J**, Control section in which the primary antibody was omitted. Bar = 16 μ m. **K**, AO mRNA localized in a longitudinal section of a *M. truncatula* nodule by in situ hybridization. Silver-black spots denote transcript accumulation. Bar = 16 μ m. **L**, Detail of the previous image. Bar = 50 μ m. **M**, AO mRNA localization in the lateral part of 21-day-old wt lupin nodule. Silver-black spots denote transcript accumulation. Bar = 16 μ m. **N**, Detail of the previous image. Bar = 50 μ m.

pinus (Fig. 3H) and *Medicago* (data not shown) root nodules. Intense fluorescence was observed on the nuclei and the periphery of infected cells, due to immunolabeling of the numerous plastids and to labeling of the cell wall regions. ABA immunogold localization on nuclei and chloroplasts of stressed pea and lavender plants has been described by Welbaum and associates (1997) and Pastor and associates (1999), respectively. The immunolocalization pattern of ABA did not display any kind of gradient over the nodule tissue as described above for the immunolabeling of AO protein.

Immunohistochemical detection of IAA was carried out in nodules fixed with EDAC, a reagent that cross-links the carboxyl group of free IAA to structural proteins while preserving the antigenicity of IAA (Aloni et al. 2003; Thomas et al. 2002). The strongest immunofluorescence signal was detected in the apical part of *M. truncatula* nodules (Fig. 4A), including the meristem, zone II, and first cells layer of zone III, as well as in the LMZ of lupin nodules (not shown). Nuclei were clearly immunolabeled and the nucleoplasm showed stronger signal than the nucleoli (Fig. 4B and C). A weak cytosol labeling, although distinguishable from background autofluorescence (Fig. 4C), was also observed in all cells, except the empty, possibly dead cells of the basal part of the nodule, in zone IV (Fig. 4D). It is noteworthy that the nuclear signal was mainly present in apical cells of *M. truncatula* nodules and in pericycle cells but it could not be observed in mature differentiated cells. Nuclear localization of IAA has been reported in root meristematic cells (Ohmiya et al. 1990) and also in cells within the morphogenic zone but not in tissues in which any cell division activity was soon to be expected, in sunflower immature zygotic embryo explants (Thomas et al. 2002). In

nodules, the highest IAA immunolocalization signal was found in the same cell types and nodule zones in which the strongest AO immunofluorescence signal was detected.

AO gene expression.

Partial cDNA clones were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA isolated from *L. albus* and *M. truncatula* nodules. Their sequence analysis revealed high homology with other plant AO genes. *L. albus* and *M. truncatula* AO partial cDNA clones showed 89 and 86% identity, respectively, with the *A. thaliana* AAO1 published sequence (Sekimoto et al. 1998). Southern blot experiments suggest the presence of three AO homologs in the *M. truncatula* genome (data not shown).

AO expression was studied with whole-mount radioactive in situ hybridization in 21-day-old *L. albus* and *M. truncatula* nodules. A total of 10 to 20 nodules were analyzed per case. A high level of expression was found in the meristem of the *M. truncatula* nodule (Fig. 3K and L) and in the lateral meristematic zone of the lupin nodule (Fig. 3M and N). The significant background observed in nodule tissue did not allow us to discriminate the signal on the mature infected cell of lupin and *M. truncatula* nodules. The expression pattern evidenced by in situ hybridization was similar to the immunolocalization pattern of AO protein. The distribution of silver granules in sense probe-labeled slides revealed a nonspecific diffuse background signal uniformly present in all cells (data not shown).

Specific primers were used to analyze AO expression in lupin primordia, nodules, and *Mesorhizobium loti*-elicited Fix⁻ nodules (Fig. 5). The highest expression level was observed in primordia and the lowest in Fix⁻ primordia, this being in agreement with the AO activity detected in gel and immunolocalization analyses.

Fig. 4. Immunolocalization of indole-3-acetic acid (IAA) in nodules of *Medicago truncatula*. **A**, Micrograph of a nodule section counterstained in red with propidium iodide (PI), showing immunolocalization of IAA. Note the strong green fluorescent signal in the meristem (M), zone II, and a lateral cell layer, corresponding to the pericycle in the vascular bundle. The yellow color is due to the addition of red and green fluorescences. Bar = 50 μ m. **B**, Meristematic zone showing strong immunolabeling of nucleoplasm. Nucleoli in red due to PI staining. Bar = 20 μ m. **C**, High magnification micrograph of meristematic cells. Bar = 5 μ m. **D**, Cells of zones III and IV in a nodule section not counterstained with PI. Note the weak immunolabeling signal in the cytoplasm region closed to the cell walls in mature cells. Empty cells in zone IV do not display the cytosolic signal. Bar = 25 μ m. **E**, Control section where the primary antibody was omitted. Bar = 50 μ m.

DISCUSSION

Phytohormones such as cytokinins and auxins are most likely involved in nodule initiation and development, because they are necessary for activation and completion of the cell cycle. High concentration of phytohormones can also modulate the sink-source relationship of developing primordia with the whole plant and favor the quick growth of nodules. This strategy, used by *Agrobacterium tumefaciens* (Zhu et al. 2000), is responsible for proliferation of transformed plant cells in tumors.

The origin of IAA in legume nodules is still not clear. It is known that the young root nodule has increased concentrations of auxins, and this high level of auxins has been attributed mostly to polar transport (Fedorova et al. 2000; Kawaguchi

Fig. 5. Aldehyde oxidase (AO) expression. **A**, Reverse transcriptase-polymerase chain reaction analysis of AO expression. **B**, Ubiquitin expression as a control. Lane 1, *Lupinus albus* wild-type (wt) nodule (21 days old); lane 2, *L. albus* wt nodule primordium (16 days old); and lane 3, *L. albus* Fix⁻ nodule (16 days old).

and Syono 1996). During lateral root and nodule development, genes related to auxin transport are expressed in primordia, particularly in cells derived from the pericycle and, in later stages, in the regions of the developing organs in which vasculature arises (de Billy et al. 2001). On the other hand, it has been suggested that elevated levels of IAA in nodules can be derived from the prokaryotic microsymbiont (Lambrecht et al. 2000). It has also been shown that inoculation with *Bradyrhizobium* mutants with low IAA production reduced the number of root nodules in soybean, and the involvement of IAA produced by the microsymbiont has been suggested (Fukuhara et al. 1994).

To further investigate the role of auxins in nitrogen-fixing symbioses, we studied the enzyme AO, involved in auxin biosynthesis via the indole-3-pyruvic acid pathway. AO catalyzes the oxidation of indole-3-acetaldehyde to form IAA (Koshiba et al. 1996). Our results show that, in legume root nodules, both the plant cell and the bacteroid have active AO that might play distinct roles in the nodule. The different AO activity-band patterns observed in the plant cell cytosol and the microsymbiont clearly indicate that the cytosolic fraction of the nodules was not contaminated with bacterial enzyme. Moreover, immunolocalization experiments showed that AO mostly accumulates in the meristematic cells of the *Medicago* nodule, devoid of bacteria, thus suggesting that the AO activity in nodules can be mainly attributed to the plant symbiont and not to the prokaryote. Isolated bacteroids and free-living bacteria displayed similar activity-band patterns. The relative abundance of isoenzymes and their substrate preference could be related to the symbiotic stage of the rhizobia. It is interesting to note that heptaldehyde acted as a good substrate for bacterial AO only. This could be an indication that rhizobial AO might be involved in metabolic pathways in the nodule other than IAA biosynthesis. Heptaldehyde may be used as a marker to discriminate bacterial AO isoenzymes from plant AO isoenzymes in the nodule. The fact that no activity was detected in either inoculated or noninoculated roots could be due to the dependence of the root system on basipetal auxin transport or to the contribution of other pathways of auxin biosynthesis to the root system auxin pool.

Existing data indicate that indole-3-acetic acid synthesis can be activated in regions other than the shoot apex (Ljung et al. 2002; Normanly and Bartel 1999). According to Ljung and associates (2002), all parts of the plant can potentially act as sites for IAA biosynthesis, and root tissue has a high capacity to synthesize IAA. In nodules, our results showed that AO expression levels did not correlate with ABA immunolocalization, which was uniform over the nodule tissues. Differently, the highest IAA levels were detected in the nodule zones, in which AO protein and mRNA accumulated preferentially. The lack of correlation between the AO and ABA location and the spatial correlation between AO and IAA immunolocalization suggest that AO might be mainly involved in local IAA synthesis.

However, we cannot exclude the possibility that nodule AO is also active in ABA biosynthesis or in aldehyde detoxification. The occurrence of different IAA biosynthesis pathways, such as the nitrilase pathway, cannot be excluded either. So far, there is no evidence of the involvement of AO in plant developmental processes, however the involvement of an AO isoenzyme in the synthesis of indole-3-acetic acid in young pea leaves has been suggested recently (Zdunek-Zastocka et al. 2004).

Medicago truncatula and *L. albus* nodules have different structure and development. However, the sites of expression of AO during the development of both types of nodules can be considered equivalent. AO was highly expressed in the meristem and the infection zone of the nodules. In *M. truncatula*, these locations correspond to zones I and II, respectively. In

lupin nodules, AO was located in the lateral meristematic zone, which is composed of noninfected cells and infected cells (with a low number of bacteria) that retain their ability to divide. For this reason, the lateral meristematic zone is comparable to zones I and II in medical barrel nodules. Lupin Fix⁻ nodules with restricted growth and early senescence displayed low levels of AO protein, no indole-3-aldehyde-oxidizing activity, and low gene expression. It is well known that the size of Fix⁻ nodules is always smaller than that of nodules elicited by Fix⁺ strains. Meristematic activity ceases early and, consequently, their growth is usually limited (Kiers et al. 2003; Vasse et al. 1990). It is possible that the host plant may play an active role in the regulation of nodule auxin synthesis and be involved in the regulation of nodule meristem growth and development. It is known that the ratio of cytokinin to auxin levels is important in the development and maintenance of the nodule (Ferguson and Mathesius 2003; Hirsch et al. 1997). Localized IAA synthesis could provide a fine regulation mechanism of the auxin/cytokinin ratio.

Phytohormones, mostly auxins and cytokinins, endogenously exert a temporal and local control on the cell cycle (Stals and Inze 2001). Mitogen-activated protein kinase (MAPK) activation by auxin treatment suggests that MAPK cascades might mediate cellular responses to auxin (DeLong et al. 2002). Mitotic B-type cyclins in yellow lupin nodules (Jelenska et al. 2000) and A2-type cyclins in alfalfa nodule meristems (Roudier et al. 2003) are auxin-upregulated. It seems likely that AO has a role in the regulation of IAA biosynthesis, and IAA can play a role in nodule organogenesis, a process that is necessarily linked to a fine regulation of the cell cycle. We cannot ascertain whether high AO expression in the nodule meristematic tissue is the cause of its high proliferation activity or a consequence of it. The modulation of auxin synthesis in the root nodule meristem may allow the plant to exert control over the growth of effective root nodules and suppress noneffective symbioses. It is possible that self-regulation of nodule development is achieved by local control of auxin synthesis via the indole-3-pyruvic acid pathway, by means of either AO expression, activity in the root nodule meristem, or both.

MATERIALS AND METHODS

Plant material, growth conditions, and rhizobial strains.

Seeds of *Lupinus albus* L. cv. Multolupa and *M. truncatula* R 108 were surface-sterilized in 1% HgCl₂ with gentle shaking, were rinsed several times with distilled water, and were allowed to imbibe water for 2 h. Seeds were sown in pots filled with vermiculite for lupin plants and sand/vermiculite (1:2) for barrel medic. Three days after sowing, lupin seedlings were separately inoculated with suspensions of *Bradyrhizobium* sp. (*Lupinus*) ISLU16 or with *Mesorhizobium loti* NZP2037 (González-Sama et al. 2004). *M. truncatula* was inoculated with *Sinorhizobium meliloti* 41.

Bacteria were grown at 28°C with shaking in a yeast extract-mannitol liquid medium (Vincent 1970). Plants were grown in a growth chamber under controlled conditions consisting of a 16-h light photoperiod, 60% relative humidity, and temperature of 25°C day and 15°C night and were irrigated with nitrogen-free nutrient solution (Hoagland and Arnon 1938).

Inoculated and noninoculated roots were collected 12 days after sowing. Lupin primordia, young nodules, and mature nodules were collected 16, 21, and 30 days after sowing, respectively. *Medicago* nodules were collected 21 days after sowing.

Enzyme extraction.

Protein extracts were prepared from fresh or from nodules frozen at -80°C that were collected 16 and 21 days after sow-

ing. Samples (1 to 2 g) were ground in a mortar with liquid nitrogen. AO extraction buffer (250 mM Tris, pH 8.3, 10 mM EDTA, 20 mM dithiothreitol, 10 mM reduced glutathione, and Sigma [St. Louis] protease inhibitor cocktail [1 µl/ml]) was added to ground samples. A ratio of 1 to 2.5 g per 1 ml of buffer was used. The homogenized tissue was centrifuged at 27,000 × g and 4°C for 15 min. The resulting supernatant was used in subsequent assays.

Protein extracts were also prepared from cultured bacteria. Bacterial culture (100 ml) was centrifuged at 17,000 × g and 4°C for 10 min. The pellet was resuspended in 1 ml of AO extraction buffer, was sonicated five times (50%, 40 s), and was ground in a mortar with liquid nitrogen. Samples were spun at 20,000 × g and 4°C for 15 min, and the supernatant was recovered.

The microsymbiont was isolated from nodules according to the following method. Nodules were ground on ice, in a mortar with 50 mM K-phosphate buffer, pH 7.5, 1 mM dithiothreitol (DTT), and protease inhibitors cocktail (Sigma). A total of 3.5 g of nodule tissue in 14 ml of buffer (1:4 wt/vol) was used. Samples were centrifuged at 400 × g and 4°C for 5 min to remove cell wall debris and nuclei. The supernatant was then further centrifuged at 8,000 × g and 4°C for 10 min. The pellet was washed twice in 7 ml of buffer containing 300 mM sucrose and 2 mM Cl₂Mg, and was resuspended in 1 ml of AO extraction buffer. After sonication, the bacteroid fraction was ground in a mortar with liquid nitrogen and was extracted as above.

AO activity.

Proteins were subjected to native PAGE in 7.5% acrylamide gels in a Laemmli buffer system. Each lane was loaded with 800 µg of nodule protein, 900 µg of root protein, 120 µg of bacteroid protein, or 800 µg of bacterial protein. In-gel AO activity was developed as described previously (Zdunek and Lips 2001), in a mixture containing 0.1 M TRIS-HCl, pH 7.5, 1 mM MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide; Sigma), 0.1 mM phenazine metosulphate (Sigma), and 1 mM indole-3-aldehyde (Aldrich, St. Quentin, France) or heptaldehyde (Aldrich) as substrates. Symmetrically loaded gels were cut in half and were used either for activity analysis or immunological analysis. Experiments were repeated at least three times.

Immunoblot analysis.

Proteins were transferred onto a nitrocellulose membrane and were visualized using two polyclonal rabbit antibodies (AAO1, AAO2) raised against *A. thaliana* 30-kDa AAO1 and AAO2 polypeptides (Akaba et al. 1999). The anti-AAO1 antibody specifically reacts with isoform AOα (Seo et al. 2000a), and the anti-AAO2 antibody specifically recognizes AOγ in *Arabidopsis* seedlings (Akaba et al. 1999), as shown by immunoprecipitation. AOα and AOγ are not related to ABA synthesis. Both primary antibodies were used at 1:4,000 dilution. Alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody at 1:20,000 dilution. The reaction was detected by a colorimetric reaction (NBT/BCIP; Sigma) according to the standard protocol.

Immunolocalization.

Indirect immunofluorescence analysis of AO was performed with samples fixed for 1 h in 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.4). Samples were embedded in 8% agarose, and 50- to 100-µm sections were cut with a vibratome. Sections were blocked with 2% bovine serum albumin (BSA) for 30 min, were incubated with the above polyclonal anti-AO antibodies (1:400) overnight at 4°C in phosphate-buffered saline (PBS) containing 0.5% Triton. Alternatively, a purified

anti-AO antibody was prepared. Bands corresponding to the electroblotted nodule proteins of 140 and 145 kDa were dissected from the membrane, and the antibody was eluted from the nitrocellulose strips with PBS-glycine buffer, pH 3.5 (Fedorova et al. 1994). For ABA localization, sections were blocked in 5% BSA for 1 h and were further incubated with a polyclonal anti-ABA antibody (Welbaum et al. 1997) (1:3,000) in PBS containing 2% BSA and 0.5% Triton. Goat anti-rabbit CY-3-labeled IgG (Molecular Probes, Leiden, The Netherlands) was used as secondary antibody (1:400 dilution, 1 h, room temperature). Plant nuclei and bacteroids were stained with SYTOX (Molecular Probes). For IAA immunolocalization, nodules were prefixed under a brief vacuum treatment in freshly prepared 4% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) (Sigma) in 0.1× PBS, pH 7, at room temperature for 30 min. EDAC cross-links IAA to structural proteins (Shi et al. 1993). Subsequently, nodules were postfixed in 4% paraformaldehyde in cacodylate buffer, pH 7.4, containing 25 mg of sucrose per ml (40 min at room temperature under vacuum). Nodule sections were incubated for 30 min in a blocking solution of 5% goat normal serum (Sigma) in PBS and were incubated overnight at 4°C with the primary antibody. A mouse monoclonal hybridoma antibody raised against IAA-BSA conjugate (IAA-17-II-A) (Agdia-Phytodetek/Linaris, Elkhart, IN, U.S.A.) was used at a concentration of 1 µg/µl. Goat anti-mouse Alexa 488-labeled IgG (Molecular Probes) was used as secondary antibody (1:100 dilution, 2 h, room temperature). Sections were counterstained with propidium iodide. Controls were carried out in the absence of primary antibodies. Sections were embedded in Fluoromount G (Electron Microscopy Sciences, Ft. Washington, PA, U.S.A.) and were examined using a Leits DM IRB inverted epifluorescence microscope connected to a Leica TCS 4D confocal system (Bannockburn, IL, U.S.A.).

RT-PCR.

Total RNA was isolated from 100 mg of tissue, using the RNeasy plant mini kit (Qiagen, Hilden, Germany), and was treated with RNase-free DNase I (Qiagen), to remove any genomic DNA contamination, according to the manufacturer's recommendations. RNA (1 µg) was reverse-transcribed into single-stranded cDNA templates using the First-strand cDNA synthesis kit (Roche, Mannheim, Germany).

Consensus primers were designed from the alignment of highly conserved sequence regions of AO or ubiquitin genes. Primers were used to amplify *L. albus* and *M. truncatula* nodule cDNA. The amplified PCR products were cloned into a pDRIVE plasmid (Qiagen), using the PCR cloning kit (Qiagen) following the manufacturer's protocol. Sequence analyses confirmed high homologies (over 85%) with other published plant AO and ubiquitin sequences. Primers were designed based on the sequence of *L. albus* cDNAs. The specific primers designed to amplify *L. albus* AO (forward primer, 5'-GAGTCAAGCTGTGAAGCAGTTAGGC-3' and reverse primer, 5'-CTAACCCCATGACAAAACCTCCTTC-3') generated a 343-bp PCR product. The specific primers designed to amplify *L. albus* ubiquitin (forward primer, 5'-CACTGGGAAGACCA TTACTCTTGAGG-3' and reverse primer, 5'-TAGTCAGCGA GAGTCCTTCCATCTTC-3') generated a 153-bp PCR product.

In situ hybridization.

Antisense [³⁵S] RNA probes and sense [³⁵S] RNA control probes were generated by in vitro transcription with T7 and SP6 RNA polymerases (Stratagene, La Jolla, CA, U.S.A.), respectively, as described by the manufacturer, using pDRIVE-cloned AO partial cDNA clones as templates. After phenol extraction and ethanol precipitation, riboprobes were separated

from free [³⁵S] uridine triphosphate with a Sephadex G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Probes were partially degraded to a length of approximately 150 nucleotides by heating at 60°C for 36 min, in a 60 mM Na₂CO₃/40 mM NaHCO₃ solution.

In situ hybridization was performed according to Scheres and associates (1990). Nodules were fixed in 4% paraformaldehyde in 0.01 M phosphate buffer overnight. Material was dehydrated in ethanol series and was left in 100% ethanol for 3 h. Ethanol was subsequently replaced with xylene. Nodules were embedded in Paraplast (Sigma). Sections (7 µm) were cut with a microtome Spencer 820 (American Optical Corporation). Prior to hybridization paraffin was removed in a xylene-ethanol series. Prehybridization included incubation of the slides at 37°C for 30 min in Proteinase K (1 µg/ml) (Sigma) in 100 mM Tris-HCl (pH 7.5), 50 mM EDTA. The hybridization mix contained 50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% polyvinylpyrrolidone, 10% dextran sulphate, 60 mM DTT, and yeast tRNA (150 µg/ml). A total of 150 µl of hybridization solution was used per slide. Slides were covered with parafilm and were incubated at 42°C overnight in a moisture chamber, then were washed, dehydrated, and dipped in Amersham nuclear-track (LM-1) emulsion. Slides were developed using a D-19 developer (Kodak) after 2 weeks of exposure, were contrasted with 0.5% eosin, and were photographed.

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