Induction and Spatial Organization of Polyamine Biosynthesis During Nodule Development in *Lotus japonicus*

Emmanouil Flemetakis,¹ Rodica C. Efrose,¹ Guilhem Desbrosses,² Maria Dimou,¹ Costas Delis,¹ Georgios Aivalakis,¹ Michael K. Udvardi,² and Panagiotis Katinakis¹

¹Agricultural University of Athens, Department of Agricultural Biotechnology, Iera Odos 75, 118 55 Athens, Greece; ²Max Plank Institute for Molecular Plant Physiology, Am Mühlenberg, 14476 Golm, Germany

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Putrescine and other polyamines are produced by two alternative pathways in plants. One pathway starts with the enzyme arginine decarboxylase (ADC; EC 4.1.1.19), the other with ornithine decarboxylase (ODC; EC 4.1.1.17). Metabolite profiling of nitrogen-fixing *Lotus japonicus* nodules, using gas chromatography coupled to mass spectrometry, revealed a two- to sixfold increase in putrescine levels in mature nodules compared with other organs. Genes involved in polyamine biosynthesis in *L. japonicus* nodules were identified by isolating cDNA clones encoding ADC (*LjADC1*) and ODC (*LjODC*) from a nodule library. Searches of the public expressed sequence tag databases revealed the presence of a second gene encoding ADC (*LjADC2*). Real-time reverse-transcription-polymerase chain reaction analysis showed that *LjADC1* and *LjADC2* were expressed throughout the plant, while *LjODC* transcripts were detected only in nodules and roots. Induction of *LjODC* and *LjADC* gene expression during nodule development preceded symbiotic nitrogen fixation. Transcripts accumulation was maximal at 10 days postinfection, when a 6.5-fold increase in the transcript levels of *LjODC* was observed in comparison with the uninfected roots, while a twofold increase in the transcript levels of *LjADC1* and *LjADC2* was detected. At later stages of nodule development, transcripts for ADC drastically declined, while in the case of ODC, transcript accumulation was higher than that in roots until after 21 days postinfection. The expression profile of genes involved in putrescine biosynthesis correlated well with the expression patterns of genes involved in cell division and expansion, including a *L. japonicus* Cyclin D3 and an α -expansin gene. Spatial localization of *LjODC* and *LjADC1* gene transcripts in developing nodules revealed that both transcripts were expressed in nodule inner cortical cells and in the central tissue. High levels of *LjADC1* transcripts were also observed in both nodule and connecting root vascular tissue, suggesting that putrescine and other polyamines may be subject to long-distance transport. Our results indicate that

polyamines are primarily involved in physiological and cellular processes involved in nodule development, rather than in processes that support directly symbiotic nitrogen fixation and assimilation.

The polyamines putrescine, spermidine, and spermine are low-molecular-weight aliphatic amines that are ubiquitous in both eukaryotes and prokaryotes (Cohen 1998; Pegg 1986). In plants, polyamines have been implicated in a variety of physiological and developmental processes, including rhizogenesis (Kakkar et al. 2000), root growth (Watson et al. 1998), development of flowers and fruits (Kakkar et al. 2000), senescence (Pandey et al. 2000), and somatic (Bastola and Minocha 1995) and pollen embryogenesis (Garrido et al. 1995). Changes in cellular polyamine content in plant tissues have also been associated with environmental stress, including mineral deficiency (Mo and Pua 2002; Watson and Malmberg 1996), chilling (Roy and Wu 2001), and osmotic stress (Borrell et al. 1996). The analysis of transgenic plants with altered levels of polyamine biosynthetic enzymes indicates that changes in internal polyamine levels can affect stem elongation, leaf morphology, and root growth (Capell et al. 1998, 2000; Kumar et al. 1996; Malmberg et al. 1998).

Root nodules of leguminous plants accumulate polyamines to levels that are five to ten times higher than in other organs, although the polyamine composition varies considerably among legume species (Fujihara et al. 1994). It has been shown that at the estimated concentration in nodule cell cytosol, polyamines have inhibitory effects on ammonium and malate transport across the peribacteroid membrane (Whitehead et al. 2001). However, the true physiological roles of polyamines during nodule development and symbiotic nitrogen fixation remain unknown.

The biosynthetic pathways for polyamines are well established in both eukaryotic and prokaryotic organisms. Eukaryotic cells synthesize putrescine directly from ornithine, through the activity of the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17). In plants, putrescine is also synthesized indirectly from arginine, via the activity of arginine decarboxylase (ADC; EC 4.1.1.19). Biosynthesis of spermidine and spermine are catalyzed by spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22) by the addition of an amino propyl moiety to the diamine, putrescine and the triamine, spermidine, respectively (Kumar et al. 1997; Malmberg et al. 1998).

Genes encoding enzymes for polyamine biosynthesis have been isolated from numerous plant species over the past few

Current address of G. Desbrosses: Laboratoires des Symbioses Tropicales et Méditerranéennes, Equipe Réponse des Plantes aux Microorganismes, Université Montpellier 2, CC 002, Place Eugène Bataillon, F-34095 Montpellier Cedex 05, France.

Corresponding author: P. Katinakis; Fax: +30-210-5294314; E-mail: bmbi2kap@aua.gr

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years. ADC cDNA clones have been isolated and characterized from oat (Bell and Malmberg 1990), *Brassica juncea* (Mo and Pua 1998), grapevine (Primikiri and Roubelakis-Agelakis 1999), carnation (Chang et al. 1996), soybean (Nam et al. 1997), pea (Perez-Amador et al. 1995), tomato (Rastogi et al. 1993), *Arabidopsis thaliana* (Watson and Malmberg 1996), and rice (Chattopadhyay et al. 1997). ODC cDNA clones have been isolated from plants such as *Datura stramonium*, tobacco (Michael et al. 1996), and tomato (Alabadi and Carbonell 1998). Spermidine synthase (SPDS) cDNA clones have been isolated from *Datura stramonium* (Hashimoto et al. 1998), pea (Alabadi and Carbonell 1999a), and tomato (Alabadi and Carbonell 1999b), while only one cDNA clone encoding spermine synthase, from *A. thaliana*, has been reported (Hanzawa et al. 2000).

The expression of ODC and ADC genes appears to be developmentally regulated in plants. In tomato plants, the highest levels of ODC gene transcripts were detected in roots and flowers, and the lowest levels were found in the leaves (Alabadi and Carbonell 1998; Kwak and Lee 2001). In contrast, ADC gene expression in pea roots was found to be very low in comparison with other tissues (Perez-Amador et al. 1995). Expression of two pea spermidine synthase genes (*SPDSYN1* and *SPDSYN2*) also exhibited an organ-specific pattern. Transcripts from both genes were found at high levels in shoot tips, young leaves, stems, and flowers, whereas low levels of expression were found in roots and adult leaves (Alabadi and Carbonell 1999a). Changes in the ADC activity and levels of accumulation of mRNA have been implicated in cell elongation, cell extension, and responses to various stresses (Malmberg et al. 1998). The expression of ADC is stimulated by numerous stress conditions, including osmotic stress, acidic pH, nutrient deficiency, UV light, and pollutants (Malmberg et al. 1998; Mo and Pua 2002; Nam et al. 1997). On the other hand, changes in the level of expression of ODC were correlated with cell proliferation and normal cell growth (Michael et al. 1996).

Despite the numerous studies reporting ODC and ADC expression levels in different plant organs, little or no information is available on the expression patterns of these genes at the tissue level, especially in legume root nodules. In order to decipher the roles of polyamines in symbiotic nitrogen fixation, we have chosen to work with the model legume *Lotus japonicus*, which is amenable to reverse genetics (Stougaard 2001). After demonstrating that putrescine accumulates in mature

nodules, we isolated and characterized two genes, *LjADC1* and *LjODC*, involved in polyamine biosynthesis in this organ, while a second ADC cDNA, named *LjADC2*, was found in public expressed sequence tag (EST) databases. The expression patterns of these two genes in nodules indicate that polyamines probably play diverse roles in developing and mature nodules. Isolation of these genes will enable manipulation of polyamine levels in transgenic *L. japonicus* plants, which should help to reveal the roles of these compounds in nodules in the future.

RESULTS

Putrescine accumulates to relatively high levels in nitrogen-fixing nodules of *L. japonicus*.

During the past years, development of new methods based on gas chromatography coupled to mass spectrometry (GC-MS) has enabled detection and quantification of hundreds of compounds from complex plant extracts (Roessner et al. 2000). GC-MS was used to profile metabolites in nitrogen-fixing nodules and other organs of *L. japonicus*. Evaluation of chromatographs obtained from these organs revealed a two- to sixfold increase in putrescine levels in mature nodules compared with that in primary and lateral roots or leaves (Fig. 1). These results are in agreement with previous reports, indicating that root nodules of leguminous plants contain five- to tenfold higher polyamine levels than other organs (Fujihara et al. 1994).

Characterization of cDNA clones coding for ornithine and arginine decarboxylase.

Recently, large numbers of ESTs from *L. japonicus* nodules have been deposited in public databases, and the corresponding clones have been used to produce cDNA arrays for transcriptome analysis. This enabled identification of genes coding for enzymes involved in diverse metabolic pathways whose expression is induced during nodule development (Colebatch et al. 2002). In silico analysis revealed the presence of ESTs encoding several enzymes involved in putrescine biosynthesis, including one ODC and two ADC isoforms in *L. japonicus*. The longest ODC and ADC cDNA clones were completely sequenced and were named *LjODC* and *LjADC1*. The *LjODC* clone (accession number AJ575746) is a full-length cDNA coding for a polypeptide of 422 amino acids, while the *LjADC1* clone (accession number AJ575747), which encodes 567 amino acids, is not a full-length cDNA. Sequence comparisons with other plant ADC sequences indicate that the *LjADC1* cDNA lacks about 400 nt of 5' coding sequence. We were able to identify only two overlapping ESTs, corresponding to *LjADC2*, in the public nucleotide sequence databases. The contig nucleotide sequence that results from these ESTs (TIGR identification number: TC5158) lacks approximately 800 nt of 5' coding sequence when compared with the *LjADC1* clone and codes for a polypeptide showing 63% similarity to *LjADC1*.

Comparative analysis of deduced amino acid sequences revealed that *LjODC* shares 57 to 58% identity with ODC from *Datura stramonium*, tomato, and tobacco (Fig. 2A). Less similarity was observed with mouse ODC (43%), as expected. The putative antizyme-binding region (corresponding to residues 117 to 140 of the mouse ODC sequence), found in all the mammalian ODCs characterized so far (Hascilowicz et al. 2002), shows a low level of homology with the corresponding region of the *L. japonicus* ODC. However, residues Tyr¹²², Asp¹³⁴, and Glu¹³⁸ of the mouse ODC, which are conserved in the antizyme-binding region of all mammalian ODCs, are also present in the *L. japonicus* sequence. Like all plant ODCs characterized so far, *LjODC* does not possess the C-terminal extension found in all mammalian ODC sequences, corresponding

Fig. 1. Putrescine accumulation in *Lotus japonicus* mature nodules, primary roots, lateral roots, and mature leaves. Metabolites were extracted, derived, and analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Putrescine accumulation is expressed as a response ratio (Roessner et al. 2000). For the quantification of putrescine, three specific mass to charge ratios (m/z) were selected: 174 (white bars), 214 (black bars), and 361 (gray bars). Bars represent the average response of three independent measurements representing three independent biological repeats + standard deviation ($n = 3$).

to the degradation element II (residues 422 to 461 of the mouse ODC sequence). In contrast, the degradation element I (residues 376 to 424 of the mouse ODC sequence), including the conserved motif FNGF found in all mammalian homologs (Hascilowicz et al. 2002), is well conserved among the plant ODCs.

Comparison of the *LjADC1* polypeptide with previously characterized plant ADC proteins revealed that it shares 72, 69, and 73% identity to pea, *A. thaliana*, and tobacco homologs, respec-

tively (Fig. 2B). Apart from the high sequence homology to other plant ODCs and ADCs, the *L. japonicus* proteins possess several motifs and amino acid residues that are conserved in all prokaryotic and eukaryotic ODC and ADC enzymes characterized so far. Thus, although comparison of *L. japonicus* ODC and ADC amino acid sequences revealed only a low level of sequence conservation (data not shown), we were able to identify residues Lys⁹⁰ and Cys³⁶⁷ of *LjODC*, and Lys²⁹ and Cys⁴²¹ of *LjADC* as putative equivalents of the mouse ODC residues Lys⁶⁹ and Cys³⁶⁰. The mouse amino acid residues have previously been shown to participate in the binding of the cofactor pyridoxal 5'-phosphate (PLP) and the substrate, respectively (Poulin et al. 1992). The motifs LDIGGGFT and IDIGGGLG found in *LjODC* and *LjADC*, respectively, are similar to the conserved motif hh(D/N)hGGGh(G/T), where h represents a small hydrophobic residue found in all of these decarboxylases from prokaryotic and eukaryotic organisms (Michael et al. 1996).

Profiling of *LjODC* and *LjADC* gene transcripts in symbiotic and nonsymbiotic organs of *L. japonicus*.

To gain insight into the metabolic pathways likely to be involved in putrescine biosynthesis in different organs of *L. japonicus*, the accumulation of *LjODC* and *LjADC* transcripts was examined. For this purpose, total RNA was isolated from different *L. japonicus* organs (young nodules at 14 days post-infection, uninfected roots from 21-day-old plantlets, young leaves, stems, flowers, and green seedpods) and was subjected to real-time and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis (Fig. 3). Both *LjADC1* and *LjADC2* transcripts exhibited a similar accumulation pattern in all organs under study. The highest levels of *LjADC1* transcripts were observed in the uninfected roots, followed by green seedpods, flowers, and developing nodules at 14 days postinfection. Lower levels of *LjADC1* transcripts were found in the stems and young leaves. Similarly, *LjADC2* gene transcripts accumulated mainly in the green seedpods, uninfected roots, and flowers and, to a slightly lower level, in developing nodules and stems. Like *LjADC1*, the lowest level of *LjADC2* gene transcripts was detected in young leaves. In contrast, *LjODC* transcripts were exclusively detected in developing nodules and uninfected roots but not in any other organ tested (stems, leaves, flowers, and seedpods). Interestingly, in developing nodules at 14 days postinfection, a greater than twofold induction of *LjODC* gene expression, as compared with that in uninfected roots, was detected.

Expression of *LjODC* and *LjADC* genes during nodule development.

To further investigate the accumulation of transcripts involved in polyamine biosynthesis during nodule development, we performed real-time RT-qPCR assays for *LjODC*, *LjADC1*, and *LjADC2* genes, on cDNA templates reverse-derived from total RNA samples isolated from nodules at 10, 14, 21, and 28 days postinfection. In order to allow direct comparison of the transcript accumulation in developing nodules and uninfected roots, total RNA obtained from the whole root of uninfected 21-day-old plantlets was used to determine the basal levels of gene expression. In the growth conditions we used, nodules are first visible at 10 days postinfection as small white bumps. At this early developmental stage, we detected a 6.5-fold increase in the transcript levels of *LjODC*, while only a moderate twofold increase in the transcript levels of *LjADC1* and *LjADC2* was observed (Fig. 4). At 14 days postinfection, the mRNA levels of *LjODC* declined but remained more than twofold higher than the basal uninfected root levels. Similarly, the mRNA levels for both *LjADC* genes decreased, but in this case,

Fig. 3. Accumulation of *LjODC*, *LjADC1*, and *LjADC2* gene transcripts in symbiotic and nonsymbiotic organs of *Lotus japonicus*. Total RNA was isolated from various organs, and poly(A)⁺ RNA was converted to cDNA and was subjected to real-time quantitative polymerase chain reaction. Small differences in the starting cDNA template were normalized by the parallel quantification of the constitutively accumulating ubiquitin transcripts. The relative mRNA level was calculated with respect to the level of the corresponding transcript in uninfected roots (=1). Bars show means + standard deviation (*n* = 3).

the expression levels were lower than in the control uninfected root. It should be pointed out that, during this developmental stage, nodules continue to grow in size and become functional, as indicated by the accumulation of nitrogenase transcripts (data not shown). At later stages of nodule development, *LjODC* mRNA levels continued to decline, reaching the basal root levels at 28 days postinfection. Remarkably, both *LjADC* transcripts greatly diminished as the nodule matured. The level of *LjADC1* mRNA was about threefold lower in nodules at 21 days postinfection compared with that of uninfected roots, while the downregulation of *LjADC1* increased to 16-fold at 28 days postinfection. A less dramatic but similar downregulation was observed for *LjADC2*, for which we measured a two- and sevenfold decrease in comparison with the uninfected root at 21 and 28 days postinfection, respectively.

As polyamines have been implicated in various plant growth and developmental processes (Kakkar et al. 2000), we tested the possible correlation between the temporal expression patterns of genes actively involved in cell division and expansion and the expression profile of the genes involved in putrescine biosynthesis during nodule development. A search in the public nucleotide databases enabled us to identify genes coding for cyclins, expansins, and extensins expressed in *L. japonicus* nodules. Transcript levels for one representative from each of these families was determined using real-time RT-qPCR analysis (Fig. 4). Transcripts for *LjCycD3*, a homolog to *A. thaliana* D3 cyclin, increased about 3.5-fold in young nodules at 10 days postinfection and then gradually decreased, reaching the uninfected root basal levels at 21 days postinfection. A similar transcript accumulation pattern was observed for *LjExp*, a

Fig. 4. Accumulation of *LjODC*, *LjADC1*, *LjADC2*, *LjCycD3*, *LjExp*, and *LjExt* gene transcripts during *Lotus japonicus* nodule development. Total RNA was isolated from uninfected roots of 14-day-old plantlets and from nodules at 10, 14, 21, and 28 days postinfection (d.p.i.), and poly(A)⁺ RNA was converted to cDNA and was subjected to real-time quantitative polymerase chain reaction. Small differences in the starting cDNA template were normalized by the parallel quantification of the constitutively accumulating ubiquitin transcripts. Relative mRNA level was calculated with respect to the level of the corresponding transcript in uninfected roots (=1). Bars show means + standard deviation ($n = 3$).

L. japonicus α -expansin homolog. *LjExp* mRNA levels increased about 3.5-fold in nodules at 10 days postinfection and subsequently dropped to basal levels at 21 days postinfection. Interestingly, both *LjCycD3* and *LjExp* mRNAs followed an accumulation pattern similar to that observed for the transcripts of *LjODC* and *LjADC* during nodule development. A different expression pattern was observed for the *LjExt* gene, a member of the extensin-like (hydroxy)proline-rich glycolprotein family. *LjExt* expression was five- to sixfold up-regulated

in nodules at 10 and 14 days postinfection, while in mature nodules, *LjExp* mRNA declined but remained two- to threefold higher than the basal uninfected root level.

Localization of *LjODC* and *LjADC1* transcripts in developing nodules.

The localization of *LjODC* and *LjADC1* transcripts in developing and mature nodules was examined, using an in situ hybridization approach. Sections of *L. japonicus* nodules at various

Fig. 5. In situ localization of *LjODC* and *LjADC1* gene transcripts in *Lotus japonicus* root nodules. **A** through **F**, Transverse thin sections (7 μ m) of nodules at 14 (**A** and **D**), 21 (**B**, **E**, **G**, and **H**), and 28 (**C** and **F**) days postinfection with *Mesorhizobium loti* were hybridized with digoxigenin-11-rUTP-labeled RNA probe in vitro, transcribed from *LjODC* and *LjADC* cDNA clones. Hybridization signal is visible as blueish purple precipitate. **A**, *LjODC* gene transcripts at 14 days postinfection were localized in the nodule inner cortex (ic) and at lower levels in the central tissue (ct), mainly in the uninfected cells. **B** and **C**, In mature nodules at 21 and 28 days postinfection, respectively, *LjODC* expression was detected in the nodule inner cortex (ic), whereas the hybridization signal in the central tissue (ct) was barely detectable. **D**, At 14 days postinfection, *LjADC1* gene transcripts were detected in the nodule inner cortex, root vascular tissue, as well as in the nodule central tissue. **E**, In mature nodules at 21 days postinfection, *LjADC1* expression was detected at high levels in the nodule inner cortex and the connecting vascular tissue (vb) and at a lower level in the nodule central tissue. **F**, In older nodules at 28 days postinfection, low levels of hybridization signal were visible in the nodule inner cortex and the nodule central tissue, whereas a strong hybridization signal was visible in the root vascular tissue. **G**, A close-up view of a nodule region in **B**; vb = vascular bundle, un = uninfected cells, and in = infected cells. **H**, A close-up view of a 21-days-postinfection nodule showing high *LjADC1* expression in the nodule inner cortex and vascular tissue. **I** through **L**, As a negative control, transverse sections of nodules (at 21 [**I** and **J**] and 28 [**K** and **L**] days postinfection) were hybridized to sense digoxigenin-11-rUTP-labeled RNA transcribed from *LjODC* and *LjADC1* cDNA clones. In this case, no significant hybridization signal was visible. Bars = 100 μ m (**A**, **B**, **C**, **D**, **E**, **F**, **I**, and **K**) or 10 μ m (**G**, **H**, **J**, and **L**).

stages of development were hybridized with 11-digoxigenin-rUTP-labeled RNA probes transcribed from *LjODC* and *LjADC1* cDNA clones. Both antisense and sense labeled RNA transcripts were used as probes. At 14 days postinfection with *Mesorhizobium loti*, detectable levels of *LjODC* transcripts were observed in the nodule inner cortical cells, whereas a low level of expression was detected in the central tissue, mainly in the uninfected cells (Fig. 5A). In nodules at 21 days postinfection, *LjODC* transcripts were still present mainly in the nodule inner cortical cells, while the hybridization signal in the cells of the central tissue was lower (Fig. 5B and G). In nodules at 28 days postinfection, a low level of hybridization signal was detected in the nodule inner cortex, whereas in the cells of the central tissue, the hybridization signal was barely detectable (Fig. 5C).

In the case of *LjADC1*, in nodules at 14 days postinfection, a strong hybridization signal was detected in the nodule inner cortex as well as in both the infected and uninfected cells of the central tissue (Fig. 5D). In addition, accumulation of *LjADC1* mRNAs was detected in the root vascular tissue. In the later stages of nodule development at 21 days postinfection, accumulation of *LjADC1* gene transcripts followed a similar spatial pattern (Fig. 5E). High expression levels were observed in the nodule inner cortical cells, whereas lower levels of expression were detected in both the infected and uninfected cells. Interestingly, exceptionally high accumulation of *LjADC1* gene transcripts was detected in the root central cylinder and the nodule-connecting vascular bundle (Fig. 5E) as well as in the nodule vascular bundle (Fig. 5H). In contrast, *in situ* hybridization failed to detect any accumulation of *LjODC* gene transcripts in the nodule vascular bundle (Fig. 5G). In agreement with the RT-qPCR results, in nodules at 28 days postinfection, expression of *LjADC1* was barely detectable, whereas strong hybridization signal was detected in the root vascular system (Fig. 5F).

As a negative control, sections of *L. japonicus* nodules at 21 and 28 days postinfection with *M. loti* were hybridized to sense digoxigenin-labeled RNA probes transcribed from *LjODC* and *LjADC* cDNA clones (Fig. 5I to L). In this case, no significant hybridization signal was detected.

DISCUSSION

In higher plants, two alternative pathways synthesize the diamine putrescine; one pathway converts ornithine to putrescine directly through the activity of ODC, the other converts arginine to putrescine indirectly via agmatine and N-carbamoylputrescine in steps catalyzed by ADC, agmatine iminohydrolase (AIH), and N-carbamoylputrescine amidohydrolase (CPA), respectively. We found relatively high levels of putrescine in mature nodules of *L. japonicus* (Fig. 1) and identified *LjODC* and *LjADC* cDNA clones in a nodule library (Fig. 2), which indicates that putrescine may be produced by both pathways in this organ. The presence of cDNA sequences encoding homologs of AIH and CPA in nodule EST libraries of *L. japonicus* and other legumes lends support to this idea.

All the eukaryotic ODCs as well as the plant and bacterial ADCs characterized so far belong to group IV of the PLP-dependent decarboxylases (Sandmeier et al. 1994). The sequence similarities between proteins of this group enable identification of amino acid residues that are likely to be important for catalytic activity. Residues Lys⁹⁰ and Cys³⁶⁷ of *L. japonicus* ODC correspond to the Lys⁶⁹ and Cys³⁶⁰ residues of mouse ODC, which are known binding sites for the cofactor PLP and the specific, irreversible ODC inhibitor difluoromethylornithine (DFMO), respectively (Poulin et al. 1992). Similarly, the PLP and difluoromethylarginine (DFMA) binding sites of

the *L. japonicus* ADC can be tentatively assigned as Lys²⁹ and Cys⁴²¹, respectively, on the partial *LjADC* sequence (Fig. 2).

The motifs LDIGGGFT and IDIGGGLG found in *LjODC* and *LjADC*, respectively, resemble motif I of methyltransferases, which are thought to participate in the formation of the S-adenosylmethionine binding pocket (Kagan and Clarke 1994). A similar motif is also present in an equivalent position in spermidine synthases (Hibi et al. 1994). Whether this decarboxylase motif can bind S-adenosylmethionine remains to be elucidated. This motif also resembles the nucleotide-binding site of FAD-binding flavoproteins (Van Beeumen et al. 1991) and the P region of GTP-binding proteins, which is involved in the hydrolysis of GTP (Kaziro et al. 1991). However, the functional significance of these similarities, if any, remains to be determined. Like all the plant ODCs characterized so far, the *L. japonicus* ODC does not possess the carboxy-terminal extension found in all mammalian ODCs, which is thought to be involved in the rapid turnover of the protein through association with an ODC antizyme (Murakami et al. 1992). Absence of a similar C-terminal domain in plant ODCs may make them more stable than the animal enzymes *in vivo*. However, certain conserved amino acid residues in the antizyme-binding region and the carboxy-terminus degradation elements, which are required for the antizyme-dependent degradation of mammalian ODCs (Hascilowicz et al. 2002), are also present in the plant homologs. So far, no plant homologs of the animal ODC antizyme have appeared in public sequence databases.

Most plant species possess the genetic potential to synthesize putrescine via both ODC and ADC pathways. A notable exception to this is *A. thaliana*, which does not possess an ODC gene (Hanfrey et al. 2001). Thus, *A. thaliana* is dependent totally on the ADC pathway for putrescine biosynthesis. Based on the relative frequency of ADC and ODC sequences in large EST collections from many different plant species, including soybean, *Medicago truncatula*, and *L. japonicus*, it appears that the ADC pathway may be the primary source of putrescine in plants in general. It is interesting, therefore, that *LjODC* is represented by five ESTs from nodule libraries, while *LjADC1* is represented by only one EST. In contrast, *LjODC* is represented by only one EST in libraries from non-symbiotic organs, while *LjADC1* and *LjADC2* are represented by eight ESTs. In addition, *LjADC1* and *LjADC2* are represented by one EST each in libraries from infected roots harboring nodule primordia. No other ODC or ADC genes are yet represented among the *Lotus* nodule ESTs. Thus, the ODC pathway may contribute more than ADC to putrescine synthesis in nodules, in contrast to other organs.

In agreement with the distribution of ODC and ADC in *L. japonicus* EST collections, expression profiling of the corresponding genes by RT-qPCR indicated that *LjADC* transcripts are present in all the *L. japonicus* tissues or organs examined, albeit at different levels (Fig. 3), while *LjODC* transcripts were detected only in nodules and roots. The absence of ODC in the other tissues of *L. japonicus* indicates that ADC is probably the sole route for putrescine biosynthesis in these tissues. However, we cannot exclude the presence of a second *L. japonicus* ODC transcript that did not amplify due to mismatches of the primers used in the RT-qPCR reaction.

The level of ADC or ODC gene expression and enzyme activity varies with organs and the stage of organ and plant development. Our data showed that in young nodules at 10 days postinfection, a developmental stage characterized by rapid nodule growth, both *LjADC* and *LjODC* transcripts exhibited maximum levels of accumulation, whereas they both rapidly declined during later stages of nodulation. A similar expression profile was observed for *LjCycD3* and *LjExp* genes, coding for a D-type cyclin and an α -expansin, respectively. Plant D-type

cyclins are classified into three groups, and although their precise function is still unknown, they have been proposed to participate in control of the cell cycle as well as other developmental programs (Meijer and Murray 2000). In *Medicago* spp., the expression of a nodule-specific *CycD3* family member has been detected in zones I and II, indicating a role in nodule meristematic activity and endocycles, respectively (Foucher and Kondorosi 2000). Although, at present, there are no reports on the involvement of expansins in nodule development, their role in cell growth by mediating cell wall expansion is well-established (Li et al. 2003). In soybean, *GmEXP1* gene coding for an α -expansin was found to be specifically expressed in the elongation zone of growing root tips, mediating cell elongation, whereas the *SbHRGP3* gene, coding for a Hyp-rich glycoprotein, was expressed in the region of maturation leading to the cessation of cell elongation (Ahn et al. 1996; Lee et al. 2003). Interestingly, the peak of *LjExt* gene transcript accumulation was found to succeed the maximum transcript accumulation of *LjExp* gene. Whether this implies roles for *LjExp* and *LjExt* genes in cell wall expansion and stiffening during nodule development, respectively, remains to be demonstrated. The correlation of *LjODC* and *LjADCs* expression patterns with the expression of *LjCycD3* and *LjExp* suggests that polyamines are primarily involved in nodule development rather than the process of nitrogen fixation, as the nitrogenase transcripts are detectable at high levels only after 14 days postinfection (data not shown).

The localization and distribution of *LjODC* and *LjADC* transcripts revealed that, in young nodules, both genes are expressed in nodule inner cortex and in both infected and uninfected cells of the central tissue. Activities of both ODC and ADC in one cell type have also been reported in cultivated poplar cells, with ADC being much higher than that of ODC (Bhatnagar et al. 2001). Since, at this stage of development, nodule growth takes place primarily through cell expansion, it could be argued that expression of both genes correlates either with cell expansion, enlargement, or both. Previous reports suggested that ODC is mainly involved in cell division (Alabadi and Carbonell 1998; Michael et al. 1996; Nam et al. 1997) while ADC is involved in cell expansion (Alabadi and Carbonell 1998; Michael et al. 1996; Perez-Amador et al. 1995). Our results do not support this idea, as *LjODC* and *LjADC* genes were coordinately up-regulated during nodule development, and *LjODC* transcripts remained high even at later stages of nodulation. Although the exact role of polyamines during cell growth is not yet understood, one possible way in which polyamine biosynthesis may contribute to cell expansion is via the action of diamine oxidase (DAO) that produces H_2O_2 , which is considered to play an active role in lignin biosynthesis and cell wall loosening (Maller and McPherson 1998). In mature nodules (21 to 28 days postinfection), a developmental stage when cell division and enlargement cease, we observed a drastic decrease of the levels of both *LjADC* and *LjODC* transcripts. These observations are consistent with the hypothesis that putrescine may be involved in cell expansion. In addition, H_2O_2 derived from DAO activity may promote cross-linking of a plant matrix glycoprotein associated with the lumen of infection threads (Wisniewski et al. 2000). Thus, putrescine, which is a preferred substrate for DAO, may be important to restrict the growth of rhizobia within infection threads. This hypothesis is supported by the observation that accumulation of *LjODC* and *LjADC* transcripts is higher at stages of nodule development when the infection process is terminating. Furthermore, studies with transgenic pea plants revealed that lines showing cosuppression of DAO activity were less sensitive to the inhibitory effects of exogenous putrescine, indicating that putrescine-derived products of DAO could retard nodule development (Wisniewski and Brewin 2000).

The nodule and root vascular system are metabolically active tissues, and a number of genes coding for enzymes involved in carbon metabolism are up-regulated there (Kavroulakis et al. 2000). The localization of *LjADC* transcripts in the root and nodule vascular system, at relatively high levels in all stages of nodule development suggests that the synthesized putrescine may be exported, possibly through the xylem, to the other plant organs. A considerable amount of putrescine and other polyamines (spermine and spermidine) have been found in phloem of *Ricinus communis* seedlings, indicating translocation between different organs (Antognoni et al. 1998). Furthermore, pulse chase experiments demonstrated that radioactive-labeled putrescine was transported from *Limonium tataricum* roots to the shoots (Duhazé et al. 2002).

The correlation between expression of polyamine biosynthetic genes and those involved in cell division and expansion observed here points to a primary role for polyamines in nodule development rather than symbiotic nitrogen fixation per se. This assumption is in agreement with the accumulating evidences implicating polyamines in various plant growth and development processes, including stimulation of cell division, senescence, regulation of rhizogenesis, embryogenesis, floral development, and fruit ripening (Kakkar et al. 2000). To our knowledge, this is the first report demonstrating the induction and spatial expression pattern of genes involved in polyamine biosynthesis during the process of nodule development. Further work needs to be done to determine the precise biochemical and physiological roles of polyamines during nodulation. Past attempts to reveal one or more roles of polyamines in plants using reverse genetics have been stymied by the apparently essential nature of these compounds in normal plant development (Kakkar et al. 2002). However the use of nodule-specific promoters to drive the expression of antisense or RNAi gene constructs in transgenic *L. japonicus* may yield important information for the role of polyamines during the process of nodule development. This task may be facilitated by the recent development of a system for loss-of-function analyses of genes involved in nodule development by the expression of hairpin RNA in *L. japonicus* root nodules (Kumagai and Kouchi 2003).

MATERIALS AND METHODS

Plant material and growth conditions.

L. japonicus (Gifu B-129) seeds were kindly provided by J. Stougaard (University of Aarhus, Denmark). The plants were grown in a controlled environment with an 18-h day and 6-h night cycle, a 22°C day and 18°C night regime, and 70% humidity (Handberg and Stougaard 1992). Prior to germination, seeds were scarified for 5 min with H_2SO_4 and then were sterilized for 20 min in a solution containing 2% NaOCl, 0.02% Tween 20. Seeds were pregerminated at 18°C in the dark for 72 h, and the small plants were grown in B&D nutrient solution (Broughton and Dilworth 1971). For the inoculation with rhizobia, 72-h-old seedlings were spot-infected with a 0.1-optical density at 600 nm suspension culture of *M. loti* (strain E1R.pMP2112), and the plants were grown in nitrogen-free B&D nutrient solution (Broughton and Dilworth 1971).

Metabolite extraction, derivation, and GC-MS analysis.

Mature leaves, primary root tips, lateral roots without nodules, and mature nodules of various sizes were harvested from 12-week-old *L. japonicus* plants, were immediately frozen in liquid nitrogen, and were stored for no more than one month at -70°C. Between 25 and 50 mg fresh weight of tissue was ground in a 2-ml Eppendorf tube containing a clean stainless steel metal ball (5-mm diameter) in a mixer mill grinder (MM200, Restch, Haan, Germany) for 2 min at 30 cycles per

s. In order to keep the sample as cold as possible, the grinding jars were cooled down in liquid nitrogen.

Metabolites were extracted according to Roessner and associates (2000), with the following modifications. Ground samples were mixed with 420 µl of a solution composed of 330 µl of 100% methanol precooled at -20°C, 30 µl of a solution of ribitol in 100% methanol (0.2 mg/ml) (Sigma, München, Germany) and 30 µl of a solution of d4 alanine in water (1 mg/ml) (Sigma). Samples were then shaken for 15 min at 70°C. The extraction was continued with the addition of 200 µl of chloroform, followed by incubation for 5 min at 37°C. The soluble phase was obtained by addition of 400 µl of water, followed by vigorous vortexing of the sample. After centrifugation for 5 min at 14,000 rpm at room temperature, two aliquots of the soluble phase were collected (80 µl) into a fresh Eppendorf tube and were dried overnight in a Centrivac, (Hereaus, Hanau, Germany) without heating.

Derivation was performed according to Roessner and associates (2000), with the following modification. Dried sample was resuspended for 90 min at 30°C in 40 µl of methoxyamin-hydrochloride (20 mg/ml in pyridine). This was followed by the addition of 70 µl of MSTFA together with 10 µl of alkane mix (Roessner et al. 2000). GC-MS measurements were performed according to Roessner and associates (2000). For all organs tested, three biological replications were contacted.

The chromatograms were evaluated automatically according to (Roessner et al. 2000). Putrescine content was confirmed manually. Results were expressed as a response that corresponds to the ratio between the area of the target metabolite divided by the area of the reference metabolite (ribitol, m/z 319) and reported relative to the fresh weight.

Determination of transcript levels using real-time RT-qPCR assay.

Organs of *L. japonicus* plants, including nodules at various stages of development, were harvested and ground in liquid nitrogen. Total RNA was isolated according to Brusslan and Tobin (1992) and was quantified by spectrophotometry and agarose gel electrophoresis. Prior to RT-PCR, the total RNA samples were treated with DNase I (Promega, Madison, WI, U.S.A.) at 37°C for 45 min, in order to eliminate any traces of contaminating genomic DNA. First-strand cDNA was reverse transcribed from 2 µg of DNase-treated total RNA. All DNase-treated total RNA samples were denatured at 65°C for 5 min, followed by quick-chilling on ice in a 12-µl reaction mixture containing 500 ng of oligo(dT)_{12-18mer} and 1 µl of 10 mM dNTPs. After the addition of 4 µl of 5× first-strand buffer (Invitrogen, Paisley, U.K.), 1 µl (40 units) RNaseOUT (Invitrogen) ribonuclease inhibitor, and 2 µl of 0.1 M dithiothreitol, the reaction was preheated at 42°C for 2 min before the addition of 1 µl

(200 units) of SuperScript II reverse transcriptase (Invitrogen). The reaction mixture was incubated at 42°C for 50 min, followed by heat inactivation at 70°C for 15 min. The resulting first-strand cDNA was diluted to a final volume of 200 µl, and SYBP green-labeled PCR fragments were amplified using gene-specific primers (Table 1) designed from the transcribed region of each gene using Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR reactions were performed on the ABI PRISM 7900HT sequence detection system, using SYBR Green master mix (Applied Biosystems), gene-specific primers at a final concentration of 0.2 µM each, and 1 µl of the cDNA as template. PCR cycling started with the initial polymerase activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 4% (wt/vol) gel. The expression levels of a *L. japonicus* ubiquitin gene, detected using LjUBQ-F 5'-TTCACCTTGCTCCGCTCTTC-3' and LjUBQ-R 5'-ACCA CCAGCACACACAGACAATCC-3' primers were used as internal standards to normalize small differences in cDNA template amounts. For the relative quantification of gene expression, a modification of the comparative threshold cycle (C_t) method was used. The ratio of the target gene transcripts (X) in the organ of interest (S), normalized to ubiquitin levels (U), and the target gene transcripts in root (R), was calculated as $(1+E)^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ was calculated as $[(C_t^{X,S} - C_t^{U,S}) - (C_t^{X,R} - C_t^{U,R})]$. PCR efficiency (E) for each amplicon was calculated employing the linear regression method on the log(fluorescence) per cycle number data, using the LinRegPCR software (Ramakers et al. 2003). All real-time qPCR reactions were performed as triplicates.

In situ hybridization.

In situ hybridization experiments were performed as previously described (Scheres et al. 1988; Flemetakis et al 2000). *L. japonicus* nodules harvested at 14, 21, and 28 days postinfection with *M. loti* (strain E1R.pMP2112) were fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) for 4 h in a vacuum aspirator. Fixed nodules were block-stained in 0.5% safranin, were dehydrated through ethanol series, and were embedded in paraffin, and 8- to 10-µm-thin sections were cut. Antisense and sense RNA probes labeled with digoxigenin-11-rUTP (Boehringer Mannheim, Mannheim, Germany) were transcribed from the *LjODC* and *LjADC* clones, using the T7 and SP6 promoters of pSport1 plasmid vector. In order to improve probe penetration into the tissue, the probes were partially degraded to an average length of 150 nt. Sections were prepared for hybridization according to Scheres and associates (1988) and were hybrid-

Table 1. *Lotus japonicus* genes studied and the corresponding primers used for real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Gene			Primer	
Whole name	Abbreviation	Accession no. ^a	Name	Sequence
Ornithine decarboxylase	LjODC	AJ575746	LjODC-470F	5'-TGCAAATCCGAGTCCGACA-3'
			LjODC-531R	5'-TCATAGGTTGCGAGGTTGACG-3'
Arginine decarboxylase 1	LjADC1	AJ575747	LjADC1-1248F	5'-GGTGTTCCTCATATCCCCATT-3'
			LjADC1-1310R	5'-GAGAGGATGCCTTTACAGTGG-3'
Arginine decarboxylase 2	LjADC2	TC5158	LjADC2-215F	5'-AGAACATGTCTTCTGCAACCATC-3'
			LjADC2-282R	5'-CTTGAAGTCTCGGCGTAGC-3'
Cyclin D3	LjCycD3	TC5715	LjCycD3-278F	5'-ATCTCGAACGAACGTGGAGAGA-3'
			LjCycD3-345R	5'-TCAACACGAGTCCCTCCATCA-3'
Expansin	LjEXP	TC3966	LjExp-367F	5'-GGAAGCATATTGGTCAACCATC-3'
			LjExp-431R	5'-CCATTGCTGTTGGAGAGTGCA-3'
Extensin	LjEXT	TC5923	LjExt-45F	5'-TCAATCACCTTGATTGACC-3'
			LjExt-113R	5'-GGCGTAGTGGTTTGCTGAAATT-3'

^a Accession numbers are from the TIGR or GenBank databases.

ized overnight at 42°C in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.025% bovine serum albumin, 10% dextran sulphate, and 60 mM dithiothreitol. Hybridization signals were visualized with antidigoxigenin antibodies conjugated with alkaline phosphatase. Images were processed and prepared for presentation with Photoshop 6 software (Adobe Systems Inc., San Jose, CA, U.S.A.).

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