Identification of new nodulin cDNAs from yellow lupine by differential display

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Abstract

In this study several sequences that represent yellow lupine nodulin genes are identified. Four different cDNA clones were isolated from a lupine nodule cDNA library and sequenced. Database search revealed that the deduced amino acid of one clone, DD3A20, shows near 80% amino acid similarity to plant stearoyl-acyl carrier protein desaturases and contains crucial amino acids in the fatty acid binding domain which are characteristic for stearoyl-ACP desaturases. The other sequences seem to be unique. Nearly identical signal peptide sequences are present at the N-termini of the DD4A9 and DD2T15 putative proteins. Northern analysis indicated that the pattern of expression of the Llldd4A9, Lldd2A18 and Llldd2T15 genes is the same as that of the lupine leghemoglobin gene LllbII, while the desaturase gene Ll3A20 is turned on at least 3 days earlier. Ll3A20 is also expressed in lupine fruits. RT-PCR analysis of the Llldd3A20 and Llldd2T15 expression confirmed organ-specific and temporal pattern of expression in nodules. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Infection of legume plants with specific strains of Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium or Mesorhizobium leads to the development of specialized nitrogen fixing organs called root nodules. Although nodulation is controlled by genetic determinants of both symbionts, the type of root nodule depends on the plant species [1]. There are two major classes of nodules that are formed on legume roots. Indeterminate nodules usually develop on temperate legumes. These nodules are characterized by the presence of a persistent apical meristem, with initial cell divisions occurring in the deeper cell layers of cortex, close to endodermis [1–3]. Growth and differentiation of new cells leads to the establishment of central tissue zonation. Four zones have been distinguished in mature alfalfa nodules: bacterium-free meristematic zone I; infection zone II; nitrogen fixing zone III and senescent zone IV [4]. Determinate type nodules are characteristic for tropical legumes such as Glycinae, Phaseolus or Vigna. In this class of nodules meristematic activity ceases after several days of development and subsequent growth is caused by cell expansion. The first cell divisions are hypodermic and in mature nodules the central tissue does not show zonation [1–3].

Nodulins were, by the original definition, plant gene products appearing exclusively during nodule formation [5]. However, the term nodulin gene is used in a less strict sense and refers to genes

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expressed in nodules, but not in uninfected roots. Intensive studies over the past two decades have identified almost 200 nodulin genes in different plant hosts [6]. Nodulins are defined as early or late according to the time of appearance during nodule development. Early nodulins are expressed well before nitrogen fixation onset and function in nodule development and infection processes [3,5]. Although the expression of several early nodulin genes has been well documented, the exact roles of the encoded proteins are still unknown [7–11]. Late nodulins participate in nodule function and appear in nodules during the onset of nitrogen fixation, after the nodule structure has developed [3,5]. This group of nodulins includes leghemo-globins; enzymes functioning in nitrogen and carbon metabolism (glutamine synthetase, uricase, scharose synthetase); peribacteroid membrane proteins and many others, of which the function remains to be elucidated [3,12–16]. Recently, it has been reported that the Vicia sativa leghemoglobin gene Vslb1, is expressed in root hairs within 1 h after Nod factor treatment indicating that the function of some nodulins may have to be revised [17].

Lupine root nodules have been described as a unique lupinoid subtype within the indeterminate class [18]. The meristem is located basi-laterally and contains bacteroids, so there is no distinctly marked infection zone. Initial cell divisions occur hypodermally. During nodule growth the connection to the root broadens causing the nodule to surround the root [18]. Several lupine nodulin genes have been identified and characterized. These include genes coding for two leghemoglobins (LbI and LbII), glutamine synthetase and ENOD2-like protein [19,20]. To identify additional nodulin genes and to increase the repertoire of usable molecular probes, the differential display approach was employed [21,22]. Differential display has been successfully used to identify new nodulin genes from Sesbania rostrata and to prepare a nodulin expressed sequence tag library from Lotus japonicus [23,24]. In this study expression analysis of several newly identified nodulin genes during lupine nodule development is presented. One of the isolated clones encodes a putative stearoyl-acyl carrier protein desaturase. A function for this enzyme in nodule development is proposed.

2. Material and methods

2.1. Plant material

Yellow lupine (Lupinus luteus cv. Ventus) seeds were surface sterilized in 0.1% HgCl2 and 0.01% Tween 20 for 15 min, washed intensively with sterile water and germinated for 3 days at 28°C in Petri dishes in full darkness. Seedlings were moved to perlite and watered with 7 ml of a freshly grown culture of Bradyrhizobium sp. (Lupinus) USDA 3045 (OD = 0.5–0.6). Plants were kept in a growth room with a 16 h photoperiod at 25°C and 8 h dark at 20–22°C. Nodules were harvested 6, 9, 13, 15, and 21 days after inoculation (DAI). Uninfected roots, leaves and stems were cut 21 DAI and fruits were harvested from plants 60 days after sowing. During growth, plants were watered with a medium containing micro- and macroelements, but the medium for nodulating plants lacked inorganic nitrogen [19].

2.2. RNA isolation and northern hybridization

Total RNA was isolated essentially according to Ref. [25], although three extractions with phenol were required to obtain RNA with A260/A280 > 1.8. RNA was dissolved in DEPC-treated water and kept at −70°C. Integrity of RNA was checked by formaldehyde agarose gel electrophoresis [26]. For northern hybridizations, 20 μg samples of total RNA were separated electrophoretically on denaturing agarose gels, stained in ethidium bromide solution and blotted to nitrocellulose filters according to the manufacturer’s protocol [Schleicher and Schuell]. DNA for probes was purified from agarose gels and then labeled in a random priming reaction with 30–50 μCi of [α-32P]dATP following the manufacturer’s protocol [Promega]. Labeled probes were purified from unincorporated nucleotides on sephadex G-50 fine [Pharmacia] columns. Filters were hybridized for 24–72 h in a standard solution containing 6 × SSC, 5 × Denhardt solution, 1% SDS, 50% formamide and 70 μg/ml of denatured salmon sperm DNA. Filters were washed using high stringency conditions (0.1 × SSC, 0.1% SDS, 60°C). If differential displayed probes were used, washing conditions were less stringent (0.3 × SSC, 0.1% SDS, 50°C).
2.3. Differential display RNA fingerprinting

RNA differential display reactions were carried out using a RNAmap kit (GenHunter, Brooklyn MA). Half microgram samples of total RNA isolated from nodules 21 days after infection with *Bradyrhizobium* sp. (*Lupinus* USDA 3045 and uninfected roots were reverse transcribed and 1 µl aliquots amplified according to the protocol provided by the manufacturer, although reactions were scaled down to 10 µl. PCR products were separated on 6% polyacrylamide sequencing gels. After 48 h of autoradiography, nodule-specific products were cut out from dried gels and the DNA eluted by boiling with 100 µl water and precipitated with 3 volumes of ethanol in the presence of 20 µg glycogen [Boehringer Mannheim]. Reamplification of 3' ends of cDNA fragments was performed according to standard protocol (40 µl volume, 20 µM dNTPs, 1.5 mM MgCl₂) [21,22]. Reamplified fragments were blunt ended by a 15 min incubation at 37°C with 1U of DNA Polymerase I Klenow fragment in the presence of 50 µM dNTPs. DNA fragments were separated on 2% agarose gels and, after purification, phosphorylated with T4 DNA kinase [Amersham] and cloned into the SmaI site of pBluescript SK-.

2.4. cDNA library screening

A yellow lupine nodule cDNA library constructed in a λ-ZAP II vector (Stratagene) was screened with the [α32P]dATP-labeled differential display probes, prepared from individual cloning products that showed nodule specificity in northern hybridization. Filters were hybridized at 55°C for 36–48 h and then washed two times in 2× SSC at room temperature, once at 55°C in 1× SSC and two times in 0.2× SSC, 0.1% SDS at 50°C.

2.5. DNA sequencing and computer analysis

cDNA clones were sequenced using a Sequenase II kit (USB Amersham), according to the manufacturer’s protocol. Homology searches were done using the BLAST and FASTA algorithms. Sequence alignment was performed using the CLUSTALW program. The PROSITE and BLOCKS databases were searched to identify conserved amino acid motifs. Prediction of the cleavage site in signal peptides was based on rules described by von Heijne [27].

2.6. RT-PCR analysis

Reverse transcription reactions were performed according to the manufacturer’s instruction [Boehringer Mannheim]. Briefly, cDNAs were synthesized from 3 µg total RNA isolated from nodules (6, 9, 13, 21 DAI), leaves, stems and young fruits for 2 h at 37°C in reaction buffer containing 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 10 mM dithiotreitol, 200 ng of oligo d'T18, 1 mM dNTPs. Reactions were stopped by heating for 5 min at 95°C, diluted 50 times and 2 µl were used in PCR to amplify cDNA fragments using 35 cycles of amplification. Primers [MWG-Biotech] used in PCR and temperature conditions of amplification reactions were as follows: 5'-atggtggctactagattacagtgactacag-3’, 3’-ctacttactacagtagttacta-5’ (94°C 30 s, 50°C 30 s, 72°C 40 s) for amplification of the yellow lupine leghemoglobin II fragment; 5’-aagttgagccacataggt-3’, 5’-acaacactgtataaaagca-3’ (94°C 30 s, 50°C 30 s, 72°C 35 s) for DD2T15 cDNA; 5’-ggatttcacggatcaggtg-3’, 5’-cgagctgtgtgcactgtg-3’ (94°C 30 s, 50°C 30 s, 72°C 40 s) for DD3A20 cDNA.

2.7. Nucleotide sequence accession numbers

The cDNA nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers AF139377 (DD3A20), AF139378 (DD2A18), AF139379 (DD4A9), AF139380 (DD2T15-S) and AF139381 (DD2T15-L).

3. Results

3.1. Identification of nodule-specific cDNAs

To identify genes specifically expressed in mature yellow lupine nodules (21 DAI) the differential display RNA fingerprinting approach was utilized [21,22]. This technique has several advantages over subtractive or differential hybridization and has been successfully applied to identify plant genes expressed under specific conditions [28,29]. It requires only a small amount of RNA and
enables systematic analysis of cDNA pools from different tissues. Thirty five differential display (DDRT-PCR) reactions were carried out using anchored oligo dT primers ending with A and T and set of decamers. Comparison of amplified products enabled us to select 50 nodule specific products, which were recovered from gels and successfully reamplified. Control reactions performed on RNase A-treated RNA samples showed no PCR products, indicating that the RNA preparations were DNA free and sufficiently purified to be used for DDRT-PCR. Fourteen products were hybridized to RNA from nodules 21 DAI and uninfected roots to verify the specificity of isolated fragments. Ten probes gave a clear nodule-specific signal. These ten sequences were ligated into the SmaI site of pBluescript SK- and verified again for their specificity by northern blot analysis. The second verification was carried out because one band on the differential display gels may correspond to several different cDNA fragments. Results obtained after Northern hybridizations with four probes that were further analyzed are shown in Fig. 1.

3.2. Screening of the cDNA library and sequence analysis

Differential display (DD) probes, assigned as DD4A9, DD2A18, DD3A20 and DD2T15, were used to screen a λ-ZAP II nodule cDNA library and the sequence of cDNAs isolated was determined. Based on the Northern analysis (Fig. 1) one expected to isolate at least two classes of DD4A9 cDNA clones. Surprisingly, it was possible to isolate only one class of ~850 base pairs in length, corresponding to the smaller band on the Northern blot (Fig. 1). Two classes of DD2T15 were isolated. In the DD2T15-L clones an additional 107 base pairs insert is present at the 3’ end that is absent in smaller DD2T15-S clones. Nucleotide and deduced amino acid sequences of the cDNA clones were compared with nucleotide databanks (GenBank, EMBL) using BLAST and FASTA algorithms. Database searches revealed significant similarity between DD3A20 and plant stearoyl-acyl carrier protein desaturases (80% similarity at the amino acid level and 65% at the nucleotide level) (Fig. 2A). A lower degree of similarity (~70% at the amino acid level) was revealed to other plant acyl desaturases, including Δ⁴-palmitoyl (C16:0)-ACP desaturase of Coriandrum sativum, Δ³-myristoyl (C14:0)-ACP desaturase of Pelargonium hortorum and Δ⁶-palmitoyl (16:0)-ACP desaturase of Thunbergia alata. These enzymes are specific for altered fatty acid and double bond positions. The plant acyl-ACP desaturases are ferredoxin/NADPH requiring enzymes that function in the stroma of plastids [30–32].

There is also a 63% identity between a 400 nucleotide region at the 5’ end of the DD2A18 sequence and the Arabidopsis thaliana EST AT39811 (accession no. R65398). This homology, however, did not suggest any function that this product could fulfill in the nodule. The remaining two sequences, DD2T15 and DD4A9 seemed to be unique. The hydrophobicity profiles of the putative proteins indicated the presence of hydrophobic N-terminal signal peptides in the DD4A9 and DD2T15 products. Putative cleavage sites for signal peptidase were identified according to von Heijne’s rules (Fig. 2B) [27]. Comparison of the signal peptides showed only two amino acid differences, suggesting that both products are localized to the same place. They may be secreted to the extracellular space or bound to the plasma mem-

Fig. 1. Northern blot analysis showing specificity of the differential display probes. Twenty micrograms of total RNA isolated from nodules harvested 21 days after inoculation (DAI) (N) and uninfected roots (R) were subjected to denaturing agarose electrophoresis, transferred to nitrocellulose and probed with 32P-labeled differential display cDNA fragments. Equal loading was checked by ethidium bromide staining (not shown).
A.

**Glycine**
MALRLN-PIPTQTPSLPQ-----MASRSPRFRMAS-----TLSRGSGKEVEIIKKPTPPR

**Thunbergia**
MALKCS-VTPHVQPSPF-----VNLQRSHRVYMAS-----TDASANVGKGAFTPPR

**Arabidopsis**
MALKFPNLSAQYFKPSRTPSSRFPKFLACSSSPLSSGPEKEVLKPTPPR

**Lupinus**
MQTCYCTIRIQILPPL-----WARRTGKMLP-----PIAIASTEPPSLK-----SK

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**Glycine**
EVHVQYTHSMPPQKIIEIPQSLDAEINILAHKPEKCVQCPQDFPLD-----PSSDGFEQ

**Thunbergia**
EVKVLQTHMPAPEKEKIFHSLHGVAEELHSLKLKPEKCVQPNDFPLD-----PSSDGFEQ

**Arabidopsis**
EVHVQYLSMKPPQKEIKFSEMIAEELHILKHDEKSVQCPQDFPLD-----PASDGFEQ

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**Glycine**
------THMSPPEIEFESLSQVSLPLPKPVEQCPQDFPSLFPFD-PTDQ

**Thunbergia**
------THMSPPEIEFESLSQVSLPLPKPVEQCPQDFPSLFPFD-PTDQ

**Arabidopsis**
------THMSPPEIEFESLSQVSLPLPKPVEQCPQDFPSLFPFD-PTDQ

**Lupinus**
------THMSPPEIEFESLSQVSLPLPKPVEQCPQDFPSLFPFD-PTDQ

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**Glycine**
VLERERAKELDDFDYFVVLGDMITEALPTYQTMLNTLDGVRDEGTASLTSWAIRRAW

**Thunbergia**
VLRLRRLKELDDEYFVVLGDMITEALPTYQTMLNTLDGVRDEGTASLTPWAIWRAW

**Arabidopsis**
VLRERARDLDDFDYFVVLGDMITEALPTYQTMLNTLDGVRDEGTASLPWAIRRAW

**Lupinus**
VKALRDRTELPEEFYLVVLGDMITEDALPTYQSMINLDDGVRDEGTSSPSWALRAW

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**Glycine**
TAENHRGDLNNKLKYLSLGRVDMPQIEKTIQYLYIGSMGDPRTENPSFYLFYTSQFGERAT

**Thunbergia**
TAENHRGDLNNKLKYLSLGRVDMPQIEKTIQYLYIGSMGDPRTENPSFYLFYTSQFGERAT

**Arabidopsis**
TAENAHGDLNNKLKYLSLGRVMQIEKTIQYLYIGSMGDPRTENPSFYLFYTSQFGERAT

**Lupinus**
TAENRHGDLNYLGRVDMPQIEKTIQYLYIGSMGDPRTENPSFYLFYTSQFGERAT

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**Glycine**
FISHGNTARLAKEHDKLAQICGMIASDEKRHETAYTKIVEKLFPFESDPDGTVMAFADMM

**Thunbergia**
FISHGNTARLAKEHDKLAQICGSAIDEKRHETAYTKIVEKLFPFESDPDGTVMALADMM

**Arabidopsis**
FISHGNTARQAKEHDKLAQICGTAIDEKRHETAYTKIVEKLFPFESDPDGTVMADMM

**Lupinus**
FISHGNTARLAKEQGDPVLRACIGTIADEKRHETAYTKIVEKLFPFESDPDGTVMADMM

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**Glycine**
RKKIAMPALMYGDQDDNFLNFDNYSSQARIQVGYTAKDYADILEFLVGRKEQLTGLSGE

**Thunbergia**
RKKVSAMAPALYDQDDNFLENLFEHFAAERVQIGVYTAAYDETILVGRVEKQLTGLSGE

**Arabidopsis**
RKKIAMPALMYGDQDDNFLNFDNSQARIQVGYTAKDYADILEFLVGRKEQLTGLSGE

**Lupinus**
RKKIAMPALMYGDQDDNFLNFDNSQARIQVGYTAKDYADILEFLVGRKEQLTGLSGE

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**Glycine**
GRKQAEYICGLPFPRRLEERAAQARVKESS-----TLKFWSIHRESTTLNAPREEHEGI

**Thunbergia**
GRNASEYICLAFPRRLEERAAQARQGA-----PVFFSWYQREVK------

**Arabidopsis**
GNKQDYLICGLAPFPRRLEERAAQARLKGQRQKPLPVPYHFLCILKLKL

**Lupinus**
GKQADVFCLAFPRRLEERAAQARLKGQRQKPLPVPYHFLCILKLKL

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B.

MSTSMVNSKEFLFFGLLLFFVPLSFQVLA*H

MSTSMVNSKEFLFFGLLLFFVPLASQVLA*H

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Fig. 2. (A) Alignment of DD3A20 amino acid sequence and stearoyl-ACP desaturases from *Glycine max* (acc. no. L34346), *Arabidopsis thaliana* (acc. no. X93461) and *Thunbergia alata* (acc. no. U07597). Underlined, bold amino acids in the middle of the sequence are those that seem to be important in the enzymatic specificity [33]. Asterisks and double dots indicate identical and similar amino acids, respectively. (B) Comparison of putative hydrophobic signal peptides in the DD4A9 and DD2T15 proteins. Asterisk indicates the signal peptide cleavage site. Double and single dots indicate identical and similar amino acids, respectively.
that contains a target site for prenylation. The leucine residue at the end of so called CAAX box (Cys-aliphatic-aliphatic-Leu) indicates that a geranylgeranyl moiety may be bound to the conserved cysteine residue [34]. Searches of the PROSITE and BLOCKS databases with the predicted proteins did not expose any conserved motifs that could shed light on the function of these nodulins.

3.3. Expression analysis of the identified genes

To analyze expression patterns during nodule development, the DD cDNA clones were hybridized with RNA isolated from nodules 9, 13, 15, 21 DAI. Hybridization to RNA extracted from other organs (leaves, stems, roots, fruits) enabled us to determine if the identified genes encode classical nodulins. As a symbiotic marker characteristic of functioning nodules, we used the lupine leghemoglobin II cDNA that has been shown to appear around 13 DAI in lupine nodules [19]. Fig. 3 shows the hybridization pattern of the analyzed cDNAs. As expected, the DD2T15 cDNA hybridized to two classes of transcript differing in size. It was assumed that the hybridizing RNAs correspond to the DD2T15-L and DD2T15-S clones. The expression of the three genes represented by DD4A9, DD2A18 and DD2T15 cDNAs resembled LlhbII gene expression. These genes seemed not to be expressed in uninfected roots or aerial organs. The gene represented by the DD3A20 cDNA clone that likely encodes a stearoyl-ACP desaturase appeared to be induced around 9 DAI. Accumulation of the transcript of this gene was also detected in young developing fruits. Expression of the genes designated LlBII and Lldd2T15 was further analyzed by RT-PCR (Fig. 4). The cDNA fragments of DD2T15 were specifically amplified in RNA preparations that hybridized in northern blots. Fragments of the stearoyl-ACP cDNA were detected 6 DAI, 3 days before it was possible to detect DD3A20 RNA by Northern blot. To distinguish between induction of the LlBII gene caused by infection processes and by development of young roots, RNA samples corresponding to 4 day old uninfected roots were included in the RT-PCR analysis. No visible products were produced in this sample after 35 cycles of amplification, indicating that the LlBII gene induction

Fig. 3. Northern blot analysis of gene expression represented by isolated cDNAs. RNA was isolated from nodules harvested 9, 13, 15 and 21 days after inoculation (DAI). R, 21-day-old uninfected roots; F, fruits; L, leaves; S, stems. Asterisks indicate that two RNA classes hybridize with DD2T15 probe. Equal loading was confirmed by ethidium bromide staining (not shown).

brane. The DD2A18 protein seems likely to be attached to a membrane because the C-terminal forty amino acids form a hydrophobic domain.
is infection specific (Fig. 4). Amplification of the leghemoglobin cDNA fragment as a control was in agreement with the hybridization (Fig. 4). Nodules 6 and 9 DAI included the area of roots where nodules would appear, or were actually forming respectively, and symbiotically induced RNA can be highly diluted in these samples (this is especially true in RNA from nodules 6 DAI). These experiments allowed us to conclude that all genes except Lldd3A20 are expressed exclusively in nodules and code for classical nodulins. The protein DD3A20 can be called a nodulin in the same sense as ENOD12, that is also expressed in stems and flowers, but not in the uninfected roots [35].

4. Discussion

The application of differential display has enabled us to carry out identification of new RNAs that are nodule specific or nodule enhanced. Using this approach mature yellow lupine nodules were compared, 21 day after inoculation with Bradyrhizobium sp. (Lupinus) USDA 3045 to uninfected roots. In a set of 35 reactions 50 nodule specific cDNA fragments were selected.

Northern analysis of 14 differentially displayed (DD) probes confirmed nodule specificity for ten selected products. The DD product designated DD4A9, hybridized specifically to at least four classes of RNA but we isolated only one class of cDNA clone from the cDNA library probably representing the smallest RNA species. cDNA clones were also isolated using three other DD probes: DD2A18, DD3A20 and DD2T15.

Comparison of the nucleotide sequences of the isolated cDNA clones and sequence databanks revealed homology between the DD3A20 cDNA and plant acyl acyl carrier protein desaturases. Highest similarity was found to Δ⁶-stearoyl-ACP desaturases. Similarity to other acyl-ACP desaturases was not surprising because despite their functional divergence these enzymes share more then 70% similarity at the amino acid level [33]. It is difficult to predict the enzymatic specificity of the lupine acyl-ACP desaturase based only on the amino acid sequence homology. It is worth noting that Cohoon et al. have altered the enzymatic specificity of the Δ⁶-palmitoyl ACP desaturase of castor bean to allow desaturation of stearic acid at Δ⁹ position by changing five amino acids in a 30 amino acid domain in the middle of the protein [33]. Some of these amino acids form the active site of the enzyme. In the DD3A20 encoded lupine desaturase all the crucial positions are occupied by the same amino acids as in the Δ⁹-stearoyl ACP desaturase of castor bean, suggesting that the identified enzyme is also a Δ⁹-stearoyl-ACP desaturase (Fig. 2A).

The products of the DD4A9 and DD2T15 sequences contain nearly identical putative signal peptides that suggest a common cellular localization perhaps the plasma membrane or extracellular space. The DD2A18 protein probably interacts with a membrane since a prenylation motif is present at the C-terminus of the hydrophobic C-terminal domain.

Northern blot analysis of nodule development showed that the expression of the Lldd4A9, Lldd2A18 and Lldd2T15 genes is similar to the expression of the leghemoglobin gene LlibII and begins around 13 DAI. These results indicate that the putative products of the Lldd4A9, Lldd2A18 and Lldd2T15 genes are classical nodulins. The

Fig. 4. RT-PCR analysis of Lldd2T15 and Lldd3A20 expression during nodule development. Amplification of lupine leghemoglobin II cDNA fragment was included as control. RNA was isolated from nodules harvested 6, 9, 13, 15 and 21 days after inoculation (DAI). R, 21-day-old uninfected roots; F, fruits; L, leaves; S, stems, U, 4-day-old uninfected roots. Asterisks represent position of specific product. Lower bands represent primers or primer dimers.
expression of the Lldd3A20 gene, which likely encodes a yellow lupine Δ⁹-stearoyl-ACP desaturase, begins before that of LlbII. The DD3A20 cDNA specifically hybridized to RNA from nodules 9 DAI or older. In RT-PCR analysis we were able to amplify the specific fragment in RNA samples from nodules 6 DAI but not in a sample from 4 days old uninfected roots. These data suggest that Lldd3A20 may be activated by infection related processes. The Lldd3A20 gene expression was also detected in green lupine fruits. Yellow lupine seeds contain a high proportion of lipids as storage material, which may account for expression of this gene in fruits [36].

Identification of a lupine stearoyl-ACP desaturase gene highly induced during nodulation draws attention to lipid metabolism in nodules. The surface area of the peribacteroid membrane is 30–100-fold greater than that of the plasma membrane indicating that nodule development requires massive production of membrane lipid components [37–39]. It has been reported that activities of some membrane building enzymes are stimulated several times in soybean nodules and nodule-specific choline kinase activity has been identified [40]. The precise composition of fatty acids in nodule membranes is not known, however, it is well established that plant membranes contain a high fraction of polyenoic fatty acids, mainly linoleic and linolenic acids [30,31]. Both are derived from the desaturation of oleic acid produced by desaturation of stearic acid by Δ⁹-stearoyl-ACP desaturase. The increase in stearoyl-ACP desaturase gene expression may be necessary to provide enough components for membrane building and could be important in establishing an effective symbiosis. Plant nodule may be a good model for analysis of organ specific expression of lipid synthesis genes. In the future we will start a characterization of the lupine nodule by in situ hybridization to determine the temporal and spatial pattern of expression of the genes identified in this study.

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