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Characterization and expression analysis of the yellow lupin (*Lupinus luteus* L.) gene coding for nodule specific proline-rich protein✪

Wojciech M. Karłowski, Paweł M. Stróżycki and Andrzej B. Legocki✉

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Z. Noskowskiego 12/14, 61-704 Poznań, Poland

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The *LlPRP2* gene coding for a proline-rich protein shows a high level of similarity to, as well as significant differences from the family of *ENOD2* nodule-specific genes. Several sequence motifs with putative regulatory function were identified in the 5′ and 3′ noncoding regions of the *LlPRP2* gene. Northern blot analysis revealed that the expression of the *LlPRP2* gene begins 9 days after inoculation of yellow lupin roots with *Bradyrhizobium sp. (Lupinus)*; the expression is restricted to symbiotic nodules and is not detected in other tissues or organs. Detailed hybridization analysis showed that, when expression is activated, the *LlPRP2* transcript is modified so as to produce at least three bands and a continuous distribution of decay intermediates. The modification of the *LlPRP2* transcript probably involves degradation from the 5′- and/or 3′-ends of the RNA molecules. Southern blot analysis indicates that only one gene is present in the yellow lupin genome. The presence of genes homologous to the *LlPRP2* gene was confirmed for three cultivars of yellow lupin and for *Lupinus angustifolius*. However, *LlPRP2* homologues were not detected in *Lupinus albus* cv. *Bac,* indicating that this plant may lack the *ENOD2* sequence.

The root nodule, plant organ a newly formed as a result of a symbiotic interaction between legumes and bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium,* and *Azorhizobium,* makes the plant independent of soil nitrogen supplies. Nodule formation is initiated by a signal exchange between the symbiotic partners and involves differential expression of a number of plant genes, referred to as nodulin genes [1]

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✉Corresponding author: Andrzej B. Legocki; Phone: (48 61) 852 8919; Fax: (48 61) 852 0532; e-mail: legocki@ibch.poznan.pl

Abbreviations: OSE, organ specific element.

(for review see [2]). Depending on the timing of nodulin gene expression, genes are commonly classed as „early", activated during nodule formation, or „late", which are expressed in fully developed nodules [3].

We are interested in studying the symbiotic interactions between the legume *Lupinus luteus* and its microsymbiont *Bradyrhizobium sp. (Lupinus)*. The nodules formed on yellow lupin roots are collar-shaped, but show some similarities to indeterminate nodules in having several distinct zones [4]. Our earlier studies indicated that expression of late nodulin genes is controlled in lupin by mechanisms that differ from those found in most legumes [5]. Additionally, lupins also differ from other legumes in that, although they are nodulated by symbiotic bacteria, they are less frequently colonized by mycorrhizal fungi [6, 7]. These two symbiotic associations share some common features [8, 9], including activation of the same plant genes [10, 11].

Here we describe the sequence analysis and characterize the expression of an early nodulin gene from yellow lupin coding for a protein that is highly homologous to ENOD2. ENOD2 is one of the most extensively studied early nodulins. DNA sequences coding for ENOD2 proteins have been published for soybean [12, 13], pea [14], alfalfa [15], *Maackia amurensis* [16], *Sesbania rostrata* [17, 18] and yellow lupin [19, 20].

The presumed products of *ENOD2* genes are proline-rich proteins built up of two repeating pentapeptides. Based on the presence of proline-rich motifs in the sequence of the putative protein, ENOD2 nodulins have been postulated to be cell wall components [12].

ENOD2 gene expression is activated between the 7th and 10th day after inoculation but the expression pattern varies from one type of nodules to another. In determinate nodules, e.g., those formed on soybean roots, *ENOD2* genes are transiently expressed [21]. In contrast, in indeterminate nodules, which form on pea or alfalfa roots, *ENOD2* expression remains at a constant level during the development of the nodule [14]. *ENOD2* gene expression takes place in ineffective nodules, as well as in bacteria-free nodules of alfalfa when induced by auxin transport inhibitors or by exopolysaccharide-deficient *Sinorhizobium meliloti* mutant strains [22]. Moreover, *ENOD2* mRNAs accumulate in both *S. rostrata* [17, 23] and alfalfa [24] roots in the absence of rhizobia in response to cytokinin treatment.

The *LlPRP2* gene from yellow lupin, described in this paper, differs significantly in structure as well as expression pattern from *ENOD2*s described for other legumes. Additionally, we have found that in one lupin cultivar a gene homologous to *ENOD2* appears to be missing. This finding might provide interesting insights into ENOD2 function in symbiotic nodule development.

MATERIALS AND METHODS

Plant materials. Yellow lupin (*Lupinus luteus*) cultivar *Ventus* plants were grown, inoculated with *Bradyrhizobium* sp. (*Lupinus*) (strain 3045 USDA, Beltsville, MD), and the nodules were collected as described earlier [25]. Lupin plants (*L. luteus* cvs. *Ventus*, *Parys*, *Popiel*; *L. angustifolius* cv. *Bar*; *L. albus* cv. *Bac*) used for genomic DNA isolation were grown under the same conditions but without the inoculation step.

DNA sequence analysis. DNA and protein sequences were analyzed using DNASIS and PROSIS computer programs (Hitachi Software Engineering Co.). A multiple sequence alignment was performed using the CLUSTAL W program [26]. DNA and amino-acid sequences were compared with sequences present in GenBank and EMBL databases using the BLAST algorithm [27].

Synthesis of 5′- and 3′-end specific probes by PCR. To obtain specific probes for the 5′- and 3′-ends of the LlPRP2 gene, the following primers were synthesized: E2s5-1: 5′-CCCCTCTATAAATGCTTGGAA-3′, E2s5-2:

5′-GTCTCAAATGGAGGAGGCTCA-3′, E2s3-1: 5′-AAGCTACGAGTCCCAACCTTA-3′ and E2s3-2: 5′-ACACAGTGGCATTGTGCTTTA-3′. PCR reactions were conducted in the presence of 50 mM NaCl, 50 mM Tris/HCl (pH 9.0), 10 mM $MgCl_2$, 50 pmoles of each oligo-primer, 150 μM of each dNTP and 1.5 units of Taq DNA polymerase (Promega). The cycling parameters were as follows: twenty-five cycles at 94°C for 1 min 30 s, at 56°C for 2 min, and 72°C for 1 min 30 s. The obtained DNA fragments were subjected to electrophoresis, eluted from the gel, and radio-labeled. All probes used in hybridization experiments were ^{32}P-labeled using the Random Primer DNA Labeling Kit under conditions specified by the manufacturer (Boehringer Mannheim).

Southern and Northern blot analysis. Genomic DNA from 3-day-old, uninoculated lupin roots was isolated as described earlier [28]. After additional purification on a cesium chloride gradient, the DNA was digested with *Eco*RI and/or *Hin*dIII restriction enzymes. Genomic DNA from *Bradyrhizobium* sp. (*Lupinus*) was isolated according to the protocol described in [29] and then digested with *Hin*dIII. The digested DNA was fractionated on a 0.8% agarose gel and blotted onto a nitrocellulose membrane (Schleicher and Schuell). Hybridization was performed with ^{32}P-labeled probes at 50°C for 48 h in a buffer containing 5 × SSC, 1 × Denhardt's solution, 0.1% SDS and 100 μg/ml of salmon sperm DNA. After hybridization, the filters were washed in 1 × SSC/0.1% SDS buffer at 50°C.

Total RNA was isolated from segments of roots or from nodules at different stages of development as described by de Vries *et al.* [30]. The poly(A)$^+$ fraction was isolated from total RNA using the Dynabeads Biomagnetic Separation System according to the manufacturer's manual (DYNAL International). RNA was separated on 1.5% agarose gel containing formaldehyde [31], transferred onto a nitrocellulose membrane and hybridized with ^{32}P-labeled probes. The hybridization experiments were performed at 42°C in the same buffer as the Southern hybridization except that it also contained 50% formamide. Filters were washed under high stringency conditions: 0.1 × SSC at 55–65°C, depending on the probe.

The DNA probes, 3′- and 5′-end specific as well as *LENOD2* cDNA, were ^{32}P-labeled using a "Random Primed DNA Labeling Kit" according to the manufacturer's instructions (Boehringer Mannheim).

5′-End transcript mapping. The synthetic primer (sttr1: 5′-GTTTGCCAGCACCGGAGGGGT -3′), complementary to the sequence located between 49 and 69 bp downstream from ATG codon in the *LlPRP2* gene, was used in the primer extension reaction. The reaction was performed on total RNA isolated from 21-day-old nodules. The sttr1 primer was radio-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham) and then incubated with RNA in annealing buffer (0.4 M NaCl, 50% formamide, 10 mM Pipes, pH 6.4, and 1 mM EDTA, pH 8.0) overnight at 35°C. After precipitation, the nucleic acids were resuspended in reverse transcriptase buffer. Then, 0.5 mM 4 × dNTPs, and 50 units of M-MLV Reverse Transcriptase (Promega) were added to the mixture. After 2 h incubation at 37°C, the RNA was degraded by ribonuclease and the sample was subjected to electrophoresis in a denaturing 8% polyacrylamide gel together with the sequencing leader initiated with the same primer.

RESULTS

Analysis of the *LlPRP2* gene

The *LlPRP2* gene was isolated from a yellow lupin genomic library using as a probe a cDNA *LENOD2* that codes for a protein showing sequence similarity to ENOD2 nodulins [19, 20]. This gene encodes a putative protein of 103 kDa with a signal sequence at the N-terminus followed by a stretch of proline-rich pentapeptide repeating units. These units belong to

two groups, appearing one after the other within the protein sequence. The sequence of the first motif, PPHEK, is identical to that of the two known motifs from the ENOD2 peptide. However, the second amino-acid motif from the LlPRP2 putative protein is less conserved, converging with the second motif characteristic for ENOD2 (PPEYQ) only in the first two proline residues. Also other motifs found in known proline-rich nodulins are absent from the amino-acid sequence of the LlPRP2 protein. A comparison of the nucleotide and deduced amino-acid sequences of the *LlPRP2* gene with various nodulin sequences reveals the highest level of homology to the *ENOD2* gene family (62–63% identity). However, this level of homology is lower than that between other proteins deduced from known *ENOD2* sequences (72% identity between ENOD2 proteins from soybean and *S. rostrata*). Moreover, the deduced LlPRP2 protein is more that 2.5 times as large as those of ENOD2 described to date [20].

In the *LlPRP2* promoter region, several putative regulatory motifs were found. These include: a 150 bp duplicated fragment that forms a direct repeated sequence, and a sequence characterized by a stretch of repeated $(TA)_{10}$ [20]. In position –97, a 38 bp fragment was identified that has 66% identity to the OSE (Organ Specific Element) from the soybean *lbc3* gene [32] (Fig. 1A). This fragment possesses both characteristic sequence motifs with single substitutions (AAAG*c*T and C*c*CTT). These sequences are separated from each other by 5 bp as it occurs in the promoter of the pea *Pslb* gene. Interestingly, the localization of these motifs, –111 bp from the transcription initiation site for the CTCTT sequence, is similar to that in leghemoglobin genes [33]. It is worth noting that this motif is characteristic for late nodulin genes from several legumes, but it is absent in the promoters of late nodulin genes from lupin, i.e. *leghemoglobin I* [34] and glutamine synthetase [35].

Our analysis of the promoter sequences of *ENOD2* genes resulted in identification of a 9-bp element that immediately precedes the AGGA-box found in the *ENOD2* gene promoters of soybean [12] and *S. rostrata* [17]. This new motif, composed exclusively of A and T residues (7 and 2, respectively), is identical in the *ENOD2* and *LlPRP2* promoter regions (Fig. 1B). The analogous sequence was absent in the corresponding position of other proline-rich proteins encoding genes: *ENOD12* and *MtPRP4* [36]. However, the AGGA-box, which is characteristic for *ENOD2* genes, is not conserved in the *LlPRP2* promoter sequence and has only 58.3% homology, compared to 91.7% between genes from soybean and *Sesbania* (Fig. 1B).

In the 3′-end UTR region of the *LlPRP2* gene, we found a high degree of similarity to several plant and animal sequences deposited in GenBank, with homology ranging from 60% to 70%. However, all of those sequences are located in the intron parts of these genes. Further analysis of this region revealed the presence of a putative intron (56 bp long), which covers the polyadenylation signal and the site where poly(A) is added to mRNA. Additionally, in the 3′ untranslated region of the *LlPRP2* gene, an 11-bp GT-rich element in a position similar to those found in of *ENOD2* sequences was identified. This element is also present in sequences of *MtPRP4* and *ENOD12* from pea but is further downstream from the stop codon (Fig. 1C). It is worth noting that the GT-rich element is more conserved in *ENOD12* than in *ENOD2* genes from pea and that it is not present in *ENOD12* sequences from other legumes. This element may be involved in stabilization of the RNA molecule [37], or may be a part of the signal for polyadenylation of mRNA [38].

Analysis of the *LlPRP2* gene expression

The expression of the *LlPRP2* gene was examined in different plant organs grown under

different conditions. A dot-blot RNA hybridization analysis revealed the presence of *LlPRP2* mRNA only in symbiotic nodules and dominant transcript of about 3000 nt in size, which is in agreement with the size of the transcribed region of the *LlPRP2* gene (Fig. 3A).

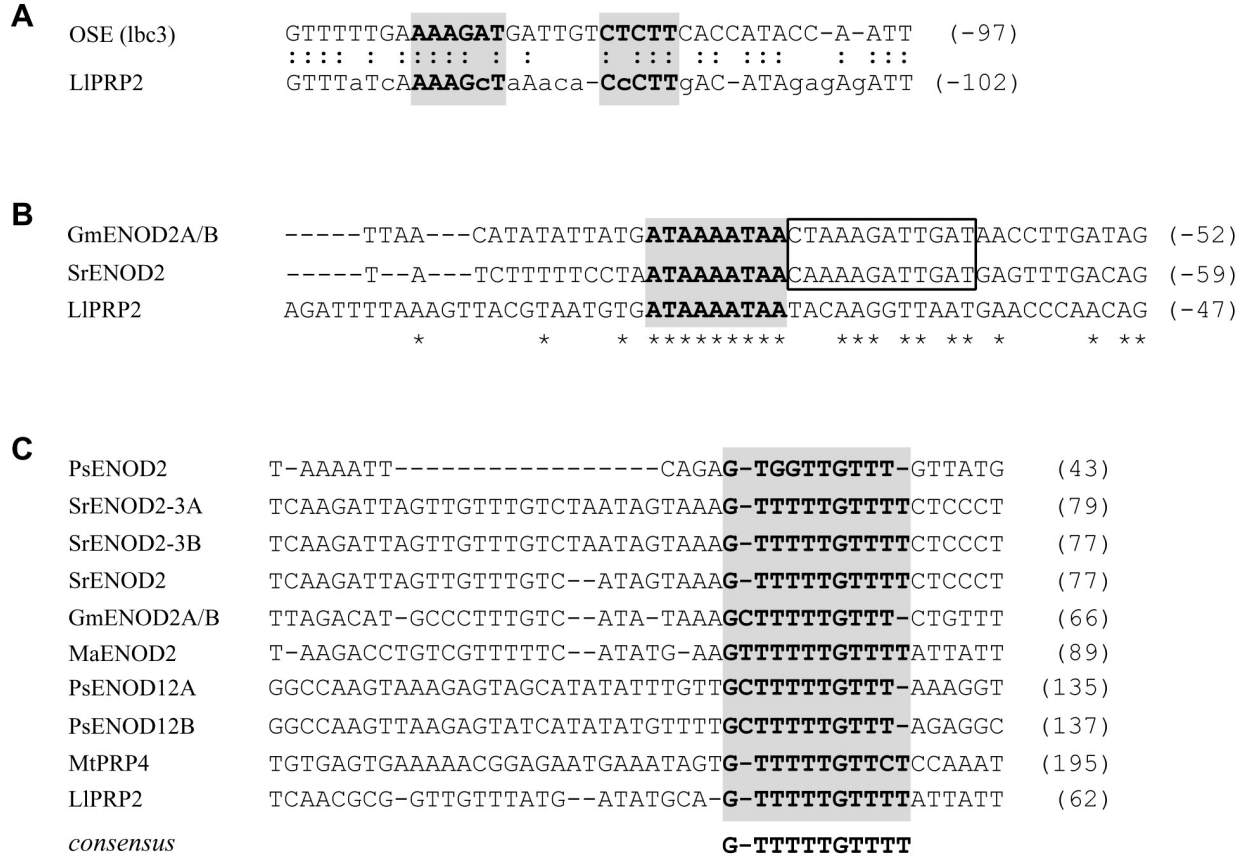

Figure 1. Analysis of *LlPRP2* DNA sequence.

(A) Alignment of the OSE sequence from the *lbc3* gene with the promoter fragment of the *LlPRP2* gene. The conserved OSE motifs (AAAGAT and CTCTT) are shown as shaded boxes. The deviations from the *lbc3* OSE sequence are written in lowercase letters. The numbers represent the distance from the transcription start site. (B) Sequence comparison of the promoter region of the *LlPRP2* gene with homologous fragments of the *GmENOD2A* and *B* (acc. nos. X16875 and X16876) and the *SrENOD2* (acc. no. X63339) promoters. The "AGGA" motif is shown as an open box, and the A+T-rich element is shown as a shaded box. The asterisks indicate the base pairs that are identical in all sequences. (C) 3′-End sequence alignment of the *LlPRP2* gene with analogous sequences of *PsENOD2* (acc. no. X51987), *SrENOD2-3A* and *-3B* (acc. no. M23314 and M23315), *SrENOD2* (acc. no. X63339), *GmENOD2A* and *B* (acc. nos. X16875 and X16876), *MaENOD2* (acc. no. AF039708), *PsEnod12A* and *B* genes (acc. nos. M60585 and X57232), and *MtPRP4* (acc. no. L23504). The shadowed box indicates the conservative GT-rich fragment. The numbers represent the distance from the translation termination codon. Dashes indicate gaps that have been introduced

not in other parts of the plant (Fig. 2). During nodule development, *LlPRP2* transcript begins to accumulate 9 days after inoculation with *Bradyrhizobium sp. (Lupinus)* and its presence is detectable at a high level until the nodule senescence (Fig. 2).

A more detailed analysis with total RNA separated on a gel revealed the presence of one However, in addition to the dominant band, a continuous distribution of smaller RNAs, in which there are at least two additional bands (of about 1600 nt and 1200 nt), was observed (Fig. 3A, arrows). The same pattern of hybridization was observed in several additional experiments using RNA isolated by two different methods (not shown).

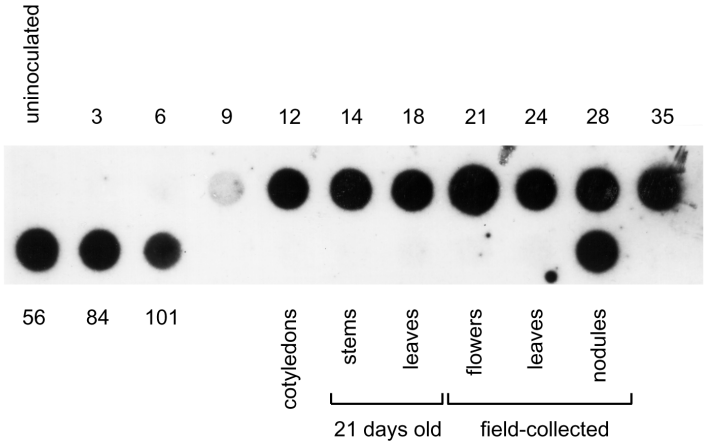

Figure 2. Analysis of the *LlPRP2* gene expression in different organs of yellow lupin.

Dot-blot hybridization of RNA (20 μg) isolated from different stages of nodule development and from different plant organs of yellow lupin. *LENOD2* cDNA was used as a probe. The positive signals were present only in "dots" representing RNA isolated from nodules. Numbers indicate days after inoculation with *Bradyrhizobium* sp. (*Lupinus*).

To verify that the *ENOD2*-hybridizing RNAs are not a product of degradation caused by the preparation method and/or manipulation of the RNA samples, several other probes including *leghemoglobin I* (Fig. 3B) and LlR18A were used. In all these experiments, only the *LlPRP2* probe gave the characteristic hybridization pattern of several bands and an RNA smear. Figure 3B shows a non-degraded, single transcript hybridization against the same RNA with a lupine *leghemoglobin I* cDNA. The *LlPRP2* gene is expressed earlier than the *Lb* gene during a symbiotic nodule development.

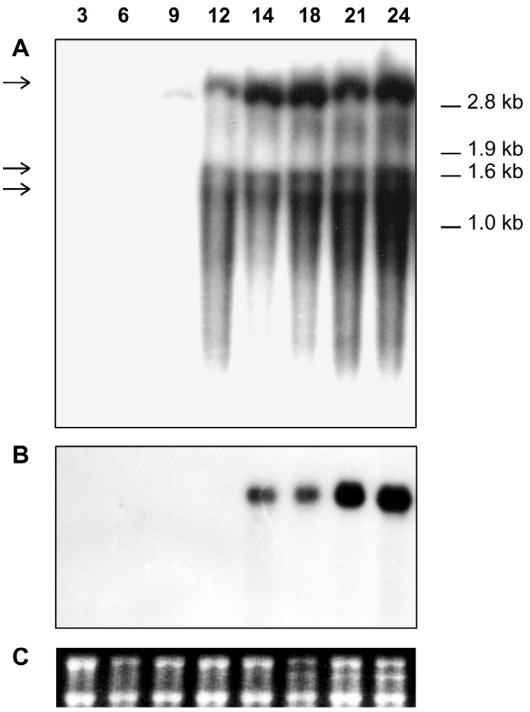

Figure 3. RNA gel blot analysis of total RNA from yellow lupin nodules.

Total RNA (25 μg) was fractionated by denaturing gel electrophoresis, blotted to nitrocellulose membrane, and hybridized with the radiolabeled cDNA *LENOD2* probe (A). The arrows indicate the dominant RNA band of the size corresponding to that of the transcribed region of the *LlPRP2* gene (3000 nt) and additional signals of about 1600 nt and 1200 nt in size. The control hybridization of the same RNA samples with cDNA leghemoglobin I (B) shows the lack of a degradation intermediates. (C) Picture of the RNA gel used in (A) and (B) showing equal loading of RNA samples.

To exclude the possibility of non-specific hybridization of RNA with repeated sequences present in the cDNA *LENOD2* clone, two specific probes, one for the 5′-end and the other for the 3′-end, were prepared by PCR on the *LlPRP2* gene (Fig. 4). The specificity of each probe was confirmed by hybridization with *Hin*dIII-digested lupin genomic DNA, resulting in each case in single-band hybridization (not shown).

Figure 5 illustrates the results of hybridization of RNA isolated from 21-day-old nodules with the 5′- and 3′-end specific probes. The hybridization pattern after incubation with the 3′-end specific probe (Fig. 5A) is similar to that observed after hybridization with the complete cDNA probe (Fig. 3A). Interestingly, after hybridization with the 5′-end specific probe (Fig. 5B), a set of degradation products was observed, but it was less intense and consisted of numerous discrete bands. The use of such specific probes eliminated the possibility of nonspecific hybridization with those RNA species, which code for other repetitive proline-rich proteins. Both sets of hybridiza-

tion experiments clearly show that, in nodule tissue, there exists a pool of RNAs, which differ in size but possess the specific, terminal 5′- and/or 3′-end sequences of mRNA encoded by the *LlPRP2* gene.

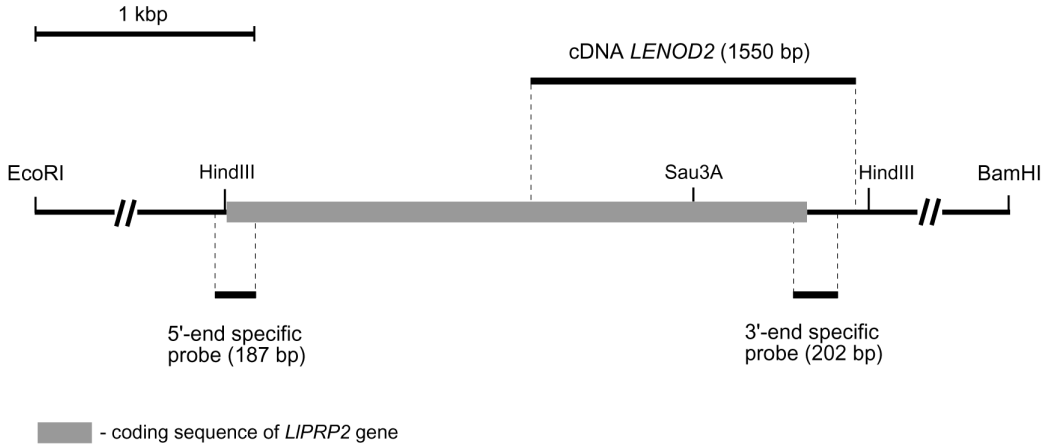

Figure 4. Schematic representation of all molecular probes used in hybridization experiments.

The whole cDNA fragment shown covers the 3′-end half (1550 bp) of the transcribed region of the *LlPRP2* gene. The probe specific for 5′-end of *LlPRP2* gene contains 40 bp of the promoter, 5′-end untranslated region and the sequence coding for signal peptide. The 3′-end specific probe contains a DNA fragment (56 bp) coding for non-repetitive end of the protein and 147 bp of the 3′-end untranslated sequence.

A different pattern of hybridization was obtained when poly(A)$^+$ RNA samples from 21-day-old lupin nodules were probed. Hybridization with the 3′-end specific probe showed only one band, which was separated by a gap from a large number of continuously migrating smaller mRNAs, in which we could not detect any distinct bands (Fig. 5C, gap in hybridization signal is indicated by a bracket). In contrast, only one band was detected after hybridization with the 5′-end specific probe (Fig. 5D). From the Northern hybridization experiments we conclude that, among the pool of RNAs specifically hybridizing to the *LlPRP2* probe, only one transcript which is polyadenylated and possesses both the 5′- and 3′-ends, can be detected. This transcript corresponds to the full-length mRNA of the *LlPRP2* gene (Fig. 5, arrow). All other signals detected on the RNA gel blots appear to represent degradation intermediates of the *LlPRP2* transcript. To additionally confirm this hypothesis, we conducted Northern hybridization experiments using single-stranded probes representing sense and antisense strands of the *LENOD2* cDNA. The hybridization signals were observed only after hybridization with antisense probe (not shown). The obtained hybridization data strongly suggest that specific degradation of *LlPRP2* gene transcript occurs during nodule development.

Mapping of the 5′-end of the *LlPRP2* transcript by primer extension reaction

A primer extension reaction was performed on total RNA to determine the transcription initiation site of the *LlPRP2* gene. The reaction was also designed to answer the question whether the transcripts are degraded from the 5′-end by exonucleases or whether an endonuclease, which would cut-off a 5′-end fragment of mRNA, is involved in this process.

The putative transcription initiation site (+1) was identified as the 15th nucleotide preceding the start of translation. On the autoradiogram, one dominant band was observed (not shown). Therefore, we conclude that an enzyme, which probably digests the fragment from the 5′-end downstream from the primer alignment site (at least 63 bp), is likely to be involved in the degradation of the

LlPRP2 transcript. If an exonuclease was involved in the degradation process, additional fragments corresponding to shortened mRNA molecules would have been generated in the primer extension reaction.

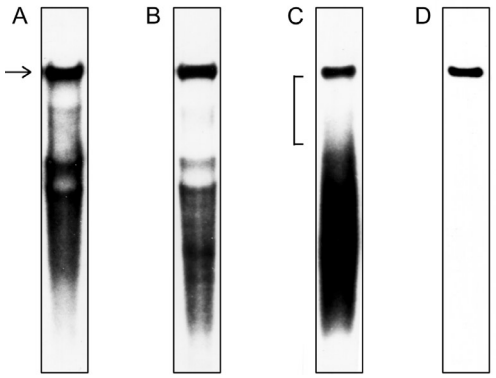

Figure 5. RNA gel blot analysis with the *LlPRP2* 5′- and 3′-end specific probes.

RNA samples isolated from 21-day-old yellow lupine nodul were fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with ^{32}P-labeled 3′- and 5′-end specific probes. The lanes (A) and (B) represent hybridization of total RNA with the 3′- and 5′-end specific probes, respectively. The lanes (C) and (D) represent hybridization of the poly(A)$^+$ RNA fraction with the 3′- and 5′-end specific probes, respectively. The arrow indicates the RNA band appearing in all analyzed lanes, its size corresponds to the size of the *LlPRP2* transcribed region. The bracket in C indicates the "gap" in the signal between the major band and the continuously migrating decay intermediates (see text).

Southern hybridization with genomic DNA

The results from Northern hybridization experiments indicated that a pool of transcripts, which are specific for the *LlPRP2* gene, exists in nodule tissue. However, as shown in Fig. 6A, only one hybridizing DNA fragment was detected on a genomic Southern. Genomic DNA isolated from *Bradyrhizobium sp. (Lupinus)* did not hybridize to the probe. Moreover, a PCR reaction on yellow lupin genomic DNA with primers surrounding the coding region of the *LlPRP2* gene resulted in amplification of a single band (not shown). The PCR and hybridization results indicate that only one *LlPRP2* gene is present in the genome of yellow lupin.

In addition, the results of Southern hybridization show that a gene with high homology and of a size similar to *LlPRP2* gene is present in three of the four analyzed cultivars of yellow lupin (Fig. 6B). Also, we identified a positive signal in the genomic DNA isolated from *L. angustifolius*, but the size of hybridizing DNA fragment was smaller. Surprisingly, using the same conditions of hybridization (see Material and Methods), we were unable to detect any positive signal in the lane corresponding to genomic DNA from *L. albus* cv. *Bac* (Fig. 6B).

DISCUSSION

The *LlPRP2* gene from yellow lupin encodes a protein with significant homology to the proteins encoded by the *ENOD2* gene family. The gene lacks introns, and the putative protein contains a signal peptide that is almost identical to those found in *ENOD2* sequences [20]. Additionally, the promoter sequence shows the presence of an AT-rich element, which so far was found only in *ENOD2* genes. Moreover, like in the case of *ENOD2, LlPRP2* gene expression precedes activation of the leghemoglobin gene. On the other hand, one of the pentapeptide motifs found in the *LlPRP2* amino-acid sequence was not homologous to any of those found in other proline-rich nodulins. Additionally, the putative protein encoded by the gene from yellow lupin is two-and-a-half-times as large as the *ENOD2* putative proteins described to date [20], which makes the *LlPRP2* gene distinct from members of the *ENOD2* gene family.

We found that *LlPRP2* gene expression was restricted to symbiotic nodules. In addition, we found that a large number of RNAs specifically hybridized to the *LlPRP2* probes. From the latter results we conclude that:

◆The *LlPRP2* gene is transcribed into a full-length RNA, which is polyadenylated and to which corresponds the majority of the signal detectable after hybridization experiments.

◆The transcript is modified to produce shorter RNAs as soon as the expression of the *LlPRP2* gene is detectable.

◆A comparison of results presented on Fig. 5C and 5D indicates that the *LlPRP2*-hybridizing transcripts are probably degraded from the 5′-end, most likely by a mechanism involving the removal of the 5′-end part of the transcript by a specific endonuclease, followed by exonuclease digestion. This mechanism might be similar to that proposed for degradation of oat phytochrome A mRNA [40]. From the primer extension experiments, we conclude that a specific endonuclease cuts off at least 63 nt from the 5′-end of the *LlPRP2* RNA molecule (behind the primer alignment site). However, the presence of the "gap" in the signal observed after hybridization of poly(A)$^+$ fraction of RNA with the 3′-end specific probe (see Fig. 5C, bracket) suggests that the removed part of the transcript might be much larger.

◆At least two additional distinct bands (see Fig. 3A, arrows; Fig 5A and 5B) and a set of discrete degradation products made up of poly(A)$^-$ RNA can be detected in total RNA isolated from nodules. These non-polyadenylated RNAs are clearly visible on comparing Figs. 5B and 5D which represent hybridization of the 5′-end specific probe to total and poly(A)$^+$ RNA, respectively. The detection of poly(A)$^-$ RNAs suggests the presence of a second *LlPRP2* mRNA decay pathway occurring at the 3′-end of the mRNA molecule. The identification of a putative intron in the noncoding, 3′-end part of the gene (not shown) may provide an alternative explanation for the presence of

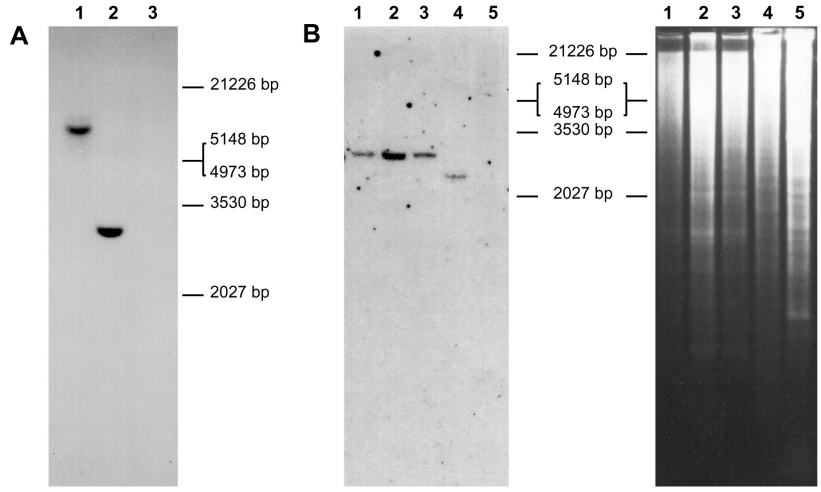

Figure 6. Southern blot analysis of lupin genomic DNA.

(A) *Lupinus luteus* cv. *Ventus* genomic DNA was digested with *Eco*RI (lane 1) and *Hin*dIII (lane 2) and loaded at 10 μg per lane. A 2 μg sample of *Bradyrhizobium sp. (Lupinus)* genomic DNA (lane 3) was digested with *Hin*dIII. The hybridizing band appeared only in lanes 1 and 2, with plant DNA. The same results were obtained after hybridization with cDNA *LENOD2* and the 5′-end specific probe (not shown). The numbers on the right show the sizes of the DNA molecular marker. (B) *Lupinus luteus* cv. *Parys* (lane 1), *Popiel* (lane 2), *Ventus* (lane 3), *Lupinus angustifolius* cv. *Bar* (lane 4) and *Lupinus albus* cv. *Bac* (lane 5) genomic DNA were digested with *Hin*dIII, loaded at 8 μg per lane and hybridized with the *LENOD2* cDNA probe. The lane corresponding to *L. albus* cv. *Bac* genomic DNA lacks a hybridization signal. The numbers between the columns show the sizes of the DNA molecular marker. Picture of the genomic DNA used for hybridization in (B) shows the amount of DNA loaded in each lane.

non-polyadenylated transcripts. The origin of the two distinct bands of 1600 nt and 1200 nt in size is not known.

The control of mRNA stability plays a fundamental role in the regulation of gene expression in plants [41]. Among the many analyzed-to-date *ENOD2* genes from different legumes, only the yellow lupin gene shows this unusual mechanism of posttranscriptional regulation. Moreover, in most plant mRNAs no naturally-occurring intermediates can be detected on gel blots, and only two exceptions to this rule have been well characterized: soybean ribulose-1,5-bisphosphate carboxylase small-subunit (*SRS4*) mRNA [42] and oat phytochrome A (*PHY A*) mRNA [40].

One of the characteristic features of the *ENOD2* genes is their tissue-specific expression. In soybean, alfalfa, and pea, *ENOD2* transcripts were localized to the nodule parenchyma (inner cortex), close to the bacteria-filled area in the central part of the nodule [14, 22]. In contrast to all known *ENOD2* genes, the *LlPRP2* gene is expressed in the infected central tissue [43], and not in the nodule parenchyma. It is possible that the expression of the *LlPRP2* gene in yellow lupin nodules is up-regulated in the "young" infected cells (prefixation zone) and is later turned off (i.e. the transcript is degraded) in "older" cells. This could explain the unusual pattern of hybridization with *LlPRP2*-specific probes.

Another interesting result was the surprising observation that a DNA fragment homologous to *ENOD2* was not detected in *L. albus* cv. *Bac*. Because of the high sequence homology of the *LlPRP2* gene to *ENOD2*, it is possible that the genome of *L. albus* cv. *Bac* lacks an *ENOD2* sequence. An analogous situation was described for the alfalfa cv. *Iroquois* genome, where the *ENOD12* gene was absent in the genome without effecting on nodulation [44]. In our case, *L. albus* cv. *Bac* would be the first legume described to be lacking an *ENOD2* gene. Thus, finding questions whether *ENOD2* is essential for the nodulation process (the *Bac* cultivar is readily nodulated) or is there a protein that could take over the function of *ENOD2* proteins. However, additional experiments will have to be conducted to answer those questions.

The results presented in this paper clearly show that the first full-length early nodulin gene isolated from yellow lupin has several unique sequence and expression features. Although the *LlPRP2* gene shows high similarity to *ENOD2* nodulins, it shows also many significant differences. The data presented here confirm the hypothesis that yellow lupin is a distinctive legume which differs from other legumes not only in its symbiotic characteristics (structure and development of the nodule and resistance to mycorrhizal colonization) but also on the molecular level.

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