Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphatases

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Abstract A cDNA encoding previously purified and characterized diphosphonucleotide phosphatase/phosphodiesterase (PPD1) from yellow lupin (*Lupinus luteus* L.) was identified. The *ppd1* gene encodes a protein containing a cleavable signal sequence. A functional expression of PPD1 in *Saccharomyces cerevisiae* confirmed the proper gene identification. A gene homologous to *ppd1*, encoding a putative membrane protein (PPD2), as well as fragments of two other genes encoding PPD3 and PPD4 proteins were also isolated. Amino acids composing the putative active center of PPD1 and PPD2 are similar to those present in known purple acid phosphatases, which suggests that the reported genes might encode a novel group of specific metallophosphatases. RT-PCR revealed that the corresponding PPD1 mRNA accumulates in stems and leaves, and PPD2 mRNA in stems, leaves and seedlings.

Key words: Lupin; Diphosphonucleotide phosphatase/phosphodiesterase; Metallophosphatase; Functional expression

1. Introduction

Plants have developed several strategies for phosphorus utilization. A major role in supply and metabolism of phosphate in plants is played by phosphatases. Acid phosphatases with broad substrate specificity, secreted outside a plant cell or located inside the vacuole, are involved in the production and recycling of inorganic phosphate from phosphate esters. The most widely studied plant purple acid phosphatases (PAPs) were purified from kidney bean, sweet potato, and soybean (see [1]). They are homodimeric with subunit molecular masses of ~55 kDa and are more diverse with respect to metal content than the mammalian enzymes [2–4]. PAPs comprise a group of metalloenzymes that contain binuclear Fe(III)–Me(II) centers where Me is Fe, Mn or Zn [4–6]. They are distinguished from other phosphatases by their acidic pH optima and characteristic purple color in solution, which is due to the presence of a metal complex. cDNAs of low molecular weight plant PAPs have been found in *Arabidopsis thaliana*, sweet potato, soybean, red kidney bean, and Easter lily [1,7]. The three-dimensional structure of PAPs has been solved [5,6]; however, their biological function in plants has not been characterized in detail. A role in phosphate acquisition of these enzymes in rice and soybean [8] or in iron transport in mature pollen [7] has been proposed.

The plant cell also produces specific phosphatases showing distinct metabolic functions, e.g. phosphoenolpyruvate phosphatase [9], phosphotyrosyl-protein phosphatase [10], adenosine 5′-tetraphosphate phosphohydrolase [11], pyrophosphatase [12,13], or phytase [14]. A widely distributed pyrophosphatase/phosphodiesterase activity has recently been found in plants that catalyze the hydrolytic breakdown of several NDP-monosaccharides [15,16].

We have previously purified and characterized an unspecific acid phosphatase from yellow lupin (*Lupinus luteus* L.) seeds [17,18] and an enzyme that specifically hydrolyzes diphosphonucleotides and phosphodiesters [19,20]. The purification procedure, characterization of enzymatic activity [19], and detailed structural analysis of carbohydrate moiety [20] of the latter enzyme were reported.

The aim of this study was to isolate, identify and characterize a gene of diphosphonucleotide phosphatase/phosphodiesterase (PPD) from yellow lupin (*L. luteus*) and to functionally express the protein. In addition, we isolated homologous genes, which might encode a novel group of specific metallophosphatases participating in phosphate metabolism.

2. Materials and methods

2.1. Plant material

Seeds of yellow lupin (*L. luteus*) from the 2000 harvest were obtained from Centrala Nasienna (Poznañ, Poland). Seeds were soaked for 6 h as described previously [19], seedlings were collected after 2 days, and leaves and stems were harvested from 35-day-old plants.

2.2. cDNA isolation and identification

The fresh plant material was frozen in liquid nitrogen and immediately homogenized using mortar and pestle. Total RNA from homogenized tissues was purified using the Plant RNeasy Mini Kit (Qiagen) as recommended by the manufacturer.

In preliminary experiments we designed a set of primers based on the sequence of previously described PPD1 peptides, generated by enzymatic and chemical digestion [19]. Fragments of cDNA were amplified using forward and reverse primers as follows: for *ppd1* 5′-AGAACAGTGGGATGGCG-3′ and 5′-TTATAGTTGTGTTTCTC-TTTAT-3′, for *ppd2* 5′-TGCAAGGACCGTGGGATGG-3′ and 5′-TCACTACTITTCTTGTACTCAA-3′, for *ppd3* 5′-GAICATGACT-GGAGAGAAGGA-3′ and 5′-TCACTACTITTCTTGTACTCAA-3′, for *ppd4* 5′-TACAAITTCATTGAICACTGC-3′ and 5′-TCACT-

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Abbreviations: PAP, purple acid phosphatase; PPD, diphosphonucleotide phosphatase/phosphodiesterase; HRP, horseradish peroxidase

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ACTITTCTTGTACTCAA-3′. The amplification products 880, 1057, 472 and 442 bp in length, respectively, were directly sequenced. The full-length PPD1 and PPD2 cDNAs were further synthesized using the 5′-RACE and 3′-RACE Amplification Systems for Rapid Amplification of cDNA Ends, according to the manufacturer's protocol (Gibco BRL). Reverse transcription (RT) was carried out at 50°C for 1 h. For amplification of 5′ end of *ppd1* the alternate protocol was used. Briefly, after RT synthesized cDNA was purified using GlassMax Spin Column (Gibco BRL) and then poly-A tailed. The tailed cDNA was used as a template for reverse transcriptase (Superscript II; Gibco BRL), using 3′-RACE Adaptor Primer (Gibco BRL). After purification on GlassMax Spin Columns, the product was used as a template for polymerase chain reactions (PCR). The design of gene-specific primers was based on *ppd1–ppd4* fragments of cDNA synthesized in the preliminary experiments. PCR reactions were performed in the presence of FastStart *Taq* Polymerase (Roche). The same conditions were applied to all PCR reactions: a 'hot start' incubation at 95°C for 5 min was followed by 40 cycles of denaturation (94°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 2 min). The reaction volume was 50 μl. The PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

For tissue-specific localization, RT was performed using 3 μg of total RNA and Thermoscript cDNA Synthesis System, according to the manufacturer's protocol (Gibco BRL). As a primer oligoT_{18} was used. The reaction was carried out at 55°C for 1 h, and 2 μl of the reaction solution were used for PCR. The *ppd1* gene was then amplified using a forward primer 5′-ATGATGGTTGAGATGGAGAAA-TC-3′ and a reverse primer 5′-AGATGCCAATGTTGTAGTTGGA-C-3′, and the *ppd2* gene using a forward primer 5′-ATGGGTGATT-CCAAGTTTGTCTTC-3′ and a reverse primer 5′-AGATGCCAGT-GTTGTTCTTGGGC-3′.

2.3. GDPase assay in cell lysates of Saccharomyces cerevisiae

Wild-type yeast cells (*S. cerevisiae*), strain BY4742, and the *gda1* null mutant strain (Research Genetics) with a deletion of Golgi GDPase were grown at 30°C in minimal medium (SD; Difco) containing 2% glucose. Yeast cells were chemically transformed (Frozen-EZ Yeast Transformation II; ZYmo Research) with an empty pYES2.1/V5-His-TOPO expression vector, containing V5 epitope and 6His tag at the C-terminus (pYES2.1 TOPO TA Cloning Kit; Invitrogen), and the vector containing *ppd1* or *ppd2* genes. The genes were amplified using the same primers as designed for mRNA tissue localization. The positive clones were selected on SD plates without uracil (SD–URA). The recombinant PPD1 or PPD2 expression was induced for 8 h at 24°C in SD–URA containing 2% galactose.

For GDPase assay, yeast cells were broken in 50 mM MES buffer, pH 6.2, containing 100 mM NaCl and Complete Protease Inhibitors (Roche), using Mini-Bead Beater-8 Cell Disrupter (Biospec Products). After homogenization (5 times for 1 min at 4°C), the lysates were separated from glass beads. Releasing of inorganic phosphate was determined as described by Olczak et al. [17]. Each lysate sample prepared in MES buffer contained 25 μg of proteins. Protein concentration was determined using the bicinchoninic method [21].

2.4. Protein electrophoresis and Western blotting

S. cerevisiae cells were homogenized using glass beads. The cells (about 5 OD_{600} per tube) were resuspended in 100 μl of 50 mM triethanolamine buffer, pH 7.2, containing 150 mM NaCl and Complete Protease Inhibitors (Roche). Then, 100 μl of ice-cold 20% TCA was added and the homogenization was performed five times for 1 min at 4°C, using Mini Bead Beater-8 Cell Disrupter (Biospec Products). The cell suspension was transferred to a fresh tube, neutralized with 1 M Tris base, and mixed with Laemmli sample buffer (1:1, v/v). Yeast lysates and culture medium containing secreted chitinase were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) on 10% gels, as described elsewhere. Each sample contained 50 μg of proteins. After electrophoresis, proteins were transferred onto nitrocellulose membranes (BioRad) in 30 mM CAPS buffer, pH 11.0 for 1.5 h at a constant current of 0.3 A. Membranes were blocked in PBS containing 5% skim milk, and then treated with mouse anti-V5 monoclonal antibody (1:5000; Invitrogen), followed by anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; 1:10 000; Promega). Secreted chitinase was detected in yeast culture medium using rabbit anti-chitinase serum (kindly provided by Dr. Charles Specht, Boston University School of Medicine), and then with anti-rabbit IgG conjugated with HRP (1:10 000; Promega). Chemiluminescence detection was performed using the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer). ECL films (Amersham Pharmacia Biotech) were exposed for about 1 min and then developed.

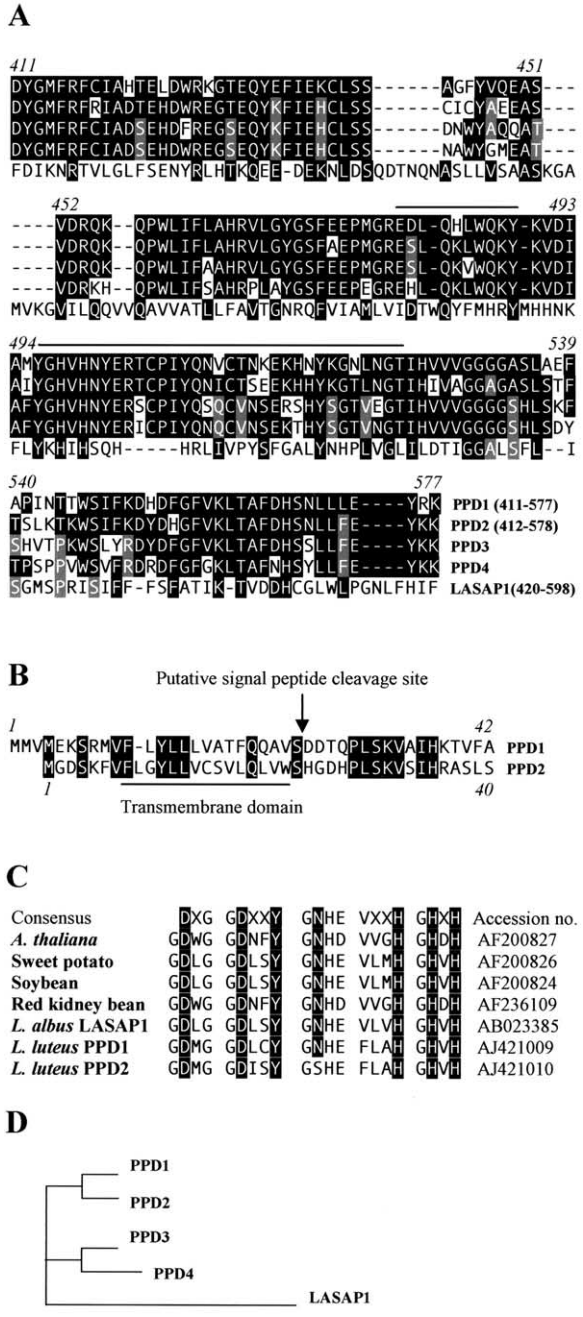

Fig. 1. Homology of yellow lupin (*L. luteus*) PPD1–PPD4 proteins and representative plant PAPs. A: Alignment of homologous yellow lupin PPD1–PPD4 (EMBL accession numbers AJ421009, AJ421010, AJ421011 and AJ421012) and white lupin (*L. albus*) LASAP1 (EMBL accession number AB023385; [24]) fragments. Identical amino acids are shown in black boxes, and partial homology is indicated in gray boxes. The lines on the top of the sequences denote the previously identified peptides derived from the native protein [19], matching the PPD1 amino acid sequence. B: Alignment of N-terminal regions of PPD1 and PPD2. C: Comparison of amino acid residues composing putative active centers. The conserved residues are shown in black boxes. D: Phylogenetic analysis showing the relationship within PPD1–PPD4 and LASAP1.

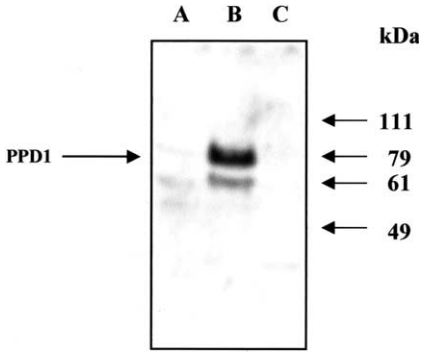

Fig. 2. Overexpression of the protein encoded by the *ppd1* in *S. cerevisiae gda1* mutant strain as examined by Western blotting. Yeast cells containing the pYES2.1/V5-His-TOPO expression vector with the *ppd1* gene before induction (lane A) and after galactose induction (lane B), and yeast cells containing the empty vector after galactose induction (lane C) were disrupted using glass beads as described in Section 2 and examined by SDS–PAGE on 10% gels. The proteins were transferred onto nitrocellulose membranes. The 79-kDa recombinant PPD1 (indicated by the arrow) was detected with mouse anti-V5 monoclonal antibody, followed by anti-mouse IgG antibody conjugated with HRP. Chemiluminescence staining was used to detect the recombinant protein.

3. Results and discussion

3.1. The PPD1 and homologous PPD2–PPD4 proteins belong to a novel group of specific metallophosphatases

We have previously purified and characterized PPD1 from yellow lupin (*L. luteus* L.) seeds [19,20]. In this report we isolated and identified a cDNA for this enzyme, based on the sequence of peptides generated by enzymatic and chemical digestion of the purified native protein [19]. In the preliminary experiments we isolated RNA from 6-h-imbibed seeds, but we were not able to amplify detectable products. In further experiments we isolated RNA from 2-day-old seedlings and detected genes homologous to *ppd1* (*ppd2*, *ppd3*, and *ppd4*); however, these sequences (Fig. 1A) did not perfectly match the previously described PPD1 peptides [19]. Finally, we isolated and identified PPD1 transcript from leaf total RNA pool. The obtained sequence matched the peptides derived from native purified PPD1. The *ppd1* mRNA is 2096 bp in length including a single open reading frame of 1848 nt that encodes 615 amino acid residues (EMBL accession number AJ421009), forming a 69.5-kDa protein. Hydropathy analysis [22] indicated that PPD1 has a cleavable hydrophobic N-terminal signal sequence between amino acid positions 25 and 26 (AVS-DD). This finding together with the presence of Lewis structures in PPD1 carbohydrate moiety [20] suggests that this protein may be secreted outside the cell.

The *ppd2* mRNA sequence is 2120 bp in length including a single open reading frame of 1839 nt that encodes 612 amino acid residues (PPD2; EMBL accession number AJ421010), forming a 69-kDa protein. PPD2 has an uncleavable N-terminal transmembrane domain (amino acid positions 6–22) and seems to be a type 2b membrane protein. The sequence analysis [22] showed that it may be bound to plasma membrane or to endoplasmic reticulum, facing the cytosol side of the membrane. Although the protein sequence of PPD2 shares high homology with PPD1 (Fig. 1A), it does not completely match the sequence of peptides from previously purified PPD1 [19].

We also isolated fragments of two other genes encoding PPD3 and PPD4 proteins (EMBL accession numbers AJ421011 and AJ421012, respectively). Unfortunately, the RACE methods failed to amplify 5′ fragments of *ppd3* and *ppd4* and 3′ fragment of *ppd3*. Further analysis of these genes utilizing different conditions is under investigation in our laboratory.

Alignments of all examined sequences were performed using CLUSTALW software [23]. The comparison of the known middle fragments of PPD1–PPD4 is shown in Fig. 1A. The amino acid sequence of the complete PPD1 sequence indicates high homology to PPD2 (73%). The differences between PPD1 and PPD2 are reflected mostly in N-terminal regions (Fig. 1B). Amino acid residues composing the putative active center of PPD1 are identical to those present in known plant PAPs (Fig. 1C; [1]). PPD1 contains all seven conserved amino acids [1], which suggests the possibility for the presence of metal complex in its active site. This was experimentally confirmed by an influence of EDTA on PPD1 activity. Prolonged incubation of the native purified PPD1 with EDTA at 37°C (data not shown) resulted in a significant decrease of its enzymatic activity, compared to the control without EDTA. This confirms that PPD1 strongly binds metal ions. The conserved amino acids of PPD2 are also similar to those present in PAPs, with the exception that asparagine in position 368 is replaced by serine (Fig. 1C). Comparison of PPD1 and PPD2 sequences to known phosphatases from white lupin (*Lupinus albus* L.; EMBL accession numbers AF309552, AB037887 and AB023385) showed low identity level (less than 27%). The sequence of the putative membrane PPD2 protein exhibited only 24% identity as compared to membrane unspecific phosphatase isolated from white lupin roots (EMBL accession number AB023385; [24]). The metallophosphatase motifs are present in PPD1; however, compared to unspecific PAPs, PPD1 is a specific enzyme. Based on high homology between PPD1 PPD2, PPD3 and PPD4 it is very likely that PPD2, PPD3, and PPD4 may exhibit similar substrate specificity to well-characterized PPD1.

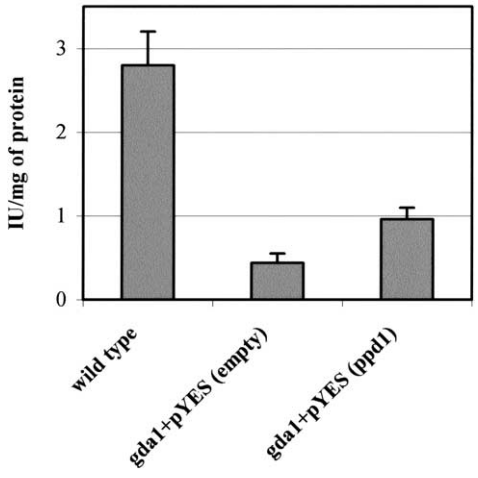

Fig. 3. GDPase activity of PPD1 recombinant protein overexpressed in *S. cerevisiae*. Wild-type, *gda1* mutant cells containing empty vector and the mutant cells expressing recombinant PPD1 were lysed using glass beads as described in Section 2. Releasing of inorganic phosphate from GDP was determined according to Olczak et al. [17]. Four independent experiments were performed. Data are shown as mean ± S.D.

We also searched databases for PPD1 and PPD2 similarity to other published sequences. Both proteins are highly homologous only to three genes present in *A. thaliana* (EMBL accession numbers AC027656, AB012248 and AL035523). The identity level is 74%, 67%, 57% for PPD1 and 72%, 67%, 58% for PPD2, respectively. However, to date, these proteins were not purified from *A. thaliana* and their enzymatic specificity was not examined.

As shown in Fig. 1A the predicted amino acid sequence of the fragments of PPD3 and PPD4 is similar to homologous fragments of PPD1 (70% and 71%, respectively) and PPD2 (71% and 74%, respectively). We could not compare the amino acid sequence of the active centers of PPD3 and PPD4, since we know only fragments of these genes.

The analysis of phylogenetic relationship [23] showed that PPD1–PPD4 proteins are closely related (Fig. 1D). In contrast, all four proteins reported in this study are evolutionarily more divergent from other plant PAPs. In Fig. 1D we show comparison of PPD1–PPD4 with well-described white lupin LASAP1 [24].

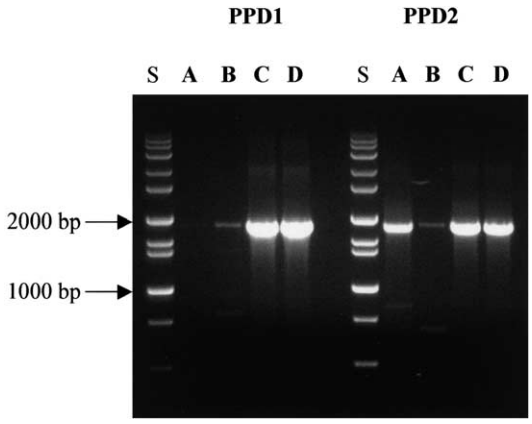

Fig. 4. Analysis of PPD1 and PPD2 transcripts in various *L. luteus* organs as examined by RT-PCR. 3 μg of total RNA isolated from 2-day-old seedlings (lane A), 6-h-imbibed seeds (lane B), stems (lane C) and leaves (lane D) from 35-day-old plants were used for RT. RT-PCR conditions are given in Section 2. Lane S, molecular weight standards.

3.2. Functional analysis of PPD1 in S. cerevisiae

To confirm the identity of the *ppd1* gene we examined substrate specificity of overexpressed PPD1 protein. In the preliminary experiments we found that the overexpressed and purified recombinant PPD1 from *E. coli* cells did not show enzymatic activity. Therefore, we utilized overexpression in eukaryotic expression system. For this purpose, the yellow lupin *ppd1* gene was PCR amplified from cDNA synthesized from total RNA isolated from lupin leaves, using gene-specific primers. The gene containing signal sequence was cloned into the yeast expression vector pYES2.1/V5-His-TOPO, and analyzed for a function in *S. cerevisiae*. As confirmed by sequencing, the inserts cloned into the expression vector were identical to the open reading frames of *ppd1* and *ppd2* amplified by RACE analysis. The PPD1 activity was examined in a *gda1* null mutant strain, which exhibits decreased endogenous GDPase (GDA1) activity. GDA1 is localized in the membrane of Golgi apparatus, and as a secondary effect of the knock-out mutation, *N*-glycosylation of glycoproteins in the mutant cell is affected.

As shown in Fig. 2 the recombinant PPD1 protein was expressed only after galactose induction in cells containing the pYES2.1/V5-His-TOPO vector with *ppd1* gene, as determined by Western blotting using anti-V5 monoclonal antibody. The molecular weight of the recombinant protein is about 79 kDa (Fig. 2). The native PPD1 purified from lupin seeds [19] migrates in SDS–PAGE as a 75-kDa protein. The predicted molecular weight of the soluble PPD1 lacking the signal sequence and containing the carbohydrate moiety is about 70.5 kDa. The difference between the calculated molecular weight and the molecular weight determined by SDS–PAGE may be explained by the fact that glycoproteins usually migrates slightly slower in polyacrylamide gels. The higher molecular weight of the recombinant PPD1 might result from the presence of the tag and different glycosylation in yeast cells. These findings highly confirm that the *ppd1* gene encodes the previously purified enzyme.

Overexpression of PPD1 in the yeast *gda1* mutant cells increased the GDPase activity (Fig. 3). The specific activity was two times higher compared to the mutant cells not expressing PPD1. However, recombinant PPD1 expression did not restore the wild-type phenotype of the mutant cells. Chitinase, which was used as *N*-glycosylation marker, did not show increased glycosylation compared to the mutant cells as examined by Western blotting using anti-chitinase antibodies (data not shown). This suggested that the PPD1 was not localized in Golgi apparatus.

Using the same protocol we amplified the *ppd2* gene and overexpressed PPD2 in yeast cells. The level of recombinant PPD2 expression in *gda1* yeast mutant cells was more than 10 times lower as compared to PPD1 (data not shown). Probably, the expression was toxic for cells, which was reflected in very slow growth of yeast cells. Although this expression did not influence the GDPase activity in the *gda1* yeast mutant strain (data not shown), we cannot rule out a hypothesis that this protein shows diphosphonucleotidase activity similar to PPD1. Similarly to PPD1, also PPD2 did not restore the wild-type phenotype of the mutant cells.

3.3. Occurrence of PPD1 and PPD2 mRNA in yellow lupin organs

To assess whether a function of proteins encoded by *ppd1* and *ppd2* is organ-specific, the occurrence of mRNA was analyzed in various lupin tissues. RT-PCR revealed that the corresponding PPD1 mRNA accumulates in stems and leaves, but not in seedlings (Fig. 4). PPD2 mRNA was present at high level in stems, leaves and seedlings (Fig. 4). Surprisingly, the PPD1 transcript level in seeds, the source from which the native enzyme was previously purified, was very low. It seems that the PPD1 protein is accumulated during seed maturation but the protein is not produced in imbibed seeds and 2-day-old seedlings.

4. Concluding remarks

To our knowledge, this is the first report describing an enzyme similar to purple metallophosphatases and expressing specific PPD activity. The native purified enzyme cleaves the pyrophosphate bond in diphosphonucleotides and phosphodiester bond in various phosphodiesters and has a high affinity towards diphosphate bond in organic and inorganic pyrophosphates, with the highest specificity towards diphos-

phonucleotides [19]. Its substrate specificity is similar to nucleotide pyrophosphatase/phosphodiesterase from soybean leaves [16], with the exception of low affinity towards nucleotide sugars (GDP- and UDP-glucose). The enzyme, despite its ability to cleave pyrophosphate bond, differs from plant soluble pyrophosphatases [13] by a preference to act in acid pH, different molecular mass and no requirement for divalent cations in vitro. Since PPD1 cleaves pyrophosphates it might supplement unspecific phosphatase activity in the plant cell. It is possible that PPD1 can be involved in hydrolysis of exogenous pyrophosphates, substrates not available for previously described yellow lupin unspecific phosphatase [17].

Our results demonstrate that PPD1, PPD2, PPD3 and PPD4 may form a novel group of specific metallophosphatases. High homology within the reported proteins indicates that they may exhibit enzymatic activity similar to PPD1. Although a possible function of these enzymes is unknown, their physiological role could be related to a regulation of diphosphonucleotides level in plant metabolism.

The isolation and identification of the full-length cDNA of PPD3 and PPD4 is under investigation. To define a physiological role of this group of enzymes our future studies will focus on PPD1 and PPD2 proteins and mRNA subcellular localization in lupin tissues.

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