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POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Aislamiento y clonación de los genes que codifican a las principales enzimas involucradas en la vía de síntesis de las poliaminas en plantas de frijol

Tesis que presenta

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Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Juan Francisco Jiménez Bremont

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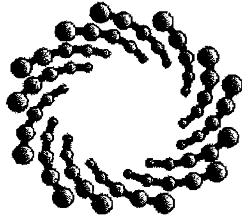

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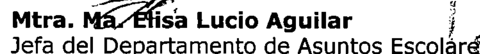

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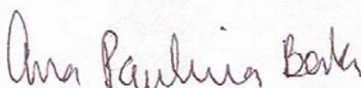

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La presente investigación se realizó en el Laboratorio de Proteómica y Expresión Génica del Departamento de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la tutoría del Dr. Juan Francisco Jiménez Bremont, fungiendo como asesores: La Dra. Ana Paulina Barba de la Rosa y el Dr. Ángel Gabriel Alpuche Solís**.**

Durante la realización de este trabajo el autor recibió una beca académica del Consejo Nacional de Ciencia y Tecnología Registro No. 172353

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Resumen

Se aislaron secuencias parciales de cDNA de la arginina descarboxilasa (*Pvadc)*, de S-adenosilmetionina descarboxilasa (*Pvsamdc*) y de espermidina sintasa (*Pvspds*) de frijol (*Phaseolus vulgaris*), utilizando oligonucleótidos diseñados en base a regiones conservadas en las secuencias de enzimas pertenecientes a diferentes especies de plantas. El análisis de las secuencias mostró que los genes *Pvadc, Pvsamdc* y *Pvspds* estaban principalmente relacionados con los ortólogos de *Glycine max*, *Phaseolus lunatus* y *Pisum sativum*, respectivamente. Los patrones de expresión de estos genes junto con el de ornitina descarboxilasa (*Pvodc*) fueron analizados en hojas jóvenes y maduras, tallos, raíces, puntas de raíces, pétalos, estigmas, ovarios, filamentos y anteras de plantas de frijol. La expresión de *Pvsamdc* fue similar en todos los tejidos. Los otros transcritos presentaron expresión tejido específica. *Pvadc* fue pobremente expresado en pétalos y casi no se observó expresión en puntas de raíces, *Pvspds* fue principalmente expresado en raíces, estigmas y filamentos, y *Pvodc* fue detectado solo en raíces.

Abstract

Partial cDNAs sequences for arginine decarboxylase (*Pvadc*), S-adenosylmethionine decarboxylase (*Pvsamdc*) and spermidine synthase (*Pvspds*) were isolated from the bean *Phaseolus vulgaris* using primers designed from conserved regions of enzymes belonging to plant species. Sequence analysis showed that the *Pvadc, Pvsamdc* and *Pvspds* genes were most closely related to the orthologous genes from *Glycine max*, *Phaseolus lunatus* and *Pisum sativum,* respectively. The expression patterns of the genes, together with that of ornithine decarboxylase (*Pvodc),* were analysed in young and mature leaves, stems, roots, root tips, petals, stigma, ovaries, filaments and anthers of bean plants. *Pvsamdc* was found to be expressed at similar levels in all tissues. The other transcripts showed tissue specific expression. *Pvadc* was barely expressed in petals and not at all in roots tips, *Pvspds* was mainly expressed in roots, stigma and filaments, and *Pvodc* was detected only in roots.

Additional key words: arginine decarboxylase, ornithine decarboxylase, *Phaseolus vulgaris*, S-adenosylmethionine decarboxylase, spermidine synthase.

Abbreviations: ADC - arginine decarboxylase; ODC - ornithine decarboxylase; ORF – open reading frame; PCR - polymerase chain reaction; Put - putrescine; RT-PCR - reverse-transcriptase polymerase chain reaction; SAMDC - S-adenosylmethionine decarboxylase; Spd - spermidine; SPDS - spermidine synthase; Spm - spermine; SPMS - spermine synthase.

Introduction

Polyamines are essential and abundant organic cations known to be involved in many basic processes in plants (Tiburcio *et al*. 1990, Kumar *et al*. 1997, Malmberg *et al*. 1998). The most abundant polyamines are putrescine (Put), spermidine (Spd) and spermine (Spm). Polyamines (free or conjugated) have been related specifically to the response to stress, rhizogenesis (Jarvis *et al*. 1985, Kakkar *et al.* 2000), root development and meristematic function, somatic embryogenesis and organogenesis (Feirer *et al*. 1984), flower and fruit development (Kakkar and Rai 1993, Galston and Kaur-Sawheny 1990), pollen formation and dormance (Kakkar and Nagar 1997), growth response induced by light (Yoshida *et al*. 1999), lateral growth of leaf-homolog organs (Watson *et al*. 1998), senescence (Muchitch *et al*. 1983, Kakkar and Nagar 1996), and response to microbial symbionts (El Ghachtouli *et al*. 1996). The first polyamine synthesized in the metabolic route is Put, derived from ornithine through the activity of ornithine decarboxylase (ODC; EC 4.1.1.17). In plants and some bacteria, Put is also synthesized from arginine by the action of arginine decarboxylase (ADC; EC 4.1.1.19). Put is subsequently converted to Spd through the activity of S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) and spermidine synthase (SPDS; EC 2.5.1.16). Conversion of Spd to Spm also requires SAMDC and spermine synthase (SPMS; EC 2.5.1.22) (Tabor and Tabor 1984).

Expression patterns of the gene encoding ADC support the idea that this is the main source of putrescine during cell elongation. Studies on ODC suggest that it may be involved in regulation of cell division in growing tissues (Michael *et al.* 1996, Nam *et al.* 1997, Martin-Tanguy 1997; Alabadí and Carbonell 1998). Several studies have shown that the *samdc* transcript is mainly accumulated in young tissues and organs where active cell division occurs, and decline to lower levels as organs age and senesce (Mad Arif *et al*. 1994, Marco and Carrasco 2002). In contrast, the *spds* transcript is expressed in all plant organs in *Arabidopsis thaliana* (Hanzawa *et al*. 2002). Plant *samdcs* possess a small upstream ORF (uORF) in the 5’ leader sequence of the mRNA. Hanfrey *et al*. (2002) demonstrated that

uORF-mediated translational control of SAMDC is essential for polyamine homeostasis and for normal growth and development.

Previously, we have reported the ODC-encoding gene (*Pvodc*) in *Phaseolus vulgaris* (Jiménez-Bremont *et al*. 2004). The present communication describes the isolation and sequencing of partial cDNA sequences encoding ADC, SPDS, SAMDC and the *samdc* uORF from *Phaseolus vulgaris*. Based on the isolated gene sequences, specific primers were designed in order to analyse gene expression patterns in different bean tissues.

Materials and methods

Plants: *Phaseolus vulgaris* L. (*cv*. Canario 60) seeds were sterilized with a 50 % commercial sodium hypochlorite solution (6 % free chlorine) for 10 min and rinsed several times with sterile, distilled water. Aseptic seeds were germinated in an agrolite/water support, and grown in a commercial substrate (*Cosmocel*, Monterrey, Mexico) in plastic pots for 45 d. Plants were maintained until flowering in a growth chamber under controlled conditions [16-h photoperiod (150 µmol m^{-2} s^{-1}), 25 ± 2 °C].

***RNA isolation and RT-PCR assays*:** Total RNA was isolated using the Trizol reagent as recommended by the manufacturer (*Invitrogen,* Carlsbad, USA), and treated with DNase I (*Invitrogen*). First strand cDNA synthesis was performed with SuperScript™ First-Strand Synthesis System for RT-PCR (*Invitrogen*). Reaction mixtures were incubated with RNase H at 42 °C for 50 min, then at 70 °C for 15 min, and finally at 37 °C for 20 min to remove RNA templates from the cDNA:RNA hybrid molecules. A control reaction for each sample was performed without reverse transcriptase. The samples were analysed by electrophoresis on 1% agarose gels. PCR products of the expected size were cloned in the pCR 2.1 vector (*TA Cloning Kit, Invitrogen*) and further sequenced. Alignments and

phylogenetic analyses were performed with the MegAlign (*DNASTAR*, Madison, USA) and CLUSTAL (Higgins and Sharp 1988) programs. Sequences were compared to those available at EMBL GenBank.

Cloning and sequencing of Pvadc, Pvspds, Pvsamdc and uORFsamdc cDNAs fragments**:** Primers (external or internal) of each gene were designed, based on conserved regions of the corresponding enzymes from several plants. Nested PCR reactions were started with the external primers followed by further amplification using the internal primers. The template was the cDNA obtained by reverse transcription using RNA isolated from bean leaves. The primers synthesized for the isolation of the *Pvadc* fragment were: 38 5'-CTCATTACCAAGGTGTTTATCCGGTGAA-3' (sense), 40 5'-GAGCGCCTCCTCATAAGCCCCACCCAAA-3' (antisense) and 42 5'-GGCTTCTCGTCCAAACGGTGAAT-3' (antisense) designed according to the conserved regions of cDNAs from: *Glycine max* (GenBank U35367), *Vitis vinifera* (GenBank X96791), *Nicotiana tabacum* (GenBank AF321137.1) and *Datura stramonium* (GenBank AJ251898.1). Primers to obtain the *samdc* fragment were: 2272 5'-GTTTCTGCAATTGGTTTTGAAGGTTT-3' (sense), 2274 5'-GAAAAGAGGTTGGAAATATCCT-3' (sense), 2273 5'-CTTCTTCATCTTCATCTTTCCAGCA-3' (antisense) and 2299 5'-CCACCCATTCCAAGC-3' (antisense) from conserved regions of cDNAs from: *Phaseolus lunatus* (GenBank AB062360), *G. max* (GenBank AF488307), *Pisum sativum* (GenBank AB087841.1) and *Arabidopsis thaliana* (GenBank U63633). Primers for synthesis of the *Pvspds* fragment were: Spe-3-5 5'-CCTGGATGGTTCTCTGAAATTAGCC-3' (sense) and Spe-3-3 5'-CTCTTCGCAAAAGATGGCAAACAGAA-3' (antisense) primers from conserved regions of cDNAs from: *P. sativum* 2 (GenBank AF043109.1), *P. sativum* 1 (GenBank AF043108), *Lycopersicon esculentum* (GenBank AJ006414) and *D. stramonium* 2 (GenBank Y08253). For the uORF isolation, we designed the primer uORF 5'-ATGGAGTCTAAAGGAGGTAAAAAG-3' (sense) located at the beginning of the small conserved open reading frame in the 5' UTR found in plants *samdc´s*

(Franceschetti *et al.* 2001) in combination with primer 2299 5'-CCACCCATTCCAAGC-3'(antisense) (Fig. 3A).

***RT-PCR analyses of gene transcripts*: ** Different sets of primers were designed to amplify each cDNA by RT-PCR: *Pvodc* (GenBank AY125817) sense 5'-ATGTTGGCTGCTATGGGTTCGAA-3' and antisense 5'-AGTATTCACGTAAATCTCCACGTA-3'. *Pvadc* (GenBank *AY671973)* sense RTADC1 5'-TCGTGGTAGAGGACATCGTCAAA-3' and antisense RTADC2 5'-CCAAACGGTGAATGGGGATGA-3'; *Pvsamdc* (GenBank AY327898) sense RTSAM1 5'-GGTTTAAGAGTTCTTACGAAATCAC-3' and antisense RTSAM2 5'-ATTTGTTCAGAGGGCTTGCTTGC-3'; *Pvspds* (GenBank AY674166) sense RTSPE1 5'-TGATCACTCATCTTCCTCTTTGCTC-3' and antisense RTSPE2 5'-CTCAGAGTTGTAAAATTTCAGTGGTC-3. For synthesis of an actin fragment (loading control) the primers used were 5-Act 5'-ATGGGGCAGAAGGATGCGTATG-3' and 3-Act 5'-AGCCTTCATAGATGGGGACCGT-3'. Equal RNA amounts were used for each RT-PCR reaction as described previously. PCR amplifications were performed in a 50 μl reaction mixture containing 1 μl of the RT reaction product as template. The PCR amplification conditions for each gene were as follows: 5 min at 94 ºC, 30 cycles of 45 s at 94 °C, 45 s at 55 °C (*Pvodc*), 56 °C (*Pvadc),* 50 ºC *(Pvsamdc*) or 58 °C (*Pvspds*), 80 s at 72 ºC and 8 min at 72 ºC for the final extension.

Results and discussion

Plant ADC, SAMDC and SPDS sequences have highly conserved regions and thus provide several candidate sites for designing PCR primers of consensus sequence in order to clone related genes. Nested amplifications by PCR were carried out using these primers to obtain partial cDNAs sequences encoding ADC, SAMDC and SPDS from *Phaseolus vulgaris*.

Isolation and sequence analysis of a partial cDNA corresponding to a bean arginine decarboxylase gene (*Pvadc*): We amplified a unique band of 1161 bp that corresponded to the gene encoding *P. vulgaris* ADC. The sequence obtained (Fig. 1A) was registered at the GenBank (AY671973). The pyridoxal phosphate binding site (Sandmeier *et al.* 1994), was identified at amino acids 7-25 [YP(V)KCN(QDRF)VV(ED)I(VKF)G] of the encoded sequence (Fig. 1A). Site-directed mutagenesis of Lys95 in *Nicotiana glutinosa* ODC causes a substantial loss of enzymatic activity (Lee and Cho 2001). The consensus sequence of the decarboxylases family responsible for substrate binding was located at amino acids 187-201 [G(AN)M(RV)IDIGGGLG] of the *P. vulgaris* encoded sequence. It has been proposed that the stretch of three consecutive glycines is the motif directly involved in substrate-binding (Moore and Boyle 1990). A probable PEST region [(sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] characteristic of proteins with high turnover rate (Rechsteiner and Rogers 1996), with PEST score of -8.45, was identified at residues 255-292 of the encoded sequence (Fig. 1A). Comparison using Clustal analysis of this fragment with other plant ADCs showed an homology of 87.6% with *Glycine max* (GenBank U35367), 73.6% with *Vitis vinifera* (GenBank X96791), 73.1% with *Nicotiana tabacum* (GenBank AF321137.1), 72.6% with *Datura stramonium* (GenBank AJ251898.1) and 72.4% with *Capsicum annuum* (GenBank AY156514.1). According to these results, bean ADC is most closely related to the *G. max* enzyme (Fig. 2).

Isolation and sequence analysis of a partial cDNA corresponding to a bean spermidine synthase gene (*Pvspds*): We isolated by PCR a single band (728 bp; GenBank AY674166) corresponding to a cDNA fragment of *Pvspds* from bean. The encoded sequence showed high similarity with other SPDSs: 88.5% with *Pisum sativum* 2 (GenBank AF043109.1), 87.2% with *P. sativum* 1 (GenBank AF043108), 84.1% with *Lycopersicon esculentum* (GenBank AJ006414), 82.3% with *D. stramonium* 2 (GenBank Y08253), 81.4% with *Arabidopsis thaliana* (GenBank AB025622.1) (Fig. 2). A consensus sequence of the synthases family [VLVIGGG(D)G(GV)L(R)E] (Sekowska *et al.* 1998), was located at amino acids 37-

51 of the encoded sequence (Fig. 1B). Six SAM or dcSAM-binding motifs were identified in the sequence. Motif I [IGGGDGGVLREIARHSS] corresponding to amino acids 40-57, shares 11 amino acids with the consensus sequence of the synthases family, motif II [EI] is located at amino acids 65-66, motif III [DG] at amino acids 96-97, motif IV [DAVIVDSS] at amino acids 110-117, motif V [GPAQELF] at amino acids 121-127 and motif VI [PGGVVCTQA] is located at amino acids 141-149 (O' Gara *et al*. 1995) (Fig. 1B). Also, a possible PEST region with a score of -1 was located at amino acids 102-129 [KNVPEGTYDAVIVDSSDPIGPAQELFEK] (Fig. 1B).

Isolation and sequence analysis of a partial cDNA corresponding to a bean S-adenosylmethionine decarboxylase gene (*Pvsamdc*): The nested PCR assays produced a single fragment of 935 bp. We compared the sequence of the polypeptide coded by *P. vulgaris samdc* (GenBank AY327898, reported in this work) with the sequences reported from other plant SAMDCs: The highest similarity was to *P. lunatus* with 95.2% (GenBank AB062360) and to *G. max* with 82.3% (GenBank AF488307) enzymes; lower homology to enzymes from *P. sativum* with 75.6% (GenBank AB087841.1), *Vicia faba* with 74.6% (GenBank AB087841.1) and *A. thaliana* with 66.6% (GenBank U63633), was shown; whereas sequence comparison with enzymes from the monocots *Oriza sativa* (GenBank Y07766) and *Zea mays* (GenBank Y07767) SAMDCs showed 53.7% and 51.4%, respectively. These results suggest that the homology of SAMDCs agrees with the phylogenetic relationships of the plant species (Fig. 2). The putative characteristic motifs of decarboxylases were identified in the encoded sequence (Fig. 1C). Accordingly, a conserved sequence from all SAMDCs was located at amino acid residues 50-61 of the encoded sequence [SYVLSESSLFV], which is also considered as the consensus proenzyme-processing site of the decarboxylases family (Ekstrom *et al.* 1999). Two putative PEST regions located at amino acids 26-65 [KSQLDEILTPAACTIVSSLSNDDVDSYVLSESSLFVYAYK] and 234-254 [HITPEDGFSYASFETVGYDFK] of the encoded sequence with scores of -6.52 and -7.17 respectively, were identified (Fig. 1C). Interestingly, the first putative PEST

box is located at the proenzyme-processing site of the enzyme (Mad Arif *et al*. 1994).

5' UTR ORF in *Pvsamdc* of *P. vulgaris*: A single 1316 pb fragment, which includes the bean *samdc* 5' UTR region and a small ORF within this sequence was amplified by PCR. The sequence obtained corresponding to the 5' UTR ORF from *P. vulgaris samdc* and was registered at the GenBanK (AY730047). As indicated above, the encoded small uORF (53 aa) has been described to be involved in the translational control of SAMDC in plants (Hanfrey *et al*. 2002). The encoded uORF from bean reported in this work, was found to be homologous to uORFs from several plants (Fig. 3B).

Differential expression of *Pvadc, Pvodc, Pvsamdc* and *Pvspds* in different bean tissues. Transcript levels of *adc*, *odc*, *samdc* and *spds* were analysed by RT-PCR in different bean tissues (Fig. 4). The maximal expression levels of *Pvadc* transcript in non reproductive tissues were observed in young leaves, stems and roots, while no expression was observed in roots tips. We also analysed the transcript levels in flower tissues during the pre-anthesis stage observing that stigma, ovaries and filaments showed the highest expression levels followed by anthers and petals (Fig. 4A). Previously it has been reported that *adc* transcript accumulation appears to be tissue-specific and developmentally-regulated in tomato (Rastogi *et al.* 1993) and soybean (Nam *et al*. 1997). In pea, *adc* gene was highly expressed in young developing tissues such as shoot tips, young leaflets and flower buds, but lower levels were detected in mature and fully expanded leaflets and roots (Perez-Amador *et al.* 1995). A similar behavior was observed in our study where the highest accumulation of the *adc* transcript occurred in young developmental organs during cell expansion or elongation. ODC activity is essential for normal cell growth and has been closely associated with cellular proliferation.

We detected *Pvodc* expression in roots, and surprisingly we observed two weak amplification products in young leaves, suggesting that two *odc* genes could be

present in bean (Fig. 4B). Our transcriprtion results correlate with those from *D. stramonium* (Michael *et al.* 1996), where *odc* mRNA accumulates to a greater extent in highly branching and fast growing transformed root cultures as compared to leaves or stems, where cell division is limited. Studies on tomato have shown similar results for *odc* (Kwak and Lee 2001), higher expression levels were detected in roots followed by stems, whereas lower levels were found in young and mature leaves. Alabadí and Carbonell (1998) found that unpollinated ovaries in tomato at 1 day post-anthesis showed a high level of *odc* mRNA, which gradually decreased with time. In contrast, we did not detect *odc* expression in any of the pre-anthesis flower tissues analysed. The *Pvsamdc* transcript was present in rather similar amounts in the majority of the analysed tissues from the bean plant, although ovaries and anthers showed slightly lower levels (Fig. 4C). In potato, *samdc* was highly expressed in young and actively dividing tissues whereas its levels were reduced in mature and non-dividing ones (Mad Arif *et al*. 1994). In a study of pea *samdc* (Marco and Carrasco, 2002) it was observed that transcript levels varied among tissues and in response to developmental conditions. The levels of expression of *Pvspds* were highest in roots and only barely present in mature and young leaves and stem, whereas no expression was observed in roots tips (Fig. 4D). In flower tissues highest expression was observed in stigma and filaments, followed by ovaries, but no expression was detected in petals and anthers. In contrast, *spds* trancripts were expressed in all plant organs of *A. thaliana* (Hanzawa *et al*. 2002).

In general, we observed highest accumulation of the analysed *P. vulgaris* transcripts in roots, followed by stigma and filaments, whereas lowest expression occurred in petals and anthers. These results indicate that expression levels of the genes involved in the biosynthetic pathway of polyamines are tissue-specific and developmentally-regulated. In addition these results support the idea that distribution patterns of genes involved in polyamine biosynthesis do not follow a common pattern in different plant species. This is probably the result of different regulation mechanisms or environmental conditions.

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Legends to figures

Fig. 1. Comparison of predicted amino acid sequences encoded by the cDNA fragments of *Phaseolus vulgaris* ADC, SAMDC and SPDS with the corresponding enzymes from different plants. **A)** ADC: *P. vulgaris*, *Glycine max* and *Arabidopsis thaliana* 2. The consensus sequence of the decarboxylases family is shown in a box; the piridoxal binding site is underlined; and double underlining marks the possible PEST regions. **B)** SPDS: *P. vulgaris*, *Pisum sativum* 2 and *A. thaliana.* The consensus sequence of the synthases family appears in a box; the six proposed binding motifs for dcSAM are gray-highlighted; and a probable PEST region is double underlined. **C)** SAMDC: *P. vulgaris*, *P. lunatus* and *A. thaliana*. The consensus sequence of the decarboxylases family and the site of proenzyme processing are shown in a box; and the probable PEST regions are shown in lowercase. Identical residues are marked by asterisks, and amino acid conserved substitutions are indicated as dots.

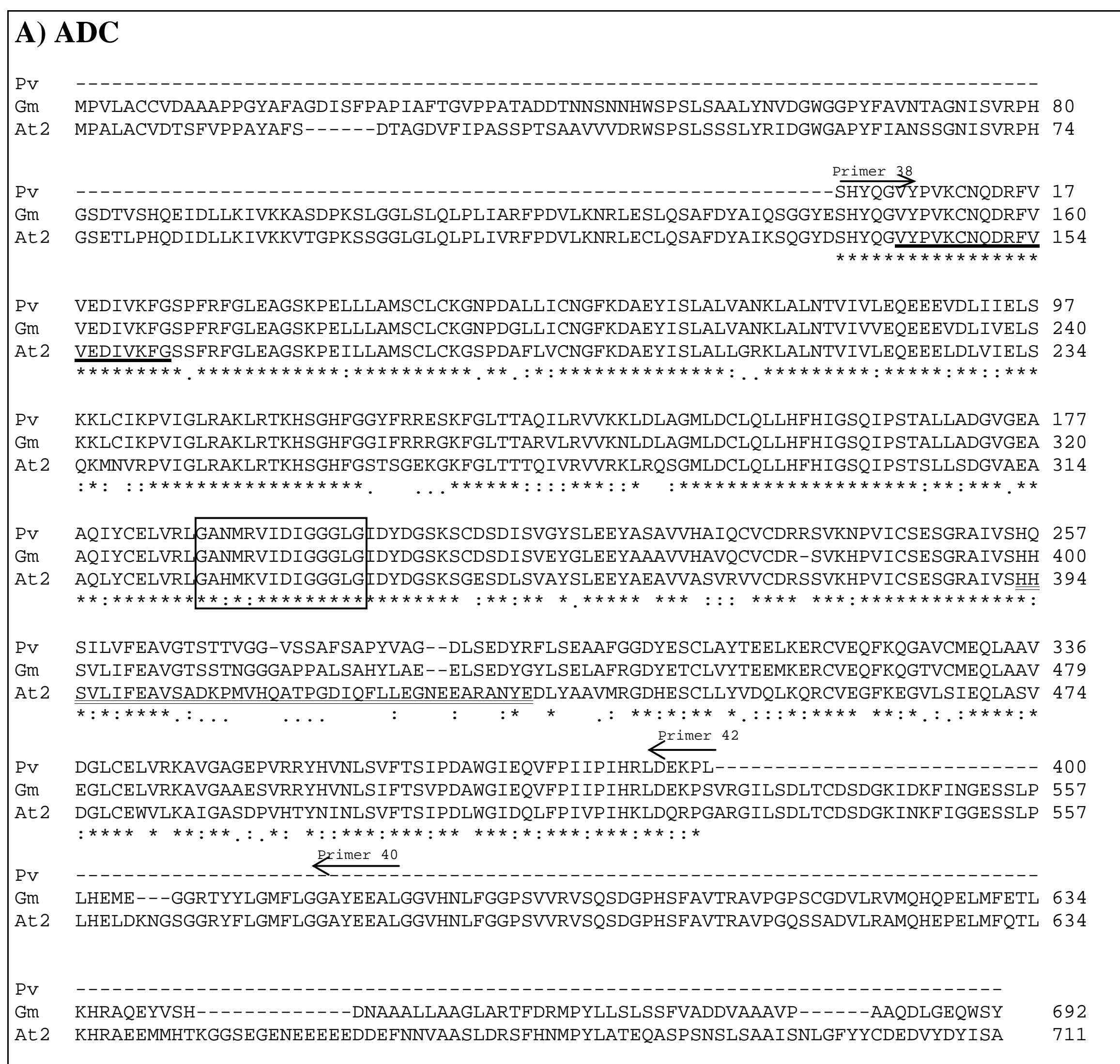
Fig. 2. Phylogenetic dendrograms of ADCs, SAMDCs and SPDSs. ADC: *Datura stramonium, Capsicum annuum, Lycopersicon esculentum, Nicotiana tabacum, Carica papaya, Nicotiana sylvestris, Arabidopsis thaliana* 1, *A. thaliana* 2, *Vitis*

vinifera, Daucus carota, Phaseolus vulgaris, Malus domestica, Glycine max, Pisum sativum and *Avena sativa.* SPDS: *D. stramonium* 2 , *Hyoscyamus niger, L. esculentum, Solanum tuberosum, C. annuum, N. sylvestris, N. tabacum, D. stramonium* 1, *O. sativa, P. sativum* 2, *P. vulgaris, P. sativum* 1, *M. domestica, G. max* and *A. thaliana*. SAMDC: *D. carota, N. tabacum, V. vinifera, M. domestica, Magnifera indica, Oriza sativa, Zea mays, Ipomoea nil, P. sativum, Vicia faba, G. max, Phaseolus lunatus, P. vulgaris and A. thaliana*.

Fig. 3.. Alignment of the deduced amino acid sequences of the small uORF harbored in the *samdc* mRNA 5' leader sequences. **A)** Scheme of the strategy followed to obtain the uORF sequence; see text for details. **B)** EST sequences of several monocots and dicots were compared (accession numbers in parentheses): Le, *Lycopersicon esculentum* (EST-AI483261); Ds, *Datura stramonium* (Y07768); Pt, *Pinus taeda* (EST-AI725223); Nt, *Nicotiana tabacum* (AF033100); Cl, *Citrullus lanatus* (EST-AI563097); Gm, *Glycine max* (EST-AI442381); Gh, *Gossypium hirsutum* (EST-AI728571); Cr, *Catharanthus roseus* (U12573); In, *Ipomoeau nil* (U64927); Pv, *Phaseolus vulgaris* (AY730047); Ps, *Pisum sativum* (U60592); Mt, *Medicago trunculata* (EST-AA661003); At2, *Arabidopsis thaliana* 2 (AJ251899), Bj2, *Brassica juncea* 2 (X95729); Bj1, *Brassica juncea* 1 (U80916); Zm, *Zea mays* (Y07767); Hc, *Hordeum chilense* x *Tritordeum turgidum* conv. Durum (X83881); Os1, *Oriza sativa* 1 (Y07766); Os2, *Oriza sativa* 2 (AJ251899); Dc1, *Dianthus caryophyllus* (U38526); Dc2, *Dianthus caryophyllus* (U38527); Mc, *Mesembryanthemum crystallinum* (EST-AI822872). Identical residues are marked by asterisks, and amino acid conserved substitutions are indicated as dots.

Fig. 4. Expression analyses of *adc, odc, samdc* and *spds* in different tissues of *P. vulgaris*. 45 d-old bean plants were used for RT-PCR analyses. 0.01 cm^3 RT-PCR product was loaded on each lane and separated by electrophoresis on 1 % agarose gel. Actin product was used as a loading control.

Figure 1



B) SPDS

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Pv      --------------------------------------------------------------------------------
Ps2     -MAAESTVELESSMKEHRDDDEKSNGFSVSAVSMDVEGGDKDPSGNGVSSVIPGWFSEISPMWPGEAHSLKIEKILFQGK 79
At      MSSTQEASVTDLPVKRPREAEEDNNGGAMETE----NGGGEIKEPSCMSSIIPGWFSEISPMWPGEAHSLKVEKILFQGK 76

                    Primer Spe-3-5                                  Motif I        Motif II
Pv      --------------NSILVLDGVIQLTERDECAYQEMITHLPLCSIPNPKKVLVIGGGDGGVLREIARHSSVEKIDICEI 66
Ps2     SEYQKVMVFQSSTYGKVLVLDGVIQLTERDECAYQEMITHLPLCSIPNPKKVLVIGGGDGGVLREVARHSSIEKIDICEI 159
At      SDYQDVIVFQSATYGKVLVLDGVIQLTERDECAYQEMITHLPLCSISNPKKVLVIGGGDGGVLREVARHSSVEQIDICEI 156
                      ..:*****************************.******************:*****:*:******
                                  Motif III          Motif IV   Motif V           Motif VI
Pv      DGMVVDVSKQYFPDVAVGYEDPRVTLTVGDGVAFLKNVPEGTYDAVIVDSSDPIGPAQELFEKPFFGSVAKALRPGGVVC 146
Ps2     DNMVVEVSKQFFPEVAVGFNDPRVTLRIGDGVAFLKAAPEGTYDAVIVDSSDPIGPAQELFEKPFFQSVARALRPGGVMC 239
At      DKMVVDVAKQYFPNVAVGYEDPRVNLIIGDGVAFLKNAAEGTYDAVIVDSSDPIGPAKELFEKPFFESVNRALRPGGVVC 236
        * ***:*:**:**:****::****.* :********.. ******************:******** ** :*******:*

Pv      TQAESIWLHMDIIEDIVSNCRHIFKGSVNYAWTTVPTYPSGMIGFMLCSTEGPPVDFKHPVNPIDENE-CQKSARPLKFY 226
Ps2     TQAESIWLHMDIIEDIVSNCRHIFKGSVNYAWTTVPTYPSGMIGFMLCSTEGPLVDFKHPVNPIDQKD-CQKSVRPLKFY 319
At      TQAESLWLHMDIIEDIVSNCRDIFKGSVNYAWTSVPTYPSGVIGFMLCSSEGPQVDFKKPVSLIDTDESSIKSHCPLKYY 316
        *****:***************.***********:*******:********:***  ****:**. ** .: . **  ***:*

                     Primer Spe-3-3
Pv      NSEIHTAAFCLPSFAKR-ITSEFA 248
Ps2     NSEIHTAAFCLPSFAKRKIGSKET 342
At      NAEIHSAAFCLPSFAKKVIDSKAN 340
        *:***:**********: * *:
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C) SAMDC

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           Primer 2272   Primer 2274
Pv      ------------KRLEISFSQPGLFADPEGRGLRVLTksqldeiltpaactivsslsnddvdsyvlsesslfvyaykIII 68
Pl      MAVSAIGFEGYEKRLEICFSQPGIFADPEGRGLRVLTksqldeiltpaactivsslsnedvdsyvlsesslfvyaykIII 80
At      MAVSATGFEGFEKRLEISFFETTDFLDPQGKSLRSLTksqldeiltpaectivsslrnsfvdsyvlsesslfvypykIII 80
                    *****.* :.  * **:*:.** ************* *******:*. **************.*****

Pv      KTCGTTKLLLSIPPILKFADMLSLKVKSVTYTRGSFIFPGAQPHPHRCFTEEVAILNDYFGKLGSGSMAFIMGSPDKK-Q 148
Pl      KTCGTTKLLLSIPPILKFADMLSLTVRSVTYTRGSFIFPGAQPHPHCSFTEEVAILNDYFGKLGSGSMAYIMGSPDKK-Q 160
At      KTCGTTKLLLSIPHILRLADSLCLTVKSVRYTRGSFIFPGAQSYPHRSFSEEVALLDDYFGKLNAGSKAFVMGGSDNNPQ 160
        ************* **::** *.*.*:** ***********.:** .*:****:*:******.:** *::**..*:: *

Pv      KWHIYSAS-ADPLISSDTNVCTLEMCMTGLDREKAQIFYREQSASAAIMTVNSGIRKILPMSDICDFDFEPCGYSMNSVE 226
Pl      KWHIYSAS-ADPLISSDTNVCTLEMCMTGLDREKAQIFYKEQSASAAIMTVNSGIRKILPKSDICDFDFEPCGYSMNSVE 238
At      RWHVYSASSTEESAVCDKPVYTLEMCMTGLDNIKASVFFKTNSVSASEMTISSGIRNILPGSEICDFNFEPCGYSMNSIE 240
        :**:**** ::     .*. * **********. **.:*:: :*.**: **:.****:*** *:****:**********:*

Pv      GDAVSTIhitpedgfsyasfetvgydfkVLNLNALVDRVLSCFLPDEFSVAVRVDGASKPSEQMCFVDVKGYCREEWIHE 306
Pl      GDAVSTIhitpedgfsyasfetvgydfkVLNLNAMVDRVLSCFLPNEFSVAVRVDGASKPSEHMCFVDVKGYCREEWCHE 318
At      GDAVSTIhvtpedgfsyasfetvgydlkALNFKELVDRVLVCFGPEEFSVAVHANLGTEVLASDCVADVNGYFSQERELE
        *******:*****************:*.**:: :***** ** *:******:.: .::     *..**:**  :*   *

         Primer 2299          Primer 2273
Pv      GLGMG------------------------------- 311
Pl      ELGMGGFVVYQKFVKISDCVSPRSTLKCWKDEVEEE 354
At      ELGLGGSVLYQRFVKTVECCSPKSTLGFC------- 349
         **:*
```

Figure 2

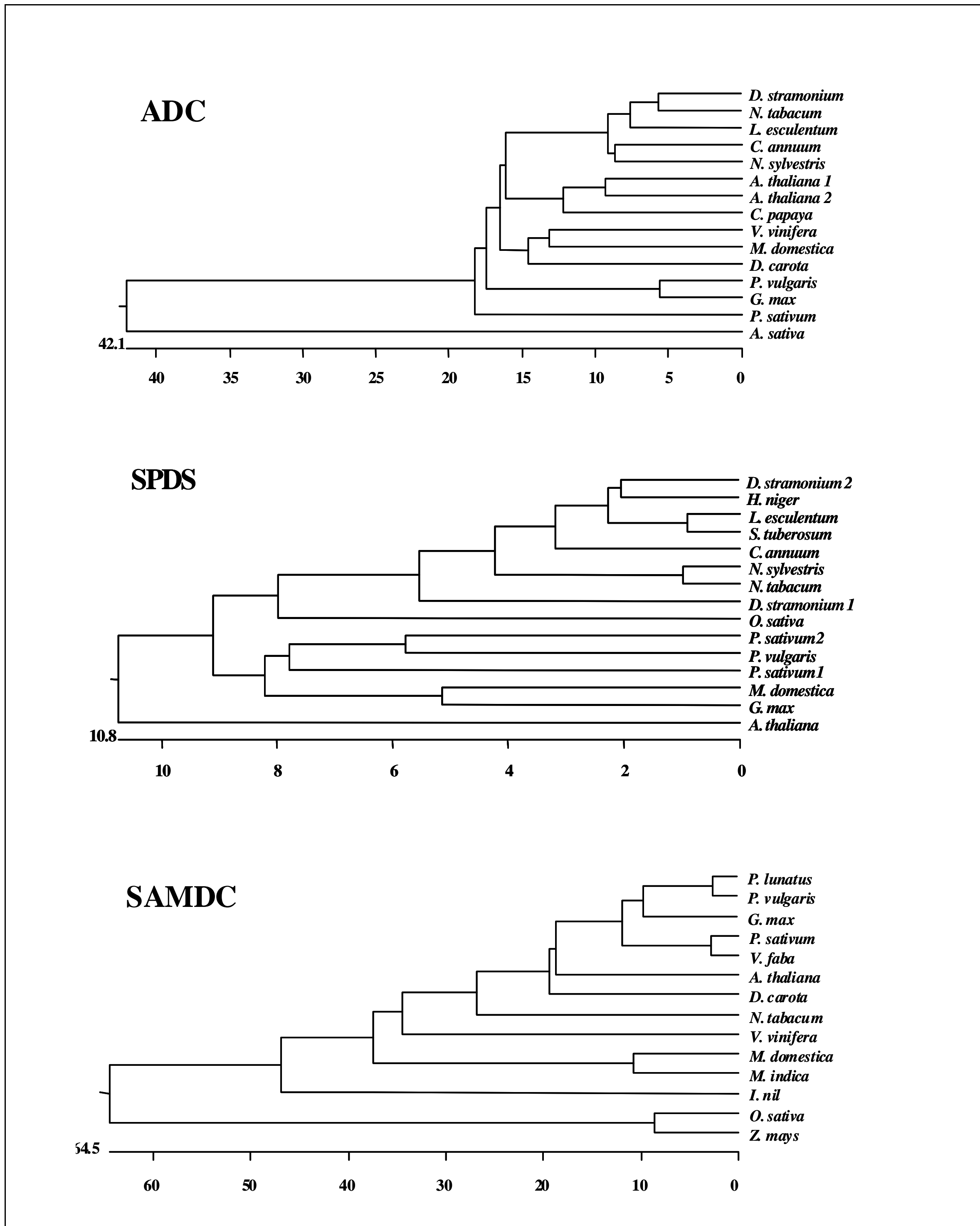

Figura 3

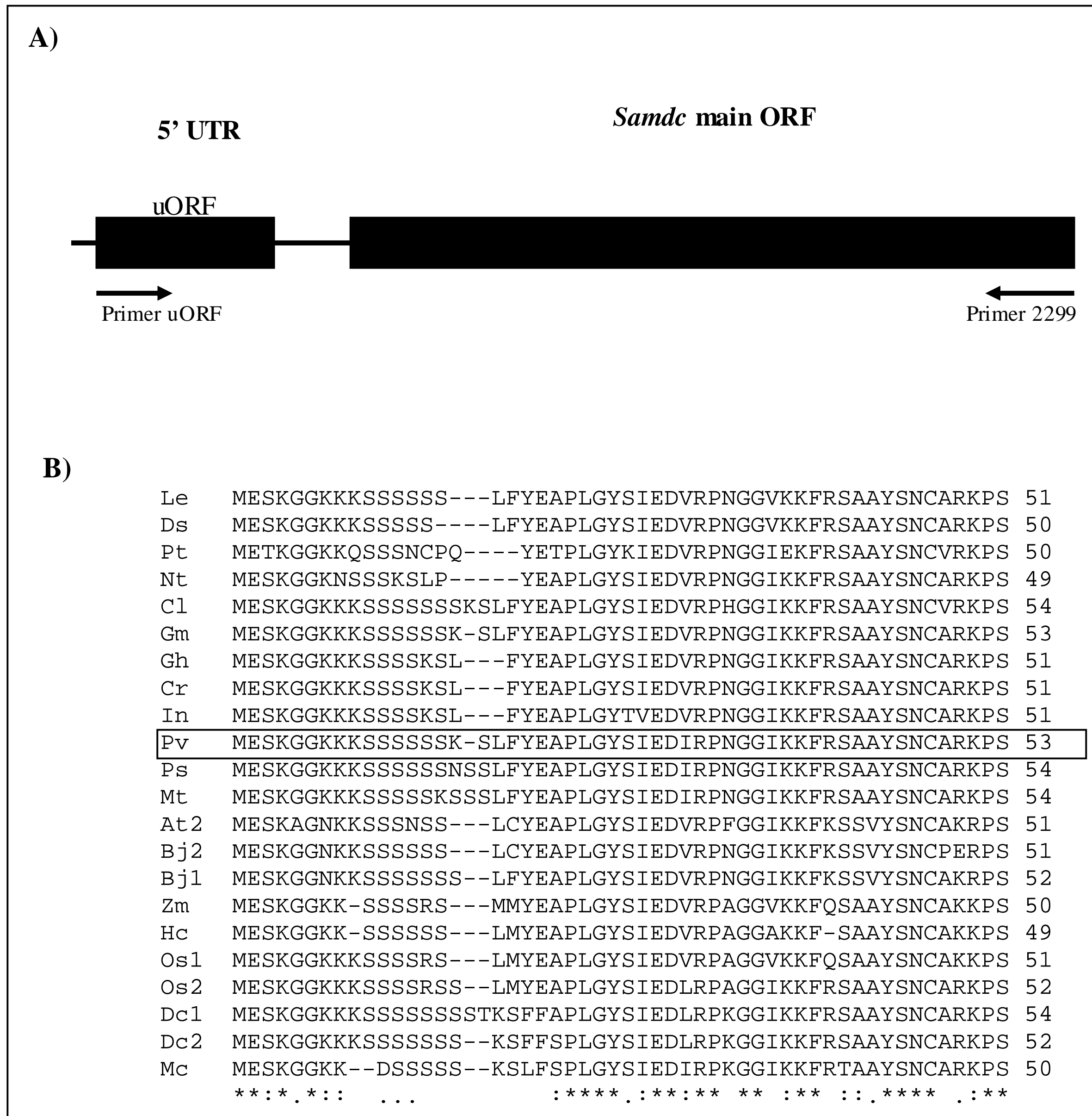

Figure 4