Plant Mol Biol Rep
DOI 10.1007/s11105-011-0381-7

Identification and Analysis of Differentially Expressed Genes During Seed Development Using Suppression Subtractive Hybridization (SSH) in *Phaseolus vulgaris*

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Abstract Interspecific hybridization in the genus *Phaseolus*, conducted to introgress desired traits into common bean (*Phaseolus vulgaris* L.), leads to the abortion of immature embryos, usually at early developmental stages. Little is known about the physiological responses of embryo dysfunction in *P. vulgaris* during the early stages of embryogenesis and the genes that are involved in these responses. Identification of the genes involved in *Phaseolus* embryogenesis may provide information that will help to understand the molecular basis of *Phaseolus* embryo dysfunction. To investigate the genes expressed during *Phaseolus* seed development, we constructed a suppression subtractive hybridization (SSH) library using cDNA from abortive seed development as the driver and those from normal seed development as tester. The differentially expressed cDNA fragments were identified by differential screening. We identified 72 unique ESTs of which we selected 12 candidates on the basis of their redundancy. These candidates were subjected to a validation procedure based on the study of their expression level by real-time PCR. Sequence analysis revealed that most of the differentially expressed genes are related to metabolism and regulation such as protein synthesis. Some genes also encoded transcription factors. These genes showed high mRNA transcript levels in seed tissues and little or no expression in other tissues (root, stem, flower, leaf, and cotyledon). Seven genes were chosen and their expression profile during seed development in *P. vulgaris* was analyzed by real-time PCR using RNA preparations originating from different seed development stages. This study revealed hitherto unknown genes putatively involved in dicotyledonous embryogenesis, which serve as a starting point for understanding *Phaseolus* embryogenesis.

Keywords *Phaseolus* · Seed development · RT-PCR · SSH

Abbreviations

DAP	Days after pollination
EST	Expressed sequence tag
RT-PCR	Reverse transcription polymerase chain reaction
SSH	Suppression subtractive hybridization

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Published online: 23 December 2011

Introduction

Common bean (*Phaseolus vulgaris* L.) is a staple food in many developing countries, where it is used daily as a primary source of protein (Broughton et al. 2003). In these countries, the average yield of bean varieties is still very low. Limiting factors include diverse cultivation systems (intercropping beans with cereals, bananas, and root or tuber crops), restricted improved cultivar adoption, and susceptibility to biotic and abiotic stresses (Mahuku et al. 2002; Caixeta et al. 2003; Rainey and Griffiths 2005). Good sources of resistance have been found in such species as *Phaseolus polyanthus* Greenm. and *Phaseolus coccineus* L. (Busogoro et al. 1999). The use of interspecific crosses in breeding is a promising tool for expanding the genetic base of the cultivated common bean and for overcoming some agronomical constraints (Broughton et al. 2003). Numerous attempts have been made to obtain interspecific hybrids between *P. vulgaris* and the two donor species, *P. polyanthus* Greenm. and *P. coccineus* L.. The transfer of genetic resistance to *P. vulgaris* through interspecific hybridization is difficult because of the premature abortion of hybrid embryos. In addition, the rate of growth and final size of these hybrid embryos seems to be influenced by the parental genotypes (Geerts et al. 2002).

P. coccineus has been commonly used in wide crosses with *P. vulgaris*, especially to introgress genes controlling traits such as cold temperature tolerance and resistance to root rot and bean yellow mosaic virus (Schmit and Baudoin 1992). Consistent success has been limited to crosses where *P. vulgaris* is used as the female parent, but it has been reported that descendants naturally reverted to the cytoplasm donor parents after a few generations (Baudoin et al. 2004). The reciprocal cross is generally unsuccessful, although some rare successes have been reported (Nguema Ndoutoumou et al. 2007). Some data indicate that this low seed set was attributable partly to the failure of complete fertilization and partly to the slow development of interspecific hybrid embryos, endothelium proliferation, and poor endosperm development (Geerts et al. 2002). The disruption of major genes involved in embryogenesis can cause the degeneration of interspecific embryos. The transcripts of these genes can be localized in the embryo proper, in the endosperm, or in the maternal tissues around the embryo. The comprehensive and integrated mechanisms controlling gene expression during *Phaseolus* embryogenesis are not yet known.

Embryogenesis has been studied for a long time, including comparative morphological observations of zygotic embryo development in various plant species (Kaplan and Cooke 1997). Several techniques, such as representational differential display (Linkiewicz et al. 2004), expressed sequence tag (EST) analysis (Sun et al. 2007; Zhou et al. 2011), and the microarrays approach (Ben et al. 2005; Yang et al. 2011a), have been used in efforts to detect genes differentially expressed during zygotic embryogenesis.

The main objective of this study was to isolate and characterize cDNAs differentially expressed during *Phaseolus* embryogenesis. The identification of specific genes required to produce a viable embryo with a normal phenotype will improve the understanding of embryo dysfunction in *Phaseolus* interspecific hybrids.

Here, we report on the use and validation of the suppression subtractive hybridization (SSH) technique to isolate and study genes from *Phaseolus*. The SSH technique is believed to generate an equalized representation of differentially expressed genes and provides a high enrichment of differentially expressed mRNA (Diatchenko et al. 1996; Marenda et al. 2004). The efficiency and reproducibility of SSH are very useful in studies of tissue-specific, developmental, or induced differentially expressed genes (Von Stein et al. 1997; Basyuni et al. 2011; Prabu et al. 2011; Yang et al. 2011b). In addition, in some plant species, SSH has proved useful for identifying genes differentially expressed during zygotic and somatic embryogenesis (Bishop-Hurley et al. 2003; Namasivayam and Hanke 2006; Legrand et al. 2007; Tsuwamoto et al. 2007; Wang et al. 2007; Geng et al. 2009). Despite its utility and efficiency in isolating differentially expressed genes, SSH has not yet been widely applied in *Phaseolus* embryogenesis. In this study, differentially expressed genes during the early stage of seed development in *P. vulgaris* were identified using SSH. The expression patterns of the isolated genes in zygotic embryogenesis were determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The paper discusses the putative roles of the identified genes.

Material and Methods

Plant Material, Hybridization, and Growth Conditions

Two cultivated genotypes, NI637 (*P. vulgaris*) and NI16 (*P. coccineus*), were grown in growth chambers under the following conditions, 27°C/23°C (day/night), 75% relative humidity, 580 μE m^{-2} s^{-1} light intensity and 12 h photoperiods. Several crosses were made between the two selected genotypes using either *P. vulgaris* or *P. coccineus* as the female partner. Seeds were collected each day at 3, 6, 8, 9, and 12 days after pollination (DAP) from auto or cross-pollinated flowers.

RNA Isolation and cDNA Synthesis

Total RNAs were extracted from degenerated seeds of interspecific hybrids (*P. coccineus* (♀) X *P. vulgaris*) and

normal seeds (*P. vulgaris*), following the protocol described by Chang et al. (1993). Apart from Tris–HCl, all the reagents were prepared with DEPC-treated water. Ovules (100 mg) were ground to a fine powder under liquid nitrogen. Then 700 µl of warm (65°C) extraction buffer (100 mM Tris–HCl pH 8, 25 mM EDTA pH 8, 2 M NaCl, 2% CTAB, 2% PVP, 2% β-mercaptoethanol) was added and the mixture was homogenized by vortexing for 2 min. An equal volume (700 µl) of chloroform/ isoamyl alcohol (24:1) was added, vortexed for 2 min, and centrifuged for 10 min at 14,000×*g* at room temperature (RT), and the supernatant was collected. For RNA precipitation, 1/4 V of 10 M LiCl was added, the mixture was inverted five times, incubated overnight at 4°C, and centrifuged for 15 min at 14,000×*g* (4°C); the pellet was re-suspended in 200 µl of DEPC-treated water and then 2.5 V EtOH and 1/10 V of 3 M NaAc pH 5.2 were added. The mixture was incubated for 60 min at −80°C and centrifuged for 15 min at 14,000×*g* (4°C); the pellet was washed twice with 500 µl of 70% EtOH, and then dried and re-suspended in 50–100 µl H_2O. Poly(A)$^+$ RNA was subsequently purified using an Oligotex^{TM} mRNA Mini Kit (Qiagen), following the manufacturer's guidelines.

Double-strand cDNAs were synthesized using the cDNA synthesis system Kit (Roche) with 2 µg of mRNA. The resulting cDNAs were dissolved in 20 µl of H_2O.

Driver and Tester Preparation

Driver and tester double-strand cDNAs were digested by *Rsa*I in a 50-µl reaction mixture containing 15 U of enzymes (Fermentas) for 3 h. The cDNAs were then phenol-extracted, ethanol precipitated, and re-suspended in 6 µl of H_2O.

The digested tester cDNA (1 µl) was diluted in 5 µl of H_2O. A PCR-Select^{TM} cDNA Subtraction Kit (Clontech, USA) was used for adaptor ligation: the diluted tester cDNA (2 µl) was ligated to 2 µl of adapter 1 and adapter 2 (10 µM) in separate ligation reactions in a total volume of 10 µl at 16°C overnight, using 15 U of T4 ligase (Roche) in the buffer supplied by the manufacturer. After ligation, the samples were heated at 70°C for 5 min to inactivate the ligase and then stored at −20°C.

Suppressive Subtractive Hybridization

Suppressive subtractive hybridization (SSH) was performed with the PCR-Select^{TM} cDNA Subtraction Kit (Clontech, USA). Two microliters of 5× hybridization buffer [50 mM Hepes pH 8, 0.5 M NaCl, 0.02 mM EDTA pH 8 and 10% (*w*/*v*) PEG 8000] were added to each of the two tubes containing adapter 1 and adapter 2-ligated tester cDNA. The solution was overlaid with mineral oil, and the samples were denatured for 1.5 min at 98°C and then allowed to anneal for 8 h at 68°C. After this first hybridization, the two samples were combined and a fresh portion of heat-denatured driver in 1.5 µl of hybridization buffer was added. The sample was allowed to hybridize for an additional 10 h at 68°C. The final hybridization was then diluted in 200 µl of dilution buffer (20 mM Hepes pH 8, 50 mM NaCl, 0.2 mM EDTA), heated at 72°C for 7 min and stored at −20°C. *Phaseolus* specific elongation factor 1α (EF-1α) primers were designed for evaluating the subtraction efficiency: EF-1αF5′: GTGCATTAAGTGTGGAGA and EF-1αR5′: TAGGCTCCTTCTCGAGCTCT.

After subtraction, two PCR amplifications were performed. The first was conducted in 25 µl and contained 1 µl of diluted subtracted cDNA, 0.3 µM of P1 primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U of Taq polymerase (Invitrogene) and 1× reaction buffer. PCR was conducted using the following parameters: 72°C for 2 min, 30 cycles at 94°C for 30s, 68°C for 30s, 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified products were diluted tenfold in H_2O. Some of the product (1 µl) was then used as a template in secondary PCR with nested PCR primers PN1 and PN2. PCR was performed for 20 cycles (94°C for 30 s, 66°C for 30 s, 72°C for 2 min). The product of the second PCR was analyzed on 2% agarose gel stained with ethidium bromide.

Cloning and Dot-Blot Analysis of the Subtracted cDNA

Amplified fragments from the second PCR were purified using the Gene Elute Gel Extraction Kit (Sigma), cloned into a pJET1.2/blunt vector using the Clone JET^{TM} PCR Cloning Kit (Fermentas) and subsequently transformed into competent *Escherichia coli* (DH5α), plated on solid Luria–Bertani (LB)/1.5 Difco agar plates supplemented with 50 mg/l ampicillin, and then grown overnight at 37°C. Some 128 colonies were randomly selected and grown in 200 µl LB medium in standard 96-well plates. Then, 1 µl of each bacterial culture was used to amplify the cDNA inserts in the recombinant plasmids with the primers: F5′-CGACTCACTATAGGGAGAGCGGC and R5′-AAGAACATCGATTTTCCATGGCAG. A 5-µl aliquot of each PCR product was added to 0.4 M NaOH and 10 mM EDTA in a final volume of 50 µl. The mixture was denatured for 10 min at 100°C and dotted onto Hybond N$^+$ nylon membranes. Altogether, two pairs of blots were prepared and subsequently cross-linked at 80°C for 2 h. The blots were prehybridized with hybridization-buffer (6× SSC, 5× Denhardt's, 0.5% SDS, and 100 µg/ml sheared salmon sperm DNA) for 4 h at 65°C. First-strand cDNAs were incorporated with ^{32}P-dCTP during reverse transcription from total RNAs (derived from tester and driver samples) using the RevertAidTM H Minus M-MuL V RT 200 U/µl

(Fermentas). Hybridizations were performed overnight at 65°C in the hybridization buffer containing ^{32}P-labeled cDNA probes, prior to autoradiographic exposure to a KODAK Storage Phosphor Screen (Bio-RAD). Finally, the membranes were scanned with a PERSONAL MOLECULAR IMAGER FX (Bio-RAD) and analyzed using the Quantity One program (Bio-RAD). The Dot blot analysis was repeated twice with two independent sets of blots.

DNA Sequencing

Plasmids were extracted using the quantum prep plasmid Miniprep Kit (BIORAD). cDNA sequencing was carried out using the GENOMELAB^{TM} CEQ/GeXP DNA ANALYSIS SYSTEM (BECKMAN COULTER). Homology searches in the GenBank database were conducted using the BLASTX and BLASTN search programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Semi-Quantitative Reverse Transcriptase-PCR

The specific organ expression of wild-type *P. vulgaris* was analyzed for leaves (2-week-old plants), flowers (0 DAP), stems (2-week-old plants), roots (2-week-old plants), cotyledons (3-day-old seedlings), and seeds (15 DAP). The samples were ground in liquid nitrogen, and total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The amount of total RNA was determined using UV spectrophotometry. Total RNA (5 μg) was treated with 1 U of RNase-free DNAseI (Roche) for 30 min at 37°C, prior to RT-PCR. The RNA was precipitated (1/10 (*v*/*v*) 3 M NH4 OAc, 2.5 (*v*/*v*) ethanol) at −80°C for 1 h. Total RNA (5 μg) was re-suspended in 11 μl RNase free water and 1 μl oligo $(dT)_{18}$ (Fermentas) and was reversed transcribed into cDNA by random priming with the RevertAidTM H Minus M-MuLV RT 200 U/μl (Fermentas): after 5 min at 70°C, the reverse transcriptase mix (5× reaction buffer, RibolockTM Ribonuclease inhibitor 20 U/μl, 10 mM dNTP mix) was added and kept at 37°C for 5 min; total pre-treated RNA was reverse transcribed at 42°C for 1 h. Primer pairs were designed using Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/) (Table 1). The 18S gene was used as a control for RNA loading.

Fragments were amplified from these cDNA pools using Taq DNA polymerase (Invitrogen).

PCR amplification was performed using the following program: 3 min at 94°C, 30 cycles for 30 s at 94°C, 30 s at 55–60°C and 30 s at 72°C. An elongation step of 72°C for 2 min was conducted for the last cycle. Reactions were performed in a Minicycler (BioRad iCycler) thermal cycler. The experiments were repeated three times. The amplified PCR products (15 μl) were electrophoresed on 1% (*w*/*v*) agarose gel, stained with ethidium bromide, and then scanned using an image analyzer.

Measuring Temporal Expression of Select Clones by Real-Time RT-PCR Analysis

Total RNA from the abortive and normal seeds of *P. vulgaris* and *P. coccineus* were reverse transcribed using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche), following the supplier's instructions. For cDNA synthesis, 5 μg of total RNA from each time point was used as a template in a 20-μl reaction mixture. Each reaction mixture contained 1× buffer reverse transcriptase (RT), 0.5 mM deoxynucleoside triphosphate, 1 μM oligo (dT) primer, 10 U of RNase inhibitor, and 4 U of AMV reverse transcriptase. The reaction mixture was incubated for 60 min at 37°C. cDNA was diluted 1:10, and 5 μl of the dilution was used in a SYBER Green RT-PCR (Fermentas).

Real-time PCR experiments were carried out using SYBER Green chemistry for amplicon detection. The SYBER Green assays were performed on the ABI7500 FAST real-time PCR instrument (Applied Biosystems). The

Table 1 The primers used in the RT-PCR analysis

Target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GW884166	TTACAGAGAGGGCCCAGAGA	GCAAGATGTTTACCGGAGGA
GW884169	GGTGCTCTAACCAGGGTGTC	TTCCATGGCAGCTGAGAATA
GW884170	ACAGGCTGATGACTCCCAAG	CATCGATTTTCCAGTTGCAG
GW884171	GCGACAGTGCAATCTCCAAC	TTCCATGGCAGCTGAGAATA
GW884172	GCACATGTGTGCTATCCTGA	ACAAAACGCTGGCTTCTGAT
GW884173	CAACGAAAAGGACAGCGAAC	GCGGGTCGAGCTTGTAGAG
GW884174	CGGAAAAAGCACCATCCAGT	TTCCATGGCAGCTGAGAATA
GW884175	GAACCCTTGGGACCTTCTTC	GGCGATAACAGGCTGATGAC
GW884176	ACTCACAACCGCGTCTCC	AGTCGGATACACCGGCATAG
GW884177	CCAATCACTATTGGGCTCGT	GGTGAACGTCGGTATTAAGCA

amplification of target genes, along with a template-minus control, was performed in triplicate in a 96-well plate. Each 20 μl reaction mixture contained 5 μl cDNA, 10 μl 2× SYBER Green Master Mix and 0.3 μM (each) of forward and reverse primers. The primers were designed using Primer3 software (they are listed in Table 1). The cycling conditions for amplification included a 10 min cycle at 95°C followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 82°C for 30 s. Fluorescence was measured at 82°C during each cycle. Real-time PCR data were plotted using 7500 Fast System Software (Applied Biosystems) by taking the mean of three replicates per time point. The relative difference in expression was measured using the Basic ΔC_T method.

Results

Generating an SSH Library from Interspecific Hybrids and Wild-type Seed Development

To identify genes differentially expressed in *Phaseolus* embryogenesis, an SSH library was constructed using cDNA from abortive seeds (interspecific hybrids; 12 DAP) as the driver, and cDNA pooled from normal seeds at 3, 6, 8, 9, and 12 DAP as the tester. These five periods corresponded to the early globular, globular, heart, torpedo, and cotyledon stages of embryo development, respectively (Abid et al. 2009). Total RNA was isolated and appeared undergraded on 1% agarose gel (Fig. 1a). Double-strand cDNA was synthesized and, after two rounds of subtraction, the subtraction efficiency was evaluated by detecting the housekeeping gene elongation factor 1α (EF-1α) in both the subtracted and non-subtracted cDNA pools, using PCR amplification. If the subtraction was efficient, the abundance of housekeeping genes in the subtracted library was greatly reduced compared with the unsubtracted tester control. As shown in Fig. 1b, the EF-1α fragment was clearly detectable in the unsubtracted tester after 23 cycles of amplification, whereas in the subtracted library, it required 25 cycles of amplification, indicating that cDNA homologous to both the tester and the driver had been subtracted. The subtractive product was amplified by PCR using oligonucleotides that were complementary to adapters 1 (PN1 and PN2). Figure 1c shows the electrophoretic analysis of the final PCR products. There are clear differences between the subtracted and unsubtracted cDNA libraries. The observable PCR products in the unsubtracted and subtracted libraries ranged between 300 and 2,000 bp and 300 and 1,500 bp, respectively (Fig. 1c). The difference in the amplification patterns between subtracted and unsubtracted cDNAs indicates a successful subtraction.

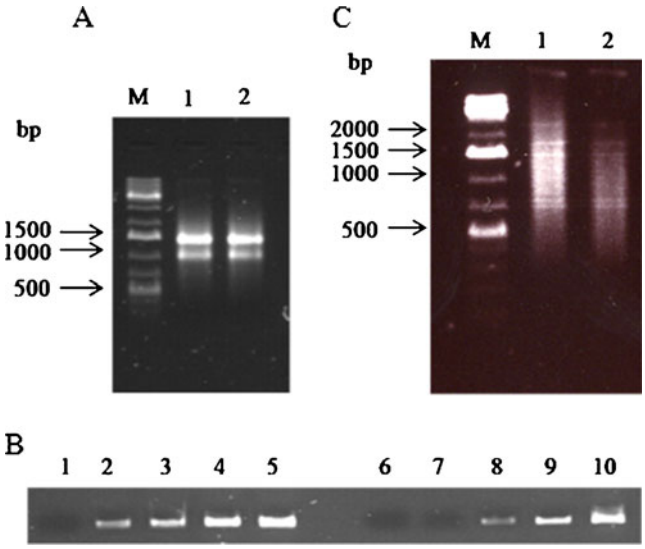

Fig. 1 The suppression subtractive hybridization (SSH) was conducted using cDNA from abortive seeds as the driver and cDNA from normal seed as the tester. **a** Total RNA of *P. vulgaris* (*lane 1*) and *P. coccineus* (*female symbol*) X *P. vulgaris* (*lane 2*). *Lane M*: DNA size markers. **b** PCR amplification of EF1α to analyze the efficiency of SSH: 1–5 were amplified from cDNA template before subtraction and 6–10 after subtraction. *Lanes 1* and *6* were the result of 20 amplification cycles, *lanes 2* and *7*, 23; *lanes 3* and *8*, 25; *lanes 4* and *9*; 28; *lanes 5* and *10*; 30. **c** Electrophoresis pattern of the second-round PCR with the unsubtracted (*lane 1*) and subtracted cDNA as template (*lane 2*). *Lane M*: DNA size markers

PCR Amplification and Dot-Blot Screening of SSH Clones

The secondary PCR products of the SSH cDNA library were inserted into a pJET 1.2/blunt vector and transformed into *Escherichia coli* (DH5α). A total of 72 recombinant clones were obtained after amplification using PCR and examination using electrophoresis. Most clone inserts ranged from 200 to 600 bp, averaging 300 bp per fragment (Fig. 2). The PCR products were spotted on hybond-N^+ nylon membranes and hybridized with probes prepared with unsubtracted tester/driver cDNA. Differences in the intensity of hybridization signals were observed for many clones (Fig. 3). Sixteen clones showing positive or stronger signals when hybridized with the tester cDNA, but having no or weaker signals with the driver cDNA, were identified and sequenced. Fourteen other clones showing a low difference signal between driver and tester clones were also sequenced.

Sequence Analysis and Identification of Differentially Expressed Genes

To identify a putative set of genes involved in *Phaseolus* embryogenesis, and to characterize function and redundancy among the putative positive clones, 30 selected individual clones were single run sequenced. After an alignment search,

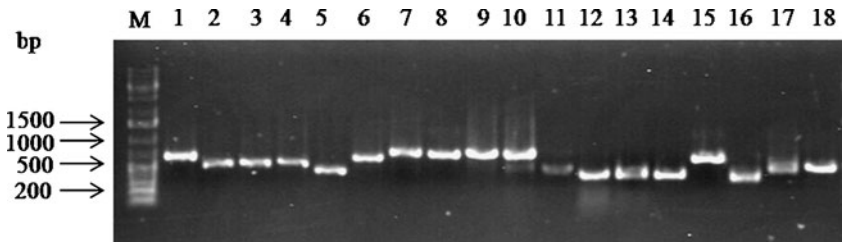

Fig. 2 PCR analysis of clones from the subtracted library. *Lanes 1–18*: PCR products from different clones. *Lane M*: DNA size markers

12 sequences were identified as unique genes, and the remaining18 sequences as repeated clones. All 12 cDNA sequences were submitted to the GenBank database (see Table 2 for the accession numbers). Using the BLASTn and BLASTx programs, the 12 cDNA sequences were used as queries to search in the GenBank database for homologies; the results are shown in Table 2. For only one EST clone (GW 884177) database searches failed to find a significant similarity with a sequence coding for a gene of known function, suggesting that this clone corresponds to an unidentified gene and could therefore be novel. The other EST clones were shown to be related to genes of known functions registered in databases. Our library contained several genes previously reported to be associated with plant zygotic embryo development, such as storage protein activator (SPA), acetyl-CoA carboxylase (ACCase), and pentatricopeptide repeat-containing protein (PPR). The library also included cDNAs not previously reported to be associated with embryogenesis, such as those coding for the esterase/lipase/thioesterase (ELT) protein or the hydrolase protein. Also in this library was cDNA coding for cell wall biosynthesis as proline-rich protein (PRP). Other genes were assigned to the ribosomal structure, biogenesis categories, and energy production such as NADH dehydrogenase, cell wall hydrolase, and peptidase M16-like protein (metalloproteases).

The most represented genes in our libraries are SPA and PPR, with five and four ESTs, respectively. BLAST analysis also showed that some cDNA clones, such as PPR and SPA, presented a high homology with two or more cDNAs isolated from different species but encoding for the same function.

Tissue-Specific Expression of the Selected ESTs

As an initial characterization of gene expression in *Phaseolus* plants, an expression pattern of the 10 selected ESTs listed in Table 2 was examined using RT-PCR (Fig. 4). Total RNA was isolated from leaves, flowers, stems, roots, cotyledons, and seeds from common bean plants. All selected ESTs showed high expression in seed tissues but less or no expression in vegetative tissues. For the PPR, transcripts were detected exclusively in seed tissues, with no expression detected in vegetative tissues. SPA and cell wall hydrolase were present at high levels in seed and flower tissues. ACCase and ELT were also detected at low levels in leaf and flower tissues, but no expression was detected in other tissues. Peptidase M16-like protein showed high expression in seed, stem, root, and cotyledon tissues and low expression in leaves and flowers. NADH dehydrogenase had about the same level of expression in the different vegetative tissues. For 18S, a

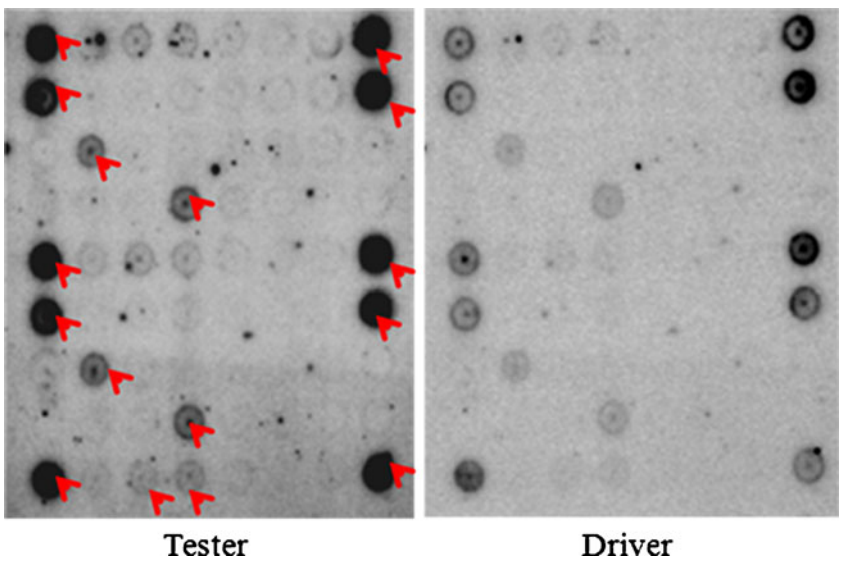

Fig. 3 Dot-blot analysis of clones from the subtracted library. cDNA from the driver and the tester were used as probes. Differentially expressed clones are marked with *arrows*

Table 2 Specification of the identified ESTs and their putative function

Clone	Length (bp)	Number of identical clones	Homology analysis	Putative identity (Accession no)	Identities (aa/aa or bp/bp)	E-value	Accession no of EST in Genbank
Pc_SSh1	259	5	BLASTn	Storage protein activator (*Triticum aestivum*) FM242578.1	157/167 (94%)	2E-64	GW884166
Pc_SSh2	212	2	BLASTn	NADH dehydrogenase subunit 5 (*Cucurbita pepo*) GQ856148.1	133/136 (97%)	7E-58	GW884170
Pc_SSh3	216	1	BLASTn	Esterase/lipase/thioesterase family protein (*Glycine max*) BM143356	112/130 (87%)	4E-41	GW884171
Pc_SSh4	256	4	BLASTn	Pentatricopeptide repeat-containing protein (*Phaseolus coccineus*) CA911930.1	127/130 (97%)	1E-54	GW884172
Pc_SSh5	292	1	BLASTx	Peptidase M16-like protein (*Sphingopyxis alaskensis*) YP_617011.1	53/69 (76%)	7E-26	GW884173
Pc_SSh6	212	1	BLASTx	Hydrolase, alpha/beta fold family protein (*Sorghum bicolor*) XP_002468316.1	20/40 (50%)	1E-05	GW884174
Pc_SSh7	133	3	BLASTn	Cell wall-associated hydrolase (*Coffea canephora*) GW397447.1	129/131 (99%)	3E-58	GW884175
Pc_SSh8	247	2	BLASTx	Proline-rich family protein (*Arabidopsis thaliana*) NP_176303.1	26/73 (35%)	4E-06	GW884176
Pc_SSh9	308	2	BLASTx	Unknown hypothetical protein (*Opitutaceae bacterium*) ZP_03725668.1	28/80 (35%)	4E-09	GW884177
Pc_SSh10	282	4	BLASTn	*Glycine max* cDNA, clone: GMFL01-47-K21 (*Glycine max*) AK245973.1	109/130 (83%)	6E-26	GW884167
Pc_SSh11	216	2	BLASTn	Ribosomal protein S26 (*Cucurbita pepo*) GQ856148.1	134/141 (95%)	1E-51	GW884168
Pc_SSh12	223	3	BLASTn	(Acc-2) cytosolic acetyl-CoA carboxylase (*Aegilops tauschii*) EU660891.1	157/167 (94%)	3E-63	GW884169

similar level of expression was detected in all tissues examined.

Confirmation of the Differential Expression of Selected Genes by Real-Time PCR

To evaluate the viability of the SSH library and Dot blot analysis, we performed real-time PCR analysis. Total RNA pools originally used as samples for constructing libraries were examined for ten selected ESTs, and the Pv 18S rRNA gene was used as an internal control.

In general, the results of real-time PCR were consistent with the differential screening data obtained by Dot blot analysis, but the Dot blot data sometimes underestimated or overestimated the real expression levels of the genes. The discrepancies observed between the two methods probably derive from cross-hybridizations that could occur on arrays between sequence-related genes from the same multigene family (Miller et al. 2002).

Real-time PCR data (Fig. 5) revealed that ESTs with significant homologies to PPR, SPA, ACCase, NADH dehydrogenase, cell wall hydrolase, PRP, hydrolase alpha/beta fold protein, and putative protein encoding to ELT were 8.09, 8.49, 7.87, 1.2, 2.9, 1.54, 1.64, and 14.56 times over-expressed, respectively, in the maternal parent compared with interspecific hybrids. Peptidase M16-like protein and the unknown putative protein were 0.54 and 0.32 times under-expressed in the maternal parent, respectively. No expression was shown for the PPR gene in interspecific hybrids. In addition, SPA, PPR, ELT, and ACCase gene expression was the most affected in interspecific hybrids during seed development, whereas PRP and cell wall hydrolase genes were the least affected. For the Hyd gene, the reduction of expression was minor compared with the parental samples.

Differential Expression of Selected ESTs During Seed Development by Real-Time PCR

In order to evaluate the differential expression of the subtracted genes during *Phaseolus* embryogenesis, the expression pattern of six selected genes related to the transcription factors and/or signal transduction and one gene with an unknown function was investigated using real-time PCR (Fig. 6). The pattern expression of the target gene transcripts was examined at different stages of common bean embryo development: 3, 6, 8, 9, and 12 DAP.

All the genes were differentially expressed during seed development in *P. vulgaris*. SPA was highly expressed during embryogenesis from the early globular stage (3 DAP) to the cotyledon stage (12 DAP), with the highest expression at 6, 8, and 9 DAP. The expression of the PRP gene and the sequence coding for the unknown hypothetical protein was higher in the early globular stage (3 DAP) and

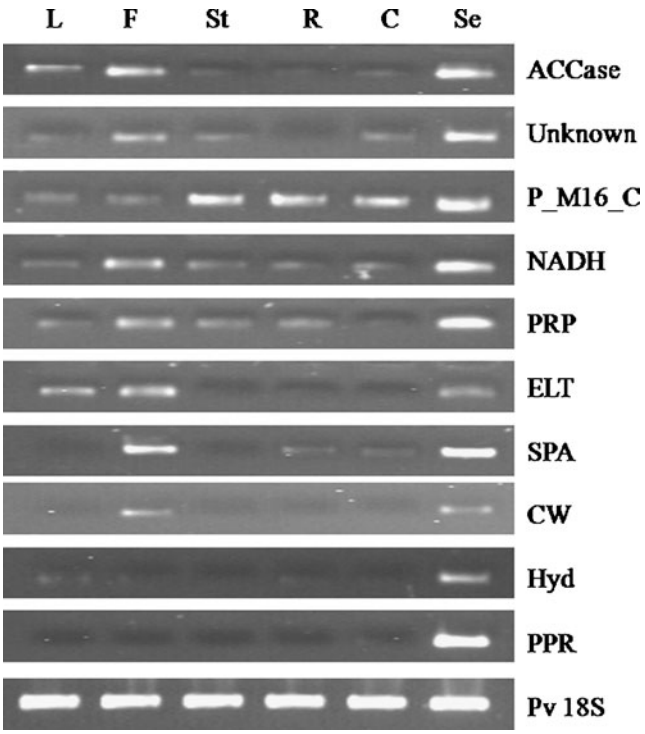

Fig. 4 RT-PCR of selected genes from common bean tissues: SPA (GW884166), NADH (GW884170), ELT (GW884171), PPR (GW884172), P_M16_C (GW884173), Hyd (GW884174), CW (GW884175), PRP (GW884176), Unknown hypothetical protein (GW884177) and ACCase (GW884169). Leaves (*L*), flowers (*F*), stems (*St*), roots (*R*), cotyledons (*C*), and seeds (*Se*). *Lower lanes*: common bean 18S internal control

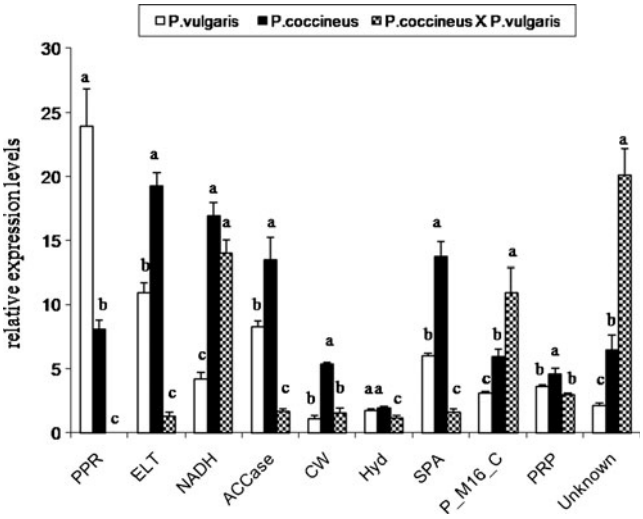

Fig. 5 Relative expression ratios of the transcripts of ten SSH candidates during seed development (six DAP) in *P. vulgaris*, *P. coccineus*, and interspecific hybrid (*P. coccineus*×*P. vulgaris*) genotypes. SPA (GW884166), NADH (GW884170), ELT (GW884171), PPR (GW884172), P_M16_C (GW884173), Hyd (GW884174), CW (GW884175), PRP (GW884176), Unknown (GW884177), and ACCase (GW884169). The relative mRNA levels of individual selected genes were normalized to the housekeeping gene 18S. Data shown represent mean values obtained from three independent amplification reactions and the *error bars* indicate standard deviation. Significant difference ($P<0.05$) detected by Tukey's multiple comparison test is shown by *different letters above the bars*

lower in other stages. ACCase and ELT showed specific expression during seed development in *P. vulgaris*. Their expression kinetic was wave-shaped, with an initial over-expression peak at about 6 DAP and the second highest peak at about 9 DAP (Fig. 6). RT-PCR analysis revealed, however, that PPR gene expression was at its maximum at 3 DAP, although at 6 DAP, expression decreased rapidly and then increased to reach high levels at a later stage (12 DAP).

Discussion

In the *Phaseolus* genus, early interspecific embryo abortion is attributed to several factors, such as nutritional barriers related to a deficient endosperm or suspensor development, endothelium proliferation and, to some extent, hypertrophy of the vascular elements (Geerts et al. 2002; Toussaint et al. 2004). However, little is known about the molecular process that alters interspecific embryo development and leads to seed abortion. The alteration in some genes involved in normal embryo development disrupts embryo formation and leads to seed abortion (Tzafrir et al. 2004). Transcripts of these genes can be localized in the embryo proper, in the endosperm, or in the maternal tissues around the embryo.

To gain insight into the molecular mechanism of embryo dysfunction from *Phaseolus* interspecific hybrids, we attempted to identify genes required in *Phaseolus* embryo development. The expression profiles of seed development in the *Phaseolus* interspecific hybrids *P. coccineus* (NI16) X *P. vulgaris* (NI637) and the maternal parent (*P. coccineus*) were compared using SSH analysis. Twelve differentially expressed transcripts were examined. Eight transcripts showed homology with previously described genes from plant species such as *P. coccineus*, *Glycine max*, *Arabidopsis thaliana*, and *Triticum aestivum*. Two transcripts showed great homology with functional protein in fungi species. Genes identified in this study were related to many aspects of plant cell metabolism, such as signal transduction, metabolism of amino acids, secondary metabolites, saccharides, and fatty acids. Mutation in these genes affects seed development and the embryogenesis process (Baud et al. 2003; Lara et al. 2003; Suda and Giorgini 2003; Schaller 2004; Saha et al. 2007). The low number of repeated annotated genes represented by single ESTs identified in this work indicates an efficient normalization of the libraries, as only the PPR and SPA genes were represented by more than four ESTs.

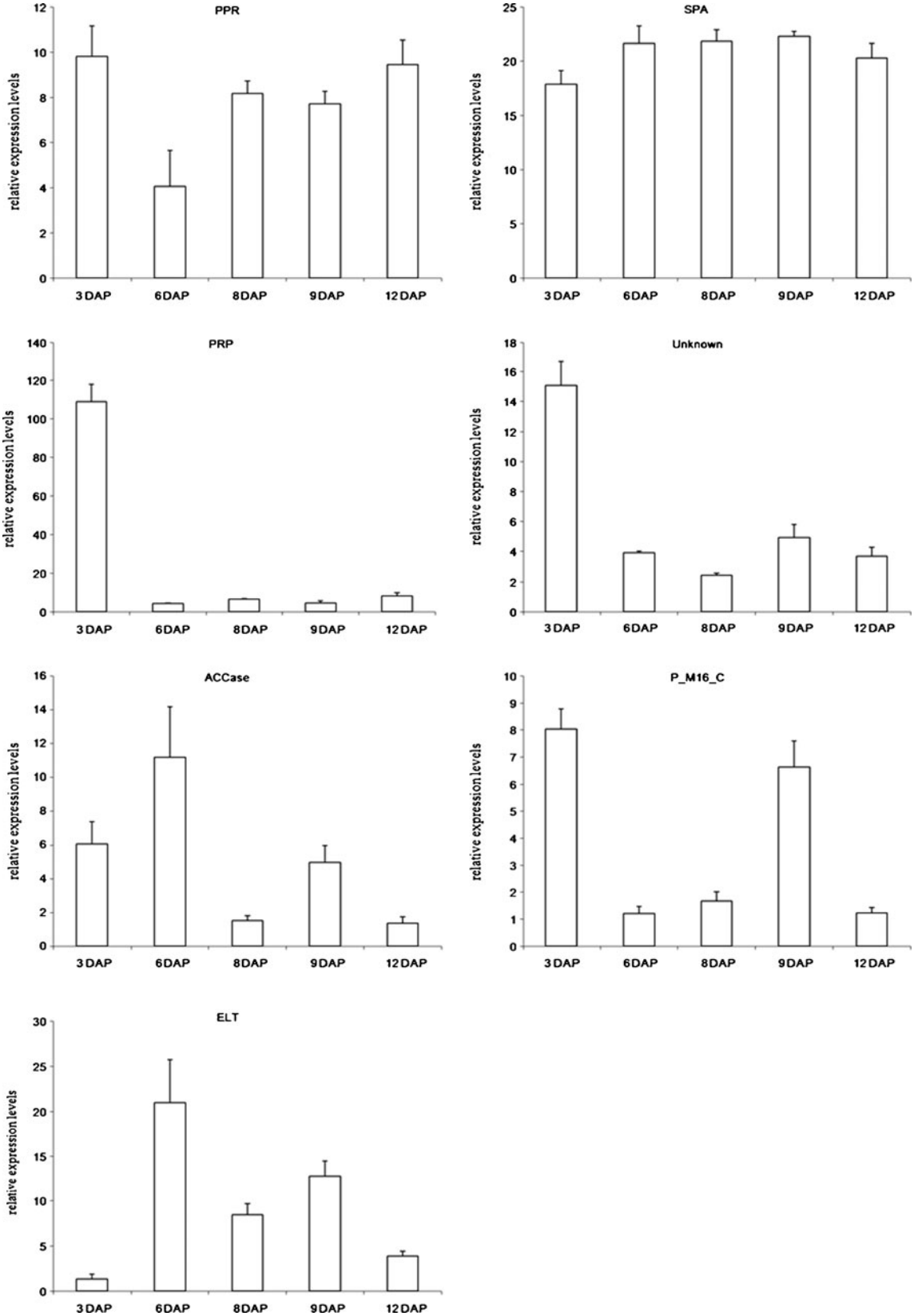

Fig. 6 Expression pattern of SPA (GW884166), PPR (GW884172), PRP (GW884176), ELT (GW884171), P_M16_C (GW884173), unknown hypothetical protein (GW884177), and ACCase (GW884169) during common bean seed development by real-time RT-PCR. The relative mRNA levels of individual selected genes are normalized with respect to the housekeeping gene Pv 18S in different tissues

Real-time PCR showed reduced expression in seven selected genes in interspecific hybrids compared with the maternal parent: this was particularly so for PPR, SPA, and ACCase. In addition, these genes are involved in normal embryo morphogenesis. ACCase catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate, which is used in the plastid for fatty acid synthesis and in the cytosol for various biosynthesis pathways, including fatty acid elongation (Konishi and Sassaki 1994). Baud et al. (2003) showed that ACCase 1 is essential for *A. thaliana* embryo development and plays a key role in apical development. Mutants lacking ACCase are embryonically lethal and showed alteration during the cell differentiation process (Baud et al. 2004). ACCase also appears to be a key factor in embryo development, although it encodes for a common and widespread enzyme of carbon metabolism.

Seed storage proteins (SSP) are specifically synthesized in developing seeds, in both the endosperm and the embryo in monocotyledons, whereas in dicotyledonous plants, the endosperm is commonly re-absorbed as maturation proceeds, and storage proteins are preferentially accumulated in the embryo (Lara et al. 2003). The bipartite endosperm box endosperm motif (EM), the GCN4-like motif (GLM), and the ACAA motif are important regulatory elements, often found in the promoters of endosperm and embryonic-specific genes, such as SSP genes (Müller and Knudsen 1993). These *cis*-elements are bound by transcription factors such as SPA, a seed-specific basic leucine zipper that activates transcription from the EM and GLM motif required for endosperm-specific gene expression (Lara et al. 2003; Albani et al. 1997). Other transcription factors regulating seed-specific expression have been well documented for some plant species, such as *Hordeum vulgare* (BLZ1 and BLZ2), *Oryza sativa* (RISBZ1 and RITA1), *Sorghum bicolor* (SBO2), and *A. thaliana* (AtbZIP). In *Zea mays*, OPAQUE2 (O2) has been shown to encode a basic domain /leucine zipper transcriptional activator that regulates a subset of zein storage protein genes (Schmidt et al. 1992). Mutation affecting endosperm storage protein in several cereal species is known to cause developmental disruptions, severely altering storage protein deposition and endosperm development (Boston et al. 1991).

PPR genes encode RNA-binding proteins that play a key role in post-transcriptional processes (including RNA editing, RNA splicing, RNA cleavage, and translation) within mitochondria and chloroplasts (Schmitz-Linneweber and Small 2008). In addition, several PPR proteins have been shown to act as fertility restorer genes in commercially important cytoplasmic male sterility systems (Bentollia et al. 2002). Interestingly, mutant PPR proteins exhibit defects in embryogenesis and the patterning of embryo morphology, resulting in embryo lethality (Cushing et al. 2005). These data suggest that some PPR genes play an essential role in plant embryo development. In *A. thaliana*, the loss-of-function phenotype of embryo-defective 175 (*emb175*) and CLUTAMINE-RICH PROTEIN23 (*grp23*) mutants altered in PPR expression produces embryonic growth arrests at a very early stage, before the globular to heart transition and at the 16-celled dermatogens state, respectively (Cushing et al. 2005; Ding et al. 2006). The mutation in *Arabidopsis AtPPR4*, a homolog of maize *PPR4*, causes embryo lethality (Schmitz-Linneweber et al. 2005). More recently, Gutierrez-Marcos et al. (2007) showed that *empty pericarp4* encodes a mitochondrion-target pentatricopeptide repeat protein required for maize seed development. Mutation in this gene confers a seed-lethal phenotype; the endosperm is severely impaired, with highly irregular differentiation of transfer cells in the nutrient-importing basal endosperm.

Eight represented genes in our libraries show homology with sequences from other plant species such as *A. thaliana* and *G. max*, encoding mitochondrial proteins such as NADH dehydrogenase, peptidase M16-like protein and putative ELT. Lurin et al. (2004) suggested that several mitochondrial mutant genes showed alteration in embryo development. These data suggests that mitochondria and plastids in general, are essential for providing energy to plant cells, particularly to the actively dividing cells of the embryo.

Another interesting result is the identification of a gene putatively encoding for basic PRP. PRP is considered to be a structural component of the cell wall, has been shown to be involved in many signaling pathways (Showalter 2001; Williamson 1994), and is reported to be essential for the formation of somatic embryos in *Cichorium intybus* (Chapman et al. 2000). A particular PRP from maize (*zmHyPRP*) is specifically expressed in embryos (José-Estanyol et al. 1992). Its expression is associated with early embryo stages, marking the initial steps of scutellum development, and it has been shown to be accumulated mostly during cell division.

Many of the genes mentioned above are similar to already known embryogenesis-related genes. In order to further confirm the expression pattern of those genes and to characterize their function so as to understand their role in the early stage of *Phaseolus* embryogenesis, six genes (PPR, SPA, PRP, ACCase, P_M16_C, and ELT) related to the transcription factors and/or signal transduction and one gene with unknown function were selected, and the expression profiles were investigated using real-time RT-PCR at different stages of seed development in *P. vulgaris*. Interestingly, RT-PCR analysis revealed that the expression of some genes, such as PPR and PRP, was clearly detected at early stages and decreased at later stages of seed development in *P. vulgaris*, suggesting a potential role of these selected genes at an early stage of common bean embryogenesis. Many of these genes were shown to be expressed in the zygotic embryos of other plant species. In

maize and *A. thaliana*, PPR is expressed in vegetative and reproductive tissues, at 6 and 12 DAP (Gutierrez-Marcos et al. 2007; Saha et al. 2007). Our data showed that PPR was expressed only in seed tissues at different developmental stages. This result suggests a possible key role of PPR during common bean embryogenesis.

In *A. thaliana*, ACCase transcripts were present in all tissues analyzed at various stages of seed development. The highest expression levels were observed at 6 DAP (Baud et al. 2003). These data are in agreement with our results. However, we revealed a low transcript of ACCase at 12 DAP. This expression pattern may be correlated with the accumulation of fatty acid at this stage of seed development in *P. vulgaris*.

In wheat, SPA, which is a member of the basic leucine zipper (bZIP) regulators of endosperm-specific gene expression such as seed storage protein (SSP), showed an early expression in the developing seed (Albani et al. 1997). In *Arabidopsis* AtZIP10 and AtZIP25, genes are expressed in roots, shoots, rosette leaves, and flowers, but intense signals are revealed particularly at the early and later stages of seed development. We showed that SPA is expressed in reproductive organs such as flowers, and is strongly up-regulated at different stages of seed development in *P. vulgaris*, suggesting that the SPA gene might play an important role during seed development in *P. vulgaris*.

Conclusion

In this study, SSH was performed to screen genes that were differentially expressed in abortive and normal *Phaseolus* seed development. The expression pattern of the genes identified in this study (SPA, PPR, PRP, ELT, P_M16_C, ACCase, and the gene encoding an unknown hypothetical protein) suggested that these genes could play a critical role during seed development in *P. vulgaris*.

In further experiments, which will be conducted to clarify whether the signals detected in real-time PCR were derived specifically from the embryo tissues or from other seed tissues, some of the genes revealed in our investigations, particularly SPA, PPR, and ACCase, will be used as probes to follow the spatial expression pattern during seed development in *P. vulgaris* using the in situ hybridization (ISH) technique for detecting gene expression at the cellular level (Bayer et al. 2009).

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