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ORIGINAL PAPER

In silico identification and characterization of putative differentially expressed genes involved in common bean (*Phaseolus vulgaris* L.) seed development

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Abstract Two genotypes of common bean (*Phaseolus vulgaris* L.) were studied to determine the structural cause of seed abortion in this species. In the non-abortive control (wild-type, cultivar BAT93), the histological analysis revealed a classical pattern of seed development and showed coordinated differentiation of the embryo proper, suspensor, endosperm tissue and seed coat. In contrast, the ethyl methanesulfonate (EMS) mutant (cultivar BAT93) showed disruption in the normal seed development leading to embryo abortion. Aborted embryos from these degenerate seeds showed abnormalities in suspensor and cotyledons at the globular, heart, torpedo and cotyledon stages. Exploring the feasibility of incorporating the available online bioinformatics databases, we identified 22 genes revealing high homology with genes involved in *Arabidopsis thaliana* embryo development and expressed in common bean immature seeds. The expression patterns of these genes were confirmed by RT–PCR. All genes were highly expressed in seed tissues. To study the expression profiles of isolated genes during *Phaseolus* embryogenesis, six selected genes were examined by quantitative RT–PCR analysis on the developing embryos of wild-type and EMS mutant plants. All selected genes were expressed differentially at different stages of embryo development. These results could help to improve understanding of the mechanism of common bean embryogenesis.

Keywords EMS mutant · ESTs · *Phaseolus vulgaris* · RT–PCR · Seed development

Abbreviations

DAP	Days after pollination
EMS	Ethyl methanesulfonate
EST	Expressed sequence tag
RT–PCR	Reverse transcription polymerase chain reaction

Introduction

Embryogenesis plays a central role in the life cycle of flowering plants. Early stages of plant embryo development are characterized by cell division and morphogenesis. After a double fertilization event, egg and sperm nuclei fuse to form the embryo. A second sperm nucleus fuses with the central cell nucleus, initiating the endosperm that supports embryo development (Jürgens 2003). A vast number of genes, such as homeobox genes, heat shock protein genes and lipid transfer protein genes that play a significant role in zygotic embryo development have been identified (Laux et al. 2004). Unquestionably, until now several other genes transcribed during the early and late stages of embryo development have not been identified or examined at the molecular level. Genetics tools provide an interesting approach to the study of plant embryo development by

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enabling genes with essential functions at this critical stage of the life cycle to be identified (Errampalli et al. 1991). Recent studies have focused on embryo mutants whose embryo development had stopped prior to the completion of the body pattern in order to determine the functional characteristics and the expression of genes that are essential for plant embryogenesis (Naway et al. 2008; Baster et al. 2009; Wu et al. 2009).

Expressed sequence tag (EST) analyses have been used extensively to discover novel genes and have proved to be a powerful and rapid way of identifying new genes that are preferentially expressed in embryo tissue during embryogenesis in plants such as *Brassica napus* (Tsuwamato and Takhata 2008), *Helianthus annuus* (Ben et al. 2005) and *Pinus radiata* (Bishop-Hurley et al. 2003). Differential gene expression, such as microarray technique, is a commonly used approach for interpreting gene expression profiles during plant and seed development (Dong et al. 2004; Liu et al. 2010; Zakizadeh et al. 2010).

Ramirez et al. (2005) showed that comparative analysis of common bean genomes with leguminous or non-leguminous species, such as *Arabidopsis thaliana* and rice (*Oryza sativa*), provides a valuable tool for in silico analysis of root nodule gene expression.

Genetic analyses have identified *Arabidopsis* genes that play essential roles during embryogenesis. Several mutations that affect the early stages of embryogenesis correspond to genes involved in the morphogenetic process that occurs during embryo development (Yamagishi et al. 2005). Genetic strategies used to identify regulators of plant embryo development, such as in the plant model *Arabidopsis thaliana*, therefore help to distinguish the gene classes that affect embryogenesis.

Over 2000 *Arabidopsis* mutants affected in different events during embryogenesis have been isolated by mutagenesis with ethyl methanesulfonate (EMS), by inserting T-DNA or by transposon tagging (Meinke and Sussex 1979; Jürgens et al. 1994; Kyjovska et al. 2003). Defective embryos represent the largest group of mutants examined in *Arabidopsis*. Many of these mutants have been described and characterized in some detail (Vidaure et al. 2007; Chandler 2008). Two complementary strategies, based on the phenotypic screening of immature siliques and germinating seedlings, were used to isolate mutants altered in the early phase of embryogenesis. Based on data from these two approaches, various estimates of the number of genes essential to seed development were made. Meinke and Sussex (1979) estimated the number of genes expressed in *Arabidopsis* embryogenesis to be about 500, whereas Jürgens et al. (1994) believed that 4,000 genes were essential for normal development of the embryo, from which about 40 genes were responsible for the establishment of the embryo morphological pattern.

Tzafrir et al. (2004) used both forward and reverse genetics as novel strategies to identify an initial dataset of 250 embryo genes required for normal embryo development in *Arabidopsis*.

The goal of this study was to explore the feasibility of incorporating the available online bioinformatic databases to discover common bean genes associated with regulatory and biological response pathways common to both embryogenesis and seed development. We focus here on 22 ESTs from common bean showing the highest homology to 22 known genes required for embryo development in *Arabidopsis thaliana* (Table 1). Disruption of all the genes selected could affect pattern embryo formation and then lead to embryo abortion in *Arabidopsis thaliana* (Tzafrir et al. 2004).

We describe the global analysis of the expression of these 22 candidate genes during common bean seed and plant development using semi-quantitative PCR. A comparative analysis of the expression of six selected genes using quantitative PCR in wild-type and EMS mutant plants defective in seed development was performed in order to highlight the putative role of these genes in common bean seed development.

Materials and methods

Plant materials

The common bean wild-type and EMS mutant plants were derived from genotype BAT93 of *Phaseolus vulgaris*. Seeds of BAT93 were mutagenized with EMS (Silué et al. 2006); M1 plants were obtained and the seeds were harvested and grown (M2 plants). From the M1 material, one mutant plant (identified 522) was selected and analysed further. Thirty three M2 seeds from M1 plant (522) were collected individually, grown and selfed; the seeds from M2 plants were collected. Among these 33 putative mutants, 24 were identified as heritable mutants: seven were homozygote (producing defective seeds), and 17 were heterozygote (producing an intermediate phenotype). The nine remaining putative mutants were not inherited (siblings were wild-type).

Seeds from the wild-type and EMS mutant were surface-sterilized in 10% sodium hypochlorite solution for 10 min, and germinated on moist sterile filter paper. Four-day-old seedlings were transferred to sterilized compost in individual pots and placed in growth chambers supplied with artificial light at 580 $\mu E\ m^{-2}\ s^{-1}$ light intensity, 27°C/23°C

Table 1 *Phaseolus vulgaris* ESTs showing homology with *Arabidopsis thaliana* genes required for normal embryo development

Arabidopsis thaliana accession number	Name	Putative function	*Phaseolus vulgaris* EST accession number	Nucleic acid identity	E-value
At4g02980	ABP1	Auxin binding protein involved in cell elongation and cell division	CV529696	(373/499) 74%	2.00E-92
At3g11940	AML1	Encodes ribosomal protein S5	EY457929	(401/485) 82%	2.00E-74
At3g60830	ARP7	Encodes an actin-related protein	FE711613	(274/351) 79%	5.00E-81
At4g02570	AXR6	Component of SCF ubiquitin ligase complexes involved in mediating responses to auxin	FE695779	(530/670) 78%	5.00E-56
At2g43360	BIO2	Catalyzes the conversion of dethiobiotin to biotin	FE705317	(534/664) 81%	5.00E-179
At4g11150	EMB2448	Encodes a vacuolar H+-ATPase subunit E isoform 1 which is required for Golgi organization and vacuole function in embryogenes	EX303864	(474/662) 72%	7.00E-99
At5g27540	EMB2473	Encodes a protein with similarity to GTPases Involved in embryogenesis, pollen tube growth and required for mitochondrial development	FE711702	(340/460) 73%	3.00E-80
At5g18580	FSI	Encodes a protein similar in its C-terminal part to B' regulatory subunits of type 2A protein phosphatases	FE707962	(644/788) 81%	0.0
At2g30410	KIS	Encodes tubulin folding cofactor A	CV540440	(198/259) 77%	5.00E-53
At1g67490	KNF	Encodes an alpha-glucosidase I enzyme that catalyzes the first step in N-linked glycan processing	CV543073	(339/478) 72%	2.00E-65
AY138461	L1L	Essential regulator of embryo development, defines a unique class of plant HAP3 subunits	CV538670	(239/296) 81%	2.00E-78
At5g10480	PAS2	Protein tyrosine phosphatase-like involved in cell division and differentiation	CV530165	(492/694) 72%	6.00E-101
At5g07500	PEI	Encodes an transcription factor required for heart-stage embryo formation	EX303834	(184/259) 71%	1.00E-31
At1g71440	PFI	Encodes tubulin-folding cofactor E	FE701414	(391/567) 69%	8.00E-57
At2g28000	SLP	Encodes chaperonin-60 alpha, a molecular chaperone involved in Rubisco folding	FE711584	(596/744) 80%	3.00E-78
At2g18390	TTN5	Encodes a member of ARF-like GTPase family	CB542010	(322/397) 81%	2.00E-47
At3g20630	TTN6	Encodes a ubiquitin-specific protease	CB542426	(417/588) 70%	4.00E-80
At2g27170	TTN7	Protein required in cohesin complex	CV541843	(338/436) 77%	2.00E-98
At5g16750	TOZ	Encodes a nucleolar localized WD-40 repeat protein that is preferentially expressed in dividing cells and is required for regulated division planes and embryo development	CV533581	(211/280) 75%	3.00E-53
At1g14610	TWN2	Required for proper proliferation of basal cells	CV540202	(424/569) 74%	1.00E-100
At1g78580	TPS1	Encodes an enzyme putatively involved in trehalose biosynthesis	CB541509	(298/356) 83%	1.00E-109
At1g63700	YDA	Member of MEKK subfamily	FE709393	(188/250) 76%	1.00E-44

(day/night) temperature, 75% relative humidity and 12 h photoperiod. The plants were watered once every 3 days.

To harvest seeds of defined age, individual flowers were tagged with threads on the day of flower opening. Mutant EMS and common bean seeds were isolated from the pods at 3, 6, 8, 9 and 12 days after pollination (DAP). For histological studies, the seeds were immediately immersed in fixation solution. For RNA extraction, the seeds were immediately frozen in liquid nitrogen and then stored at −80°C.

Histological studies

Seeds from both the wild-type and EMS-induced mutant were freshly harvested at 3, 6, 8, 9, and 12 DAP and fixed in 4% (w/v) formaldehyde (freshly made from paraformaldehyde) and 1.2% glutaraldehyde in phosphate buffer (7.4 g NaH2PO4 2H2O; 8.4 g Na2HPO4 2H2 O) for 24 h at 4°C, rinsed in phosphate buffer (PH 6.6), dehydrated in a graded ethanol series (30, 50, 70, 90, 95, 100% (v/v) and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH) for 48 h.

Sections were cut at 3 μm on a Zeiss HM 360 microtome fitted with a tungsten-carbide knife, transferred to poly-L-lysine-treated slides (Sigma), and dried overnight at 40°C. Staining was performed with Toluidine blue O, as described by Gutmann (1995), and viewed with a Nikon Eclipse E800 fluorescence microscope (Japan). Pictures were taken with a JVC 3-CCD color video camera and images were handled with the Sony image Archive Plus program.

Database searches

The *Arabidopsis* sequenced genes published by Tzafrir et al. (2004) were retrieved using the lasted version of The *Arabidopsis* Information Resource (TAIR10) database (http://www.arabidopsis.org). These sequences were used as queries against the Genome Data-base (http://www.plantgdb.org). BLASTn searches were carried out to identify homologous *Phaseolus vulgaris* ESTs with a cutoff value of e^{-4}.

RNA extraction and RT–PCR

Organ-specific expression of wild-type common bean was analyzed for leaves (2-week-old plants), stems (2-week-old plants), roots (2-week-old plants), and cotyledons (3-day-old seedlings), as well as flowers (0 DAP) and seeds (15 DAP) from 7-week-old plants. Samples were ground in liquid nitrogen, and total RNA was isolated by the Trizol reagent (Invitrogen), following the manufacturer's protocol.

For RT–PCR, 10 μg of total RNA was treated with 10 U of DNaseI (Fermentas) in DNAseI assay buffer for 30 min at 37°C. Five microgram of total RNA was reverse transcribed using the Revert Aid^{TM} H Minus M-MuLV RT and Random hexamer primers (Fermentas) at 42°C for 1 h according to the manufacturer's instruction. Fragments were amplified from these cDNA pools using *Taq* DNA polymerase (Biolab's), following the manufacturer's procedure. An amplification of 18S was used as an internal control.

Primer pairs were designed by Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://www.frodo.wi.mit.edu/primer3/).

PCR amplification was performed for all genes using the following program: 3 min at 94°C, 30 s at 94°C, 45 s at 55–60°C (depending on the primers used, see Table 2) and 1 min at 72°C. An elongation step at 72°C for 5 min was conducted for the last cycle. ABP1, FSI, L1L, TPS and Pv 18S were amplified for a total of 25 cycles. ARP7, PFI, AML1, AUXR6, KIS, TTN6, TOZ and SLP were amplified under the same conditions for a total of 30 cycles. TTN5, TTN7, TWN2, YDA, BIO2, KNF, PAS2, PEI, EMB 2473 and EMB 2448 were amplified under the same RT–PCR conditions for a total of 34 cycles.

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 a 1% (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer.

Real-time RT–PCR analysis

Samples from the wild-type and EMS mutant of cultivar BAT93 were collected at 3, 6, 8, 9 and 12 DAP. Plant materials were grown in a glasshouse in pots.

Total RNA isolated from seed tissues was treated with DNAse I (Fermentas) to remove potential genomic DNA contamination. Five micrograms of DNA-free total RNA was then used to synthesize first-strand cDNAs using an oligo-p$(dT)_{15}$ primer and AMV reverse transcriptase (Roche), following the manufacturer's protocol.

Specific primers were designed using Primer3 (Rozen and Skaletsky 2000). PFI, (F5′-TCCCTGAGCATCTTTCCAGT-3′; R5′-TTGAATGAAGGGATGGTGGT-3′); PEI, (F5′-CAGACTCCGATGTTCCGTCT-3′; R5′-ACACTCGGTCCAGTCATGTG-3′); AML1, (F5′-TTCATCAGCCAAGCATTCAG-3′; R5′-AATTGGATCTGCTGGAGTGG-3′); L1L, (F5′-AGGCAGGTAGCAGGAGACAA-3′; R5′-ATCTTGGCATGTGGAGGAAG-3′); TOZ, (F5′-AGATAAGGTTTGGGCCTTGG-3′; R5′-TTTGTCAGCTGCAGTGGAAT-3′) and TTN6, (F5′-TAGCTGCCAGTCATCCTGAA-3′; R5′-TTTCCCAGCATTAGCTCGTT-3′) were used in PCR to generate fragments of 200 bp. Real-time PCR reactions were performed using the ABI7500 FAST real-time PCR instrument (Applied Biosystems). As a control, the housekeeping gene Pv 18S was amplified (F5′-TCGAAGCGATCTTTTCGTAGA-3′; R5′-TTCTCAGTCGACTCGCTTTTT-3′). PCR was conducted in 20 μl of reaction volume containing 10 μl syber-green mix (Fermentas), 0.1 μM of each primer and 4 μl of cDNA template under the following conditions: 94°C for 3 min, and 40 cycles of 94°C for 30 s, 59°C for 45 s, and 70°C for 1 min. Real-time PCR reactions were carried out in triplicate and the results were analyzed following the manufacturer's advice (Applied Biosystems). Methods determining the relative gene expression levels were as described by Livak and Schmittgen (2001).

Results

Histological studies of the embryo development of *Phaseolus vulgaris* EMS mutagenized plants deficient in seed development

In order to characterize *Phaseolus* plants deficient in seed development, we isolated seven plants showing homozygous failure in seed development from the second

Table 2 Primers used for RT–PCR analysis

GenBank (db EST) accession number	Forward primer 5′-3′	Reverse primer 5′-3′	Tm (°C)
CV529696	F-GCACTCGTTCTGGCTTCTTC	R-TGACCAGTCCTCGTATGCAA	57
EY457929	F-CCAGAGAAGAGCATTGCACA	R-CCACTGTGTGCCACCAATAC	59
FE711613	F-GACCGATAACCCAACATTCG	R-TGTGGTTAACAGCACCCTCA	55
FE695779	F-GAGTACCAAAAAGGCGGAGA	R-TGACAAGGAGTGGAGCAGTC	55
FE705317	F-GCTGCATGGAGGGATACACT	R-GAGCTTTTGGGAGAAGTCCA	55
EX303864	F-GGTGAGGTGGTGTGGGTTAG	R-ACCCCACCAGACCAGTACAT	59
FE711702	F-TATTGAAAAGGGGCGTCTTG	R-CCAATCCAAGGACTTTCAGG	56
FE707962	F-GAGTCCAGAGATTGGCGAAG	R-GATGCAAGCTTTTCCTCGTC	55
CV540440	F-CAACACAGTGTGCAACACCA	R-GAGAAGAGGGCTCATGCAAC	57
CV543073	F-GAAAGAAGTGGAGGCAGGAA	R-CTTACTGCCTTGGTGGCACT	60
CV538670	F-CTGTCACCATGGAAAGTGGA	R-GCGAGCATTCGGTTCAGTAT	55
CV530165	F-TAACCCTCTTCGTTGGATGG	R-GTTGGGCATCCTTATGCAGT	57
EX303834	F-TCCTCACCGGAGATGTTTTC	R-GGCAGAACCCTTAGTTGCTC	55
FE701414	F-CTGCCCTGAGGACAATCAAT	R-CAGGACTAGTATGCAACCTGGA	60
FE898061	F-GAGGATTCCTGGTCTTTGGA	R-TAGCAAGGCGGTCTTCTTGT	55
CV533581	F-AGATAAGGTTTGGGCCTTGG	R-ATGATCTTCAGCCGCTCTCT	55
CV540202	F-TGCACTGGTCTCATACACAGC	R-CACAGTAACCGCCTGTGTTC	57
FE705839	F-TCAAGCGGAAAGAGAAGGAA	R-GCTTCCAAGTTCAGCACCTT	60
CB542426	F-AGCTGCCAGTCATCCTGAAT	R-GTACCCATCCTGCCTCCATA	57
CV541843	F-CTTCGAAGAGTGCCCAGTGT	R-ATCCGGAGGGGAGAGTAGAA	59
CB541509	F-GAGGATTCCTGGTCTTTGGA	R-TAGCAAGGCGGTCTTCTTGT	55
FE709393	F-GGACTACACAAGGGGCAAAA	R-CCCTATTTGCATTCCTCGAA	55

generation of an EMS-mutagenized M1 plant (522) of *Phaseolus vulgaris* L. (Silué et al. 2006). We then characterized these selected plants by comparing their embryo development pattern to the *Phaseolus* wild-type embryo during seed development.

In the wild-type plants, the pattern of seed development is similar to many flowering plants. In contrast, the EMS mutant showed disruption in normal seed development, leading to embryo abortion. Mutant embryo-defective seeds are readily distinguishable as shriveled and brown seeds from the wild-type green seeds in immature pods (Fig. 1).

The average fresh weight of the seeds is shown in Figure 2. During the first week after fertilization, both abortive (EMS mutant) and control (wild-type) seeds showed similar weights. At 12 DAP, the fresh weight of control seeds increased twofold (Fig. 2). At 30 DAP, the maximum fresh weight was attained for the wild-type, with an average weight of 488 mg seed^{-1}. For abortive seeds the maximum fresh weight (32 mg seed^{-1}) was obtained at 15 DAP (Fig. 2).

To determine at what stage of embryogenesis the EMS mutant phenotype was first observed, a developmental series of EMS mutant embryo sections was obtained and compared with those of wild-type embryos. The embryogenesis of a normal and mutant embryo viewed with Nomarski optics is shown in chronological order in Fig. 3. Development of the EMS mutant embryos was considerably delayed compared with wild-type embryos.

In the wild-type, the embryo began to develop shortly after fertilization. At about 3 DAP, the embryo reached the early globular stage of development (Fig. 3a). The cells of the embryo proper had dense cytoplasm. The suspensor attached directly to the inner surface of the inner integument. The cells of the suspensor were more vacuolated than the cells of the embryo proper. At about 8 DAP, the embryo reached the heart-shaped stage with the formation of cotyledons (Fig. 3e). The cotyledons expanded rapidly by vacuolation and gradually extended towards the chalazal end of the seed (Fig. 3g). About 12 DAP, the cotyledons continued to expand with a well-defined embryo axis (Fig. 3i). Both the root and the shoot apical meristems were well defined. At this stage, the embryo grew rapidly; it occupied most of the endosperm cavity and storage products began to accumulate within embryo cells.

In abortive genotypes (EMS mutant), the first stage of seed development was similar to the control genotype. At 3 DAP, embryos were at the early globular stage (Fig. 3b), and their tissue organization appeared to be normal. After this stage, embryo development was delayed or arrested. At 8 DAP, EMS mutant embryos did not exhibit the characteristic

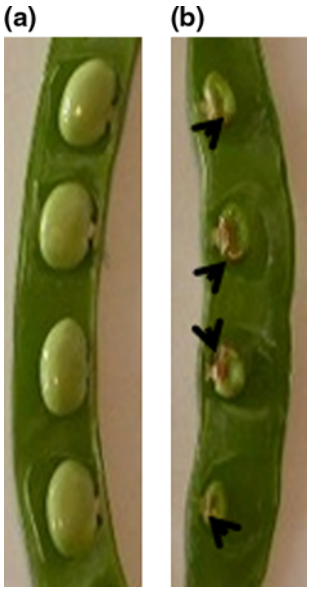

Fig. 1 Phenotypical analysis of wild-type and EMS mutant seeds of *Phaseolus vulgaris* (15 DAP). **a** Immature pod obtained by self-pollination of a wild-type plant. **b** Immature pod obtained by self-pollination of EMS mutant

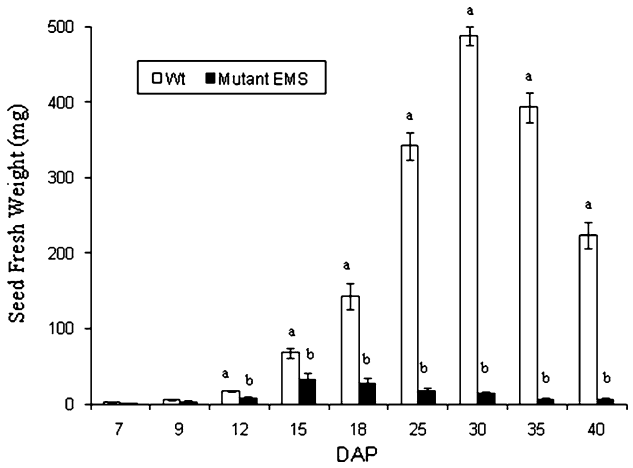

Fig. 2 Fresh weight accumulation of wild-type and EMS mutant seeds. Data shown represent mean values obtained from three different plants and the *errors bars* indicate standard deviation. Significant difference ($P < 0.05$) detected by Tukey's multiple comparison test is shown by *different letters above the bars*

heart shape and appeared elongated (Fig. 3f) by the time the wild-type embryo had become heart-shaped (Fig. 3e).

Mutant embryos appeared wider than wild-type embryos and were defective at the heart and later stages, with abnormalities in cell morphology. Unlike the torpedo shape observed for wild-type embryos (Fig. 3g), EMS mutant embryos displayed a heart-like shape (Fig. 3h). The characteristic wild-type bent-cotyledon or mature embryo stages were not observed in mutant EMS.

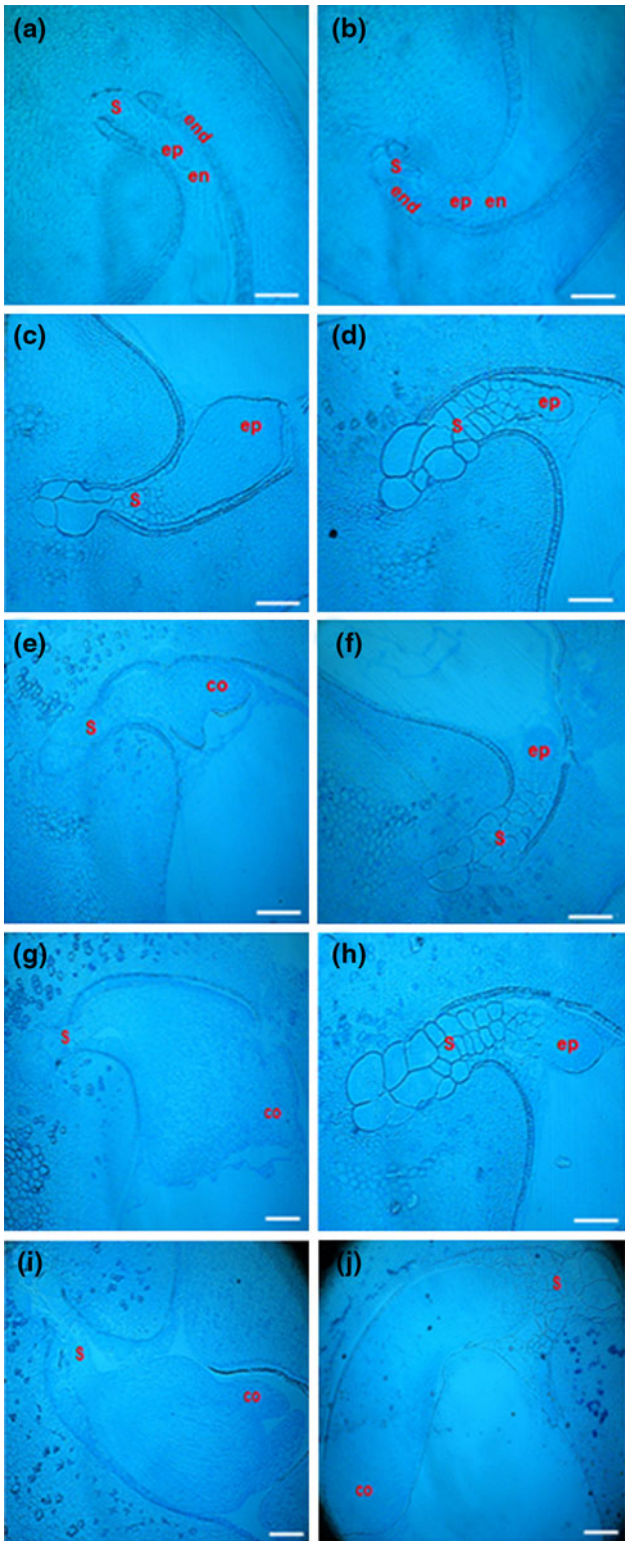

Fig. 3 Embryo development of EMS mutant in comparison to wild-type common bean (*wild-type* **a**, **c**, **e**, **g**, **i**; *EMS mutant* **b**, **d**, **f**, **h**, **j**). Embryos reached the early globular stage at 3 DAP (**a**, **b**), the globular stage at 6 DAP (**c**, **d**), the heart stage at 8 DAP (**e**, **f**), the torpedo stage at 9 DAP (**g**, **h**), and the bent cotyledon stage at 12 DAP (**i**, **j**). *Co* cotyledon; *en* endosperm; *end* endothelium; *ep* embryo proper; *s* suspensor. *Scale bars* 100 μm

In all embryo stage development, the suspensor appeared to develop but showed dramatic evolution. At the early globular stage, the embryo contained a morphologically normal suspensor and embryo (Fig. 3b). Abnormal divisions in the suspensor first appeared at the globular stage (Fig. 3d) and continued during subsequent growth (Fig. 3f, h, j). Cell divisions continued in both embryo and suspensor through to the torpedo stage, resulting in an elongated embryo and a suspensor often being the size of the embryo (Fig. 3f, h). At 12 DAP (Fig. 3j), the embryo formed elongated small cotyledons, but did not occupy the whole seed as in the wild-type samples.

Expression of selected ESTs in different organs

An in silico analysis was performed in order to identify genes involved in *Phaseolus* embryo development and specifically expressed in immature seeds. A total of 250 genes required for normal embryo development in *Arabidopsis thaliana* were used as an initial dataset (Tzafrir et al. 2004). Using the BLASTn program, the coding regions of the different genes from *Arabidopsis* were used as queries to search the EST sequences available from *Phaseolus* for homology. A total of 22 ESTs expressed in *Phaseolus* showed significant homology with their homolog in *Arabidopsis thaliana* (Table 1). Specific primers were designed for each of the 22 selected ESTs (Table 2) and the transcript expression in various tissues was analyzed by RT–PCR (Fig. 4). All 22 selected ESTs were found to be expressed in *Phaseolus vulgaris* with different but overlapping expression patterns, confirming that all genes are active in common bean tissues.

The expression level of EST *Phaseolus vulgaris* ABP1, FSI, L1L and TPS is relatively high in this species. The exponential stage for these genes and internal control Pv18S was detected from about 25 cycles (Fig. 4). The signals of other selected ESTs are lower in this species and were detected from 30 to 34 cycles. Most *Phaseolus vulgaris* ESTs had a higher expression in seeds at 15 DAP compared with other tested tissues. The expression profiles revealed that the seed, flower, cotyledon and leaf tissues exhibited a higher expression of common bean EST than stem and root tissues. AML1, L1L and PAS2 were highly expressed only in seeds at 15 DAP. Transcripts from PFI and TWN2 were more readily detected in seeds and leaves than in other tissues. SLP, TOZ and ARP7 transcripts were detected in all tissues except stems. Interestingly, the expression of ABP1, SLP, KIS and TPS was highly detected in seed and cotyledon tissues, suggesting a possible role of these genes in embryo organogenesis. YDA, PEI, L1L and PAS2 transcripts were not detected in leaf tissues. AUXR6, BIO2, EMB2448, KNF and TWN2 transcripts were detected in seed, leaf and floral tissues, but not in stems and roots or cotyledons. ARP7, TOZ and TTN5 transcripts were detected in all tested tissues except stems. Transcripts were not detected in stem or cotyledon tissues for EMB2473 or the FSI gene.

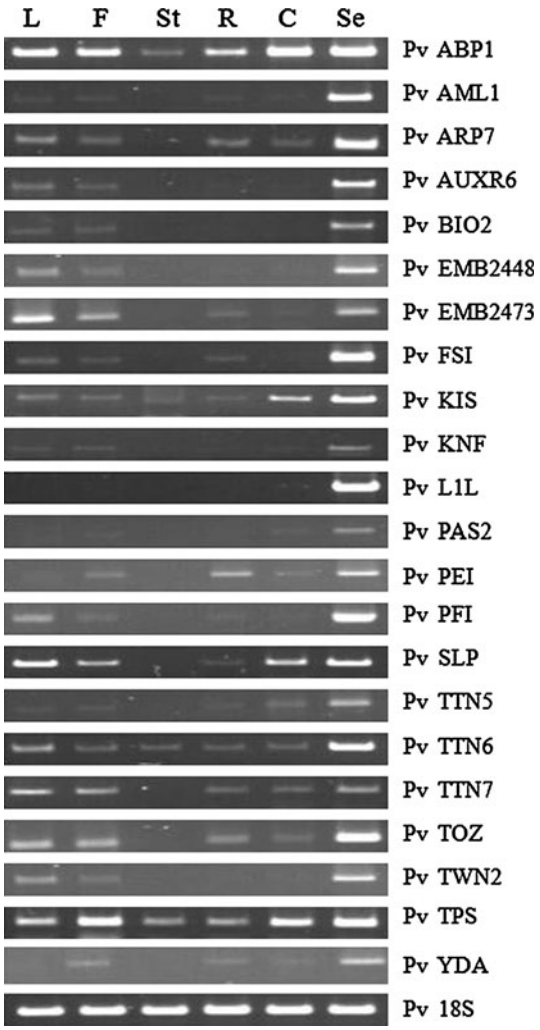

Fig. 4 Expression patterns of selected genes in various tissues by RT–PCR. RT–PCR products using gene-specific oligonucleotide primers to assess expression levels of Pv ESTs and Pv 18S in tissues from *Phaseolus vulgaris* were analyzed on 1% agarose gel. Various numbers of PCR cycles were tested, as described in "Materials and methods". *L* leaves; *F* flowers; *St* stems; *R* roots; *C* cotyledons; *Se* seeds

TPS, KIS, TTN6 and ABP1 transcripts were detected in all tissues, although at different levels. Transcripts corresponding to all selected genes were abundant in seeds, but were low, scarce or undetectable in other tissues (Fig. 4).

Real-time RT–PCR pattern analysis of the differential expression genes during seed development

Six ESTs which are related to transcription factors and/or signal transduction and highly expressed in common bean

seed tissues compared with other tissues (L1L, PEI, TOZ, AML1, PFI, and TTN6) were selected for further quantitative real-time RT–PCR analysis in order to evaluate the expression profile of these genes during common bean embryogenesis. In order to investigate if these six genes could be involved in *Phaseolus* embryogenesis, their expressions in wild-type *Phaseolus vulgaris* and the EMS mutant were compared from 3 to 12 DAP (Fig. 5).

Low relative transcript levels were shown for TTN6, PEI and PFI genes in various stages of seed development compared with AML1, TOZ and L1L. The transcript level of all genes studied changed more than twice during the time course. In general, the corresponding transcripts for selected genes showed diverse temporal expression patterns throughout seed development. In EMS mutant, the expression of TOZ, TTN6, AML1, PFI and L1L followed a bell-shaped pattern that increased through the early developmental stages, peaked at 8 DAP, and then decreased. In the wild-type, TTN6, L1L and PFI genes were characterized by a distinctly different profile with maximum expression later in development (12 DAP). In contrast, AML1 showed high expression at 3 DAP and low expression for other stages. All genes showed lower expression at 6 DAP. Our analysis also revealed that in EMS mutant TOZ, L1L, TTN6, PFI and AML1 genes showed maximum expression at 8 DAP, whereas for PEI it was 12 DAP.

The expression pattern of all tested genes was affected in EMS mutant compared with the wild-type during seed development, particularly at later stages (9 and 12 DAP) for TOZ, L1L, TTN6 and PFI. Alteration in expression occurred at an early stage (3 DAP) for all selected genes, but particularly for the AML1 gene, with a 350-fold reduction in expression.

Discussion

The *Arabidopsis* embryo-defective mutant approach is particularly useful for identifying important genes involved in embryo development and for understanding the function of expressed genes and defining their role in developmental processes (Tzafrir et al. 2003). Some embryo-defective mutants are blocked at a particular stage of the process necessary for embryo viability and development; other mutants have defects in important developmental regulators or in simple metabolism functions.

Embryo-defective mutations can be induced by physical, biological and chemical means such as EMS mutagenesis, which is a standard technique used to induce point mutation in DNA. EMS mutations can be difficult to interpret, but they can also be informative because they can reveal common genetic components of seemingly unrelated developmental processes and can indicate multiple roles for a gene in development (Vernon et al. 2001).

In order to study the mechanism of *Phaseolus* embryogenesis by identifying and characterizing the genes involved in embryogenesis, we obtained mutant plants producing degenerate seeds at different stages in seed development (Silué et al. 2006) using EMS mutagenesis.

We first characterized these selected mutant plants by comparing their embryo pattern with *Phaseolus* wild-type embryos during seed development. In an abortive genotype, embryo development progresses normally until the globular stage. After this stage, embryo development is delayed or arrested. The marked difference in embryo development between the control and the abortive genotype is probably caused by the differences observed in suspensor development. Abnormality of suspensor development is one of the main causes of embryo abortion in flowering plants (Schwartz et al. 1994). The suspensor is the first differentiated structure produced by the developing embryo (Yeung and Sussex 1979). More recent evidence suggests that the suspensor plays an active role in supporting early development of the embryo proper by providing nutrients and growth regulators (Laux and Jürgens 1997; Rademacher and Weijers 2007). Interestingly, Liu et al. (1993) suggested that the suspensor and embryo proper constituted an attractive system for identifying molecular mechanisms that mediate developmental interactions during early embryogenesis.

Our data indicate that the EMS mutant embryos develop normally but more slowly than wild-type embryos during the early stages of seed development and fail to activate a wide range of embryo-specific programs after the heart stage. In general, EMS mutant embryos showed a suspensor hypertrophy, resulting in a smaller and delayed embryo proper, compared with the wild-type embryos (Fig. 3). During the last stages of seed development in the wild-type samples, the suspensor size started to decline after 9 DAP, until it disappeared completely. In contrast, in EMS mutant, the suspensor continued to growth after 9 DAP (Fig. 3). These observations support the hypothesis that the continued growth of the suspensor in the wild-type samples is inhibited by the embryo proper during the final stages during normal development (Yeung and Meinke 1993). In EMS mutant, the embryo proper lost the capacity to inhibit the suspensor growth. The latter continued to grow and contained longer cells in the basal part than in the wild-type samples. In the wild-type common bean, the embryo proper became longer than the suspensor at 6 DAP (Fig. 3c), whereas in the EMS mutant this characteristic was not evident. Our observations suggest that EMS mutant disruption of development in the embryo proper can lead to suspensor disruption and partial transformation of the embryo.

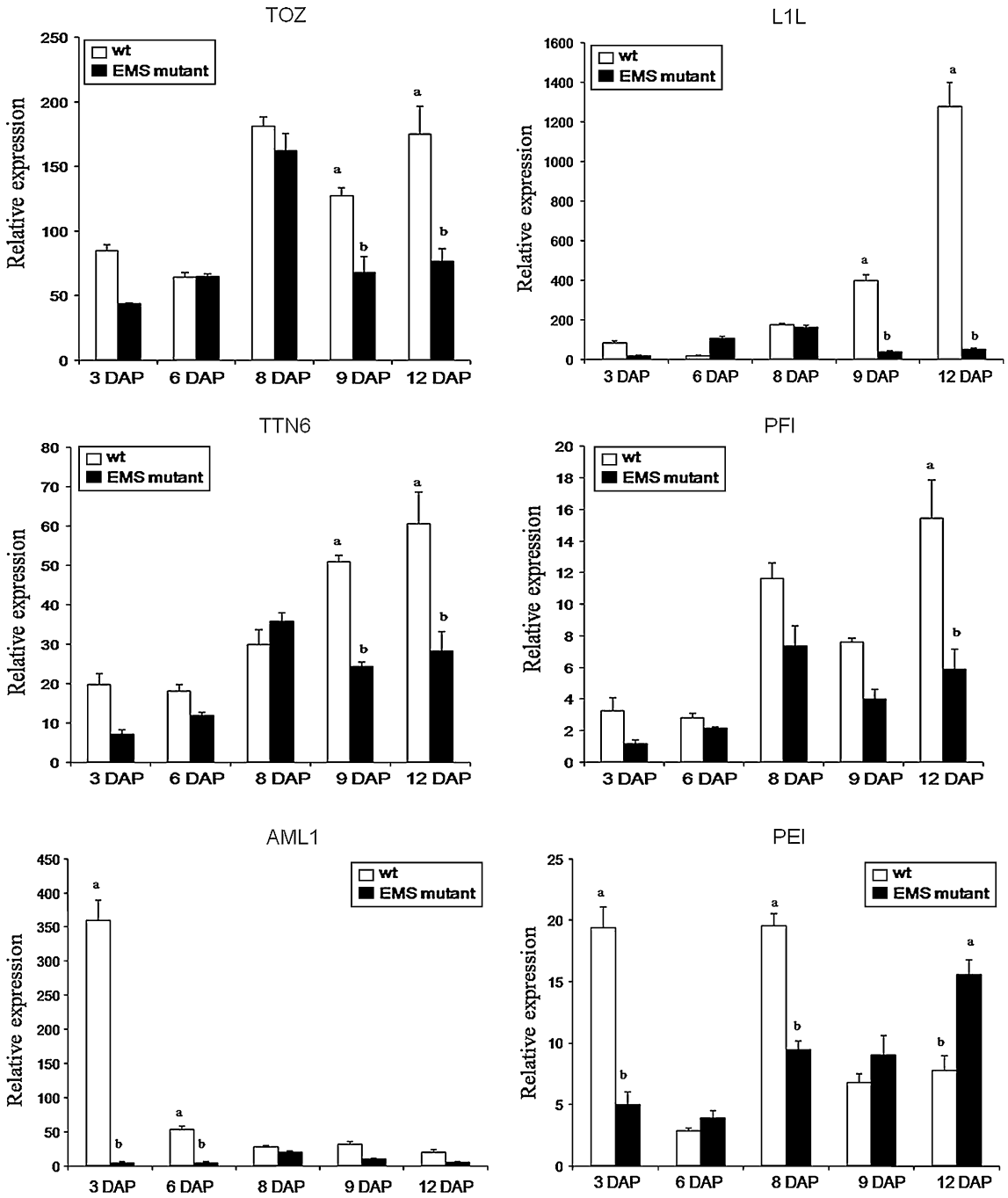

Fig. 5 Real time RT–PCR measurements of TOZ, L1L, PFI, AML1, TTN6 and PEI expression in developing wild-type and EMS mutant seeds. The relative mRNA levels of individual genes were normalized with reference to the housekeeping gene 18S. Data shown represent mean values obtained from three independent amplification reactions and the *errors bars* indicate standard deviation. Significant difference ($P \leq 0.05$) detected by Tukey's multiple comparison test is shown by *different letters above the bars*

The altered pattern of suspensor development in the EMS mutant might have further shifted the equilibrium of nutriment competition in favor of the maternal tissues. Suspensor abnormalities could be a direct result of reduced ability to transfer nutrients from the maternal tissue. The synthesis and accumulation of nutrients and storage protein bodies in the maternal tissue layers could result in the failure to translocate nutrients to the developing embryo, leading to embryo abortion.

Identical results were obtained in abnormal suspensor (*sus*) mutants (Schwartz et al. 1994), and raspberry (*rsy*) mutants (Apuya et al. 2002) of *Arabidopsis thaliana*.

The objective of the second stage of this work was to identify genes whose expression is specific in *Phaseolus*

immature seeds. Despite the importance of common bean as a food crop legume, very little EST information is publicly available (Graham et al. 2004). Only 575 ESTs from common bean and 20120 ESTs from the related species, runner bean (*Phaseolus coccineus*), have been deposited in the GenBank EST database (Ramirez et al. 2005).

We used in silico analysis to search these publicly available *Phaseolus* ESTs with *Arabidopsis* sequences known to be involved in seed development. Twenty-two ESTs showing significant homology with the *Arabidopsis thaliana* genes were selected (Table 1). The morphological defects associated with mutations in each of the 22 corresponding genes had been described and their expression during zygotic embryo development had been analyzed in *Arabidopsis* and other species, but not reported for common bean.

Arabidopsis LEAFY COTYLEDON1-LIKE (L1L) gene is a central regulator of embryogenesis that plays key roles in processes occurring in both the early and late phases. L1L is a member of the CCAAT binding factor (CBF) class of eukaryotic transcriptional activators (Alemanno et al. 2008). It is also required for normal embryo development (Kwong et al. 2003). *l1l* RNAi mutants provoked the arrest of embryo development at different stages with a range of morphological phenotypes (Kwong et al. 2003). Some embryos arrested at the globular stage. Other mutants arrested at later embryonic stages and had reduced cotyledons. The expression of L1L was strongly detected in the protoderm, confirming the role of L1L genes in coordinating primary events leading to embryonic competence (Harada 2001; Kwong et al. 2003) and their involvement in establishing a cellular environment that promotes embryo development (Lotan et al. 1998). AXR6 is involved in auxin response during early seed development. Mutants *axr6* show dramatically aberrant patterns of cell division, leading to defects in the cells of the suspensor, root and hypocotyl precursors and vascular pattern in cotyledons (Hobbie et al. 2000). The ABP1 gene encodes an auxin receptor mediating auxin-regulated cell expansion and is required for organized cell elongation and division in *Arabidopsis* embryogenesis. Mutants *abp1* showed slow proliferation and reduced cell division, and abnormal seeds arrested at the globular stage (Chen et al. 2001). The PEI gene encodes an embryo-specific transcription factor required for the globular-to-heart stage transition. Mutant *pei1* interrupts embryogenesis at the heart stage (Li and Thomas 1998). Sugars such as trehalose play a pivotal role in embryogenesis, controlling many aspects of seed growth and development. The biosynthesis of trehalose involves the formation of trehalose-6-phosphate from glucose-6-phosphate and UDP-glucose by the enzyme trehalose-6-phosphate synthase (TPS). Mutant *tps* showed perturbation in trehalose signalling that led to the disruption of cell division and deregulation of cell wall biosynthesis, with the arrest of embryo development at the torpedo stage (Eastmond et al. 2002; Gomez et al. 2006). The valyl-tRNA synthase gene (TWN2) mutation induces a defect at an early stage of embryogenesis and arrests apical cell development, followed by abnormal proliferation of the basal cell to produce secondary embryos (Zhang and Somerville 1997). Mutant *bio2* shows embryo arrest at the globular stage related to deficiency in biotin synthesis as a support to rapid cell division (Patton et al. 1998). The KIS gene encodes an ortholog of mammalian proteins involved in tubulin folding co-factors that play an essential role in the polymerization of microtubules from α/β tubulin heterodimers. Microtubules are intracellular structures required for a variety of processes, particularly the establishment of the special organization of cells, and cell morphogenesis. Mutant embryo *kis* consists of only one or a few grossly enlarged cells with one or more large nuclei and abnormal endosperm that fails to cellularize and contains only a few enlarged nuclei, although embryonic development stopped (Steinborn et al. 2002). The generation of homozygous mutant *Arabidopsis* Minute-like 1 *aml1* showed disruption in most cell division processes and development stopped completely at an early embryonic stage (Weijers et al. 2001). Alteration in mitosis was reported for titan (*ttn*) mutants, which represent another interesting class of mutants with defects in embryo and endosperm development (Liu and Meinke 1998). Nine TITAN genes have been identified in *Arabidopsis*, including TTN5. Embryo development in TTN5 mutant seeds is limited to the formation of a few giant cells. The number of endosperm nuclei is reduced and endosperm cellularization is blocked (McElver et al. 2000). Another TITAN gene product (TTN6) is related to the isopeptidase T class of deubiquitinating enzymes that recycle polyubiquitin chains following protein degradation. Mutant *ttn6* is reported to stop embryo development at the globular stage (Tzafrir et al. 2002). The TORMOZ (TOZ) gene encodes a nucleolar protein containing WD repeat domains. Disruption of the TOZ gene results in cell division defects and an arrested embryo at the globular stage (Griffith et al. 2007). ARP7 are actin-related proteins that are localized in the nucleus and involved in chromatin dynamics and transcriptional regulation. Homozygous mutants showed embryos that arrested at or before the torpedo stage of development (Kandasamy et al. 2005).

We used RT–PCR to validate our selection procedure. The expression of the 22 selected genes was confirmed in different organs (Fig. 4). Transcripts corresponding to L1L, AML1, BIO2 and PAS2 were abundant only in seeds and were slightly detectable or undetectable in leaves, flowers, roots, stems and cotyledons. The transcripts of the others

genes were not particularly detected in seed tissues but also in leaves, flowers, stems, roots and cotyledons, although the expression levels were generally lower in these five organs than the seed tissues. The RT–PCR experiments and the presence of genes known to be specifically expressed in seed demonstrate that the in silico selection procedure identifies genes specifically, or at least predominantly, expressed in developing *Phaseolus* seeds.

In order to have a better insight into the involvement of the identified genes in the complex process of *Phaseolus* embryogenesis, we monitored the expression of represented genes such as TTN6, TOZ, PFI, AML1, PEI and L1L which are related to transcription factors and/or signal transduction, during the *Phaseolus* embryogenesis process using quantitative real-time PCR in the wild-type and EMS mutant. All these genes have been reported to be expressed in *Arabidopsis* zygotic embryogenesis and are important for cell survival, as described above. Mutation in these genes affects embryo development and leads to their abortion. Our results showed that the expression of all six selected genes was significantly affected in the the EMS mutant compared with the wild-type. Highly significant and specific down regulation was revealed for the AML1 gene in EMS mutant seeds at 3 DAP, whereas L1L, TOZ and TTN6 genes showed significant down regulation at 9 and 12 DAP.

We do not know, however, if one of these genes is directly affected by the EMS mutation. These results indicate that these genes are differentially expressed during common bean seed development; their regulation is likely to occur down-stream of genes more directly involved in initiating embryo development. The delay in the increase of their expression could be due to alteration in many biological processes. Interestingly, our data showed that cell division was markedly reduced in EMS mutant and this may be enough to account for the smaller size of the mutant embryos and seed fresh weight (Fig. 2). This reduction in cell division could be directly attributable to perturbation of the cytokinesis mechanism (Sollner et al. 2002). In addition, the control of embryo growth during seed development is tightly regulated at the level of cell division (Raz et al. 2001). Alternatively, TOZ and TTN6 genes were found to be required for normal mitotic division during *Arabidopsis* embryogenesis (Tzafrir et al. 2002; Griffith et al. 2007). The altered cell division in EMS mutant as well as the level of TOZ and TTN6 gene transcripts at different stage of seed development suggested that TOZ and TTN6 genes could also play a direct role in *Phaseolus* in regulating cell division. Interestingly, the transcript levels of several genes required for controlling embryo and seed development, such as PEI (Li and Thomas 1998), and for coordinating the morphogenesis phases, such as L1L (Chiappetta et al. 2009), were altered in EMS mutant relative to the wild-type (Fig. 5). The observed alteration in cell division and cell differentiation, and the defect altering embryo development in EMS mutant, could result from the down regulation of several genes required in embryo development, such as TOZ, TTN6, L1L, AML1, PFI and PEI. Altogether, all genes were down regulated in EMS mutant versus wild-type seeds, possibly reflecting the lower biosynthetic and metabolism activity of EMS mutant seeds. In addition, TTN6, L1L, TOZ, PFI, AML1 and PEI represent various functional categories during seed development, as shown in higher plants such as *Arabidopsis*. We therefore suggest that the effect of EMS mutagenesis on *Phaseolus* embryogenesis induces a general alteration of the mechanism of seed development.

Our study demonstrates the reliability of in silico analysis methods for identifying genes involved in *Phaseolus* seed development.

Conclusion

Few studies have been carried out in order to gain better insight into the mechanism of *Phaseolus* embryogenesis; the details of molecular events occurring during its developmental process are unclear. The present study was therefore undertaken to understand of the structural pattern of *Phaseolus* embryo development.

Embryos from EMS mutant showed abnormalities mainly in suspensors and cotyledons at the globular, heart and cotyledon stages. During embryo development in mutagenized samples, the suspensor with large cells disturbed the development of the embryo, which aborted before seed maturity, which was not the case in the wild-type samples.

The in silico analysis approach used in this study provides new information about *Phaseolus* embryo development. We described the identification and tissue-specific expression of 22 new highly expressed mRNAs during the early stages of seed development in common bean.

Pattern analysis for six selected genes, demonstrated that their expression profiles were quantitatively inconsistent for wild-type and EMS mutant. RT–PCR analysis suggested that some selected ESTs might provide new targets for the molecular characterization of *Phaseolus* seed development.

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