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cDNA-AFLP profiling in the embryo axes during common bean germination

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Abstract

cDNA-AFLP fingerprinting was used to identify genes with modulated expression during germination in common bean (*Phaseolus vulgaris* L.). The analysis was performed on the embryo axes. Nine time points covering the whole germination were considered and 800 transcript-derived fragments (PvTDFs) were scored. Among them, 80 % showed no changes during germination. The 97 PvTDFs showing differential expressions during germination were sequenced along with 14 constant transcripts that were randomly chosen. The expression of seven variable PvTDFs was confirmed by real-time RT-PCR. We observed that 92 % of the transcript changes, including 35 % of appearing mRNAs, took place before radicule protrusion, 0 - 17 h after imbibition (HAI). A major shift in gene expression was observed between 9 and 14 HAI, suggesting a key moment of cell re-programming. Sequence homologies were found for 52 % of the sequenced PvTDFs. The identified transcripts encode proteins belonging to several functional groups including transcription factors, proteins involved in storage compound hydrolysis, cell elongation or oxidative stress protection.

Additional key words: early radicle growth, gene expression, gene markers, *Phaseolus vulgaris*.

Introduction

The breeding objectives for common bean (*Phaseolus vulgaris* L.) are generally focused on the improvement of yield, disease resistance as well as seed protein content and quality. Beans are susceptible to abiotic stress and under field conditions, emergence is particularly affected by soil temperatures at sowing (Kolasinska *et al.* 2000). Germination is generally poor when the soil temperature is under 15 °C and some traits are strongly dependent on germination potential in sub-optimal conditions (Zaiter *et al*. 1994, Nleya *et al*. 2005). In the general aim of improving bean germination, the identification of key genes differently expressed during the germination time-course is a first step before exploring their regulation under fluctuating temperatures in different genotypes.

Germination starts with the uptake of water by the quiescent dry seed and ends with the emergence of the embryo axis including radicle (Bewley and Black 1994). During this period, energy metabolism resumes, repair processes occur, and the cell cycle is reactivated (Bray 1995, Hilhorst *et al*. 1998). During early imbibition, mRNAs present in dry seeds allow rapid protein synthesis. The importance of stored mRNAs in the germination has been shown in *Arabidopsis* by Rajjou *et al*. (2004). These authors have underlined that even in absence of *de novo* transcript synthesis, germination occurred. However neo-synthesized mRNAs are important for a proper germination rate, and without them, early seedling growth is blocked (Rajjou *et al*. 2004).

Information concerning gene expression during germination was initially based on approaches using one or two candidate genes. With the onset of -omics technologies, several studies were carried out at transcriptomic and at proteomic level (*e.g*. Gallardo *et al*. 2001, Ogawa *et al*. 2003, Fu *et al*. 2005, Nakabayashi *et al*. 2005). Gallardo *et al.* (2001) developed *Arabidopsis*

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Abbreviations: *BLAST* - basic local alignment search tool; cDNA-AFLP - cDNA-amplified fragment length polymorphism; Ct - cycle threshold; HAI - hour after imbibition; PCR - polymerase chain reaction; PvTDF - *Phaseolus vulgaris* transcript-derived fragment; RE - relative expression; RT-PCR - reverse transcription-PCR.

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reference maps of seed proteins and focused on the effects of environmental changes and developmental stages during seed maturation, desiccation and germination. Fu *et al*. (2005) studied proteomic analysis of dry and germinating *Arabidopsis* seeds and young seedlings. Using microarrays, germination-related gene expression was analysed in *Brassica oleracea* (Soeda *et al*. 2005) and barley (Watson and Henry 2005). The other works at the transcriptome level were carried out on model plants for which full-genome analysis could be performed. In *Arabidopsis* Ogawa *et al*. (2003) have focused their genome-wide profiling on gibberellic acid biosynthesis and response whereas Nakabayashi *et al*. (2005) have focused their analysis on stored mRNAs in seed germination. In legumes, transcriptome profiling was performed by Buitink *et al*. (2006) during *Medicago truncatula* germination in relation to desiccation tolerance.

Without the availability of gene arrays in *Phaseolus vulgaris*, exhaustive gene expression analyses can not be performed at the moment for this species. As an alternative, we performed cDNA-AFLP, an efficient method for genome-wide expression analysis (Bachem *et al*. 1996) that allows quantitative transcript profiling without prior information about the genome sequence (Breyne *et al*. 2003). cDNA-AFLP allowed us to visualise on the same gel the steady state transcript levels along germination progress. In a preliminary study based on the comparison between samples of embryo axes and cotyledons harvested at 9 and 24 h after imbibition (HAI), cDNA-AFLP proved its usefulness by allowing the identification of a new germin-like protein sequence encoding *Phaseolus vulgaris* transcript-derived fragment 1 (PvGLP1), which is specifically expressed during bean germination (Aubry *et al*. 2003). This technique was also applied to identify transcripts potentially involved in barley seed dormancy (Leymarie *et al*. 2007), in *Nicotiana plumbaginifolia* dormancy breaking (Bove *et al*. 2005) and in *Arabidopsis* seed germination (De Diego *et al*. 2006). Even though embryo axes represent the future plantlets, they are not often considered separately from cotyledons or endosperm tissues in germination studies. Because of the importance of embryo axes, the present work focuses on the identification of genes with modulated expression correlated with germination of common bean in this seed organ. cDNA-AFLP was particularly interesting because it allowed gene expression profiling with a great number of time points spread out during the whole kinetics of germination. This strategy has permitted the identification of several genes induced in the axes before radicle protrusion. The potential role of the corresponding proteins during germination is discussed as well as their potential as gene markers of radicle protrusion in common bean.

Materials and methods

Plants and germination time-course: Experiments were carried out with the *Phaseolus vulgaris* L. cv. Fin de Bagnols (FB). FB was first subjected to five rounds of selfing to ensure genetic homozygosity. The water content of the dry seeds used was 13.8 %. Seeds were germinated in moisted sand (8 % distilled H_2O, m/v) in darkness at 22 °C. Seeds were declared germinated when radicles emerged (Bewley and Black 1994). Three batches of 30 seeds per time point were analysed. Whole embryo axes, *i.e.* plumules, hypocotyls and radicles, were separated from cotyledons and were collected at 0, 4, 9, 14, 17, 21, 24, 26 and 36 h after imbibtion (HAI). We also collected cotyledons harvested at 0, 17 and 36 HAI as well as fresh leaves and roots harvested from 5 week old plants grown in a greenhouse. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until used.

RNA extraction and integrity: Total RNA was extracted from tissues ground in liquid nitrogen. The frozen powder was subjected to a hot phenol extraction and a selective precipitation with 4 M LiCl to remove DNA (Verwoerd *et al*. 1989). RNAs were quantified by spectrophotometry and 2 µg were checked on 1 % agarose gel to ensure their integrity and quantification homogeneity before synthesising cDNA (Fig. 1*A*).

cDNA-AFLP procedure: The cDNA-AFLP fingerprints were obtained on embryo axes according to Bachem *et al*. (1996) with few modifications. mRNAs were isolated from total RNA using biotinylated oligo (dT) and streptavidin magnetic beads (*PolyAtract IV*, *Promega*, Madison, USA). Double strand cDNAs were synthesised with 200 ng of mRNAs with *MMLV* reverse transcriptase (*Promega*), RNase H and DNA polymerase I according to the manufacturer protocols. Half of the cDNAs obtained was checked on 1 % agarose gel. The remaining sample was subjected to standard AFLP template production (Vos *et al*. 1995). Restriction enzymes used for template production were *Eco*R I and *Mse* I. Fifteen cycles of pre-amplification were carried out using primers without extension with $1/10^{th}$ volume of the restriction/ligation mix (94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min). The products of pre-amplification were diluted 10-fold and the final PCR was carried out with 22 *Eco*R I + *Mse* I primer combinations including two base extensions (NN) as follows: 41 cycles, including cycle touchdown where annealing temperature was reduced from 65 °C to 56 °C in 0.8 °C steps for 11 cycles and subsequently maintained at 56 °C for 30 cycles.

All PCR experiments were carried out using a *BioRad* (Marnes-la-Coquette, France) thermocycler with *Taq* DNA polymerase from *Promega*. The adaptors ligated to

the restriction fragments and the primers used in the pre-amplification and final PCR were described by Vos *et al*. (1995) except that only two selective bases were added in the final amplification step. Oligonucleotides were designed by *MWG-Biotech* SA (Ebersberg, Germany). PCR products were separated on 6 % acrylamide/ bisacrylamide (19/1) gels and visualised by silver staining (Creste *et al*. 2001).

Isolation, sequencing and sequence analysis of *Phaseolus vulgaris* transcript-derived fragments (PvTDFs): 111 PvTDFs were rehydrated in 0.05 cm^3 of water, excised from the gel and incubated 15 min at 37 °C. The PvTDFs were then re-amplified by PCR using 30 cycles and the primers used for the final amplification. The PCR products were checked on 1.4 % agarose gel. The fragments were cloned using a pGEM-T easy vector system (*Promega*), 10 clones *per* PvTDF were PCR-amplified using M13 universal primers, checked for insert size on 2 % agarose gels and one among them was chosen for sequencing. Minipreparations of plasmids were realized using *Qiaprep* spin miniprep kit (*Qiagen*, Valencia, CA, USA). Inserts were then sequenced using T7 amplification primer and *ABI Prism Big Dye* terminator (3.0) cycle sequencing reaction kits (*Applied Biosystems*, Forster City, CA, USA). Sequences were determined on *ABI Prism 310* genetic analyser (*Applied Biosystems*). Putative functions were attributed on the basis of sequence homologies between translated PvTDF nucleotide sequences and protein databases. In this aim, *BLASTX* searches (Altschul *et al*. 1997) were performed using the NCBI website (http://www.ncbi.nlm.nih.gov/blast/) and the TIGR *Medicago truncatula* gene index database (http://tigrblast.tigr.org/er-blast/index.cgi?project=mtbe). Except for sequence size < 100 bp, hits were considered significant when E-value was < 1.0 e^{-5}. Otherwise a *FASTA* (Pearson and Lipman 1988) search was performed on GenBank with the same significant level. Sequences were then classified using the functional catalogue of Bevan *et al*. (1998).

Real-time RT-PCR: Seven cDNA-AFLP profiles were validated by real-time RT-PCR. An independent RT was performed according to Chateigner *et al*. (1999) using the total RNA samples previously extracted. Primers were designed based on the PvTDF sequences, using *Primer-Express* software version 2.0 (*Applied Biosystems*). All primer sequences are listed in Table 1. In order to explore if the gene expression is organ-specific, we also analysed the abundance of these 7 PvTDFs in cotyledons, leaves and roots. PCR reactions were performed in triplicate using *SYBR Green* ready reaction mix (*Applied Biosystems*) in 0.025 cm^3 reaction volumes containing 300 nM primers on an *ABI Prism 7000* sequence detection system (*Applied Biosystems*). Melting curve analysis was performed using *ABI Prism 7000* sequence detection system software version *1.0.1* (*Applied Biosystems*) to exclude the occurrence of primer dimers and unspecific PCR products.

For the relative quantification of gene expression, we used the "delta-delta method" presented by *Applied Biosystems*. The average cycle threshold (Ct) value of the embryo axes of dry seeds was used as calibrator to calculate the relative expression of the target gene transcript at any development time. The relative expressions (RE) were then calculated using the formula: $RE_{target} = 2^{-[\Delta Ctarget - \Delta Ctref]}$, where ΔCt_{target} was determined by subtracting the average calibrator Ct value from the average sample Ct value of the target gene transcript, and ΔCt_{ref} was determined by subtracting the average calibrator Ct value from the average sample Ct value of the endogenous reference (18S rRNA).

Table 1. Primers used for real-time RT-PCR experiments. F stands for forward primer and R for reverse primer.

Sequence name	Primer sequence (5' - 3')	
18S rRNA	F	CTGTCGGCCAAGGCTATAGACT
	R	TCTGTGATGCCCTTAGATGTTCTG
PvTDF130 (unidentified)	F	CCACCACTGCCACTGCTTCT
	R	GTTGATGAAAAAAGGGATTTACATTACAG
PvTDF11 (IAA-protein conjugate)	F	CAAAACGAGCGAGACGAGAAC
	R	CGGCTCGTGCTTGCCTAA
PvTDF58 (Cyt P450)	F	AAAAGGAACTCGACTAATCACAAATCT
	R	TCCAATGGATCTTCCCAAACA
PvTDF3 (PvLOX2)	F	GAAGGGTCATGGCGACTTGA
	R	TGCTGCAAATGTGAATCAGATTT
PvTDF76 (PvGLP1)	F	GTGTGTGTGCATTGGGATTGA
	R	GGTGTGAATGGTCTTGGTGTATCA
PvTDF151 (cyclin)	F	GTGGAGTATATTGATGACATTTACAAGTTCT
	R	CAAAATAGCTCTCATCCTCTCATTTATC
PvTDF31 (rab GTPase)	F	ACGGATTCTGTTTTGGCTTTGT
	R	CAACAGTGATAGGTGAAGCCTCAA

Results and discussion

The seed germination time-course could be divided into three phases (Fig. 2*A*). Germinated seeds were not obtained between 0 to 17 HAI so the axes recorded during this first phase were originated from a population of imbibed seeds. In the second phase, between 17 and 31 HAI, all radicles emerged. Thus, the axes collected during this phase represent a mix of imbibed and post-germinative seeds. 50 % of the seeds were germinated at 24 HAI (T_{50}). The third phase of the time-course corresponded to time points later than 31 HAI and only concerned post-germinative seeds. Final germination percentage was 100 %.

Because cDNA-AFLP analysis was used to identify differentially expressed genes in the axes during the whole germination process, we analysed axes at 9 times chosen along the germination time-course (Fig. 2*A*). Gene expression patterns were obtained randomly with 22 primer combinations. 800 transcript-derived fragments of *Phaseolus vulgaris* (PvTDFs) were detected. One cDNA-AFLP pattern was obtained across the germination time-course with one primer combination (Fig. 1*B*). In this pattern, several PvTDFs showed a variation of intensity during germination. PvTDFs that were considered in our study appeared, disappeared, were constant, decreased, increased or were transiently accumulated during germination. PvTDFs present in only one track were not considered. More than 80 % of the PvTDFs showed constant abundance during all the germination time-course. The 97 PvTDFs that displayed a clear-cut variation of abundance across the 9 time points were classified into groups of different gene expression profiles: 9 were transiently accumulated, 34 appeared, 24 showed a significant increase, 16 disappeared and 14 decreased during germination. Among them, 88 (92 %) showed abundance changes before the T_{50} (24 HAI) and for 54 (56 %) these changes occurred between 9 HAI and 17 HAI, *i.e*., prior to radicle protrusion (Fig. 2*A*).

All the 97 PvTDFs that showed a clear-cut variation of abundance as well as the 14 randomly chosen PvTDFs with a constant abundance were isolated from acrylamide gels, cloned, sequenced and submitted to sequence analysis. Significant homologies were found for 52 %. Based on these sequence homologies, we classified the studied PvTDFs in functional categories (Table 2). The main categories represented were “metabolism” (15 %), “protein destination and storage” (13 %) and “transcription” (12 %). 12 % of sequences were “unclassified” and 10 % remained with an “unclear classification”.

All along the paper, PvTDFs were annotated with their type of profile (A - appearing; T - transiently induced; In - increasing; Di - disappearing; De - decreasing; C - constant) followed by the time of abundance changing (*i.e*. De14 means decrease at 14 HAI). According to PvTDF sequence homologies and bibliography, we hypothesized the role of the corresponding proteins in the axes during germination, assuming that transcripts are translated.

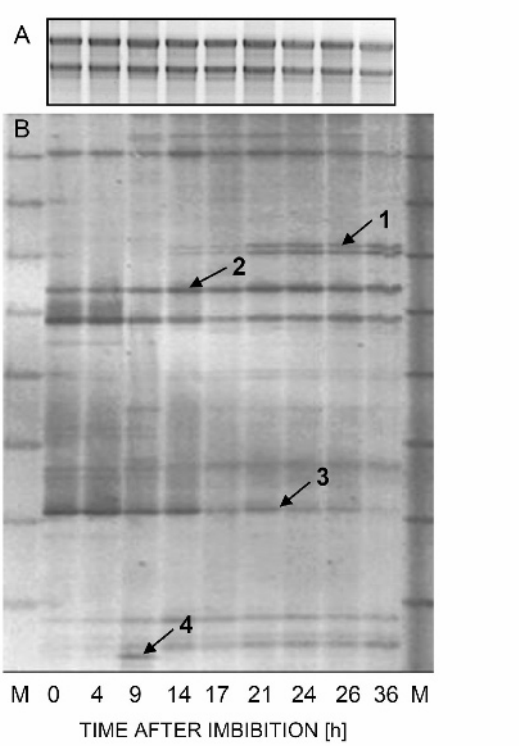

Fig. 1. cDNA-AFLP pattern displaying different expression kinetics in the embryo axes of *Phaseolus vulgaris* during the germination time-course. *A* - Image of 2 μg of total RNA from 0 to 36 h after the start of imbibition. *B* - cDNA-AFLP pattern with *arrows* pointing out different kinetics: *Phaseolus vulgaris* transcript-derived fragment (PvTDF) which appeared (*1*), constant PvTDF (*2*) and PvTDF which decreased (*3*). Bands observed in a unique panel are considered as artefacts (*4*). *Lane M* - 10 bp DNA ladder.

In coherence with intense metabolic activities expected during the first hours of imbibition, most of the selected transcripts already present in dry seed axes, with a constant or decreasing abundance during germination are associated with catabolism, translation, protein post-modification and molecule trafficking activities (Table 2). Most PvTDFs connected with catabolism decreased or disappeared before radicle protrusion and the putative function of the corresponding proteins suggested a possible storage compound mobilisation activity in the axes. In this way, the predicted products of PvTDF16-Di14, PvTDF89-De9 and PvTDF88-De17 were homologous to a storage protein (β-conglycinin), a cysteine proteinase precursor and a peptidase respectively, and could be regarded as involved in storage protein catabolism. With regard to sugars, PvTDF119-Di21 and PvTDF70-De14 were homologous to genes encoding

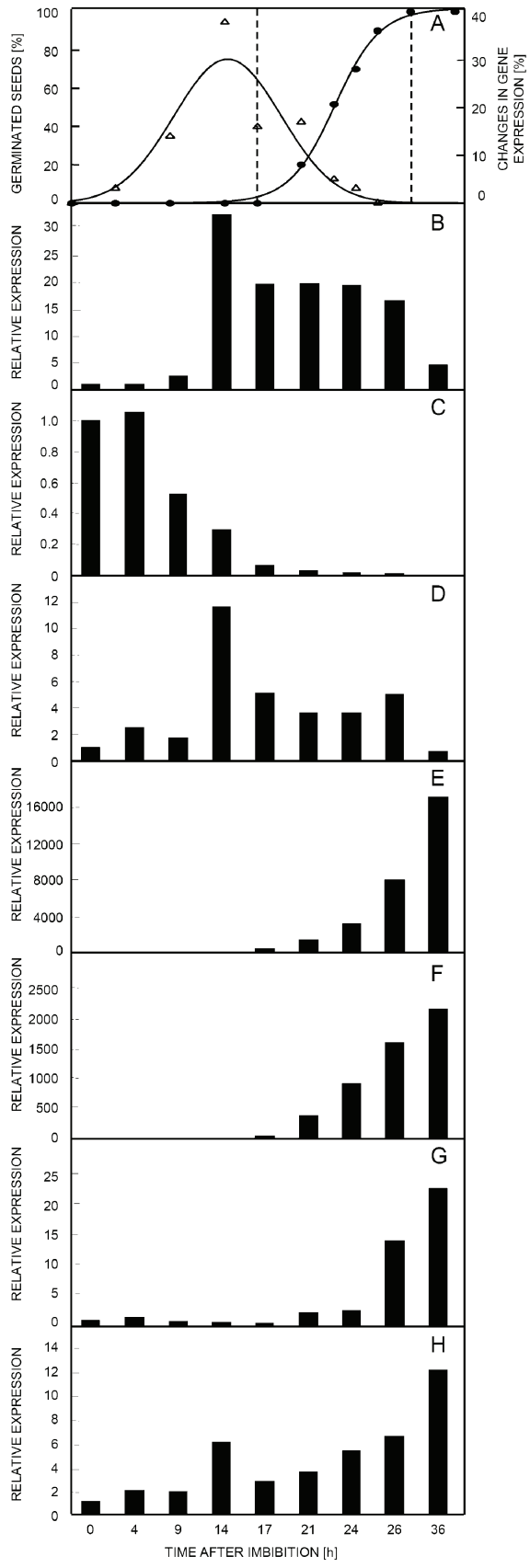

Fig. 2. *A* - Time-course of seed germination (*circles*) and changes in gene expression (*triangles*) in *Phaseolus vulgaris*. The germination time-course is divided into three phases separated by *dashed lines*. Axes for mRNA extractions were collected at 0, 4, 9, 14, 17, 21, 24, 26, 36 HAI. Changes in gene expression at a given time were calculated as the ratio between the number of PvTDFs which abundance varied at this time and the total number of PvTDFs with modulated abundance. *B* to *H* - Validation of seven cDNA-AFLP gene expression profiles during the germination determined by real-time RT-PCR. The selected genes correspond to: an unidentified PvTDF (*B*), an IAA-protein conjugate (*C*), Cyt P_{450} (*D*), PvLOX2 (*E*), a cyclin (*F*), PvGLP1 (*G*) and a rab GTPase (*H*). The level of expression of the target gene transcript during germination was calculated relative to its expression in ungerminated seed.

an alkaline α-galactosidase and a glycosyl hydrolase (α-mannosidase), respectively. Concerning protein syntheses and modifications, translation activities were indicated by the identification of PvTDF15-C (translation initiation factor), PvTDF147-C (ribosomal protein) and both PvTDF129-Di21 and PvTDF71-De14 (amino-acyl-tRNA synthetases). Post-translational modification or degradation of proteins was suggested by the identified PvTDF150-C (a heat-shock protein) and PvTDF104-C (protein with an HECT domain, *i.e.*, a catalytic domain of a subclass of ubiquitin protein ligase, which activity results in the degradation of ubiquitinated target proteins). Finally, molecule trafficking activities were suggested by the presence of PvTDF82-C (nuclear transport factor).

Protein synthesis, post-translational modification or degradation were also suggested by some transcripts that increased or appeared in the first hours of imbibition. This was the case for PvTDF14-T9-26 (translational activator), PvTDF91-In9 and PvTDF63-A14 (TCP1/cpn60 chaperonins), PvTDF127-A14 (a glyco-transferase) and PvTDF92-T4-24 [one subunit of the COP9 signalosome which controls protein degradation, including during growth at the seedling stage (Wei and Deng 2003)].

Among the genes co-activated between 9 and 14 HAI (Fig. 2*A*, Table 2), several were related to signalisation and transcription regulation. For example, the protein corresponding to PvTDF61-A14 was homologous to the ACC synthase, the key enzyme of ethylene synthesis. Ethylene negatively regulates seed dormancy by inhibiting the action of abscisic acid (Beaudoin *et al*. 2000, Ghassemian *et al*. 2000). It has a role in the initiation of germination directly or indirectly by acting on several responsive genes. In particular, it was shown that ethylene production was activated with the radicle protrusion (Puga-Hermida *et al.* 2006). The product of PvTDF44-A14 is similar to a trehalose-6-phosphate synthase. Endogenously formed trehalose was shown to act as a signal molecule in the regulation of plant metabolism and development (Vogel *et al*. 2001). PvTDF86-A14 and PvTDF93-In14 were predicted to

Table 2. Sequence similarities of the transcript-derived fragments of *Phaseolus vulgaris* (PvTDFs). PvTDFs are grouped by expression profiles during germination. [a] - *BLASTX* was used to search homologies between translated PvTDF nucleotide sequences and proteins present in NCBI and TIGR *Medicago truncatula* gene index databases. When *BLASTX* did not reveal match, comparisons were performed using *FASTA* to search for the GenBank databases. Excepted for sequence size < 100 bp, E-values < than 1.0e-5 were retained. [b] - accession number.

Expression profile	Sequence	Length [bp]	Sequence homology [a]	Organism	Accession[b]	E-value
Constitutive	PvTDF15	128	translation initiation factor eIF-3b	*M. truncatula*	AC137830	7.40e-07
	PvTDF69	171	EIL1	*P. persica*	ABK35085	3.00e-09
	PvTDF82	261	RNA-binding region RNP-1; nuclear transport factor 2	*M. truncatula*	AC171804	8.30e-10
	PvTDF103	114	unnamed protein product	*M. truncatula*	*Fasta* (ABE80840)	1.20e-15
	PvTDF104	118	zone clone mth2-21. HECT; Armadillo-like helical	*M. truncatula*	*Fasta* (ABE85276)	2.50e-23
	PvTDF106	217	BRCT; Pescadillo N terminal	*M. truncatula*	ABE93958	3.50e-23
	PvTDF147	83	ribosomal protein L4/L1e. archeabacterial like	*M. truncatula*	AC174281	9.90e-09
	PvTDF150	214	heat-shock protein DnaJ. N-terminal	*M. truncatula*	AC144517	7.10e-14
Transient						
9-26 HAI	PvTDF14	138	similar to translational activator	*O. sativa*	AAT77858	2.00e-09
9-24 HAI	PvTDF58	147	cytochrome P450 82A4 (P450 CP9)	*G. max*	O49859	3.00e-18
4-24 HAI	PvTDF77	210	disease resistance protein; zinc finger C2H2 type	*M. truncatula*	AC137995	6.80e-12
4-24 HAI	PvTDF85	183	hypothetical protein	*V. vinifera*	CAN66950	9.00e-11
4-24 HAI	PvTDF92	145	COP9 complex subunit 6 (signalosome)	*A. thaliana*	*Fasta* (AY048692)	4.50e-11
Appeared at						
9 HAI	PvTDF18	116	cytokinin-O-glucosyltransferase 1	*M. truncatula*	ABE92063	1.70e-08
9 HAI	PvTDF37	140	unknown protein	*A. thaliana*	NP_567383	2.00e-13
9 HAI	PvTDF39	48	pectolytic enzyme; pectin lyase fold	*M. truncatula*	CT030174	2.50e-03
9 HAI	PvTDF80	128	zone clone; cyclic peptide transporter	*M. truncatula*	*Fasta* (ABE85045)	2.50e-10
14 HAI	PvTDF32	67	pathogenesis-related prot 2 (PvPR2)	*P. vulgaris*	*Fasta* (X61364)	1.30e-10
14 HAI	PvTDF44	92	trehalose-6-phosphate synthase	*P. patens*	ABO61744	6.00e-09
14 HAI	PvTDF61	153	ACC synthase	*V. radiata*	CAA78122	1.00e-19
14 HAI	PvTDF63	109	GroEL-like chaperone; ATPase	*M. truncatula*	AC140915	1.80e-07
14 HAI	PvTDF64	298	protein of unknown function DUF566	*M. truncatula*	ABE79495	1.00e-32
14 HAI	PvTDF65	227	homeodomain-related	*M. truncatula*	AC135565	3.60e-20
14 HAI	PvTDF86	133	putative phosphatidylinositol-4-phosphate-5-kinase	*A. thaliana*	*Fasta* (AAL69491)	8.40e-08
14 HAI	PvTDF111	113	TAZ zinc finger; transcriptional coactivation	*M. truncatula*	ABE91077	3.00e-13
14 HAI	PvTDF112	198	NAD binding site; nucleotide sugar epimerase	*M. truncatula*	AC152937	1.90e-30
14 HAI	PvTDF123	76	transcription factor jumonji	*M. truncatula*	*Fasta* (ABE79698)	1.00e-05
14 HAI	PvTDF127	135	putative dolichyl-di-phosphooligosaccharide protein	*O. sativa*	NP_001059165	1.00e-13
14 HAI	PvTDF136	286	Roc1	*O. sativa*	XP_480435	7.00e-32
14 HAI	PvTDF153	156	Zinc finger; C2H2-type	*M. truncatula*	AC126006	1.20e-14
17 HAI	PvTDF3	164	lipoxygenase (PvLOX2)	*P. vulgaris*	AAB18970	7.00e-24
17 HAI	PvTDF121	114	polyprenyl synthetase	*M. truncatula*	AC141866	7.20e-13
21 HAI	PvTDF35	251	hypothetical protein	*O. sativa*	EAY92035	2.00e-09
21 HAI	PvTDF66	112	Gag/pol polyprotein-like	*P. vulgaris*	DQ117565	2.20e-23
21 HAI	PvTDF135	321	multicopper oxidase; type 1	*M. truncatula*	CR932966	7.30e-39
21 HAI	PvTDF151	329	cyclin	*M. truncatula*	AC169076	6.70e-28
Increased from						
9 HAI	PvTDF91	142	T complex protein	*C. sativus*	2206327A	4.00e-06
9 HAI	PvTDF124	301	hypothetical protein	*V. vinifera*	CAN68024	2.00e-19
9 HAI	PvTDF152	240	epoxide hydrolase	*M. truncatula*	AC167403	5.60e-07
14 HAI	PvTDF12	148	TET8; Tetraspanin 8	*A.. thaliana*	NP_850045	4.00e-12
14 HAI	PvTDF29	180	conserved hypothetical protein	*R. communis*	EEF51057	5.00e-06
14 HAI	PvTDF30	140	α-amylase	*M. truncatula*	AC150245	4.70e-16
14 HAI	PvTDF31	128	rab GTPase	*M. truncatula*	AC161399	1.80e-17
14 HAI	PvTDF73	126	phosphoglycerate mutase; 2. 3-bisphospho-glycerate-independent	*S. epidermidis*	NP_764115	2.00e-16
14 HAI	PvTDF93	101	seed calcium-dependent proteine kinase c	*G. max*	AAP03014	8.00e-12
17 HAI	PvTDF68	323	fibronectin; type III-like fold	*M. truncatula*	ABE86602	6.00e-23

continued

17 HAI	PvTDF113	200	ATMDAR4: monodehydroascorbate reductase 4 (NADH)	*A. thaliana*	NP_189420	3.00e-28
21 HAI	PvTDF72	168	uncharacterised plant-specific domain	*M. truncatula*	AC155101	6.80e-07
21 HAI	PvTDF76	233	germin-like protein (PvGLP1)	*P. vulgaris*	CAB77393	5.00e-32
24 HAI	PvTDF56	175	phosphatidylinositol transfer-like- prot IV	*L. japonicus*	AAK63248	4.00e-22
Disappeared at						
14 HAI	PvTDF16	121	α-conglycinin storage protein	*G. max*	*Fasta* (AAB23463)	2.40e-11
21 HAI	PvTDF119	258	alkaline β-galactosidase	*C. sativus*	*Fasta* (ABD52008)	2.10e-10
21 HAI	PvTDF129	265	putative lysyl-tRNA synthetase	*O. sativa*	ABF97356	5.00e-22
26 HAI	PvTDF11	163	IAA-protein conjugate (IAP1)	*P. vulgaris*	AAG01035	7.00e-08
Decreased from						
9 HAI	PvTDF89	260	cysteine proteinase precursor	*P. vulgaris*	CAB17077	5.00e-06
14 HAI	PvTDF70	146	glycosyl hydrolase family 38 protein	*M. truncatula*	AC155894	6.10e-18
14 HAI	PvTDF71	151	aspartyl-tRNA synthetase; class Iib; tRNA synthetase class II	*M. truncatula*	AC144766	7.10e-08
17 HAI	PvTDF48	143	ankyrin	*M. truncatula*	AC142395	3.50e-13
17 HAI	PvTDF88	122	peptidase M14. carboxypeptidase A; protein of unknown function	*M. truncatula*	AC152447	2.60e-10
17 HAI	PvTDF149	137	putative calmodulin-binding protein	*M. truncatula*	AC174353	6.70e-16

encode kinases (a phosphatidylinositol-4-phosphate-5-kinase and a seed calcium-dependent protein kinase c, CDPKc, respectively). Kinases modulate signal transduction pathways in eukaryotes (*e.g.* Kapranov *et al*. 2001), and a CDPK activity was identified in germination embryos of barley (Ritchie *et al*. 1998). All genes associated with transcription encode transcription factors (TFs) of different types. Both PvTDF65 and 136 showed homologies to homeobox genes. Those genes are generally associated with the regulation of key eukaryotic developmental processes. PvTDF136 sequence matches with *Roc1*, a *GL2*-type homeobox gene specifically expressed during protoderm differentiation and radial pattern formation in early embryogenesis (Ito *et al*. 2002). The others correspond to one TAZ (PvTDF111), one Jumonji (PvTDF123) and one C2H2 (PvTDF153) TF type. TAZ zinc finger sequences are present in p300 and CBP. These nuclear transcriptional regulatory proteins participate as transcriptional co-regulators controlling cell differentiation, growth and homeostasis (Bordoli *et al*. 2001, Kim *et al*. 2003). Fu *et al*. (2005) found high level of Jumonji TF in *Arabidopsis* dry seeds and low but persistent level in germinating seeds.

Among transcripts that increased or appeared, some were related to cell elongation or cell division, defence mechanisms and responses to stress. PvTDF39-A9, PvTDF112-A14 and PvTDF68-In17 encode a pectolytic enzyme, a nucleotide sugar epimerase and fibronectin III, respectively, and PvTDF151-A21 is similar to genes encoding cyclins. Concerning defense, it should be mentioned that PvTDF32-A14 and PvTDF77-T14-24 are encoding the bean pathogenesis-related protein PvPR2 (Walter *et al*. 1990) and a disease resistance protein, respectively. The sequence of PvTDF3-A17 matches perfectly with the bean LOX-2 mRNA. This lipid metabolism enzyme can produce important regulatory molecules such as jasmonic acid or traumatin in plants. It is required during development and under stress conditions (Porta *et al*. 1999). Free radicals could be generated under cellular stress conditions. PvTDF113-In17 and PvTDF58-T9-24 were homologous to a monode-hydroascorbate reductase and a cytochrome P_{450} encoding sequences, respectively; these proteins are involved in cell detoxication mechanisms.

Seven cDNA-AFLP profiles in the embryo axes with varying PvTDF abundance during germination were validated by real-time RT-PCR (Fig. 2*B-H*, Table 2). The concerned sequences were characterized by transiently accumulated mRNAs (unidentified PvTDF58, P_{450}), appearing mRNAs (PvLOX2, one cyclin), increasing mRNAs (a rab GTPase, PvGLP1) and disappearing mRNAs (IAA-protein conjugate).

To investigate the putative organ specificity of gene expression during germination in the axes, real-time PCR experiments were extended for the selected genes to other plant organs: cotyledons (three times tested: 0, 17, 36 HAI), leaves and roots (Table 3). Regarding the expression in seeds, transcripts homologous to *PvLOX2* (PVTDF3-A21), to *PvGLP1* (PvTDF76-In21) and to one cyclin (PvTDF151-A17) were not detected in cotyledons. For the other PvTDFs, the highest quantity of transcripts detected in cotyledons was at 17 HAI, and the transcript level was 2 to 5-fold higher than the maximum detected in the axes. Except PvTDF11-Di26 and PvTDF76-In21, mRNAs were detected in leaves and roots but with a very low abundance compared to the seed organs analysed. The genes corresponding to PvTDF11-Di26 and PvTDF76-In21 were not detected in the non-seed plant organs studied. *PvGLP1* transcripts (PvTDF76-In21) were already shown to be specifically accumulated in embryo axes (Aubry *et al*. 2003).

In the present study cDNA-AFLP profiling was carried out to identify genes with modulated expression during common bean seed germination. These

quantitative analyses were performed without prior knowledge of the sequence studied. Seven cDNA-AFLP profiles were validated by real time RT-PCR. In bean seeds, the embryo axes represent a low proportion of the mature fresh mass (approximately 2 %). In our study, we carefully collected them to identify genes expressed in this part of the embryo representing the future plants. In addition, collecting axes samples on a great number of time points during the whole germination allowed to have informative data on gene expression profiles (Fig. 2*A*). In particular, this strategy highlighted a switch in the modulation in gene expression before any seed germinates. This suggests a key moment of cell re-programming in the axes before radicle protrusion. A subset of genes that were up-regulated constituted a first pool of potential markers for bean radicle emergence. No variation in gene expression was recorded during early post-germinative stage (after 31 HAI, Fig. 2*A*). Sequence homologies were found for half of the transcripts analysed (52 %). Based on these homologies some physiological and cellular events associated with germination in the axes could be discussed.

Dry mature seeds contain stored mRNAs accumulated at the end of seed development. Some of them are maintained during the first hours of germination (Walbot 1971). The degradation of these stored mRNAs could provide a part of the nucleotides needed for *de novo* RNA synthesis without the energetic cost needed for nucleotide synthesis. Bewley (1997) suggested that mRNAs down-regulated during germination are generally associated with previous developmental processes and that some of them may be transiently used during early germination. Rajjou *et al*. (2004) have shown that many of these stored mRNAs are translated to germination-specific proteins. The majority of the PvTDFs generated in our study were already present in dry seeds. The abundance of a large proportion of these PvTDFs did not change during the whole germination time-course: around 80 % of the PvTDFs (600) were constant. This proportion suggested that numerous non-modulated genes could be necessary in the axes to accomplish germination and early axis growth. The proportion of modulated gene expression obtained was comparable with the one observed by Nakabayashi *et al*. (2005) and Watson and Henry (2005) who, using arrays, described 20 % and 10 % of modulated gene expressions in germinating seeds of *Arabidopsis* and barley, respectively. In the present study, some of the transcripts found in dry seeds declined during the first hours of imbibition or disappeared before radicle protrusion (Fig. 2*A*, Table 2).

Among the disappearing transcripts, a consistent number could concern storage compound mobilisation. Nakabayashi *et al*. (2005) have also identified this kind of stored mRNAs in *Arabidopsis* seeds. Major reserve mobilisation occurs in storage organs. Storage protein mobilisation in legume cotyledons becomes usually measurable after radicle protrusion (Bewley and Black 1994). However, Minamikawa *et al*. (1983) have shown that axes of *Vigna unguiculata* seeds at early stages of germination may degrade the self-sustained reserve proteins and use them for the synthesis of new proteins. Schlereth *et al*. (2000) mentioned that cysteine proteinases and their mRNAs were already present in both axes and cotyledons of vetch dry seeds. These proteinases, stored in protein bodies, seemed important to initiate globulin breakdown and mobilization in embryo axes independently of cotyledons during germination (Schlereth *et al*. 2000, Müntz *et al*. 2001). In addition to the breakdown of storage proteins, α-amylase and α-D-mannosidases are known to hydrolyse storage sugars in cotyledons (Lahuta *et al*. 2000). However, Minamikawa *et al*. (1983) reported increasing amylolytic activities occurring in *Vigna unguiculata* dry seeds in both embryo axes and cotyledons as germination proceeded. In our study, several genes down-regulated and one up-regulated (PvTDF30-In14; α-amylase) encode proteins for sugar breakdown. If the transcripts encoding the mentioned enzymes are translated, this could reflect a mobilisation of proteins and sugars in the axes in the first hours of imbibition to initiate axis elongation in bean.

Among the transcripts with changing abundance during germination, 65 % increased or appeared. The majority appeared before radicle protrusion (before 17 HAI). During the first hours of imbibition (0 to 9 HAI), the abundance of few mRNAs changed, showing that *de novo* mRNA synthesis occurred early in embryo axes. For example, seven transcripts appeared between 4 and 9 HAI (Fig. 2*A*, Table 2). However, most transcript increase or appeareance occurred between 9 and 14 HAI (Fig. 2*A*). These data were consistent with those obtained by Walbot (1971) who followed RNA synthesis through incorporation of ^{32}P during *Phaseolus vulgaris* germination and showed that it was initiated from 12 to 14 HAI. Bewley and Black (1994) also postulated that the phase preceding germination is generally characterised by the transcription of newly induced mRNAs.

Several PvTDFs that appeared or increased between 9 and 21 HAI encode transcription factors or proteins that could be related to defence and oxidative stress conditions prevailing in germinating seeds. Rajjou *et al*. (2006) have demonstrated that early defence mechanisms are established during germination. Defence proteins could be accumulated to prevent damages caused by pathogens, insects or herbivores during seedling establishment. Moreover, the reactivation of metabolism following seed imbibition may be an important source of reactive oxygen species (ROS) leading to oxidative stress and cellular damages (Bailly 2004). For example, some enzymes of the lipid metabolism, such as epoxide hydrolase (PvTDF152-In9) or lipoxygenase (LOX) 2 (PvTDF3-A17), could participate in lipid peroxidation, a major source of ROS. Epoxide hydrolase was shown to be expressed in cotyledons and hypocotyls of *Euphorbia*

Table 3. Presence or absence of gene expression in cotyledons, leaves and roots measured by real-time RT-PCR for the same genes which expression profiles were validated during germination in the axes (Fig. 2*B-H*). (-), (+/-) and (++): mRNA not detected, detected in low or in high quantity respectively.

	PvTDF130 (unidentified)	PvTDF11 (IAA-protein conjugate)	PvTDF58 (Cyt P_{450})	PvTDF3 (PvLOX2)	PvTDF151 (cyclin)	PvTDF76 (PvGLP1)	PvTDF31 (rab GTPase)
Cotyledons (17 HAI)	++	++	++	-	-	-	++
Leaves	+/-	-	+/-	+/-	+/-	-	+/-
Roots	+/-	-	+/-	+/-	+/-	-	+/-

lagascae during germination (Edqvist and Farbos 2003), and LOX 2 during the early stage of seedling growth in pea (Chateigner *et al*. 1999). Plant cells contain efficient mechanisms to remove ROS during germination. It is the case of enzymes of the ascorbate-glutathione cycle (Garnczarska and Wojtyla 2008). In our study, two transcripts, encoding one monodehydroascorbate reductase (PvTDF113-In17) and a cytochrome P450 (PvTDF58-T9-24), could be involved in such detoxication mechanisms. Gidrol *et al*. (1994) suggested that ROS, although toxic at high concentrations, might also be beneficial at lower concentrations in promoting important changes in plant development such as seed germination and radicle growth. To illustrate this, Carol and Dolan (2006) underlined that ROS are required for cell expansion particularly for root hair growth. Early radicle growth must be initiated concomitantly by several mechanisms including intracellular vesicular transport of proteins between different organelles of the endocytic and secretory pathways or cell elongation. Rab GTPases (PvTDF31-In14) are regulators of these endocytic and secretory pathways (Zerial and McBride 2001). It has been reported that endocytosis and vesicle trafficking characterise tip growth of root hairs (Ovečka *et al*. 2005) and several GTPases have been found to play an important role in root development (Chong and Zhuang 2007). For the initial radicle expansion, modifications leading to cell walls loosening needed for cell expansion prior to radicle protrusion are expected. Three identified transcripts could reflect such activity: PvTDF39-A9 (pectolytic enzyme), PvTDF112-A14 (nucleotide sugar epimerase) and fibronectin (PvTDF68-In17). Finally, several works underlined that cell division in radicles starts after protrusion in *Arabidopsis* (Barrôco *et al*. 2005) and in *Medicago truncatula* (Gimeno-Gilles *et al*. 2009). The appearance of one transcript (PvTDF151) at 21 HAI in the axes similar to genes encoding cyclins may indicate a regulation of cell divisions relative to radicle growth.

In the aim of identifying markers of radicle emergence from seed coats, transcripts appearing or increasing in the axes when radicle protruded (17 - 21 HAI) were of particular interest. Four of those potential markers could be pointed out in our study: PvTDF3-A17 (LOX2), PvTDF151-A21 (cyclin), PvTDF76-In21 (PvGLP1) and PvTDF68-In17 (fibronectin III). In addition to their gene expression profiles, these genes were interesting because of the function of their corresponding proteins. For example, fibronectin III is a major building block of the extracellular matrix. It could help radicle protusion and elongation by counter-balancing mechanical tensions in order to break the seed coat. The gene expression was analysed in other organs for three of these genes; their gene expression in seeds seemed specific to the axes (Table 3). *PvGLP1* expression was already shown to be associated with the early stages of embryo axis growth (Aubry *et al*. 2003) and one LOX appeared to be a marker of axis growth resumption in pea (Chateigner *et al*. 1999). As previously mentioned, the accumulation of cyclin transcripts from 21 HAI could indicate a start of cell divisions relative to radicle growth. Consistent with this hypothesis, the gene was shown to be specifically expressed in axes in the present study, and mRNAs of the homologous gene in soybean were located in proliferating parts of seedlings (Hata *et al*. 1991). In order to precise their relevancy as potential markers of radicle protrusion in common bean, the expression patterns and allelic variability of these four genes will be further studied during seed germination of several genotypes under different germination conditions.

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