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ORIGINAL ARTICLE

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Differentially expressed genes associated with dormancy or germination of *Arabidopsis thaliana* seeds

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Abstract Differential display analysis using dormant and non-dormant *Arabidopsis thaliana* (L.) Heynh seeds resulted in a set of genes that were associated with either dormancy or germination. Expression of the germination-associated genes At*RPL36B* and At*RPL27B*, encoding two ribosomal proteins, was undetectable in the dry seed, low in dormant seed, and high under conditions that allowed completion of germination. Expression of these genes was also found to be light-regulated and to correlate with germination speed. Expression of the dormancy-associated genes *ATS2* and *ATS4*, encoding a caleosin-like protein and a protein similar to a low-temperature-induced protein respectively, was high in the dry seed and decreased during germination. Expression of *ATS2* and *ATS4* was high in primary and secondary dormant seed but low in after-ripened or chilled seed. The expression of both genes was also light-regulated, but no relationship with temperature-dependent germination speed was found.

Keywords Arabidopsis · Caleosin · Germination · Ribosomal protein · Seed dormancy

Abbreviations ABA: Abscisic acid · GA: Gibberellic acid · L*er*: Ecotype Landsberg *erecta* · Cvi: Ecotype Cape Verde Islands

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Introduction

Germination *sensu stricto* starts with the uptake of water by the seed and ends with the protrusion of the radicle. It is commonly associated with activation of metabolism and degradation of reserves (Bewley and Black 1994). Germination in Arabidopsis is light-dependent, and is under the control of phytochromes. Upon light perception, gibberellin 3ß-hydroxylases are transcribed that catalyse the final step in the synthesis of bioactive gibberellins (GAs), whereas in the absence of a light pulse these enzymes are not produced (Yamaguchi et al. 1998). Light induces high levels of GA_4, the active GA in Arabidopsis germination, and, when kept in the dark at 24°C, seeds produce no GA_4 (Derkx et al. 1994). Production of GA is required for the completion of germination and application of exogenous GAs can complement the light-requirement (Derkx and Karssen 1993).

A (viable) seed is called dormant if it does not complete germination under conditions favourable to germination (Bewley and Black 1994). Changes in dormancy are primarily controlled by temperature. Elevated temperatures during dry after-ripening of seeds accelerate the breaking of dormancy. Similarly, imbibed seeds may quickly lose dormancy at low temperature (pre-chilling). Also the expression of dormancy depends on the temperature. Deeper dormancy is characterised by a narrower germination temperature 'window' whereas the absence of dormancy permits germination

at a wider range of temperatures (Bewley and Black 1994).

The suppression of seed dormancy by after-ripening and pre-chilling has been attributed to an increase in the sensitivity to germination stimulating factors, including light, nitrate and gibberellins, and a widening of the temperature window for germination (Hilhorst and Karssen 1992). When non-dormant seeds are placed in conditions that do not provide the necessary factors for germination they will enter a state of secondary dormancy (Bewley and Black 1994). In Arabidopsis seeds, secondary dormancy is readily induced at elevated temperatures when seeds are deprived of light (Derkx and Karssen 1993).

The signalling pathways between the perception of dormancy modifying signals and the final germination response are largely unknown. A number of genes that are associated with dormancy have been identified in Arabidopsis, particularly in relation with the acquisition of dormancy during seed development. Most of these are under control of abscisic acid (ABA) (e.g. Johnson et al. 2002). However, information is lacking on genes involved in the control of secondary dormancy. In the present study, we characterised expression of differentially expressed genes with respect to secondary dormancy and germination. The roles of light and temperature in the regulation of germination and dormancy were studied.

Materials and methods

Plant materials

Plants of *Arabidopsis thaliana* (L.) Heynh, ecotype Landsberg *erecta* (L*er*), were grown in a greenhouse. Seeds were harvested and, upon after-ripening, stored dry at 4°C. Plants of ecotype Cape Verde Islands (Cvi) were grown in a plant growth chamber at 21°C during the day and 18°C during the night, with 16 h of white light per day. Seeds were harvested and stored dry at room temperature. Two replicates of approximately 100 seeds were germinated in 9-cm Petri dishes on two layers of Whatman no. 1 filter paper soaked with 4 ml distilled water. Germination was scored using binoculars; seeds were regarded to have completed germination when a gravitropically bent protruded root of 1 mm was detectable. Seeds were incubated at 2°C, 5°C, 10°C, 15°C, 20°C, 25°C, 30°C or 36°C (±1°C) in the dark with 30 min of white light per day, or in a growth chamber at 21°C (±1°C) with 16 h of white light per day. Alternatively, for the experiment described in Figs. 5 and 6, dishes were wrapped in two layers of aluminium foil for incubation at 21°C in total darkness. Chilling was performed for 8 days at 2°C in the dark. Red light (620–700 nm), used in one germination experiment, was obtained from red fluorescent tubes (Philips TL 20 W/15, Eindhoven, The Netherlands). For RNA isolation, 1,000–2,000 seeds were sown in a Petri dish.

RNA isolation

RNA isolation was performed according to Wan and Wilkins (1994) with modifications. Seed and other plant material were frozen in liquid nitrogen and ground in a micro-dismembrator U (B.Braun Biotech International, Melsungen, Germany) at 2,000 rpm for 2 min. The homogenate was suspended in XT-buffer (0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1% SDS and 1% sodium deoxycholate, pH 9.0) with 1 mg dithiothreitol and 14 mg polyvinyl pyrrolidone per sample of 700 µl, added to 1 mg proteinase K and incubated at 42°C for 1 1/2 h. Nucleic acids were precipitated with potassium chloride (final concentration 0.15 M) on ice for 1 h. Samples were centrifuged in a microfuge at 5°C for 20 min and lithium chloride was added to the supernatant in a final concentration of 2 M. The samples were incubated overnight on ice, specifically to precipitate the RNA. Samples were centrifuged in a microfuge at 5°C for 20 min to pellet the RNA. The pellet was suspended in 2 M cold lithium chloride and the samples were centrifuged at 5°C for 20 min. Upon decanting the supernatant the pellet was washed in 2 M cold lithium chloride twice by re-suspending the pellet and centrifuging at 5°C for 10 min. The pellets were re-suspended in 10 mM Tris, potassium acetate was added to a concentration of 0.2 M and samples were centrifuged at 5°C for 10 min to precipitate polysaccharides. To the supernatant, cold ethanol was added and the RNA was precipitated for 3 h at −80°C. The samples were centrifuged at 5°C for 30 min to pellet the RNA. The pellet was washed with 70% cold ethanol, dried and re-suspended water. For further purification, sodium acetate, in a final concentration of 0.3 M, and ethanol were added, and RNA was precipitated overnight at −20°C. The samples were centrifuged at 5°C for 20 min to pellet the RNA. The pellet was washed with 70% cold ethanol, dried and re-suspended in water. RNA was quantified with a spectrophotometer at 260 nm.

Differential display analysis

Differential display analysis was performed with the RNAmap kit (GenHunter Nashville, TN, USA). Samples were separated on a vertical polyacrylamide gel. Differentially expressed bands were excised from the gel, re-amplified with the same combination of primers and samples were separated on a 2% (w/v) TAE-buffered agarose gel. The bands were excised from the gel, purified with the QIAEX kit (Qiagen, Valencia, CA, USA) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Samples were sequenced and similarity searches were performed with the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/).

Northern analysis

RNA was separated on a 1.5% (w/v) MOPS-buffered agarose gel. The gel was stained with ethidium bro-

mide and a digital image of the total RNA was produced with a Kodak EDAS120 camera on an UV trans-illuminator. The gel was blotted onto positively charged nylon membrane (GeneScreen Plus; NEN Life Science Products) with 10× SSC, baked in a vacuum-oven at 80°C for 30 min, and hybridised with ^{32}P-labelled fragments. The partial cDNAs, including their 3′ untranslated region (UTR), were used as probe. DNA probes were labelled with $\alpha-^{32}$ P-dATP, using the random primed DNA labelling kit (Roche Diagnostics) and purified over a Sephadex G50 column (Boehringer Mannheim). The Northern blots were pre-hybridised for 2 h at 42°C in a solution of 50% formamide, 6× SSC, 5× Denhardt's reagent and 0.5% SDS and hybridised overnight in the same solution after addition of labelled probe. The Northern blots were washed 5 min at room temperature in 2.0× SSC + 0.1% SDS, 20 min at 60°C in 1.0× SSC + 0.1% SDS, 20 min at 60°C in 0.2× SSC + 0.1% SDS and rinsed briefly in 0.2× SSC + 0.1% SDS before auto-radiography. Digital images of auto-radiographs were produced with a Kodak EDAS120 camera and a white light trans-illuminator. Total RNA was shown as a control for equal loading.

RT-PCR and PCR analysis

cDNA was prepared from RNA samples, independent of the ones used for differential display analysis, using the 'Superscript Preamplification System for First Strand cDNA Synthesis' (Gibco BRL). For semi-quantitative PCR assays, reactions were performed with 1:5, 1:25, and 1:125 dilutions of cDNA with 14–17 cycles typically, to ensure that amplifications were within the linear range. For PCR with At*RPL27B* homologues At*RPL27A*and At*RPL27C* more cycles were required to visualise the PCR product (21 and 26, respectively). Only the results of the 1:25 dilutions were presented, since linearity was confirmed consistently.

Gene-specific primers were designed with the Lasergene software package DNASTAR ensuring that the reversed primer was located in the 3′ UTR, in order to prevent similar cDNAs being amplified. The following gene-specific PCR primers were used, forward and reverse, respectively: 5′-TAGCCAGCGAATCTTGAG TTGA-3′ and 5′-GCGTGTTGCTGCTTTGAATAC-3′ for *ATS2*; 5′-CAATCGGAGGAGGTGGAGAAAA-3′ and 5′-CCACAACGCACATTAGAAAGGATT-3′ for *ATS4*; 5′-AGTGTTCTCCGCAAGATGAGGTCT-3′ and 5′-GAGGCAAATAGTTTAAAGGCAGAG-3′ for At*RPL36B*; 5′-TGAAGAAGTACCCAAGCAAAGT-3′ and 5′-GCAAAAACAATTCATGGTAGATAA-3′ for *RPL27A*; 5′-GAAGACGGCGAAGAAATCAAG-3′ and 5′-TAAATTCCAAACCAAAAGGTCTTA-3′ for At*RPL27B*; 5′-TCCGCAAAGACTCAGCTAAGA AGA −3′ and 5′-CACGATAACAAAAC GCTCAAA ATG −3′ for *RPL27C*. The amplified sequence length was 325 bp for *ATS2*, 374 bp for *ATS4*, 219 bp for At*RPL36B*, and 325 bp for At*RPL27B*.

The PCR products were separated on a 1.5% (w/v) TAE-buffered agarose gel, blotted onto positively charged nylon membrane with 10× SSC and baked and hybridisation took place as described above for Northern blots. Digital images of auto-radiographs were produced with a Kodak EDAS120 camera on a white light trans-illuminator, and bands were quantified with the Kodak 1D image analysis software. RT-PCR expression results were verified by Northern analysis using At*RPL36B* and AtRPL27B (Fig. 3, upper panel) or *ATS2* and *ATS4* probes (Fig. 5, upper panel).

Whole-mount mRNA analysis

For whole-mount in situ hybridisation, germinating seeds and young seedlings were fixed in PBS solution containing 0.1% Tween 20, 0.08 mM EGTA, 10% DMSO and 5% paraformaldehyde. After dehydration, the samples were stored at −20°C until hybridisation. Digoxigenin (DIG) labelling of RNA probes was carried out by using a nucleic acid labelling kit (Roche Diagnostics) following the manufacturer's instructions.

During hybridisation, samples were incubated in hybridisation solution (HS: 50% formamide, 5× SSC, 50 $\mu g ml^{-1}$ heparin) containing denatured DIG-labelled probe (approximately 30 ng μl^{-1} kb^{-1}) and 5 mg ml^{-1} herring sperm DNA for 16 h at 60°C. For signal detection, the tissue was incubated with gentle agitation over-night in PBT (1× PBS, 0.1% Tween 20) containing a sheep anti-DIG alkaline phosphatase-conjugated Fab fragment antibody (Roche Diagnostics) diluted 1:2,000. Detection solution was prepared by adding 5 µl nitro-blue tetrazolium chloride (100 mg ml^{-1} stock solution) and 3.75 µl 5-bromo-4-chloro-3-indolyl-phosphate (50 mg ml^{-1} stock solution), both from Roche Diagnostics, to 1 ml detection buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 0.1% Tween 20). Formation of the stable colour precipitate took up to 16 h, revealing a blue/purple colour, and the change was monitored with a stereomicroscope. Upon reaction, the material was cleared with chlorolactophenol (chloral hydrate/phenol/acid lactic 2:1:1) and observed under a light microscope or stereoscope.

Results

Germination response to temperature

Germination speed and maximum germination of L*er* seeds was optimal at 21°C (Fig. 1). Germination was near maximum at lower temperatures, dropped to intermediate levels at 25°C and dropped further to zero with increasing germination temperatures. Seeds incubated at a high temperature of 30°C or 36°C did not complete germination, even if subsequently placed at

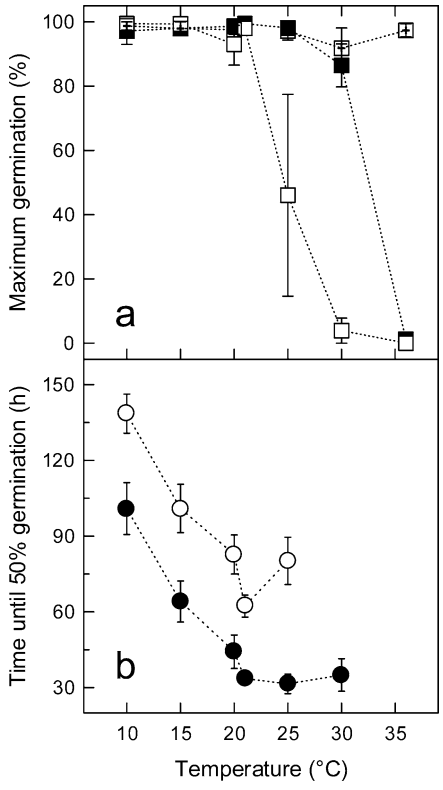

Fig. 1 Germination characteristics of L*er* seeds. **a** Maximum germination (*squares*), **b** time until 50% germination or germination speed (*circles*). *Dark symbols* represent seeds that were chilled before incubation, *open symbols* represent unchilled seeds. Upon germination at the indicated temperature seed viability was tested by exposure to an additional (second) chilling period followed by incubation at 21°C with 16 h of white light per day. The maximum germination was plotted (*cross-haired squares*). Data points are average, *error bars* indicate standard error of means ($n = 3$)

25°C (result not shown), owing to secondary dormancy. If followed by a chilling treatment and germination at 21°C near-maximum germination for every incubation temperature was achieved, indicating that the high temperature-induced secondary dormancy was broken by chilling and that seeds were fully viable. All parameters indicated that the incubation temperature can be used to create large variation in the germination response. The large standard error at 25°C could be attributed to loss of residual primary dormancy owing to dry after-ripening, which caused an increase in the maximum germination from 3% in 6-week-old seeds to 76% in 10-week-old seeds (results not shown). Fully after-ripened seeds reached 100% germination at this temperature. Maximum germination, at temperatures other than 25°C, was hardly affected by after-ripening and remained high at 10°C, 15°C and 20°C, or low at 30°C and 36°C. Chilling before germination resulted in faster germination at all temperatures and caused a decrease over time until 50% germination (t_{50}) of 29–49 h over the entire temperature range. Germination was lifted to near maximum values at 25°C and 30°C, but remained low at 36°C.

Isolation and expression of cDNAs

Differential display analysis was performed using RNA isolated from seeds incubated for 24 h at 10°C, 21°C and 30°C and seeds incubated for 24 h at 30°C and subsequently chilled. All seeds were exposed to 30 min of white light prior to sampling. These samples represent conditions that display different behaviours of germination: slow and maximal at 10°C, fast and maximal at 21°C, minimal at 30°C due to secondary dormancy and maximal at 30°C with subsequent chilling due to breaking of secondary dormancy. Four clones were isolated that were differentially expressed under these conditions. Expression was verified using RT-PCR with gene-specific primers, as well as by Northern analysis.

Clone DD1-1 was found to be identical to a gene encoding an embryo-specific protein with a putative calcium-binding site (accession NM_124906, Table 1). The cDNA was also fairly similar to *ATS1* (accession AF067857, Table 1), another gene encoding an embryo-specific protein (Nuccio and Thomas 1999). Lower se-

Table 1 Gene names, accession numbers and nucleotide sequence similarity of the four clones that were retrieved by differential display analysis

Clone	Name	Homologues	Genbank accession no.	Origin	Sequence similarity
DD1-1	ATS2	Putative embryo-specific protein	NM_124906	Arabidopsis chr.5	444/452, 98%[a]
		ATS1	AF067857	Arabidopsis chr.4	213/261, 81%
		SOP1, caleosin	AF109921	S. indicum	134/182, 73%
DD1-2	ATS4	Protein similar to low temperature induced protein (LTI-65)	NM_118690	Arabidopsis chr.4	475/476, 99%[a]
DD1-5	RPL36B	60S ribosomal protein L36	NM_180366	Arabidopsis chr.3	260/261, 99%[a]
DD1-10	RPL27B	60S ribosomal protein L27	NM_113120	Arabidopsis chr.3	358/360, 99%[a]
DD1-10 homologue	RPL27C	60S ribosomal protein L27	NM_117587	Arabidopsis chr.4	206/233, 88%
DD1-10 homologue	RPL27A	60S ribosomal protein L27	NM_128781	Arabidopsis chr.2	84/102 + 80/97, 82%

All sequences were identified as mRNA in the NCBI database
[a]Marked similarity includes the 3′ UTR, which strongly suggests that the four clones are identical to these accessions. Two additional sequences, homologous to DD1-10, were investigated and their similarity to clone DD1-10 was shown

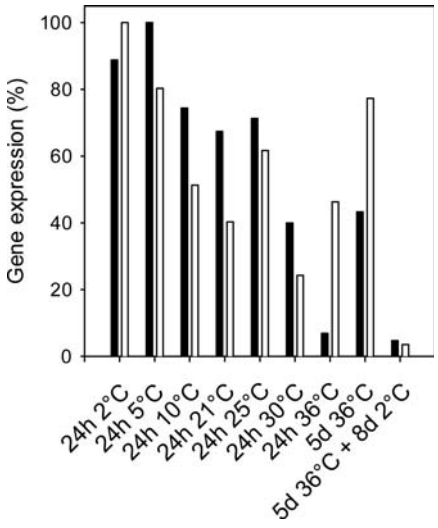

Fig. 2 Expression of *ATS2* (*black bars*) and *ATS4* (*open bars*) in L*er* seeds incubated for 24 h at various temperatures, in secondary dormant (5 days, 36°C) and in chilled (5 days, 36°C + 8 days, 2°C) seeds. The result was obtained with semi-quantitative RT-PCR. RNA was sampled after the indicated incubation period

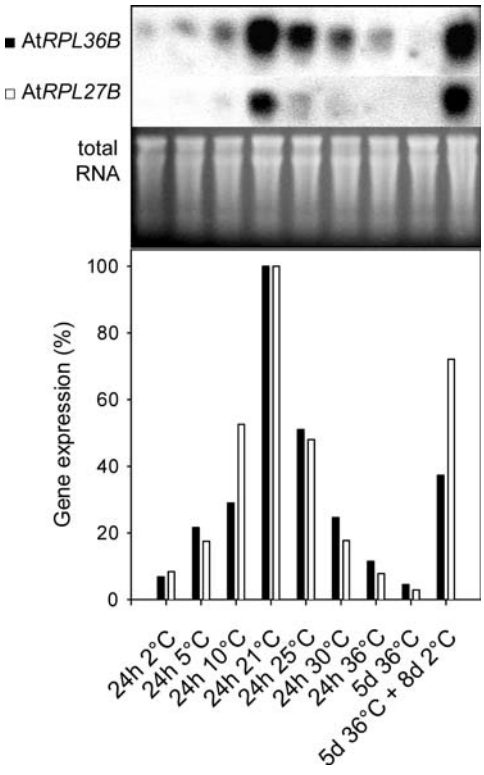

Fig. 3 Expression of At*RPL36B* (*black bars*) and At*RPL27B* (*open bars*) in L*er* seeds incubated for 24 h at various temperatures, in secondary dormant (5 days, 36°C) and in chilled (5 days, 36°C + 8 days, 2°C) seeds. The *top panel* displays the gel with total RNA and northern blots; the *lower panel* displays the result of semi-quantitative RT-PCR. The samples in the *top panel* correspond to the data points in the *lower panel*. RNA was sampled after the indicated incubation period

quence similarity was found to the 3′ end of the coding sequence of SOP1 from *Sesamum indicum*, coding for a caleosin which is a calcium-binding protein in oil bodies of the embryo (Chen et al. 1999; Naested et al. 2000). Clone DD1-1 is hereafter referred to as *ATS2*. Transcript abundance was high after 24 h imbibition in water at low temperatures, and tended to decrease with an increasing germination temperature. Secondary dormant seeds (5 days, 36°C) showed intermediate expression, abolished by a subsequent dormancy breaking treatment by chilling (5 days, 36°C + 8 days, 2°C) (Fig. 2). Primary dormant Cvi seeds that were imbibed for 1 day showed high transcript levels, whereas 1-day-imbibed after-ripened seeds showed lower, intermediate transcript levels (Fig. 4).

Clone DD1-2 was found to be identical to a gene encoding a putative protein that was similar to a gene encoding a low-temperature-induced protein (accession NM_118690, Table 1). Clone DD1-2 is hereafter referred to as *ATS4*. Like for *ATS2* transcript levels of *ATS4* tended to decrease with an increasing incubation temperature after 24 h of imbibition, though not as dramatically as *ATS2* (Fig. 2). Dormancy-related expression was very similar to *ATS2*.

Clone DD1-5 was identified as the cDNA for a ribosomal protein 60S rpL36 (accession NM_180366, Table 1), and is hereafter referred to as At*RPL36B*. During germination expression was low at extreme incubation temperatures and showed an optimum at 21°C (Fig. 3). Secondary dormant seeds (5 days, 36°C) showed negligible expression, whereas in subsequently chilled seeds (5 days, 36°C + 8 days, 2°C) that have broken dormancy, expression rose to intermediate levels.

Clone DD1-10 was identified as a cDNA for a ribosomal protein 60S rpL27 (accession NM_113120, Table 1) and is hereafter referred to as At*RPL27B*. The expression profile was very similar to At*RPL36B*, showing an optimum in expression at a germination temperature of 21°C (Fig. 3). Both At*RPL27B* and At*RPL36B* showed intermediate levels of expression in 1-day-imbibed primary dormant Cvi seeds (Fig. 4). In 1-day-imbibed after-ripened seeds that showed no primary dormancy, although the germination speed was slow, expression was unchanged. The 3′ UTR of all four clones appeared to be identical to the 3′ UTRs of their matched sequences, which strongly supported the identification of the isolated clones.

The expression of two homologues of At*RPL27B* was studied, and was found to be very similar to At*RPL27B* expression (data not shown). The expression of all four ribosomal protein genes clearly was high when germination was imminent, and low in the case of slow germination or dormancy. Expression of At*RPL27B* and At*RPL36B* was also low in the dry seed (Fig. 4). Both *ATS2* and *ATS4* appeared to be abundant in the dry seed, and although there seemed to be a clear relationship with dormancy temperature-dependent expression was not related to temperature-dependent germination speed.

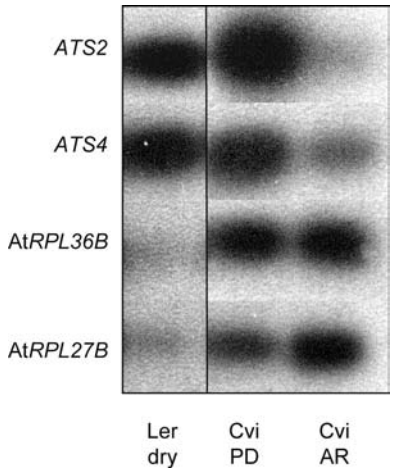

Fig. 4 Expression of *ATS2, ATS4*, At*RPL36B* and At*RPL27B* in Cvi seeds incubated for 24 h at 21°C with white light that were either primary dormant (*PD*) or partially after-ripened (*AR*), and in dry seeds of L*er*. The result was obtained with semi-quantitative RT-PCR

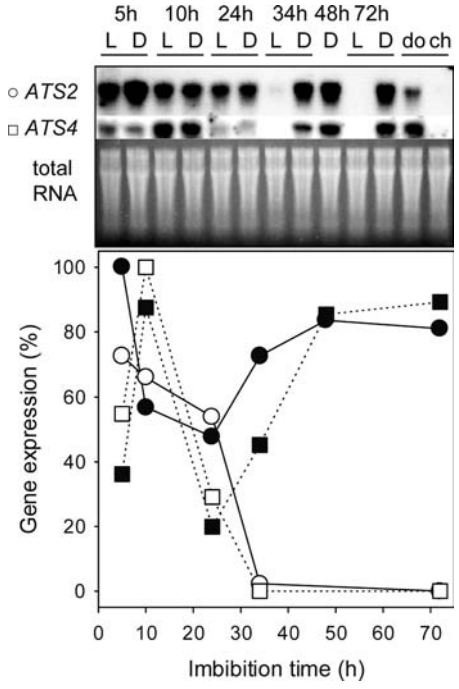

Fig. 5 Expression of *ATS2* (*circles*) and *ATS4* (*squares*) during imbibition of L*er* seeds incubated at 21°C in the dark (D: *dark symbols*) and in a 16-h light regime (L: *open symbols*). RNA samples from secondary dormant (*do*) seeds induced by 5 days incubation at 36°C and from seeds that were subsequently chilled (*ch*) by 8-day incubation at 2°C, in which dormancy was broken, were included as controls. The *top panel* displays the gel with total RNA and Northern blots; the *lower panel* displays the result of semi-quantitative RT-PCR. RNA was sampled from seeds prior to radicle protrusion except for 72 h L-incubation, when germination was completed and RNA was sampled from seedlings

Effect of light on gene expression and germination

The expression of At*RPL36B*, At*RPL27B* and its homologues displayed an inverse correlation with temperature-dependent germination speed. A surprisingly high expression was observed at 21°C with transcript abundance twice as high as at any other temperature after 24 h, which was not reflected in the germination speed. Since seeds sampled at 21°C were exposed to light for 16 h, as opposed to the other incubations that were sampled with only a 30-min exposure to light, the stimulating effect of light on expression was investigated. Seeds were incubated at 21°C either with 16 h of light per day (L) or in total darkness (D). D-incubated seeds did not complete germination (0%), whereas L-incubated seeds reached 100% germination within 72 h. Expression of *ATS2 and ATS4* in L decreased during 10 h and 24 h of imbibition. For *ATS4* this decrease was preceded by an increase between 5 h and 10 h of imbibition. After 34 h of imbibition, before the start of radicle protrusion, both transcripts were reduced to very low levels. Upon radicle protrusion (72 h in L) transcript levels remained marginal. The expression pattern of both *ATS2 and ATS4* in the dark was similar to that in light until 24 h. However, the decrease in expression became transient after 24 h when kept in the dark and high transcript levels were restored after 48 h of imbibition (Fig. 5). Therefore, light not only stimulated germination but also degradation of *ATS2* and *ATS4* transcripts. D-incubation resulted in reversion to high transcript levels.

The expression of both *AtRPL27B* and *AtRPL36B* in the dark increased from 5 h of imbibition and reached a maximum after 24 h, followed by a strong decline (Fig. 6). During L-incubation expression of both ribosomal protein genes increased similarly, but expression rose to higher levels after 34 h of imbibition. After 72 h L-incubation, when radicle protrusion had occurred, a small decline in expression was observed, possibly owing to a burst in de novo transcription at the start of the seedling stage. The expression of At*RPL27B* after 24 h was strongly reduced by the light deprivation, whereas expression of At*RPL36B* was marginally affected (Fig. 6). These results confirmed that light enhances expression of the two ribosomal proteins, which was high when completion of germination was imminent. Inhibition of germination by light deprivation resulted in transient expression of the two ribosomal proteins and reversion to very low transcript levels.

A germination test was performed to investigate how D-incubation related to dormancy induction and gene expression. Seeds were pre-incubated in the dark at 25°C for variable times, subsequently given a 60-min saturating red light (R) pulse, and incubated in the dark at 25°C for another 7 days. A window between 24 h and 38 h displayed an optimum response. Shorter pre-incubation only allowed a marginal light response, whereas longer pre-incubation resulted in a loss in responsiveness and induction of secondary dormancy (Fig. 7). The peak in the expression of the two ribosomal proteins and the transient drop in the expression of *ATS2* and *ATS4* during D-incubation seemed to precede the peak in the light response.

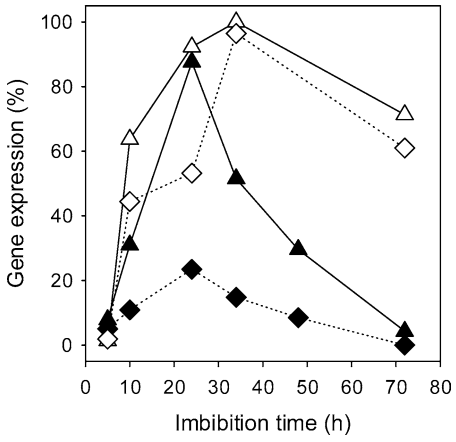

Fig. 6 Expression of At*RPL36B* (*triangles*) and At*RPL27B* (*diamonds*) during imbibition of L*er* seeds incubated at 21°C in the dark (D: *dark symbols*), and in a 16 h light regime (L: *open symbols*). The result was obtained with semi-quantitative RT-PCR. RNA was sampled from seeds prior to radicle protrusion except for 72 h of light incubation, when germination was completed and RNA was sampled from seedlings

Expression in other plant organs

Northern analysis revealed that At*RPL27B* transcripts were most abundant in root, leaf and flower tissues, less abundant in siliques and nearly absent in stem tissue. Expression of At*RPL36B* was lower than that of At*RPL27B*, although the expression profile was very similar (Fig. 8). RT-PCR showed that expression of *ATS2* and *ATS4* in plant tissues other than seed was restricted to siliques, which is likely due to the presence of developing and maturing seeds (Fig. 8).

Interestingly, in situ hybridisation experiments showed that expression of both *ATS2 and ATS4* was detected in the hypocotyl of young seedlings and in some cotyledon cells (Fig. 9). In the root tip of young seedlings also *ATS4* was detected. Although expression upon

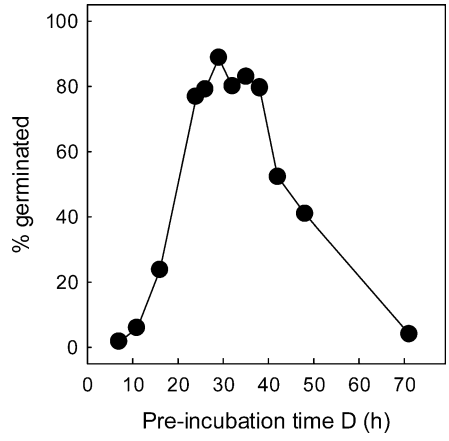

Fig. 7 Maximum germination percentage of L*er* seeds after pre-incubation at 25°C in the dark for the indicated period, a subsequent 1-h red light pulse and further incubation in the dark for 7 days at 25°C

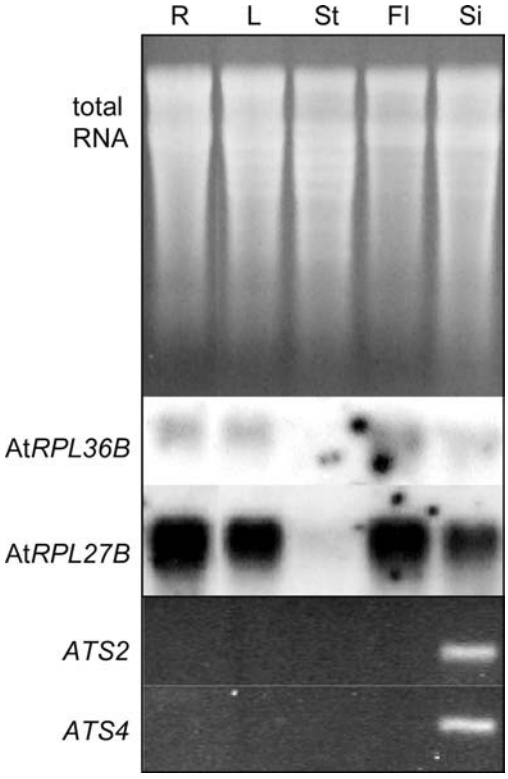

Fig. 8 Expression of At*RPL36B* and At*RPL27B*, using Northern analysis, and of *ATS2* and *ATS4*, using RT-PCR, in other parts of L*er* plants. *R* Root, *L* leaf, *St* stem, *Fl* flower, *Si* silique. For RT-PCR saturating conditions were used: 36 cycles of 1:125 diluted cDNA

radicle protrusion seemed almost constitutive at a low level in the shoot *ATS4* expression was clearly stronger in the root meristem and leaf primordia (Figs. 9e, f).

Upon radicle protrusion, At*RPL36B* expression was low in the root tip and in the hypocotyl and was distinct in the lateral root initial cells and lateral root meristem, implying a role in cell division associated with lateral root formation (Fig. 10). Expression of At*RPL27B* was only detected in the tips of the apical root and lateral roots, implying a role in meristematic activity (Fig. 10).

Discussion

Ribosomal proteins form an essential part of the ribosome complex, and are commonly found throughout the plant. However, heterogeneity in the distribution of ribosomal proteins is not uncommon (Barakat et al. 2001; Szick-Miranda and Bailey-Serres 2002). The expression pattern displayed by At*RPL36B* and At*RPL27B* during imbibition of L*er* seeds appeared to be correlated with completion of germination. When incubated at a range of temperatures, high transcript abundance seemed to coincide with high germination performance, taking into account germination speed and maximum germination. Light, essential for the completion of germination, appeared to be an important factor

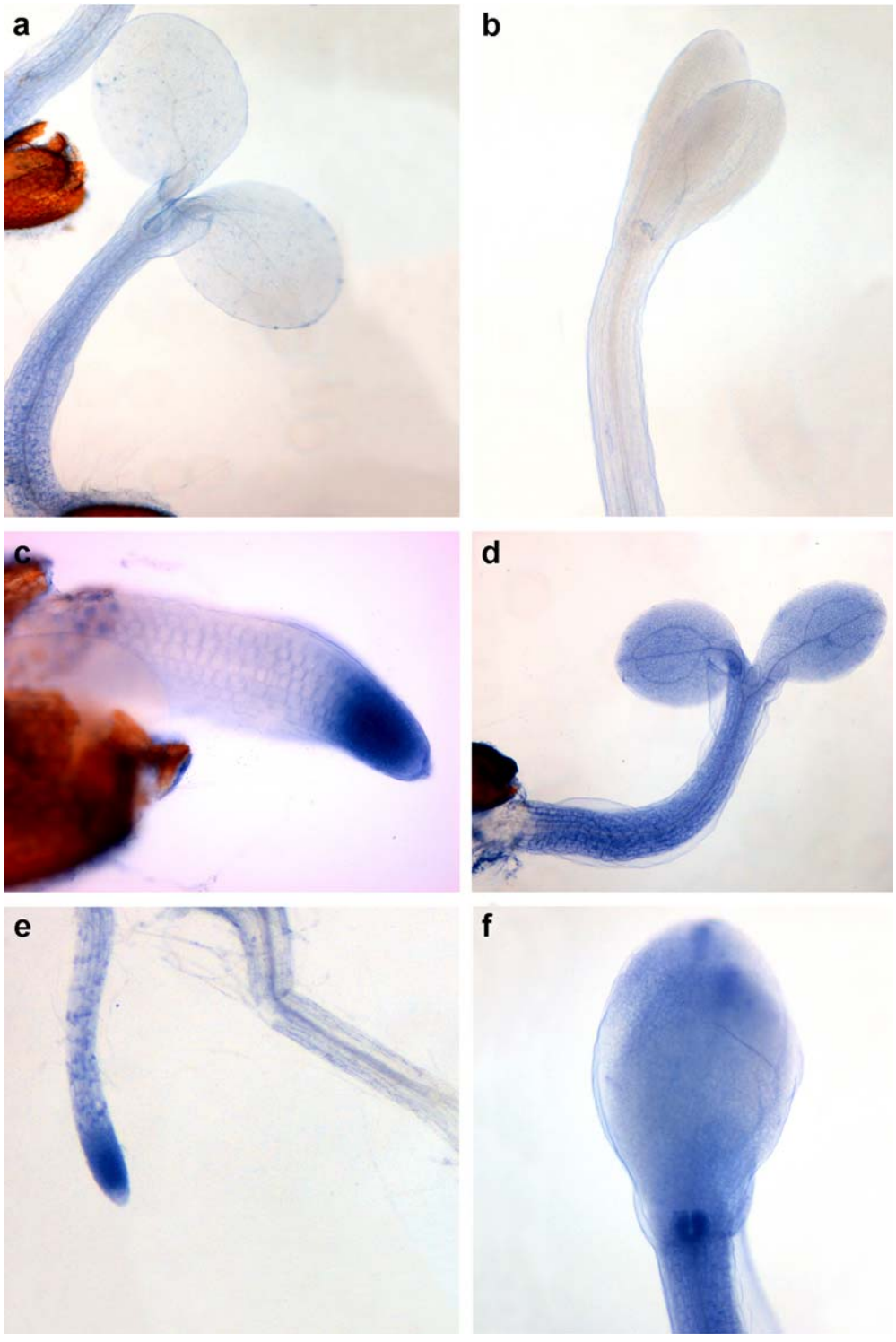

Fig. 9 Analysis of *ATS2* and *ATS4* transcripts in young L*er* seedlings by in situ hybridisation using an antisense probe. Expression of *ATS2* in hypocotyl (**a**) and cotyledons (**b**) was low, whereas expression of *ATS4* was more abundant (**d**). More specific expression was shown by *ATS4* in the root tip immediately upon radicle protrusion (**c**) and in the slightly older seedling (**e**), as well as in the leaf primordia (**f**). Sense probes showed no detectable expression

for high transcript levels as well. Although the first 24 h of imbibition showed light-enhanced expression, the strongest effect was seen from 24 h onwards, resulting in maintained, high transcript levels in the light and a complete reversion of the expression in the dark. Light-enhanced expression has been reported for other ribosomal proteins (Zhao et al. 1999). Our results showed that ribosomal protein transcript abundance was partly light-regulated and seemed to be related to completion of germination. The latter was also true for two homologues of At*RPL27B*, which displayed the same temperature-dependent expression pattern. The concurrence of higher ribosomal protein expression with completion of seed germination corresponds to the situation in bud growth: growing axillary buds of pea seedlings display an increase in *RPL27* transcript abundance, and

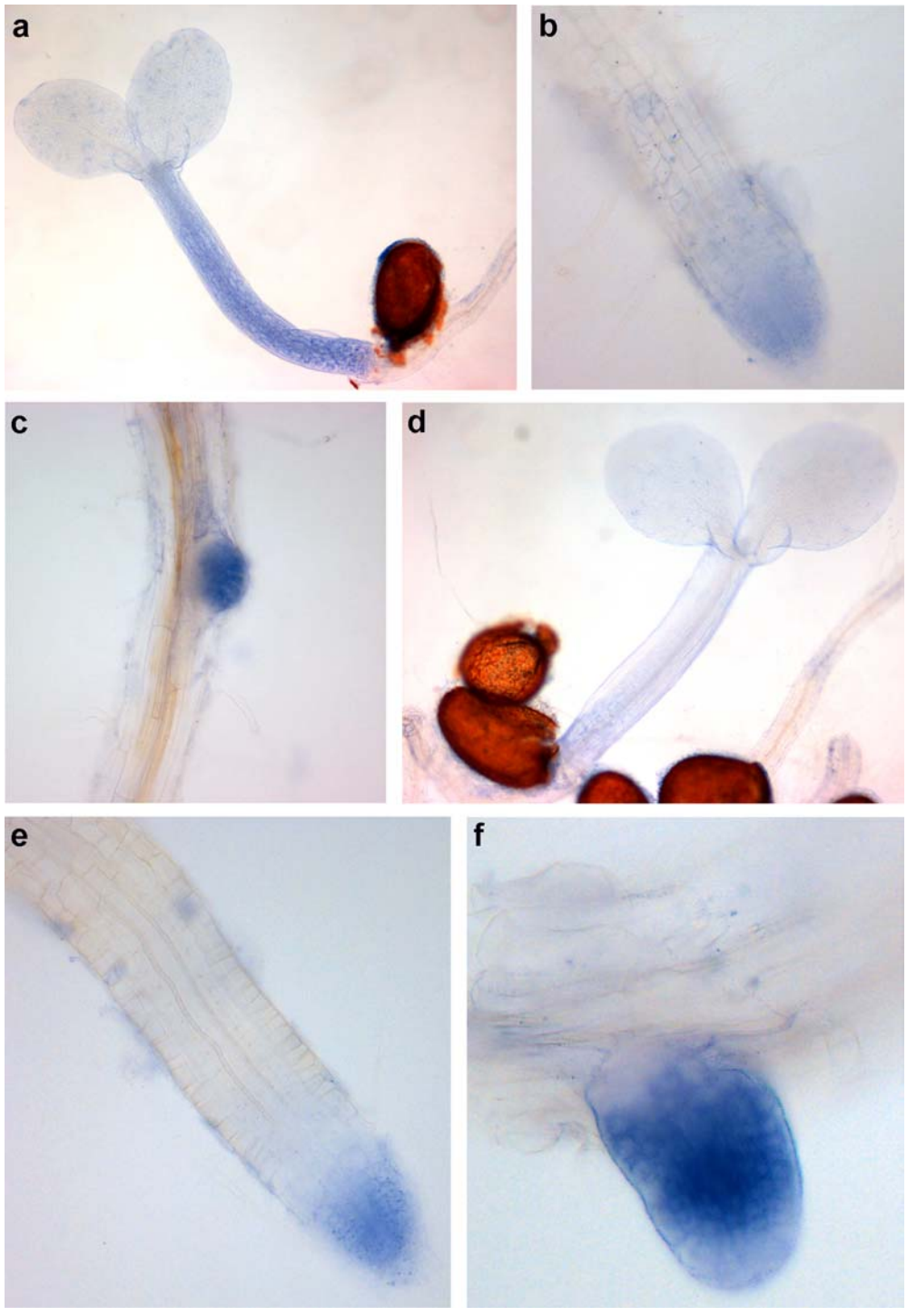

Fig. 10 Analysis of At*RPL36B* and At*RPL27B* transcripts in young L*er* seedlings by in situ hybridisation using an antisense probe. Transcripts of At*RPL36B* were present in the hypocotyl (**a**), the apical root tip (**b**) and lateral root meristem (**c**). There was no expression of At*RPL27B* in the shoot (**d**), only in the tips of the apical root (**e**) and of the lateral roots (**f**). Sense probes showed no detectable expression

expression is down-regulated with induction of dormancy (Stafstrom and Sussex 1992). Apparently, expression of these ribosomal proteins coincides with the preparation for growth. Contrasting results have been reported, which showed both high and low abundance of different ribosomal protein mRNAs during active growth, indicating that activation of ribosomal protein genes is not necessarily associated with tissue proliferation (Beltran-Pena et al. 1995).

An identification of *ATS2* as a previously described putative embryo-specific protein (accession NM_124906) was made. A different embryo-specific gene *ATS1*, with a slightly lower degree of similarity to our *ATS2*, was also described as a gene with seed-specific expression (Table 1, Nuccio and Thomas 1999). A fair degree of similarity was found with *SOP1*, a caleosin from *S. indicum* seeds, and it was hypothesised that SOP1 functions in the mobilisation of oil bodies during

germination (Chen et al. 1999). A caleosin from Arabidopsis, AtCLO1, was found to be highly expressed in seeds and also in the hypocotyl of seedlings. In the young seedling, the caleosin was closely associated with lipid bodies (Naested et al. 2000). Seed-specific expression also appeared to be displayed by *ATS2*, but additional in situ hybridisation analysis showed a low degree of expression in the young seedling (Figs. 8, 9). The *ATS2* expression pattern seemed to be consistent with that for clo-1. Homology to genes coding for proteins involved in desiccation (rdA29 and rdB29) and cold shock (cor-78, lti-65, lti-78 and lti-140, results not shown) was shown by *ATS4*, all of which displayed an ABA-independent response to both water stress and low temperature (Nordin et al. 1991; Nordin et al. 1993; Yamaguchi-Shinozaki and Shinozaki 1993; Shinozaki and Yamaguchi-Shinozaki 2000). These findings suggest that ATS4 is involved in a stress response. No information is available on the ABA induction of ATS4, but it is likely that its expression is ABA-independent as well.

Expression of *ATS2* and *ATS4* showed an opposite pattern when compared to the two ribosomal proteins. During germination in the dark a transient decrease was observed, whereas, in light, this decrease had a permanent character. Clearly, lower levels of *ATS2* and *ATS4* transcripts coincided with the completion of germination. The turning point in imbibition time for the expression of all four clones was at 24 h, when expression either reversed in the dark or continued to decrease/increase in the light (Figs. 5, 6). The germination response seemed to somewhat lag behind the change in expression (Fig. 7). The escape from dark-induced secondary dormancy fell in the time window between 24 h and 38 h of imbibition, and showed the plasticity of the light response. A re-induction of *ATS2* and *ATS4* transcript abundance may reflect the induction of (secondary) dormancy. Since *ATS2* is likely to code for a caleosin, one might presume that the seed prepares itself for the new production of oil bodies, thus getting ready for an escape from germination. Expression of *ATS4*, like that of *ATS2*, seemed to be important for seed maturation since transcripts were found to be abundant in the dry seed. The high homology to desiccation responsive proteins and cold shock proteins implies that *ATS4* is a stress-related protein. During maturation, seeds desiccate and develop primary dormancy. It is tempting to conclude that induction of *ATS4* is required for the seed to prepare for desiccation. However, its expression during induction of secondary dormancy leads us to conclude that *ATS4* is dormancy-related. Re-induction of expression of both *ATS2* and *ATS4* strongly suggests that induction of secondary dormancy shares pathways that overlap with seed maturation and with induction of primary dormancy.

Not much difference in expression was observed for At*RPL36B* and At*RPL27B* in primary dormant and partly after-ripened Cvi seeds, although there was a tendency for lower expression in dormant seed. None of the dormant seeds completed germination within the time frame of the experiment. The partly after-ripened seeds still required 16 days to reach 50% germination; these seeds were not fully dormant and completion of germination was very slow. Given the very slow germination speed, the non-dormant seeds, at this point, might not yet have reached the phase of increased expression of the ribosomal proteins. The expression of *ATS2* and *ATS4* was clearly higher in Cvi seeds with primary dormancy, implying that a larger decrease in expression of *ATS2* and *ATS4* is indicative of a lower degree of dormancy. The expression of the two clones that are abundant in the dry seed is not correlated with germination speed, and perhaps can be explained by higher metabolic turnover at a higher temperature.

Expression of At*RPL36B* and At*RPL27B* was not unique to seeds; transcripts were found in root, leaf, flower and silique tissues. Expression of At*RPL27B* was consistently higher than that of At*RPL36B*. In seedling organs, both At*RPL27B* and At*RPL36B* were detected in the tips of the apical and lateral roots, and At*RPL36B* but not At*RPL27B* was also expressed in the hypocotyl. This differential expression indicates that there are tissue-specific differences in the composition of the translational machinery, although all expression patterns in seeds showed a remarkable similarity. High expression of ribosomal proteins in rapidly proliferating tissues, such as shoot and root tips, was also found for L16 (Williams and Sussex 1995) and for L25 and L34 (Gao et al. 1994).

Both *ATS2* and *ATS4* were expressed in siliques. Transcripts of both genes appeared to be abundant in the dry seed, making it likely that expression is high in the developing and/or maturing seed, and explaining their presence in the silique. Expression of *ATS4* was detected in the seedling as well with a fair degree of tissue-specificity, but not in other tissues of the mature plant. Despite being described as a putative embryo-specific gene *ATS2* transcripts were also detected in some seedling organs. This observation enforces the conclusion that both *ATS2* and *ATS4* are not truly seed-specific, and play an unknown role in other parts of the life cycle, perhaps related to stress signalling as was proposed for *ATS4*.

Although expression of all four described genes was not unique to seeds, it could still function as a marker for the physiological state of the seed. Expression of more genes has been described during seed germination and dormancy (e.g. Johnson et al. 1995; Footitt et al. 2002; Downie et al. 2003). These and the discovery in this paper of two sets of genes showing complementary expression in germination and dormancy could prove to be a useful tool in diagnosing the dormancy status of seeds.

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