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Short communication

A molecular study of dormancy breaking and germination in seeds of *Trollius ledebouri*

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Abstract

A cDNA library was generated from seeds of *Trollius ledebouri* cv. Golden Queen after GA_3 treatment. Five clones encoded mRNAs which were down-regulated during dormancy breaking and the initial stages of germination. Two of these showed homology to storage proteins (pPCB3 and pPCB4) and one each to the late-embryogenesis-abundant (LEA) group 2 dehydrin proteins (pPCB2), a barley glucose dehydrogenase (pPCB6) and the glutathione S-transferase (GST) superfamily (pPCB7). Transcript levels declined over 8 days in GA_3-treated seeds. In dormant imbibed seeds transcript levels were relatively unchanged over the same period except for the PCB3 transcript, the level of which increased.

Seeds of *Trollius ledebouri* cv. Golden Queen (Nuttings and sons, Leicester) exhibit a deep dormancy with only 20% germination occurring over a six month period. Dormancy breaking and germination are tightly linked in this species and can only be stimulated by treatment with gibberellins (GAs) or testa removal [15]. These treatments have been used in physiological experiments which provide evidence for a single dormancy-breaking mechanism that involves the leaching from the imbibing seed of an inhibitor which is antagonistic to a germination promoter (gibberellin) [16]. In *Trollius*, GA-induced germination is visibly well defined. Initially, the embryo and endosperm expand causing testa rupture. This is followed by accelerated growth of the embryo resulting in radicle emergence and then, a few days, later hypocotyl elongation takes place. These clear-cut stages of germination provide an ideal system for studying particular stages of germination and the aim was to identify changes in gene expression underpinning the processes of dormancy-breaking and early germination.

Exogenous GAs stimulate germination of a wide variety of dormant seeds and can often act as a substitute for stratification, after-ripening or red light [3]. However, few studies have been carried out to examine changes in gene expression that may accompany the process of GA-induced germination. In the present investigation we have extended our work with *Trollius* to the molecular level by differentially screening a seed cDNA library to characterize mRNAs whose levels of expression change during GA-induced germination. At the outset of this project it was important to maximize and synchronize germination of the seed population in order to be able to extract RNA at precise stages of early germination before and after testa rupture.

The low rate of germination of imbibed seeds could be significantly increased if they were exposed to gibberellic acid (GA_3). The effectiveness of GA_3 in

The nucleotide sequence data reported will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession numbers Z69994 (PCB2), Z69995 (PCB6) and Z69996 (PCB7).

stimulating germination was dependent upon the concentration used. Seeds were imbibed for 24 h on filter paper in 10^{-5}, 10^{-4} and 10^{-3} M GA_3 at 20 °C and then in water. It can be seen from Fig. 1A that the maximum germination response was ca. 65% and 20% for 10^{-3} M and 10^{-4} M respectively with no recorded germination for 10^{-5} M. Seeds were also germinated by exposure to 10^{-3} M for 72 h. This longer exposure time not only increased the final percentage attained but also its rate of achievement (Fig. 1B).

Exposure of seeds for 72 h to 10^{-3} M GA_3 stimulated germination almost synchronously and the number of dormant seeds remaining was minimal after 8 days. Total RNA was isolated as described [13] from testaless seeds that had been germinated for 1, 2, 4, 6 and 8 days after the start of this treatment (Fig. 1B). The most advanced stage of germination used to extract RNA was day 8 because it was clear that dormancy had been broken at this point as the majority of seeds had undergone testa rupture. Purified mRNA from the five stages was pooled prior to preparation of poly(A)$^+$ RNA.

cDNA was synthesized from 5 μg of poly(A)$^+$ RNA using the λZap-cDNA synthesis kit (Stratagene, USA). cDNA clones for genes with changed levels of expression during the early stages of germination were identified by differential screening of the seed cDNA library. One filter from each replicate plaque lift was hybridised with a cDNA probe synthesized from 0.5 μg poly(A)$^+$ RNA extracted from dry seed. The second filter was hybridised to a cDNA probe synthesized using mRNA isolated from seeds at one of two stages of germination: seeds germinated for 4 days (10^{-3} M GA_3 for 3 days before transfer to water for 1 day before harvesting) or seeds germinated for 8 days (10^{-3} M GA_3 for 3 days before transfer to water for 5 days). An estimated 400 000 clones were screened. No up-regulated clones were detected using these probes but a number of clones hybridised only to the dry seed probe. These clones were selected and 13 of them were classified into gene families based on DNA hybridization homology (Table 1). The largest representative of each family was partially sequenced and the results of searches for similar proteins in the databases are also presented in Table 1.

DNA sequences for the cDNA inserts of pPCB2, 6 and 7 were obtained for both strands. The predicted amino acid sequence of the PCB2 protein exhibited convincing similarity to several group 2 LEA/RAB/dehydrin proteins (Fig. 2) in two different regions one near the C-terminus of the protein (amino acids 53–86) and one 20 amino acids away (amino acids 15–49). The sequences correspond well to the consensus sequence: EKKGIMDKIKEKLPGQH [10] known to be present in members of this class of protein near the carboxyl terminus and in additional copies upstream of the terminus. The number of repeats varies between group 2 proteins as does the number of amino acids spanning each repeat. Not only are these two lysine-rich motifs characteristic of the group 2 proteins but regions close to these are rich in threonine and glycine as are those in PCB2. As well as their proposed role as desiccation protectants [31] it has also been suggested that some LEA proteins may protect dormant seeds during repeated imbibition and redrying [29] and may play a role in the maintenance of dormancy itself [9, 37]. Some of these proteins have been shown to be actively synthesized for a longer period in dormant but not in non-dormant embryos of wheat [28] and *Avena sativa* [4].

Detailed analysis of the expression pattern of mRNAs was determined using northern gel blots. The steady-state levels of the PCB2 transcript were assessed at specific times after the start of imbibition in water or GA_3 to allow for a direct comparison of the amount of each transcript between dormant and germinating seeds. Dormant seeds contained relatively high amounts of the transcript compared to GA_3 treated seeds (Fig. 5). In the latter case, transcript levels declined between 2 and 4 days after the start of GA_3 treatment. The pPCB2 cDNA hybridised to two transcripts at high stringency suggesting that its corresponding gene belonged to a small gene family. The *Trollius* dehydrin transcripts changed only slightly in level over the 8 days following the start of imbibition but were lost after only 4 days in GA_3-treated seeds. However, the dehydrin transcript was not maintained in *Trollius* seeds imbibed for 30 days when the population was still dormant. This suggests that there is no strong link between dehydrin steady-state mRNA levels and the maintenance of dormancy in this species. In addition, the dehydrin transcripts did not show more pronounced stability during dormancy relative to other stored mRNAs investigated. This might have been expected if dehydrin mRNAs encoded proteins which functioned to restrict the germination of the imbibed seed throughout the period of dormancy. However, recent studies of wheat embryos showed that steady-state levels of LEA mRNAs were maintained and even increased upon imbibition of dormant embryos, but declined during germination of non-dormant embryos [24]. Furthermore, application of GA_3 to dormant

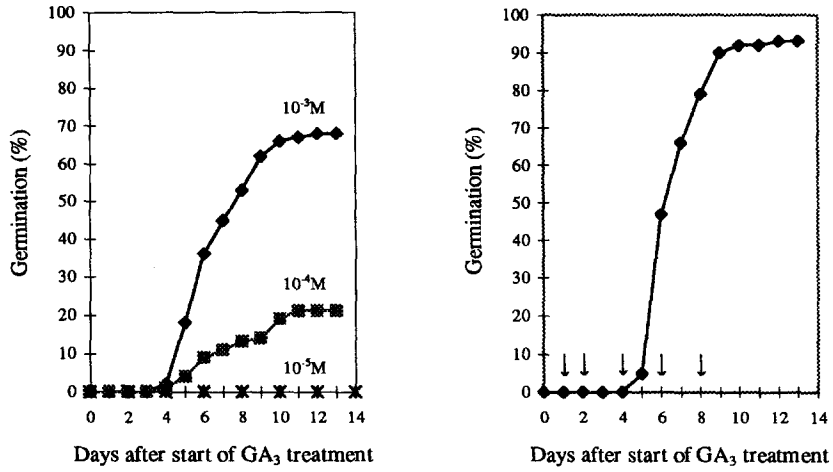

Figure 1. Time course of germination as assessed by testa rupture after a 24 h treatment with the concentrations of GA_3 indicated (left) and after treatment with 10^{-3} M GA_3 for 72 h (right). The arrows indicate the time at which seeds were harvested for RNA isolation.

Table 1. Characterization of isolated cDNA clones into gene families and their putative gene function.

Family	Number of members	Insert size of largest clone (bp)	Transcript size(s) (bp)	Name of clone	Putative gene function
2	7	482	1200 and 1000	pPCB2	group 2 LEA protein
3	2	1900	1900	pPCB3	7S seed storage protein
4	2	700	1600	pPCB4	7S seed storage protein
6	1	599	1300	pPCB6	glucose/ribitol dehydrogenase
7	1	656	1000 and 900	pPCB7	protein of the GST family

embryos of *A. fatua* has recently been shown to not only promote germination but also result in a substantial decline in the transcript level of a mRNA encoding a LEA protein [22].

The putative amino acid sequence of PCB6 exhibited similarity to several 'insect-type' alcohol dehydrogenases (ADHs) from bacteria, the strongest similarity being with two partial open reading frames from *Bacillus subtilis* [35] (Fig. 3). These proteins belong to a superfamily of dehydrogenases which have diverse functions in plants, animals and bacteria [2]. However, the PCB6 amino acid sequence showed closest identity with a seed EST from *A. thaliana* (79%) and a novel embryo-specific barley cDNA (pG31) which encodes a protein with homology to bacterial glucose and ribitol dehydrogenases (71%) [1]. Therefore it seems likely that these proteins are the homologues of PCB6. Glucose dehydrogenase activity in protein extracts of developing barley embryos was shown to be inhibited by antiserum raised against the recombinant pG31 protein [1]. This suggests that the enzyme is involved in carbohydrate metabolism specific to pathways in seeds that have not yet been characterized. The pattern of expression of the PCB6 message was similar to that of PCB2 except that there was only one band present (Fig. 5).

The deduced PCB7 amino acid sequence showed a limited amount of similarity to proteins of the GST superfamily (Fig. 4). Strongest homology was with the In2-1 protein from maize [17] which also shared similarity to these proteins and had 44% identity with PCB7. None of these have been shown to be expressed in seeds. The In2-1 transcript was induced in maize specifically by a benzenesulfonamide herbicide safener. When applied to a plant, safeners function to increase the plant's tolerance to the toxic effects of a herbicidal compound. GSTs are known to catalyse the detoxification of xenobiotics, such as herbicides in plants, by conjugating them to glutathione. As the In2-1 protein shares some homology to members of the GST superfamily, it may be involved in the metabolic detoxification of herbicidal sulphonylureas in maize. Some members of this family have been shown to be induced

(a)

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PCB2      DTGPAGGTGY GTGTGTEKKG MVENIKEKLP GAHKD 49
TaCS120   QHGHTGMTGA GTHSTGEKKG VMENIKEKLP GGHSD 296
HvDHN5    QHGHTGVTGT GTHGTGEKKG VMENIKEKLP GGHSD 500
OsRAB21   TTGTTGAHGT TTTDTGEKKG IMDKIKEKLP GQH   153
LeTAS14   KMPGQHEGEY GQTTGEEKKG MMDKIKDKIP GMH   220
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(b)

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PCB2      QCTTGGTGTA HVHPHEKKGM AENIKEKLPG PHNN   86
RsLEA     TTTTTTTGAA ADQHHEKKGI LEKIKEKLPG HHNHHP 185
OsRAB21   TGTTGAHGTT TTDTGEKKGI MDKIKEKLPG QH     164
GhD147    TTTPGQGATY HQQHHEKKGV MDKIKEKLPG HHNP   136
TaCS120   TGTTGTGTHG SDGIGEKKSL MDKIKDKLPG Q      391
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Figure 2. Comparison of LEA protein sequences with 2 regions of the putative PCB2 protein. Common amino acids are in bold and the consensus sequences are overlined. Accession numbers are shown below in parentheses. (a) amino acids 15 to 49 of PCB2 are aligned with the wheat CS120 protein [18] (sp:p46525; 67% identity), the barley Dhn5 protein (pir:s27761; 67% identity), the rice RAB21 protein [25] (sp:p12253; 53% identity) and the TAS14 protein from tomato [12] (sp:p22240; 50% identity). (b) Comparison of the C-terminus of PCB2 with a radish LEA protein [27] (sp:p21298; 63% identity), Rab21 (60% identity), the cotton LEA3-D147 protein (gp:m81655; 57% identity) and CS120 (47% identity). Double stranded plasmid DNA was sequenced by the dideoxy chain termination method using either Sequenase II (United States Biochemical) or TaqTrack (Promega) for resolving GC compressions. The sequences were aligned by computer using the Pileup program in the University of Wisconsin Genetics Computer Group package [6]. Accession numbers are shown in parentheses with the level of identity.

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PCB6      YETPSNDVID EARLERVFRT NFFSYLFVTR HALKHMREGS 40
pG31      YVRPCITEIT EQDLERVFRT NIFSYFLMTK FAVKHMGPGS 177
AtNodG    .......... .......... .......... ......EGSS 4
BsCOMKX   .......... .......... .......... ..........
BsCOMKY   .......... ........EV NVFSLYWVVK AALPYLPEGA 22

PCB6      SIINTASVNA YKGNPIVLDY TSTKGAVVGF TRSLALQLVS 80
pG31      SIINTTSVNA YKGNATLLDY TATKGAIVAF TRALSMQLAE 217
AtNodG    SIINTTSVNA YKGNASLLDY AATKGAIVAF TRGLALQLAE 44
BsCOMKX   .IINTASITA YKGNKTLIDY SATKGRIVTF TRSLSQSLVQ 39
BsCOMKY   SIITTTSVEG YNPSPMLLDY SATKNAIIGF TVGLGKQLAS 62

PCB6      RGIRVNGVAP GPVWTPL.IP ASLSEEVISK FGLE...VPM 116
pG31      KGIRVNGVAP GPIWTPL.IP ASFPEEKVKQ FGSE...VPM 253
AtNodG    KGIRVNGVAP GPIWTPL.IP ASFNEEKIKN FGSE...VPM 80
BsCOMKX   QGIRVNAVAP GPIWTPL.IP ASFAAKDVEV FGSD...VPM 75
BsCOMKY   KGIRVNSVAP GPIWTPLQIS GGQPTENIPK FGQGTPPAPL 102

PCB6      KRARQPHEIA PSYVFLASND CSSYFTGQVL HPNGGDVVNA 156
pG31      KRAGQPSEVA PSFVFLASEQ DSSYISGQIL HPNGGTIVNS 293
AtNodG    KRAGQPIEVA PSYVFLACNH CSSYFTGQVL HPNGGAVVNA 120
BsCOMKX   ERPGQPVEVA PSYLYLASDD .STYVTGQTI HVNGGTIVNG 114
BsCOMKY   NRAGQPVELA DVYVFLASEN .SSYVTSQVY GITGGIPTA  140
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Figure 3. Alignment of the putative PCB6 amino acid sequence with the barley pG31 protein [1] (gp:s72926), an *A. thaliana* seed EST (pir:s38443) and two open reading frames in the COMK locus of *B. subtilis* [35] (sp:p40397/8). Common amino acids are in bold and accession numbers are shown in parentheses.

by auxin [26] and stress responses such as heat shock and exposure to heavy metals [5], pathogen attack [33] and dehydration [20]. PCB7 also showed 12 to 24% amino acid identity to some of these proteins, therefore

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PCB7      LDLIKYLDSH FEGGPSLYPS DDHPAKREFA DELLSYTDTF 40
In2-1     LDLIRYIDSN FD.GPALLPE D..AAKRQFA DELFASANAF 134
StGST1    MVILEYIDEA FE.GPSILPK D..PYDRALA RFWAKYVEDK 103
GmHSP26-A LVIVEYIDET WK.NNPILPS D..PYQRALA RFWSKFIDDK 107
NtGST1    MIILEYIDET FE.GPSILPK D..PYDRALA RFWAKFLDDK 103
TcAc2     DLISRYIDRI SSPANALMGS S..PYQRHRV EFFLGEIGDL 105

PCB7      IASVISSFK. .....GEDA. ..DVGAPFDY LENALGKFAE 71
In2-1     TKALYSPLL. .....SHAAV SDEVVAALDK LEADLSKF.D 167
StGST1    .GAAVWKSFF SKGE.EQEKA KEEAYEMLKI LDNEF....K 137
GmHSP26-A IVGAVSKSVF TVDEKEREKN VEETYEALQF LENEL....K 143
NtGST1    .VAAVVNTFF RKGE.EQEKG KEEVYEMLKV LDNEL....K 137
TcAc2     VKAYFGLVRD PFNEEKRKSV DNNTAYIEDI IAEHQ....G 141

PCB7      EGPFFLGKEF SLVDIAYAPF LERFQPFLLE VKGYDVTVGR 111
In2-1     DGPFFLG.QF SLADVAYVTI LERVQIYYSH LRNYDIAQGR 206
StGST1    DKKCFVGDKF GPADI.VANG AALYLGILEE VSGIVLATSE 176
GmHSP26-A DKKFFGGEEF GLVDI.AAVF IAFWIPIFQE IAGLQLFTSE 182
NtGST1    DKKFFAGDKF GFADI.AANL VGFWLGVFEE GYGDVLVKSE 176
TcAc2     DGPYFLDDTF SMAEVMVVPF LACFRPVLSY YCGYDIFHNA 181

PCB7      ..PKLVKWFE EVNGIESYRQ TKWDAKELLA LHKKKWLL   147
In2-1     ..PNLQEFID EMNKIEAYAQ TKNDPLFLLD LAKSHLKIA  242
StGST1    KFPNFCAWRD EY..CTQNEE YFPSRDELLI RYRAYIQPVD 214
GmHSP26-A KFPILYKWSQ EFLNHPFVHE VLPPRDPLFA YFKARYESLS 222
NtGST1    KFPNFSKWRD EYINCSQVNE SLPPRDELLA FFRARFQAVV 216
TcAc2     ..PRLKKMYV TSMQRTTVKE TISKPEEYII GFKSKVPKSH 219

PCB7
In2-1
StGST1    ASK                                         217
GmHSP26   ASK                                         225
NtGST1    ASRSAPK                                     223
TcAc2     VTWSLAPGYV                                  229
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Figure 4. Comparison of the putative amino acid sequence of PCB7, the deduced amino acid sequence of the maize In2-1 protein [17] (pir:s17743; 44% identity) and 4 sequences belonging to the GST superfamily from potato [33] (GST1; 20% identity), soybean [5] (GmHSP26-A: sp:q03663; 26% identity), tobacco [36] (GST1: sp:q03663; 23% identity) and *T. cruzi* (TcAc2: gp:l07519; 20% identity). Common amino acids are shown in bold and accession numbers are shown in parentheses with levels of homology.

it is conceivable that PCB7 is a stress protein related to GSTs but specialized for a role in desiccation tolerance in seeds. The pattern of expression of the PCB7 message was similar to that of PCB2 including the indication of a gene family (Fig. 5).

The putative amino acid sequences of PCB3 and PCB4 both shared homology to vicilin-like (7S group) storage proteins, but the cDNAs did not cross-hybridize at high stringency (data not shown). The presence of two vicilin transcripts in *Trollius* is consistent with the vicilin gene family of other species, for example in pea, where the vicilin protein is synthesized as several polypeptides encoded by distinct mRNAs [11, 23]. It was found that the transcripts corresponding to pPCB3 and pPCB4 cDNAs showed different steady-state levels in dormant imbibed seeds (Fig. 5). The steady-state level of the PCB4 transcript remained constant for at least 12 days during imbibition but that of PCB3 increased between 2 and 4 days after the start of imbibition. It is not clear why the steady-state

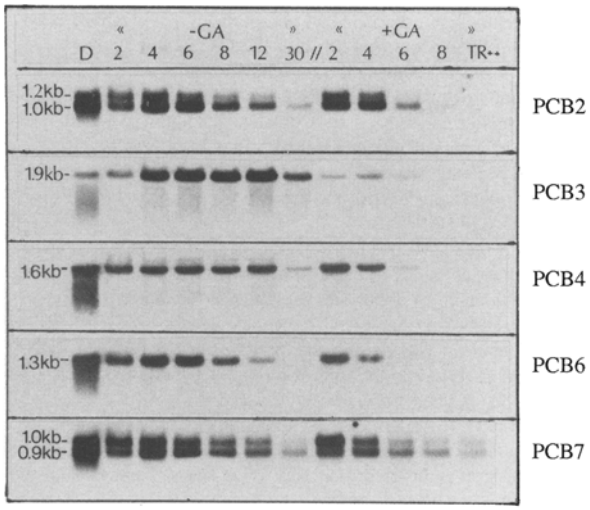

Figure 5. Northern analysis of transcripts hybridising to [^{32}P]-labelled probes made as described [8] from cDNA inserts of 5 clones as indicated. Total RNA (12 μg/lane) was extracted as described [32] from dry seed (D) and at the days indicated after imbibition (−GA) and GA-induced germination (+GA) including a stage of advanced testa rupture just prior to radicle emergence. RNA was separated on a 1% agarose gel containing 8% formaldehyde and 20 mM Na_2HPO_4, then transferred to GeneScreen membrane (Dupont). Hybridization of the blot was carried out in solution containing 50% deionized formamide, 1 M NaCl, 1% SDS, 10% dextran sulphate and 20 μg/ml salmon sperm DNA. Filters were washed to a final stringency of 0.1 × SSPE, 0.1% SDS at 65 °C for 30 min and exposed to Kodak X-AR5 film. The transcript sizes are indicated in kilobases. Data represent successive reprobings of the same membrane. Equal loading of lanes was confirmed by ethidium bromide staining to check the intensity of the ribosomal bands.

level of this storage protein mRNA increases following imbibition. The individual subunits which make up the vicilin storage protein may be mobilized at different stages of germination as part of a controlled breakdown of reserves as has been suggested for pea vicilins [23]. Some of these subunits may also be mobilized at a slow rate during imbibition. Such breakdown of stored reserves can be seen quite distinctly even in freshly imbibed *Trollius* seeds adjacent to the embryo and in peripheral parts of the endosperm [15]. *De novo* synthesis of mRNAs corresponding to these subunits may appear during imbibition to allow their resynthesis using the free amino acids accumulating as a consequence of the slow breakdown of the protein subunits. Protein may also be synthesized in the embryo from the amino acids released in the endosperm. The resynthesis of storage proteins described above may help to maintain them so that their constituent amino acids are unavailable for germination-related events. This may be part of a process preventing germination and would support the hypothesis that dormancy is actively maintained [24].

Differential screening of the library demonstrated that there was no increased steady-state level of any mRNA up to 8 days from the start of germination. At this stage testa rupture had appeared in the majority of seeds which implied that dormancy had been broken and the initial events of germination had begun. The absence of any detectable increases in specific mRNAs contrasts with recent evidence from both monocot and dicot seeds with different dormancy mechanisms. The use of two dimensional gel electrophoresis to resolve mRNA *in vitro* translation products has provided evidence for changes in the steady-state levels of particular mRNAs when comparing dormant and non-dormant seed tissue. In embryos of *A. saccharum* and *P. menziesii* a number of proteins were induced during dormancy breaking brought about by seed stratification [14, 34]. Schneider and Gifford [30] found that new proteins were synthesized *de novo* in the megagametophyte of *Pinus taeda* coinciding with that period of stratification which released dormancy in the majority of the seed population. Dyer [7] and Li and Foley [21] both found several mRNAs that were up-regulated in non-dormant embryos of *A. fatua* before visible germination commenced but did not appear in imbibed dormant embryos. In addition, Johnston *et al.* [19] were successful in pinpointing several mRNAs that were specific to non-dormant (and also dormant) embryos of *A. fatua* using the technique of differential display. In *Trollius* our results indicate that if new mRNAs are being expressed prior to or during testa rupture these are occurring at low levels or are expressed to sufficiently abundant steady-state levels but only in specific cells, such as those of the embryo or in particular regions of the endosperm. Alternatively, it is possible that an increase in steady-state levels of particular mRNAs is not characteristic of dormancy breaking and early germination in all species but is dependent upon the process by which dormancy is broken.

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