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ORIGINAL ARTICLE

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Characterization of the genes for two soybean aspartic proteinases and analysis of their different tissue-dependent expression

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Abstract We isolated and characterized two cDNAs for aspartic proteinases (APs; EC 3.4.23) in soybean [*Glycine max* (L.) Merr.]. The encoded enzymes, soyAP1 and soyAP2, share 55% amino acid sequence identity. Northern analysis demonstrated that *soyAP1* is expressed specifically in seeds, especially in dry seeds, while the expression of *soyAP2* takes place in various tissues such as roots, stems, leaves and flowers, but not in dry seeds. *SoyAP1* is highly expressed even at an early stage of germination, with a subsequent decrease in expression intensity. In contrast, the *soyAP2* mRNA level increases 48 h after imbibition. To elucidate the physiological functions of soyAPs, we investigated the localization of *soyAP* expression in seeds germinating for 48 h at 25°C. *SoyAP1* shows cell-type-specific expression in sieve tube cells of the hypocotyl. At the root tip, *soyAP1* is expressed in immature tracheary elements and sieve tube cells, and its expression pattern changes with distance from the tip; strong signals observed throughout phloem converge gradually to sieve tube cells, whereas those observed in tracheary elements disappear while the elements are still immature. On the other hand, *soyAP2* signals were detected broadly in the boundary region between the cortex and the central cylinder. These results suggest that *soyAP1* and *soyAP2* are functionally different from each other.

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Keywords Aspartic proteinase · *Glycine* · In situ hybridization · Programmed cell death · Sieve tube cell · Tracheary element

Abbreviations *AP* Aspartic proteinase · *PCD* Programmed cell death · *hai* Hours after imbibition · *daf* Days after flowering

Introduction

Aspartic proteinases (APs; EC 3.4.23) occur in a wide variety of plants (Mutlu and Gal 1999). There are multiple APs in rice (Asakura et al. 1995a, 1995b) and *Arabidopsis thaliana* (Chen et al. 2002). Some APs are ubiquitously expressed in various tissues, as in the case of phytepsin, a barley AP that is expressed in resting seeds, roots, stems, leaves and flowers (Runeberg-Roos et al. 1991; Törmäkangas et al. 1994). Similarly oryzasin 1, a rice AP, is expressed in seeds, leaves and roots (Asakura et al. 1995b), and AtPasp-A1, an *A. thaliana* AP, is expressed in seed pods, flowers, stems, leaves, roots and dry seeds (Chen et al. 2002). However, the expression of other APs is tissue-specific. These include cardosin A and cardosin B occurring only in young flowers of *Cynara cardunculus* (Veríssimo et al. 1996; Vieira et al. 2001). Also, AtPasp-A2 occurs only in dry seeds and seed pods (Chen et al. 2002), and AtPasp-A3 only in flowers and seed pods (Chen et al. 2002). All these findings of different tissue-specific expression of APs may indicate their different physiological functions in plants. Several attempts have thus been made to elucidate the functions of plant APs. Investigations of the enzymatic reactions of APs in vitro have demonstrated their involvement in processing of storage-protein precursors into mature proteins. For example, a *Brassica napus* AP (D'Hondt et al. 1993) cleaves the *Arabidopsis* 2S albumin precursor. Phytepsin (Runeberg-Roos et al. 1994) and an *A. thaliana* AP (Mutlu et al. 1998) process probarley lectin. Oryzasin 1

(Asakura et al. 2000) digests the rice storage protein glutelin. Some functions of APs can also be deduced by observing their enhanced expression under unusual conditions for plant growth. For example, APs were expressed in daylily petals (Panavas et al. 1999) and *B. napus* leaves (Buchanan-Wollaston and Ainsworth 1997) during senescence. Some APs may be involved in self-protection, as in the case of tomato leaf AP whose expression is induced by wounding (Schaller and Ryan 1996).

Other functions of APs are suggested by their localization in plant tissues. The co-localization of phytepsin and barley lectin in root cells indicates that this proteinase is a lectin processor (Runeberg-Roos et al. 1994). Moreover, phytepsin is expressed in developing tracheary elements and sieve tube cells undergoing autolysis (Runeberg-Roos and Saarma 1998). Cardosin A, which contains an Arg-Gly-Asp (RGD) motif, is expressed in protein storage vacuoles of the stigmatic papillae and vacuoles of epidermal cells of the style (Ramalho-Santos et al. 1997). The RGD motif is known as an integrin-binding sequence in mammalian tissues. It is also involved in facilitating many cell recognition functions such as adhesion, migration, signaling, differentiation, growth and other cognitive functions of cells. This suggests the involvement of an adhesion-mediated proteolysis in pollen recognition and growth (Faro et al. 1999).

While information is thus available about plant APs and their possible functions, little is known about APs in soybean, which is an important food protein resource. It is reported that soybean AP occurs in both the embryonic axis and the cotyledon (Bond and Bowles 1983), but no information has been provided so far on its molecular entity and functions. With this as a background, we undertook a study to investigate soybean APs in detail. Here we report the primary structures of APs isolated from soybean and their possible functions deduced from their tissue- and stage-specific expression.

Materials and methods

Plant materials and cultivation

Seeds of the soybean *Glycine max* (L.) Merr., cultivar Enrei, were obtained from Takinogawa-Syubyo Co., Tokyo. The seeds were allowed to germinate prior to sampling 5 days, 10 days, 2 weeks and 3 weeks after imbibition. Mature and immature plants were grown in the Experimental Field at the University of Tokyo and in its greenhouse controlled at 30°C, respectively.

Isolation of soybean AP cDNA clones

Soybean seeds sampled 1 day and 3 days after imbibition were frozen in liquid nitrogen, and ground with a mortar and a pestle. Total RNA was extracted from soybean seeds by the phenol/SDS extraction method (Brawerman et al. 1972). The single-strand cDNA was synthesized from total RNA with a First-Strand cDNA Synthesis Kit (Amersham Biosciences, UK) using *Not* I-d(T)$_{18}$ primer. Two oligonucleotide primers, P1 (5′-GAT CGA ATT CGA CAC TGG CAG CTC CAA CCT CTG G-3′, sense primer with an *Eco*RI cloning site) and P2 (5′-GAT CAA GCT TGA AAA CGT CAC CCA GGA TCC A-3′, anti-sense primer with a *Hin*dIII cloning site), were synthesized according to the amino acid sequences ^{103}Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp and ^{482}Trp-Ile-Leu-Gly-Asp-Val-Phe (oryzasin 1 numbering), which are consensus sequences of plant APs. PCR was performed with P1 and P2 primers using first-strand cDNA synthesized from total RNA from 1-day- or 3-day-germinating seeds as templates. PCR was carried out for 25 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing and 60 s at 72°C for polymerization. After two clones, frag-soyAP1 and frag-soyAP2, were obtained, 5′- and 3′-RACE (rapid amplification of cDNA ends) experiments were performed to obtain the full-length clones. An oligo(dA)-tail was added by use of terminal deoxynucleotidyltransferase. The soyAP1 gene-specific oligonucleotide primer, P3 (5′-TCC AAG TCC CAG TAT ACC ATC-3′), was used to amplify the 5′-end of the *soyAP1* cDNA clone through ten cycles of the polymerase reaction. Next, nested PCR was carried out using the P4 primer (5′-AAG TAC TTG ATT TGC TAG A-3′) as a 5′-upstream region of P3 and *Not*I-d(T)$_{18}$ primer.

3′-RACE oligonucleotide primers were designed based on the 3′-end sequence of frag-soyAP1. The 3′-RACE primer (5′-GAA TCA GCT GTT GAC TGT GGG-3′) was used to amplify the 3′-region of the cDNA. Full-length *soyAP1* was obtained by PCR with the 5′-end primer (5′-ATG GGG AAC AGG ATG AAT GCG-3′) and 3′-end primer (5′-AGT CCA TTT TGA CTG CAA CGG-3′). The full-length *soyAP2* clone was obtained by 5′- and 3′-RACE under the same conditions applied to soyAP1. 5′-RACE primers P6 (5′-TCC AAG TCC CAG TAT ACC ATC-3′) for the amplification of the *soyAP2* cDNA clone and P7 (5′-TGT CTT TGA TTT CTT AGC CGT-3′) nested PCR primer were used. The resulting fragments were cloned into pBluescript SK-vector (Stratagene, La Jolla, CA, USA).

Northern blot analysis

Plant tissues after sampling were immediately frozen in liquid nitrogen, and stored at −80°C until use. Seed coats were removed from seeds during the germination process. Total RNA was isolated from various plant tissues as described above. A 15-μg portion of total RNA was applied to a 1.0% formaldehyde-containing agarose gel, and then blotted onto a BIODYNE nylon membrane (Pall, East Hills, NY, USA) overnight at room temperature. The blot was prehybridized at 42°C for 3 h, and hybridization was done with [α-^{32}P]dCTP-labeled *soyAP1* or *soyAP2* full-length probes at 42°C overnight. The membrane was then washed three times in 2× SSC containing 0.1% SDS for 20 min at room temperature and twice in 0.1× SSC containing 0.1% SDS for 15 min at 65°C. The membrane was exposed overnight on an imaging plate (Fujifilm, Tokyo, Japan), and then the signals on the imaging plate were detected for analysis with an image analyzer FLA-3000G (Fujifilm).

Genomic southern analysis

Genomic DNA of soybean was digested with *Eco*RI, *Hin*dIII or *Pst*I at 37°C overnight. The blotted membranes were hybridized with [α-^{32}P]dCTP-labeled *soyAP1* or *soyAP2* full-length probes at 60°C overnight and washed at 60°C with 0.1× SSC containing 0.1% SDS for 1 h.

In situ hybridization

Fresh plant materials were fixed overnight in 4% (w/v) paraformaldehyde in 100 mM phosphate buffer (pH 7.3) at 4°C. Fixed tissues were dehydrated through a 50%, 70%, 90% and 100% ethanol series, placed in xylene, and embedded in Paraplast plus (Oxford, St. Louis, MO, USA). Sections (10 μm in thickness) were

cut and mounted on APS-coated slides (Mastunami, Osaka, Japan). Anti-sense and sense RNA probes labeled with DIG RNA Labeling Mix (Roche Biochemicals, Indianapolis, IN, USA) were transcribed from cDNA clones with T7 and T3 polymerases (Stratagene). Full-length cDNAs for *soyAP1* and *soyAP2*, and cDNA fragments for acyl CoA oxidase, *SCARECROW*, *ATHB-8* homologue, and *VAHOX1* homologue were used as templates. Deparaffinized sections were incubated with 1.5 μg ml^{-1} proteinase K in 100 mM Tris–HCl buffer (pH 7.5) containing 50 mM EDTA at 37°C for 30 min, and fixed in 4% (w/v) paraformaldehyde in 100 mM phosphate buffer (pH 7.3) at room temperature for 10 min. After acetylation with 100 mM triethanolamine (pH 8.0) containing 0.25% acetic anhydride, sections were dehydrated through a 50%, 70%, 90% and 100% ethanol series. Hybridization was performed in 50% (v/v) formamide, 5× SSC, 5× Denhardt's solution, containing 250 μg ml^{-1} tRNA, 500 μg ml^{-1} salmon testis DNA and 1 mM dithiothreitol overnight at 58°C. After hybridization, the slides were washed twice in 0.2× SSC at 58°C for 30 min. After the blocking reaction, sections were reacted for 1 h with anti-DIG-AP (Roche) and allowed to stand overnight at room temperature for visualization.

Isolation of four tissue-specific molecular markers

i. Acyl CoA oxidase (*ACX1;1* and *ACX1;2*; Agarwal et al. 2001). Two oligonucleotide primers coinciding with both *ACX1;1* (GenBank#: AF404403) and *ACX1;2* (GenBank#: AF404404), sense primer, 5′-TGC ATT GCT ACA AGA TAT AGT GC-3′, and antisense primer, 5′-AAC CAA GGT AGT GAT CAG TGT AG-3′ were synthesized.
ii. *SCARECROW* (*SCR*; Di Laurenzio et al. 1996; Lim et al. 2000; Sassa et al. 2001). *SCR* was searched for in the EST database for soybean (The TIGR Soybean Gene Index, http://www.tigr.org/tdb/tgi/gmgi/). Two oligonucleotide primers coinciding with the sequence of *SCR* (TC86110), sense primer, 5′-ACG AGG CCA CGG GAA AAC GCT TG-3′, and antisense primer, 5′-ATG GAA AGG TGG TCT CCA AGC TG-3′ were synthesized.
iii. *Arabidopsis* homeobox gene (*ATHB-8*; Baima et al. 1995) homologue. A soybean *ATHB-8* homologue was searched for in the EST database described above. Two oligonucleotide primers coinciding with the sequence of soybean *ATHB-8* (TC81646), sense primer, 5′-GCA CCG ATC AGA ATG GGC AGA CA-3′, and antisense primer, 5′-GGA TAA ACA GAT ACC ACC TTG AA-3′ were synthesized.
iv. Tomato homeobox gene (*VAHOX1*; Tornero et al. 1996) homologue. A soybean *VAHOX1* homologue was searched for in the EST database described above. Two degenerate oligonucleotide primers, designed from the consensus amino acid sequences deduced from the TC78881 and TC79817 nucleotide sequences, sense primer, 5′-T(A/G)(T/C) TT(T/C) CA(T/C) CA(A/G) CCI G(A/G)I AA(A/G) AA-3′, and antisense primer, 5′-GA(A/G) TCI AA(A/G/T) AT(A/G) TCA CTI (T/C)TI GC-3′ were synthesized.The fragments of these genes were amplified by PCR using first-strand cDNA synthesized from the total RNA from 5-day-old plants.

Results

Isolation and characterization of soybean APs

Two cDNA fragments encoding soybean APs, *soyAP1* and *soyAP2*, were isolated. Nucleotide sequencing indicated that soyAP1 and soyAP2 comprise 514 and 508 amino acid residues, respectively, sharing 55% amino acid identity. Two aspartic acid residues regarded as the active site and a plant-specific insertion (PSI) were found to exist in both soyAPs (Fig. 1a). A low similarity exists between soyAP1 and soyAP2 with respect to the PSI and the N-terminal pre-pro-sequence. SoyAP1 shows 87% amino acid identity to cowpea AP, while soyAP2 shares 63–65% amino acid identity with APs of the carnivorous plant *Nepenthes alata* (An et al. 2002) and a tomato AP which is induced by wounding (Schaller and Ryan 1996). The classification of various plant APs by an unrooted dendrogram shows that soyAP1 and soyAP2 belong to different groups (Fig. 1b).

An EST database search revealed the existence of the at least three other genes for soyAPs, *soyAP3* (GenBank # AW621056), *soyAP4* (TC47825 in the TIGR Soybean Gene Index) and *soyAP5* (TC52366 in the TIGR Soybean Gene Index). As far as the results of cDNA fragment sequencing are concerned, *soyAP3* differs from both *soyAP1* and *soyAP2*, while *soyAP4* and *soyAP5* resemble *soyAP1* and *soyAP2*, respectively. Considering these data, we investigated soyAP1 and soyAP2 as representative soybean APs.

Comparison of *soyAP1* and *soyAP2* transcripts by northern blot analysis

At least five APs were found in soybean seeds. We first conducted genomic Southern analysis of *soyAP1* and *soyAP2*, and confirmed that *soyAP1* and *soyAP2* clones do not cross-hybridize with each other (Fig. 2a). Our preliminary northern analysis showed that the expression pattern of *soyAP1* differed absolutely from that of *soyAP4* (data not shown). This indicates that the *soyAP1* cDNA probe does not hybridize with other *soyAP* mRNAs. On the other hand, the expression pattern of *soyAP5* was quite similar to that of *soyAP2* (data not shown). Because *soyAP2* closely resembles *soyAP5* (nucleotide sequence identity: 83%, E-value: 1e−79), we cannot exclude the possibility of cross-hybridization of the *soyAP2* probe with *soyAP5* mRNA.

Northern analysis was then carried out with 5-day-old stems, 2-week-old roots and 2-week-old leaves as well as with fully developed flower tissues, with the result that in all these samples the *soyAP2* mRNA is more strongly expressed than the *soyAP1* mRNA (Fig. 2b). Similar results were obtained with roots and leaves aged 5, 10, and 21 days (data not shown).

The expression of *soyAP1* and *soyAP2* in seeds during maturation was investigated (Fig. 2c). Phenomenologically, soybean seeds began developing shortly after flowering, gradually increased in size, and reached a maximum size by about 30 days after flowering, with a subsequent gradual decrease in size due to water loss. *SoyAP1* expression was weak at an early stage of the water loss that began about 35 days after flowering. At the middle stage of water loss (45 days after flowering), *soyAP1* transcripts had accumulated, and kept on increasing until the seeds reached a dry stage (50 days after flowering) (Fig. 2c). Apparently, the water loss from seeds is related to the strong expression of *soyAP1*. In contrast, *soyAP2* expression was not detected during the water loss or the subsequent dry-seed stage (Fig. 2c).

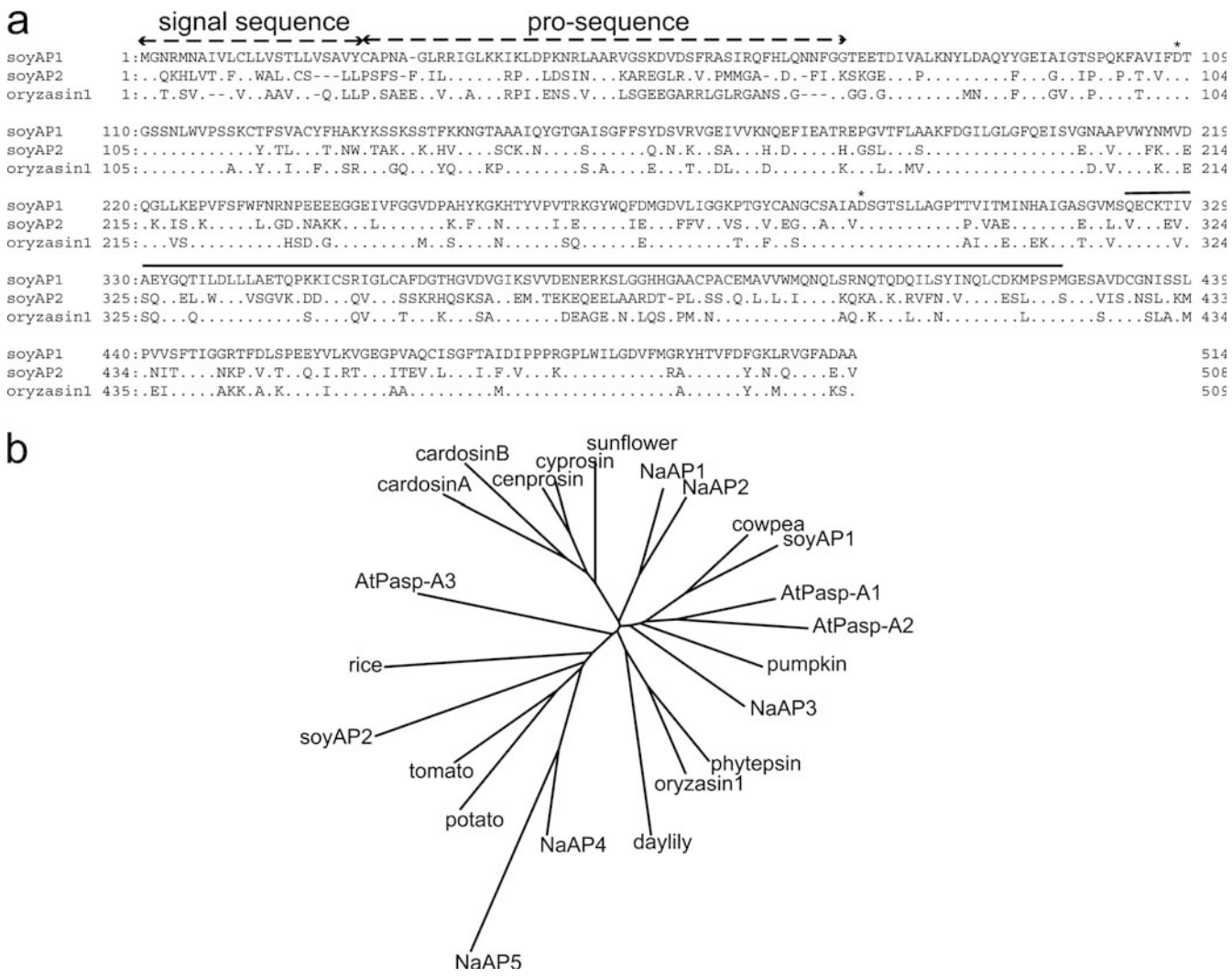

Fig. 1a,b Comparison of two soybean (*Glycine max*) APs with other plant APs. **a** Amino acid sequences deduced from cDNA clones. GenBank database accession numbers are: soyAP1, AB069959; soyAP2, AB070857. These sequences are aligned together with that of rice (*Oryza sativa*) oryzasin 1 (accession no. D32144). Amino acid residues identical to soyAP1 are denoted by *dots*; *gaps* were inserted to improve alignment. The *asterisks* indicate the aspartic acid residues in the active sites. The plant-specific insertion is *overlined*. **b** Phylogenetic relationships among several plant APs. The unrooted dendrogram was generated by CLUSTAL W version 1.81. The sequences of APs from various sources are as follows: potato (*Solanum tuberosum*), AF259982; tomato (*Lycopersicon esculentum*), L46681; *Nepenthes alata* NaAP1, AB045891; *N. alata* NaAP2, AB045892; *N. alata* NaAP3, AB045893; *N. alata* NaAP4, AB045894; *N. alata* NaAP5, AB045895; rice, D12777; pumpkin (*Cucurbita pepo*), AB002695; cowpea (*Vigna unguiculata*), U61396; phytepsin, X56136; daylily (*Hemerocallis*), AF082029; cardosin A, AJ132884; cardosin B, AJ237674; cenprosin, Y09123; cyprosin, X81984; sunflower (*Helianthus annuus*), AB025359; *Arabidopsis thaliana* AtPasp-A1, At1g11910; *A. thaliana* AtPasp-A2, At1g62290; *A. thaliana* AtPasp-A3, At4g04460

The temporal expression of the two APs during the germination process was examined at 25°C (Fig. 2d). Dry seeds gradually increased in size by imbibition and, within 48 h after imbibition (hai), most seeds had germinated, with the seed coats broken by the roots. The expression level of *soyAP1* at 3 hai was slightly lower than that in dry seeds. The amount of *soyAP1* mRNA decreased with time and, at 24 hai, the amount of *soyAP1* mRNA decreased remarkably. The expression level of *soyAP1* was also very low at 48 hai. In contrast, *soyAP2* mRNA was not detected until 8 hai, but was slightly detected at 24 hai. At 48 hai, the amount of *soyAP2* mRNA increased dramatically, suggesting that the dominant AP in seeds during the germination process changes from *soyAP1* to *soyAP2* (Fig. 2d).

In situ localization of *soyAP1* and *soyAP2*

In order to study the localization of *soyAP1* and *soyAP2*, in situ hybridization was performed with the 48-hai root and hypocotyl (Fig. 3a). Strong *soyAP1* signals were detected at the boundary region between the cortex and the central cylinder in the root, and the strongest signals was observed at a distance of about 800 μm from the root tip (Fig. 3b). In contrast, no signal was detected by a sense probe for *soyAP1* (Fig. 3c). As for the transverse

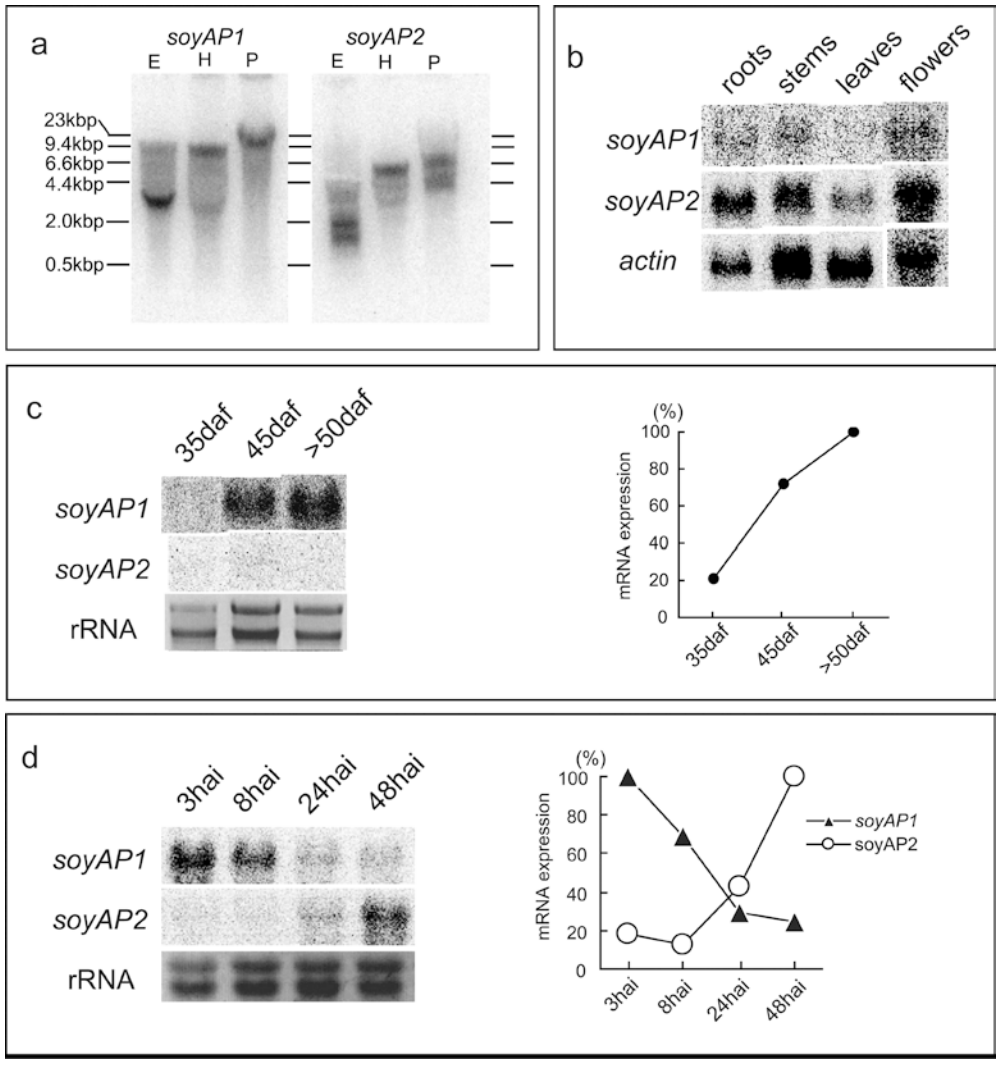

Fig. 2a–d Genomic Southern and northern analysis of *soyAP1* and *soyAP2*. **a** Genomic Southern analysis of *soyAP1* and *soyAP2*. Genomic DNA of soybean was digested with *Eco*RI (*E*), *Hin*dIII (*H*) and *Pst*I (*P*). **b** Expression of mRNA in various tissues. Roots and leaves of 2-week-old plants, and stems of 5-day-old plants were used. **c** Expression of *soyAP1* and *soyAP2* mRNA in seeds at different maturation stages. Seeds sampled more than 50 days after flowering (*>50 daf*) were dry seeds. *Left panel* The *bottom row* shows rRNA. *Right panel* Accumulation of *soyAP1* mRNA in seeds during maturation. The amount of mRNA was calibrated relative to rRNA amount. Normalized intensities of soyAP1 are shown. **d** Expression of *soyAP1* and *soyAP2* mRNA in seeds at different germination stages. Seeds were incubated in the dark at 25°C. Seed coats were removed from the seeds. *hai* Hours after imbibition. *Left panel* The *bottom row* shows rRNA. *Right panel* Changes in the amount of mRNA of *soyAP1* (*solid triangles*) and *soyAP2* (*open circles*) during the germination process. The amount of mRNA was calibrated relative to that of rRNA. Normalized intensities of soyAPs are shown

hypocotyl sections, the boundary region between the cortex and the central cylinder was dotted with strong signals of *soyAP1* (Fig. 3d). The results indicate that *soyAP1* is highly expressed specifically in certain cells.

On the other hand, *soyAP2* signals were detected in the boundary region between the cortex and the central cylinder in the root (Fig. 3e), whereas no signal was detected by a sense probe for *soyAP2* (Fig. 3f). Unlike *soyAP1*, *soyAP2* signals looked unclear around the boundary, and were concentrated near the apical meristem (Fig. 3e). As for the hypocotyl, broad signals were observed in the boundary region between the cortex and the central cylinder (Fig. 3g).

We then identified the cells in which *soyAP1* is highly expressed. For identification, tissue-specific molecular markers were used, such as acyl CoA oxidases, ACX1;1 and ACX1;2 (Fig. 3h), which localize in phloem cells of the soybean hypocotyl (Agarwal et al. 2001). Using a mixture of these marker genes and *soyAP1* as probes, we found that the strong *soyAP1* signals coincide with those of the markers (Fig. 3i). This shows that *soyAP1* is highly expressed in the phloem. Furthermore, using a soybean gene similar to *VAHOX1*, which is a phloem-specific marker gene in tomato (Tornero et al. 1996), we confirmed that *soyAP1* is highly expressed in the phloem (data not shown). Next, *SCR* (*SCARECROW*), which is specifically expressed in the endodermis (Di Laurenzio et al. 1996; Lim et al. 2000; Sassa et al. 2001), was used as a marker (Fig. 3j), and strong *soyAP1* signals were found inside the endodermis (Fig. 3k). A magnified view (Fig. 3l) clearly shows *soyAP1* expression in the cells constituting the second or third layer internal to the endodermis. Considering the signal location and cell shape, we conclude that *soyAP1* is expressed specifically in phloem sieve tube cells.

Expression of *soyAP1* and *soyAP2* in developing root tips

In order to clarify the relationship between distance from the root tip and the site of expression, experiments were carried out with transverse sections of the root tip region obtained 48 hai at 25°C (Fig. 4a–e).

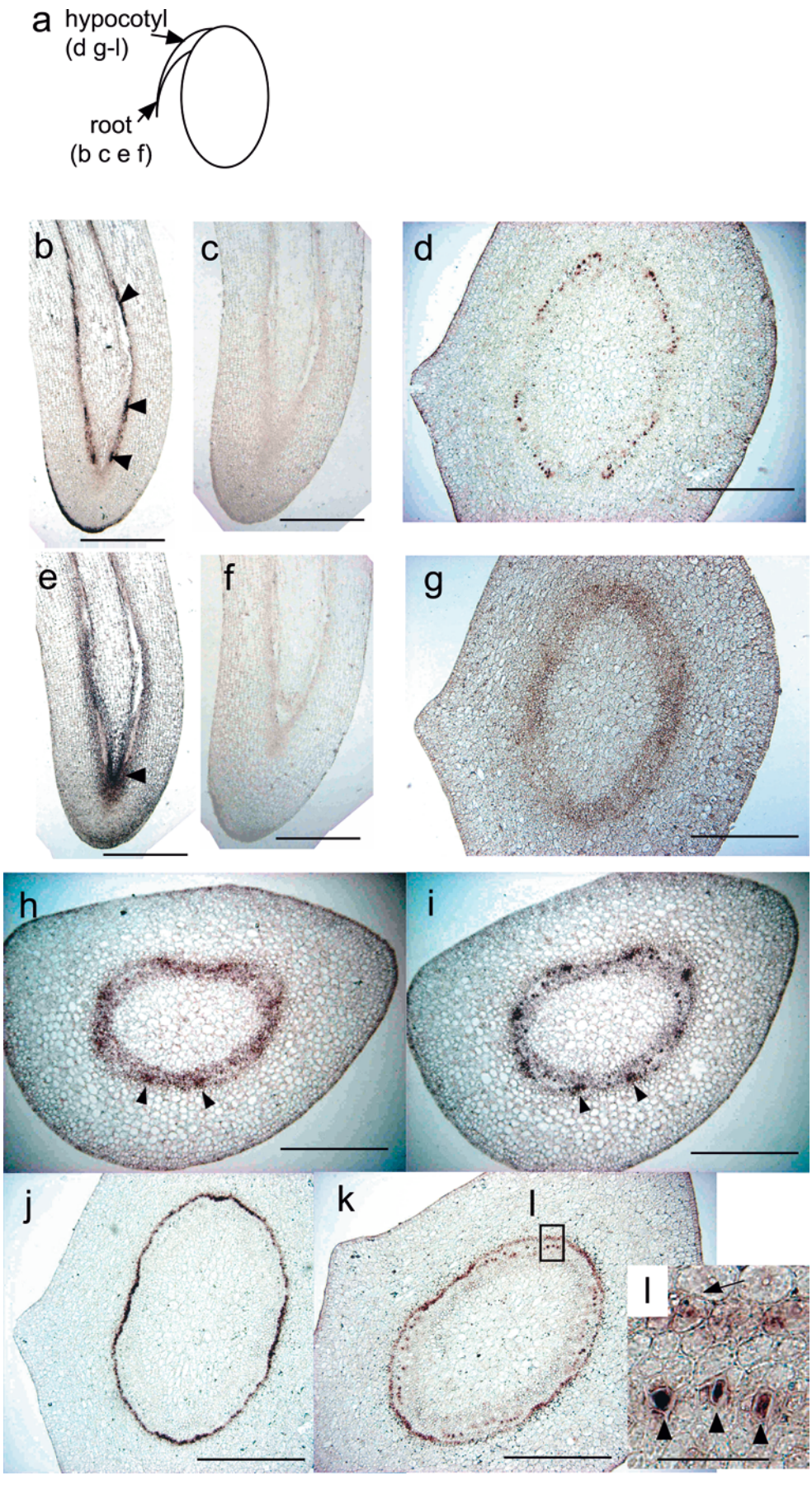

Fig. 3a–l In situ hybridization of *soyAP1* and *soyAP2* to the roots and hypocotyls of soybean seeds imbibed for 48 h at 25°C. **a** Illustration of sample. **b–d** Localization of *soyAP1*. Longitudinal root sections (**b,c**) and a transverse hypocotyl section (**d**) were hybridized with a DIG-labeled antisense *soyAP1* full-length probe (**b,d**) or a sense *soyAP1* full-length probe (**c**). Strong *soyAP1* signals were detected at the boundary region between the cortex and the central cylinder (*arrowheads*). **e–g** Localization of *soyAP2*. An antisense *soyAP2* full-length probe (**e,g**) and a sense *soyAP2* full-length probe (**f**) were used. Concentrated signals were observed near the apical meristem (*arrowhead*). **h,i**, Comparison of the expression of acyl CoA oxidase with that of *soyAP1*. A mixture of *ACX1;1* and *ACX1;2* (**h**) or a mixture of *ACX1;1*, *ACX1;2* and *soyAP1* (**i**) were used as probes. Strong signals for *ACX1;1* and *ACX1;2* in the phloem (*arrowheads* in **h**) overlay strong signals for *soyAP1* (*arrowheads* in **i**). **j–l**, Expression of *soyAP1* in sieve tube cells. *SCR* (**j**) or a mixture of *SCR* and *soyAP1* (**k,l**) were used as probes. Strong *soyAP1* signals (*arrowheads*) were observed in sieve tube cells internal to *SCR* signals expressed in the endodermis (*arrow*). Bars = 1 mm (**b,c,e,f**), 500 μm (**d,g,h–k**) and 50 μm (**l**)

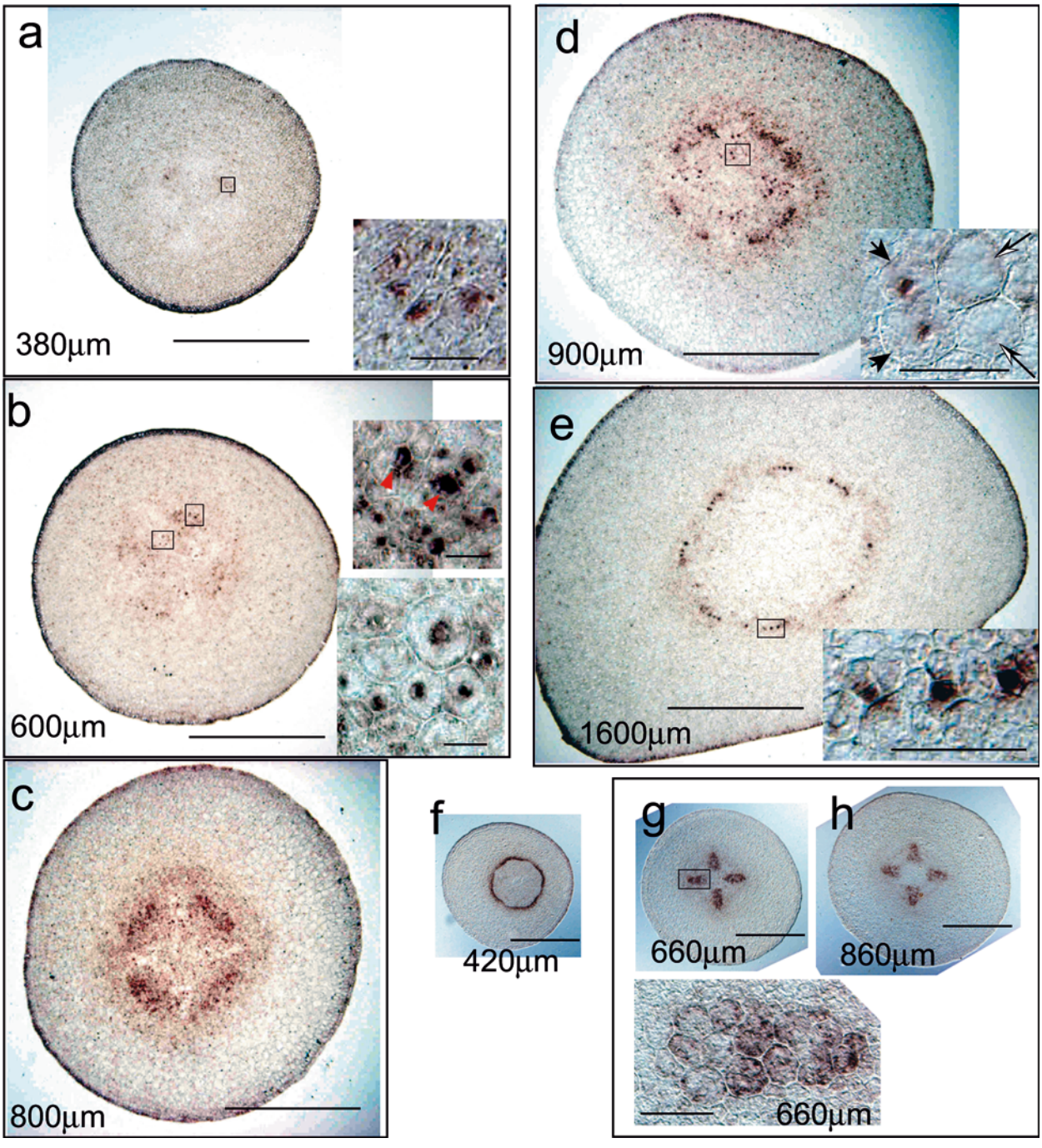

Fig. 4a–h In situ hybridization of *soyAP1* to the roots of soybean seeds imbibed for 48 h at 25°C. **a–e**, Expression of *soyAP1* according to the distance from the root tip. Transverse root sections were hybridized with an antisense *soyAP1* full-length probe. The distances from the root tip are given on each picture. The expression pattern changed with distance from the tip. At 600 µm (**b**, *upper insert*), *red arrowheads* indicate sieve tube cells. At 900 µm (**d**, *insert*), *soyAP1* was expressed in some tracheary elements (*arrows*) and not in others (*half-arrows*). **f** Localization of *SCR* at 420 µm from the root tip. **g,h** Localization of the soybean *ATHB-8* homologue. Bars = 500 µm (**a–h**), 20 µm (inserts **a,b**) and 50 µm (inserts **d,e,g**)

SoyAP1 expression in the phloem

Weak *soyAP1* signals appeared in sieve tube cells at a position 380 µm from the root tip (Fig. 4a and insert). At 420 µm from the root tip, ring-like *SCR* signals were detected in the endodermis (Fig. 4f). This indicates that the vascular system is formed at this position. Generally, the vascular system is immature at the root tip, and becomes mature as it gets further from the tip. At a position 600 µm from the tip (Fig. 4b), *soyAP1* was clearly

expressed in sieve tube cells (Fig. 4b, insert top). At 800 μm, the signal intensity became strong (Fig. 4d). To confirm the localization of *soyAP1*, we used the organ-specific molecular marker, *ATHB-8* homeobox gene, which is expressed in the procambium of *A. thaliana* (Baima et al. 1995). It was found that a soybean *ATHB-8* homologue gene is expressed in the xylem (Fig. 4g,h). By comparing the expression pattern of *soyAP1* with that of the soybean *ATHB-8* homologue, it was confirmed that *soyAP1* is expressed throughout the phloem at 800 μm from the root tip (Fig. 4c). The signals appeared throughout the phloem, comprising companion cells, sieve tube cells and parenchyma cells, in the range 380 μm to 800 μm from the root tip (Fig. 4a–c), and their positions converged to the sieve tube cells with distance from the root tip (Fig. 4d,e; e, insert).

SoyAP1 expression in the xylem

SoyAP1 signals were not detected at the 380-μm position from the root tip in the tracheary elements, but they began to appear at 600 μm or onwards (Fig. 4b and lower insert). Signals were observed in part of the xylem (Fig. 4g), and their intensity did not become stronger at 800 μm, but remained at a constant level (Fig. 4c). At 900 μm from the root tip, the xylem (Fig. 4h) was dotted with *soyAP1* signals (Fig. 4d). Observation at higher magnification showed that some tracheary elements contain signals while others do not (Fig. 4d, insert). At the 1,600-μm position, no *soyAP1* signal exists (Fig. 4f), though the tracheary elements were immature.

In summary, *soyAP1* is expressed in both immature tracheary elements and sieve tube cells, with the expression pattern depending on the developmental stage of the vascular system.

SoyAP1 expression in mature vascular system

An in situ hybridization experiment was carried out with 1-week-old plant roots (Fig. 5a) to look at the expression of *soyAP1* in their mature tracheary elements and sieve tube cells. Each of the roots at this age has developmentally different zones. There is an immature vascular system at the root tip (Fig. 5b), a developing vascular system further back from the tip, and a fully developed vascular system very far (> 5 mm) from the tip (Fig. 5c). In the latter zone, there are mature tracheary elements and sieve tube cells in which no *soyAP1* signal appears (Fig. 5c), though the signal was observed in the immature vascular system (Fig. 5b).

SoyAP2 expression

The expression pattern of *soyAP2* in the region 340–1,400 μm from the root tip 48 hai at 25°C, differs from that of *soyAP1*. *SoyAP2* signals were detected broadly in the central cylinder and the expression pattern did not change with the distance from the root tip (Fig. 6a–c).

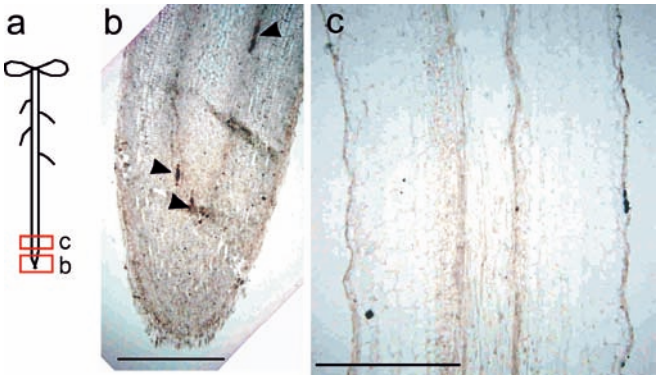

Fig. 5a–c In situ hybridization of *soyAP1* to the root of a 1-week-old plant. **a** Illustration of sections for hybridization. **b** Expression of *soyAP1* in the root tip. **c** Expression of *soyAP1* in the mature vascular system in the root. *SoyAP1* signals were observed in immature vascular systems (*arrowheads*). Bars = 500 μm (**b,c**)

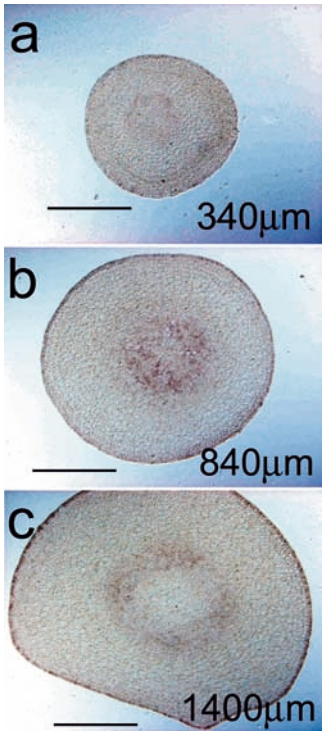

Fig. 6a–c In situ hybridization of *soyAP2* to the root of a soybean seed imbibed for 48 h at 25°C. **a–c** Expression of *soyAP1* according to the distance from the root tip. Transverse root sections were hybridized with an antisense *soyAP2* full-length probe. The distances from the root tip are given on each picture. Bars = 500 μm

Expression of *soyAP1* and *soyAP2* at an early stage of germination

We then examined *soyAP* expression at the early stage of germination, although obtaining sections from this stage was difficult because of insufficient imbibition. Thus, imbibition was done for 48 h at 4°C, because germination progresses very slowly at this temperature. Though seeds treated by this procedure increased in size due to

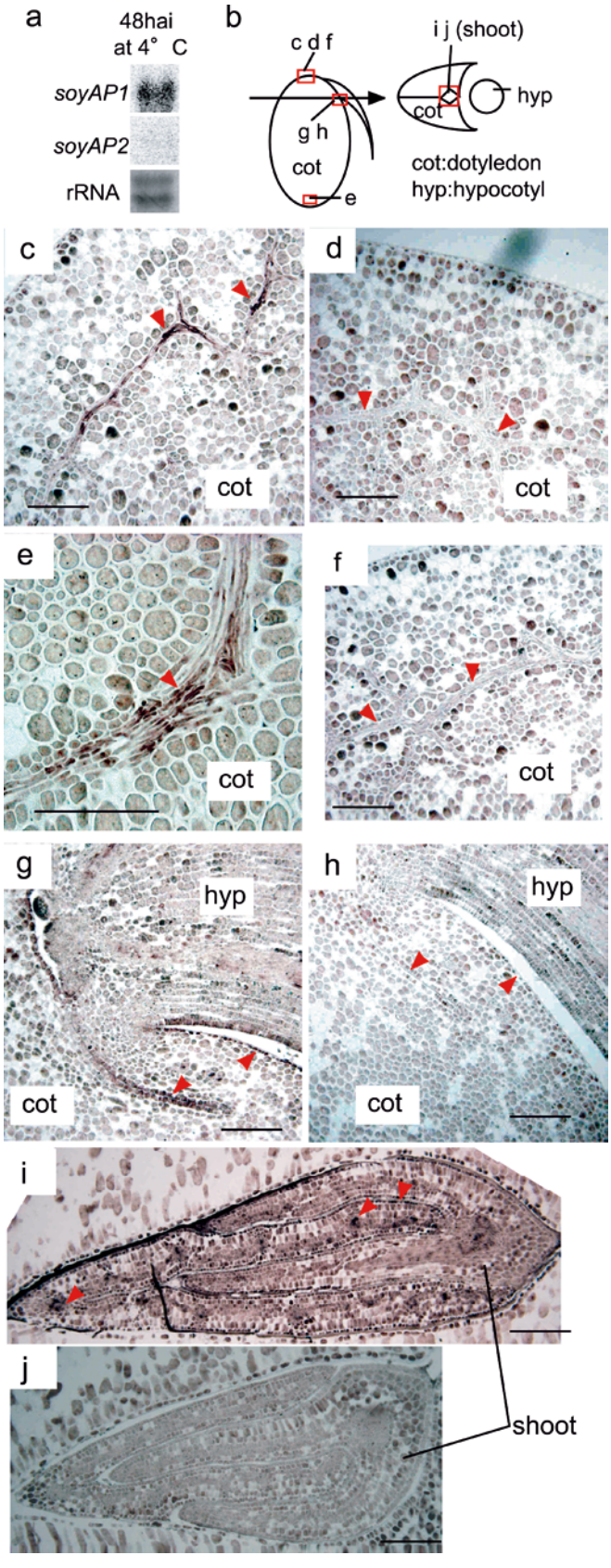

Fig. 7a–j In situ hybridization of *soyAP1* and *soyAP2* to soybean seeds at the early stage of germination. **a** RNA gel blot analysis of *soyAP1* and *soyAP2* from seeds imbibed for 48 h at 4°C. Seed coats were removed from the seeds. The *bottom panel* shows rRNA. **b** Longitudinal and transverse illustrations of soybean seed samples. **c–e** Expression of *soyAP1* in the cotyledon. Sections close to the hypocotyl (**c,d**) and distant from the hypocotyl (**e**) were hybridized with an antisense *soyAP1* fell-length probe (**c,e**) or a sense *soyAP1* full-length probe (**d**). **f** Expression of *soyAP2* in the cotyledon. The section was hybridized with an antisense *soyAP2* full-length probe. Clear signals for *soyAP1* (*red arrowheads* in **c–f**) were detected in the provascular strands, while no *soyAP2* signals were detected there. **g,h** Expression of *soyAP1* in the cotyledon. Sections were hybridized with an antisense *soyAP1* full-length probe (**g**) or a sense *soyAP1* (**h**). *Red arrowheads* indicate the epidermis. **i,j** Expression of *soyAP1* in the shoot. Sections were hybridized with an antisense *soyAP1* full-length probe (**i**) or a sense *soyAP1* full-length probe (**j**). *SoyAP1* signals were observed in the provascular strands and the epidermis (*red arrowheads*). Bars = 200 μm (**c–j**)

imbibition, they did not germinate. Northern blot analysis showed that *soyAP1* is expressed much more strongly than *soyAP2* (Fig. 7a). This expression pattern was the same as found at 3 hai and 8 hai at 25°C (Fig. 2d). Considering this result, we allowed seeds to imbibe for 48 h at 4°C to use them as early-stage-germinating seeds, and prepared sections from the portions close to and distant from the hypocotyl (Fig. 7b). As a result, provascular strands were observed throughout the cotyledon of both types of section, and these sections were used for in situ hybridization as follows.

SoyAP1 signals were detected strongly in the provascular strand of the cotyledon close to the hypocotyl (Fig. 7c), whereas no such signal was detected when the sense probe for *soyAP1* was used (Fig. 7d). Not only the sample close to the hypocotyl (Fig. 7c) but also that distant from it (Fig. 7e) showed *soyAP1* expression in the provascular strand. However, *soyAP2* expression was not observed in the provascular strand (Fig. 7f). Then, we observed the epidermis of the cotyledon close to the hypocotyl using sections including both the hypocotyl and the cotyledon. *SoyAP1* signals were also detected in the epidermis (Fig. 7g,h), whereas no *soyAP2* signal was detected in the epidermis (data not shown). Though the seeds did not germinate, the shoot was observed between the parts separated from the cotyledons. Upon observation of the shoot, *soyAP1* was found to be expressed in the provascular strands and in the epidermis (Fig. 7i,j). These results show that *soyAP1* is already expressed in the provascular strands of the seed, including the shoot and the cotyledon, and in the epidermis.

Discussion

Two soybean APs with different expression patterns

During the germination process, the levels of *soyAP1* mRNA and *soyAP2* mRNA are reversed. *SoyAP1* mRNA is dominant at the early stages of germination when the seed coats are unbroken, while *soyAP2* mRNA becomes dominant at a later stage of germination, when the seed coats are broken. Thus *soyAP1* and *soyAP2* show contrasting expression patterns during germination.

These characteristic modes of expression have been reported for cysteine proteinases in germinating vetch (Fischer et al. 2000; Schlereth et al. 2000) and rice seeds (Watanabe et al. 1991), but our finding of such a phenomenon for APs is apparently the first.

It was observed that the expression of *soyAP2* rapidly increases 48 h after imbibition. This time (48 h) is consistent with the stage in which soybean seed protein bodies begin to fuse and decrease in number (Diaz et al. 1993), with the result that vacuoles undergo a dramatic morphological change. This may suggest some involvement of soyAP2 in such a vacuole change. Also, in considering that *soyAP2* is expressed in roots and leaves with developed vacuoles, it is possible that soyAP2, as well as barley phytepsin (Runeberg-Roos et al. 1994; Paris et al. 1996) and *Arabidopsis* AtPasp A1 (Mutlu et al. 1999; Chen et al. 2002), functions as a vacuole enzyme.

Expression of *soyAP1* in immature tracheary elements and sieve tube cells

SoyAP1 is expressed in immature tracheary elements and sieve tube cells that are differentiated from the cambium or procambium. Immature tracheary elements and sieve tube cells are autolyzed by proteinases during development into their mature forms (Beers 1997; Fukuda 1997, 2000). Thus, it is expected that soyAP1 is involved in programmed cell death (PCD) during the autolysis of immature tracheary elements and sieve tube cells. Barley phytepsin is highly expressed during the autolysis of developing tracheary elements and sieve tube cells (Runeberg-Roos and Saarma 1998). We thus propose that, in general, certain plant APs are expressed in immature tracheary elements and sieve tube cells of all higher plants, monocotyledonous and dicotyledonous, and also that they should be involved in assisting the maturation of these tissues.

In the present study, we made an in-depth analysis of soyAP1-positive cells by using the tip (up to 1,600 μm position) of a root whose tracheary elements and sieve tube cells are morphologically immature. In an early immature stage when the tip length is ca. 600 μm, a strong intensity of *soyAP1* expression was observed in tracheary elements and sieve tube cells (Fig. 4b). At a more developed stage when the tip length changes from 600 μm to 1,600 μm, no soyAP1-positive tracheary elements were observed at all, though soyAP1-positive sieve tube cells are clearly observed. Neither tracheary elements nor sieve tube cells are hollow at the position 1,600 μm from the tip. It may deserve note that there is a difference between immature sieve tube cells and tracheary elements in terms of the *soyAP1* expression stage. The difference may be due to a discrepancy in development between tracheary elements and sieve tube cells and/or due to some difference in soyAP1 substrates. Considering all these results confirming that *soyAP1* is expressed in both sieve tube cells and tracheary elements at an early, immature stage, we suggest that this enzyme is involved in the maturation of the vascular system. It is noted that non-AP enzymes with a similar function do exist. Cysteine proteinase (Minami and Fukuda 1995; Ye and Varner 1996; Xu and Chye 1999; Funk et al. 2002) and serine proteinase (Ye and Varner 1996; Beers and Freeman 1997; Groover and Jones 1999) are reported to be tracheary element proteinases involved in autolysis (Beers et al. 2000). Hence, AP might be involved in autolysis in cooperation with cysteine and serine proteinases, and it might also process these enzymes into mature forms.

There are various phenomena observed for plant PCD (Beers 1997; Pennell and Lamb 1997). Some APs are reported to be involved in PCD (Beers et al. 2000), as in the case of senescence-associated genes encoding AP (Buchanan-Wollaston and Ainsworth 1997; Panavas et al. 1999). In addition, nucellin, an AP belonging to another AP family and known to be expressed specifically in nucellar cells during degeneration after ovule fertilization in barley, is suggested to be involved in PCD (Chen and Foolad 1997). We cannot exclude the possibility that soyAP1 is also involved in various types of PCD besides the autolysis of immature tracheary elements and sieve tube cells.

In the present study, we investigated the expression of two soybean AP genes, *soyAP1* and *soyAP2*, suggesting that soyAP1 is involved in the autolysis of immature tracheary elements and sieve tube cells, probably in the form of PCD. To elucidate the relationship of maturation of the vascular system with soyAP1, we plan to purify soyAP1 protein and investigate the digestion of immature tracheary elements proteins with this *soyAP1* gene expression product.

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