

***Arabidopsis* profilins are functionally similar to yeast profilins: identification of a vascular bundle-specific profilin and a pollen-specific profilin**

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Summary

Four members of the *Arabidopsis* profilin (*pfn*) multigene family have been cloned, sequenced and analyzed. By RNA gel blot analysis it has been shown that these four genes fall into two groups: one group (*pfn1* and *pfn2*) is expressed in all organs of the plant and the other group (*pfn3* and *pfn4*) in floral tissues only. Based on amino acid sequence alignment *Arabidopsis* profilins can be divided into the same two groups: PFN1 and PFN2 are 89% identical and PFN3 and PFN4 are 91% identical. Between these two groups they are 71–75% identical. The *Arabidopsis* profilins bind poly-L-proline and can complement both the *Saccharomyces cerevisiae* profilin deletion mutant and the *Schizosaccharomyces pombe* *cdc3-124*/profilin mutation, showing that the plant profilins are functionally similar to yeast profilins despite the low amino acid sequence homology. Analysis of *pfn* promoter–GUS fusion genes in transgenic *Arabidopsis* shows that *pfn2* is specifically expressed in the vascular bundles of roots, hypocotyls, cotyledons, leaves, sepals, petals, stamen filaments and stalks of developing seeds, whereas expression of *pfn4* is restricted to mature and germinating pollen grains.

Introduction

Profilins, are small proteins (molecular mass of 14 kDa) involved in the regulation of the actin cytoskeleton. Besides being actin-binding proteins, profilins also bind poly-L-proline (Bjørkegren *et al.*, 1993) and phosphatidylinositol 4,5-bisphosphate (PIP2) (Sohn *et al.*, 1995) and via this interaction with PIP2 inhibit phospholipase C (Drøbak *et al.*,

1994; Goldschmidt-Clermont *et al.*, 1990). *In vivo* studies have shown that they are essential for cytokinesis and development (Haugwitz *et al.*, 1994; Sun *et al.*, 1995). For example, in the fission yeast, *Schizosaccharomyces pombe*, a mutation in the *cdc3*-profilin gene prevents the formation of the F-actin contractile ring and arrests the cell cycle at cytokinesis leading to non-viable cells (Balasubramanian *et al.*, 1994). Assembly of the actin filament in response to extracellular signals is regulated by G-actin sequestering proteins, capping proteins and profilin (Carlier and Pantaloni, 1994). Many of the *in vivo* observations are consistent with both positive and negative regulation of actin polymerization by profilin (Sun *et al.*, 1995; Theriot, 1994). In this regard, it is interesting to note that profilin can prevent as well as stimulate actin polymerization *in vitro* (Pantaloni and Carlier, 1993). Although much progress has been made, the function of profilin in the regulation of the actin filament *in vivo* is not yet fully understood (Perelroizen *et al.*, 1995).

Cytoskeletal proteins in general have been shown to be important for cell morphogenesis and are therefore likely to be important for plant development. Yet, very few genes encoding plant cytoskeletal proteins have been isolated so far. Profilin was recently detected in plants as a pollen allergen from white birch (Valenta *et al.*, 1991). All plant profilins isolated to date were identified on the basis of sequence homology. In some cases, they were shown to bind to actin (Valenta *et al.*, 1993) as well as poly-L-proline *in vitro*; however, it is not known whether plant profilins can perform the functions similar to those of yeast profilins *in vivo*.

So far, profilin genes characterized from maize (Staiger *et al.*, 1993) and tobacco (Mittermann *et al.*, 1995) were found to be expressed specifically in pollen grains. On the other hand, as a regulatory component of the actin cytoskeleton, profilin is expected to be present in all cells, although the expression levels may vary depending on the physiological demands of the specific cell types. Expression of profilin in other parts of the plant than pollen has been reported recently. Using immunocytochemical techniques, Vidali *et al.* (1995) demonstrated the presence of profilin in bean hypocotyls and symbiotic root nodules. This observation raises the questions whether the pollen-specific profilins serve any specialized functions and whether they are distinct from other general profilins present in other plant tissues.

In this study, we have cloned and sequenced four genes

(*pfn1*, 2, 3 and 4) encoding profilin (PFN) from *Arabidopsis thaliana*, including three of the cDNAs. By complementation in *Saccharomyces cerevisiae* and *S. pombe* we showed that *Arabidopsis* PFNs are functionally similar to yeast profilins. RNA gel blot analysis demonstrated that *Arabidopsis* profilins fall into two groups; one group is expressed in all plant organs whereas the other group is in floral tissues only. Analysis of transgenic *Arabidopsis* plants harbouring *pfn* promoter–GUS fusion genes showed that *pfn2* was expressed in vascular bundles whereas *pfn4* was expressed in mature and germinating pollen grains.

Results

Cloning of four members of the *Arabidopsis* profilin multigene family

A whole-plant cDNA library of *A. thaliana*, ecotype Columbia was screened with a 399 bp probe of the birch profilin coding region (Valenta *et al.*, 1991). Forty six hybridizing clones were obtained from approximately 200 000 plaques screened. Purification followed by sequencing of nine of the initial clones established that they contained two different open reading frames encoding proteins with high homology to the birch profilin. These two different profilin cDNAs were designated as *pfn1* and *pfn2*. The clones encoding PFN1 contained two different lengths of 3' untranslated regions.

A genomic library of *A. thaliana*, ecotype Columbia was then screened using the two cDNA clones. Twelve hybridizing clones were purified, mapped with restriction enzymes and the hybridizing fragments subcloned and sequenced to establish the identity of the clones. These genomic clones contained the genes for *pfn1* and 2 cDNAs as well as two additional *pfn* genes designated *pfn3* and 4. By further subcloning, overlapping clones covering the entire regions of these four *pfn* genes were sequenced. The complete nucleotide sequences of all four *pfn* genes were deposited with the GenBank and the following accession numbers have been assigned U43322 (*pfn1*), U43323 (*pfn2* and *pfn3*), U43324 (*pfn4*), U43325 (cDNA for *pfn1*) and U43326 (cDNA for *pfn2*). We found that *pfn2* and 3 are linked in a head-to-tail orientation with 1.7 kbp in between the stop codon of *pfn3* and the start codon of *pfn2* (data not shown). This region is shown to be an active promoter for *pfn2* (*vide infra*). We do not know whether *pfn1*, *pfn2* and 3, and *pfn4* are located on the same or different chromosomes.

Each profilin gene has two introns at conserved positions

Figure 1 shows that all four *Arabidopsis pfn* genes have two introns at conserved positions. However, the sizes of the introns differ from 85 to 575 bp. The intron positions

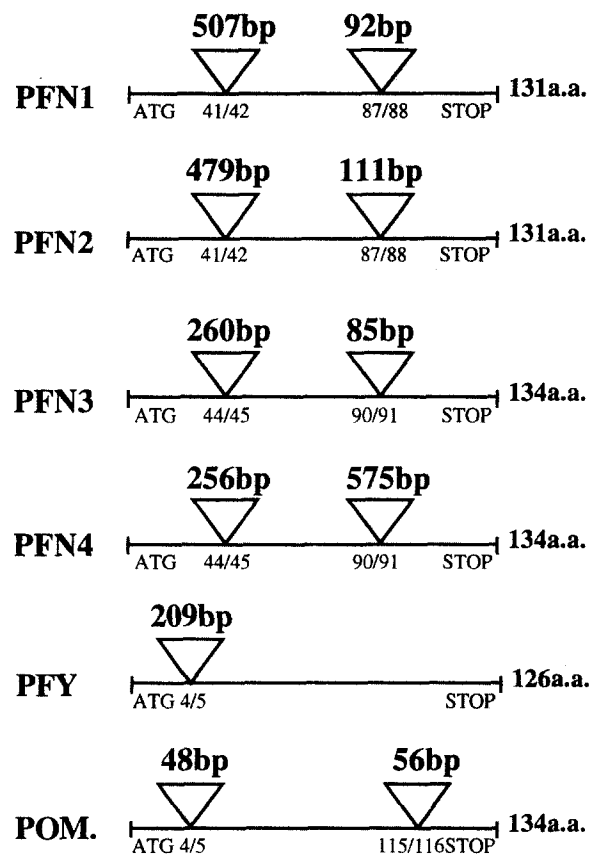


Figure 1. Position and size of introns in the profilin genes of *Arabidopsis* and yeast.

▽ represents an intron. The size of the intron is given above and the position given below. PFN1–4, *Arabidopsis* profilin 1–4 (this study); PFY, *S. cerevisiae* profilin (Magdolen *et al.*, 1988); POM., *S. pombe* profilin (Balasubramanian *et al.*, 1994). The number of amino acids for each profilin is given on the right.

are different from the intron positions in the *S. cerevisiae* and *S. pombe* profilin genes which are the only other known genomic sequences. The nucleotide sequences of *pfn1* and 2 cDNAs are in complete agreement with the genomic DNA sequences.

The profilin isoforms are divided into two groups

Figure 2 shows the deduced amino acid sequences of the four *Arabidopsis* profilins (PFN1–4) as well as their alignment with a selection of other known plant and non-plant profilins. Based on the amino acid sequence alignment they can be divided into two groups, PFN1 and PFN2 being 89% identical and PFN3 and PFN4 being 91% identical. Whereas, between these two groups the identity is between 71 and 75%. *Arabidopsis* profilins have a high degree of identity (more than 69%) with other plant profilins.

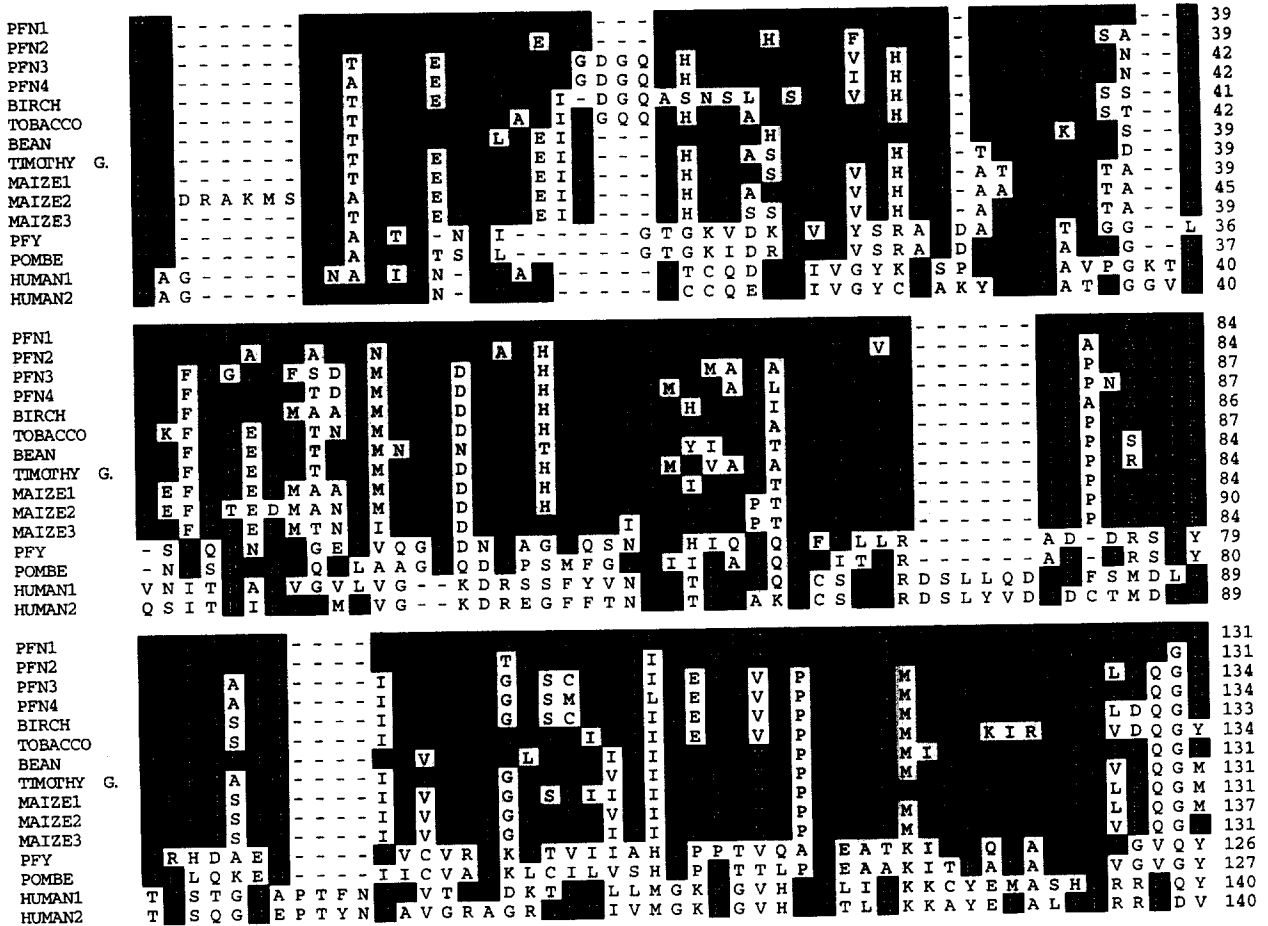


Figure 2. Comparison of deduced amino acid sequences of plant and selected non-plant profilins. The amino acid sequences of PFN1, PFN2 and PFN3 were deduced from the cDNA sequences whereas that of PFN4 was deduced from the genomic sequence. Dashes indicate insertions. Shaded areas indicate identical amino acids. Numbers indicate the positions of the amino acid residues. See text for references to the various profilin sequences.

Arabidopsis profilins bind poly-L-proline

One profilin gene from each of the two groups described above, *pfn1* and *pfn3*, was expressed in *E. coli* from the T7 promoter and the bacterial extracts were loaded on to a poly-L-proline column. We found that both PFN1 and PFN3 bound to poly-L-proline and can be eluted with 3 M urea (data not shown). The purified *E. coli* expressed *Arabidopsis* profilins were recognized by a yeast profilin antibody (data not shown).

Arabidopsis profilins are functionally similar to yeast profilins

The profilin gene *PFY* of the budding yeast *S. cerevisiae* has been shown to be important for vegetative growth, because cells deficient in *PFY* function grow very poorly (Haarer *et al.*, 1990). To determine whether *Arabidopsis* profilins could complement the *PFY* deficiency in yeast, we first confirmed the phenotype of cells lacking the *PFY* gene. A heterozygous diploid strain (22ABΔ1) containing a

complete deletion of one of the copies of *PFY* marked with LEU2, was sporulated on potassium acetate plates. In most of the 12 tetrad dissected, only two out of four spores in each tetrad gave rise to normal colonies, which were Leu⁻ auxotrophs (Figure 3a). Microscopic examination revealed that the other two spores (by inference, Leu⁺ and hence deleted in PFY) had germinated and undergone only a few divisions to give rise to microcolonies, suggesting that the deletion of PFY gene severely retarded vegetative growth. To test whether *Arabidopsis* profilins could substitute for the loss of yeast profilin function, a URA3 selectable plasmid bearing *pfn1* expressed from the yeast profilin promoter was introduced into the 22ABΔ1 diploid strain and the resultant transformants were sporulated. Tetrad dissection yielded Leu⁺ segregants (i.e. deleted in PFY), all of which grew well and were also Ura⁺ (Figure 3b), suggesting that *Arabidopsis pfn1* can complement the yeast profilin deletion. Similar results were obtained upon tetrad analysis of 22ABΔ1 harbouring either plasmid-borne yeast profilin or *Arabidopsis pfn3* (Table 1). Figure 3(c)

shows that haploids of the 22AB Δ 1 strain carrying plant profilin (either *pfn1* or 3) grew as well as the same strain carrying the native yeast profilin at elevated temperature, suggesting that the *Arabidopsis* profilins are functionally similar to the *S. cerevisiae* profilin despite the low sequence homology between them.

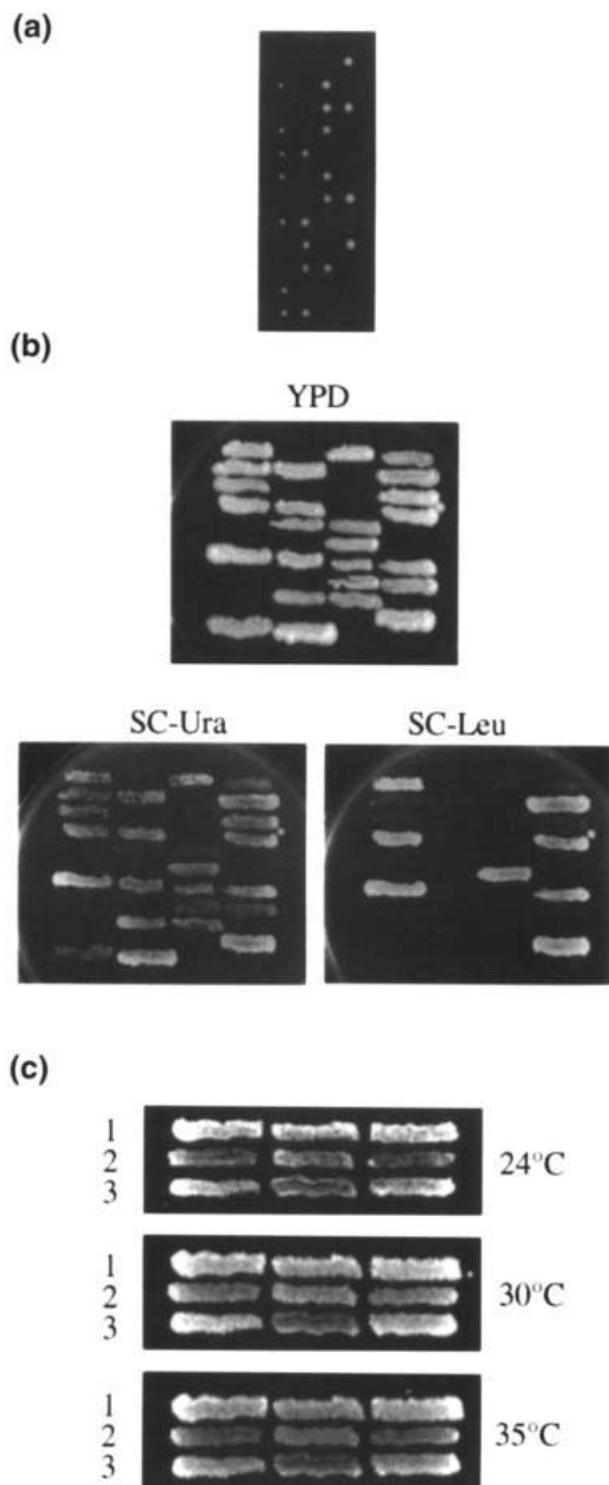


Figure 4 shows that *Arabidopsis* profilins also complemented the temperature-sensitive *S. pombe* *cdc3*/profilin mutation (E43K) when the *Arabidopsis* *pfn* cDNAs were expressed at low levels under repressed conditions (basal level of expression) from the *nmt1* promoter. When the promoter was induced by thiamine removal, overexpression of the *Arabidopsis* profilins arrested the cells as elongated and dumbbell-shaped cells, with a phenotype similar to that of the *cdc3-124* mutant at the restrictive temperature. This is not surprising because a similar phenotype was previously reported with overexpression of *Drosophila* profilin in *S. pombe* (Edwards *et al.*, 1994). It is likely that excess heterologous profilins in the cell might lead to non-productive interactions or bind to and thereby deplete components essential for normal cytokinesis.

pfn1-4 are differentially expressed

Expression of the four *Arabidopsis* *pfn* genes was analysed by RNA gel analysis using 3' untranslated regions as gene-specific probes. Figure 5 shows that whereas *pfn1* and *pfn2* were expressed in all organs of the plant *pfn3* and *pfn4* were only expressed in the flowers. Interestingly, the expression pattern divides the four *pfn* genes into two groups (1 and 2 in one group and 3 and 4 in the other), which are distinguishable on the basis of their amino acid sequences as described above. To obtain cellular resolution of the expression pattern one *pfn* gene from each group was analysed by promoter-GUS fusion in transgenic *Arabidopsis* and tobacco.

Expression pattern of pfn2 and pfn4

Figure 6 shows histochemical localization of GUS in transgenic *Arabidopsis* carrying either the *pfn2* promoter-GUS fusion or the *pfn4* promoter-GUS fusion. *pfn2* expression

Figure 3. Complementation of a *S. cerevisiae* profilin deletion mutant by PFN1.

(a) Twelve tetrads from the profilin deletion strain 22AB Δ 1, sporulated on potassium acetate plates were dissected and incubated at 22°C for 3 days. In most tetrads two spores gave rise to normal colonies, which were Leu⁺. On prolonged incubation the remaining spores formed very tiny colonies. When restreaked, the colonies produced subclones with very slow and heterogeneous growth rates, suggesting that the delayed poor growth was due to compensating spontaneous mutations that arose during the prolonged incubation.

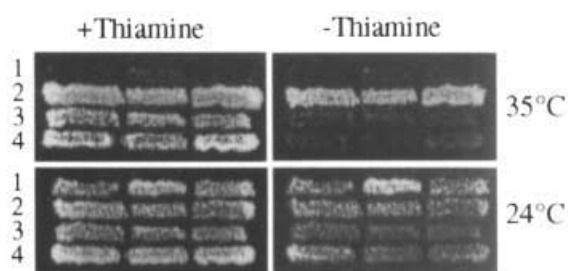
(b) Dissection of tetrads from the profilin deletion strain 22AB Δ 1 carrying plasmid-borne PFN1. After 40 h incubation the segregants were patched on a YPD plate and replica-plated on to SC-Ura and SC-Leu. The ability to grow on the dropout plates was scored at 22°C.

(c) Growth of the *S. cerevisiae* profilin deletion strain expressing *Arabidopsis* profilin cDNAs at elevated temperatures. Three independent segregants of each haploid 22AB Δ 1 strain carrying plasmid-borne PFY, PFN1 and PFN3, respectively, were patched and replicated on YPD and then incubated at the indicated temperatures. Haploid 22AB Δ 1 carrying plasmid-borne PFY, 1; PFN1, 2; PFN3, 3.

Table 1. Complementation of the *S. cerevisiae* profilin deletion strain 22ABΔ1 by *Arabidopsis* profilins

Strains	No. of tetrads dissected	Genotype of various segregants		
		Leu ⁺	Ura ⁺	Leu ⁺ /Ura ⁺
22ABΔ1 + no plasmid (negative control)	12	0	0	0
+ PFY (positive control)	12	12	26	12
+ PFN1	11	8	20	8
+ PFN3	4	3	8	3

Yeast profilin gene *PFY* deletion is marked with *LEU2*; hence all Leu⁺/Ura⁺ are deficient in *PFY* and non-viable in the case of the negative control. Cells which are Ura⁺ indicate that they harbour either the *PFY*, *PFN1* or *PFN3* gene on the plasmid. Leu⁺/Ura⁺ cells are deficient in endogenous *PFY* gene but they are viable because they carry either *PFY*, *PFN1* or *PFN3* on *URA3* selectable plasmid.

**Figure 4.** Complementation of the temperature-sensitive *S. pombe cdc3-124*/profilin mutant by *Arabidopsis* profilins.

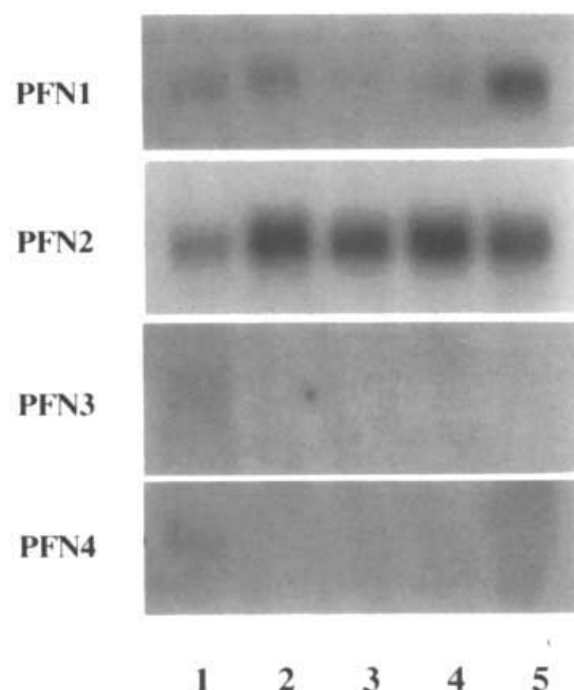
1, vector control (negative control); 2, *S. pombe cdc3* (positive control); 3, *PFN1*; 4, *PFN3*. The individual profilin genes were placed under the control of the *nmf1* thiamine-repressible promoter. Three independent transformants of each were patched and replicated on minimal medium with 2 μ M thiamine (basal level of expression) and without thiamine (overexpression) and then incubated at the indicated temperatures.

was seen in the vascular bundles of roots, hypocotyls, cotyledons, leaves, sepal and petals as well as in the vascular bundles of the stamen filaments and the stalks of the developing seeds. By contrast, expression of *pfn4* was observed only in the pollen grains. In some seedlings from some of the lines carrying the *pfn4* promoter–GUS fusion gene, GUS staining was also observed in the transition zone.

We have also introduced the profilin 4 promoter–GUS gene fusion into tobacco. Analysis of different stages of pollen development revealed GUS expression only in mature pollen; furthermore, *in vitro* germination of the transgenic pollen shows GUS expression in the growing pollen tube (data not shown).

Discussion

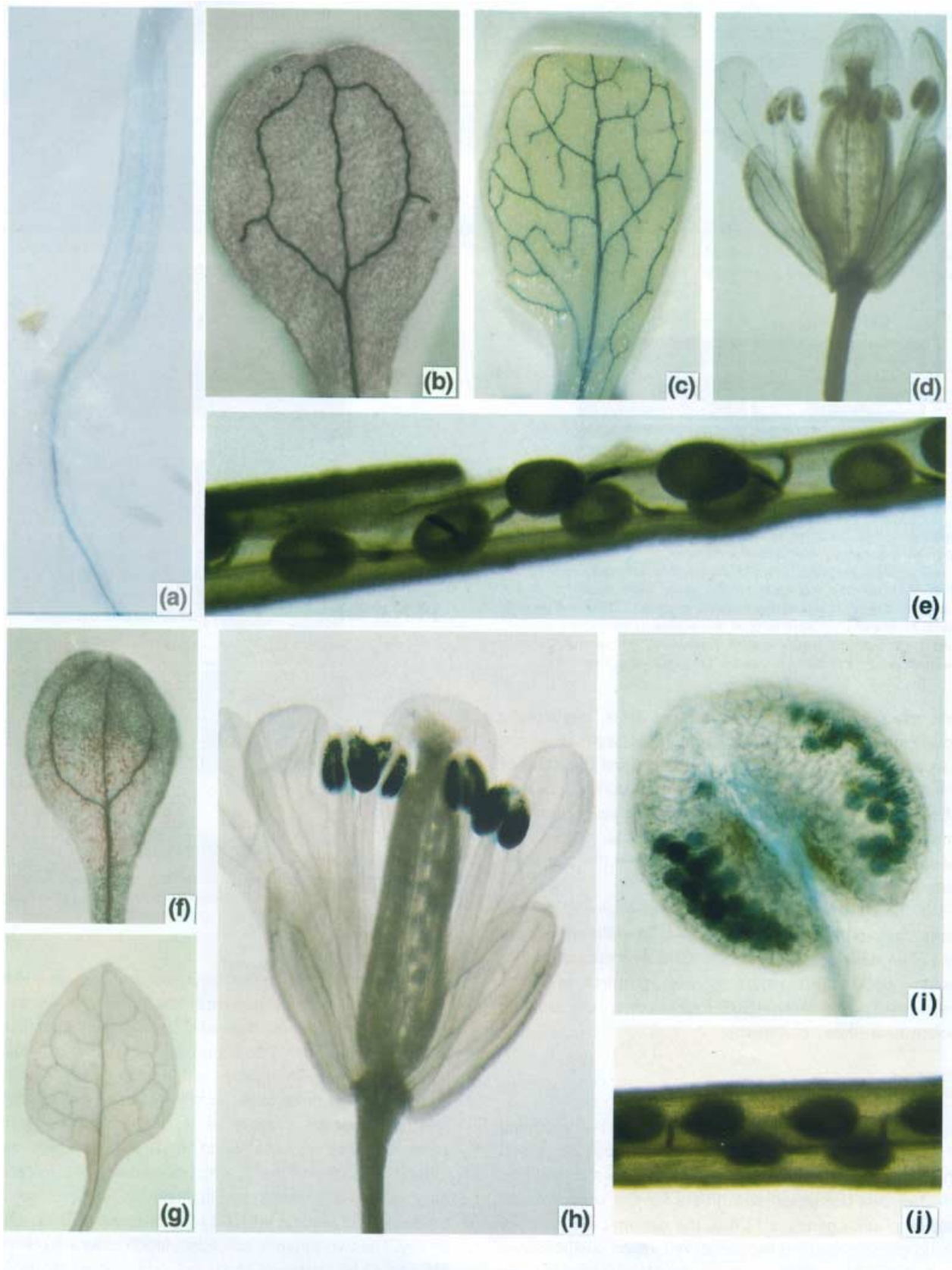
In this study, we have cloned four genes (*pfn1*–*4*) encoding *Arabidopsis* profilin. Several attempts to clone additional members of the family have been unsuccessful. Utilizing the fact that the intron size differs for the known *Arabidopsis* profilin genes, a PCR of the genomic DNA library using primers flanking the conserved intron positions was performed. Nine PCR products could be separated on agarose gel. However, cloning and sequencing of these

**Figure 5.** RNA gel blot analysis.

Total RNA (20 μ g per lane) from 1, flower; 2, silique; 3, leaf; 4, root; 5, peduncle were hybridized to ³²P-labelled 3' gene-specific profilin cDNA probes. Exposure times for *pfn1*, 2, 3 and 4 were 48 h, 48 h, 22 days and 5 days, respectively.

nine products did not reveal any new profilin genes (data not shown). Genomic Southern at low stringency also gave a few additional bands; however, cloning and sequencing of these bands showed no amino acid homology to profilin (data not shown). The maize profilin gene family is estimated to contain three to six members based on Southern blot hybridizations (Staiger *et al.*, 1993), which is in the same range as we have found for *Arabidopsis*. The two different lengths of the 3' untranslated regions for *pfn1* may reflect differential polyadenylation *in vivo*, which produced two distinct mRNAs and, thus, two cDNAs in the library. The two putative polyadenylation sites are present 118 and 43 bp upstream of the polyadenylation sequence.

Alignment of known plant profilin amino acid sequences



(Figure 2) shows that they are more than 69% identical. A notable difference is observed in the N-terminal region of the proteins where pollen profilins from dicotyledonous plants have a two to three amino acid insertion, not found in monocotyledonous profilins. Interestingly, vertebrate profilins have a three amino acid deletion at this position compared with the monocotyledonous profilins. This six amino acid insertion in dicotyledonous plant pollen profilins compared with human profilins (Honore *et al.*, 1993; Kwiatkowski and Bruns, 1988), might be an important epitope for the reported allergy of pollen profilins. It would be interesting to see whether non-pollen profilins can react with human IgE from profilin-allergic patients. The N-terminus of human profilin 2 is more similar to the plant profilins than human profilin 1, which might be important in understanding profilin allergy.

All four members of the *Arabidopsis* profilin family are acidic proteins (with theoretical pI-values of 4.54, 4.78, 4.93 and 4.93 for profilin 1, 2, 3 and 4, respectively) consistent with the previous notion that all plant profilins are acidic, compared with profilins from other species which exist in both acidic and basic forms (Drøbak *et al.*, 1994). It is known that basic forms of profilin bind PIP2 10–50 times more strongly than the acidic ones (Machesky *et al.*, 1990). However, the arginine (number 84 in *Arabidopsis* PFN1) recently implicated in PIP2 binding (Sohn *et al.*, 1995) is conserved in all plant profilins. Therefore, it is likely that local charges are more important for PIP2 binding by plant profilins, which might play a signalling role. Profilins also bind poly-L-proline *in vitro* and recently the vasodilator-stimulated phosphoprotein (VASP) has been shown to be a natural ligand for profilin by utilizing a triple repeat Gly(Pro)₅ motif (Reinhard *et al.*, 1995). So far, no proline-rich proteins such as VASP have been identified in plants, however.

Despite the sequence divergence, we found that *Arabidopsis* profilins were able to complement both the growth defects of the *S. cerevisiae* profilin deletion strain and the *S. pombe cdc3*/profilin mutant strain. These results indicate that *Arabidopsis* profilins can interact with actin and signalling molecules in a similar fashion as yeast profilins and substitute for the latter in maintaining cell division and growth. Perelroizen *et al.* (1996) recently reported that *Arabidopsis* profilins, like other profilins, can bind G-actin and promote assembly of actin filaments at the barbed end *in vitro*. In contrast to other profilins, however, *Arabidopsis* profilins do not accelerate nucleotide exchange on G-actin.

The enhancement of nucleotide exchange by profilin is therefore not involved in its promotion of actin assembly. Taken together with the fact that *Arabidopsis* profilins can functionally substitute for yeast profilins, as shown above, the results strongly suggest that the function of profilin *in vivo* is not dependent on its ability to promote nucleotide exchange on G-actin.

Profilin is expected to be ubiquitously expressed in all cells, although the expression levels may differ in a tissue-specific manner (Sohn and Goldschmidt-Clermont, 1994). It was therefore surprising to see the vascular bundle-specific expression of the *Arabidopsis pfn2* (Figure 6). We can not rule out very (extremely) low expression levels of *pfn2*-GUS in other cell types, which are not detectable by histochemical staining. In the case of bean, immunolocalization of profilin revealed a diffuse distribution in the cytoplasm of hypocotyl and nodule cells, but enhanced staining in the vascular bundles (Vidali *et al.*, 1995). It is possible that in *Arabidopsis*, *pfn1* might be expressed in all other cell types or in all cells. Experiments to investigate this point are in progress.

Using promoter-GUS fusions in transgenic plants, at least three genes, SUC2 encoding a sucrose transporter (Sauer *et al.*, 1994), *sam-1* gene encoding an S-adenosyl-methionine synthetase (Peleman *et al.*, 1989) and *GRP 1.8* encoding a french bean glycine-rich cell wall protein (Keller and Baumgartner, 1991), have been shown to be preferentially expressed in vascular bundles. Comparison of the *Arabidopsis pfn2* and the bean *GRP 1.8* promoter sequences uncovers the presence of some common elements, for example, the negative regulatory element of *GRP 1.8* (Keller and Baumgartner, 1991). On the other hand, the *pfn4* promoter region contains an ACTGT box identified as a pollen specificity element of the tomato LAT52 and LAT59 genes (Eyal *et al.*, 1995).

Although a number of *Arabidopsis* actin genes (*act1*, *act2*, *act3*, *act8* and *act11*) are expressed in the vascular bundles (Meagher and Williamson, 1994) none shows a vascular bundle-specific pattern of expression (Meagher and Williamson, 1994). The number of actin isoforms in *Arabidopsis* is more than twice the number of profilin isoforms, suggesting that the molecular interaction between profilin and actin is not isoform specific. However, the *Arabidopsis* actins can be placed into two to five different groups based on their amino acid sequences (Meagher and Williamson, 1994), which might be functionally equivalent.

Figure 6. Histochemical localization of GUS expression in transgenic *Arabidopsis* plants carrying the *pfn2* promoter-GUS fusion gene or the *pfn4* promoter-GUS fusion gene.

pfn2 promoter-GUS fusion gene. (a) Root/transition zone/hypocotyl of a 5-day-old seedling showing GUS expression in the vascular bundles. (b) Cotyledon of a 16-day-old seedling showing GUS expression in the vascular bundles. (c) Leaf of a 16-day-old seedling showing GUS expression in the vascular bundles. (d) Flower showing GUS expression in the vascular bundles of the stamen filaments, sepals and petals. (e) Silique showing GUS expression in the funiculus. *pfn4* promoter-GUS fusion gene. (f) Cotyledon of a 16-day-old seedling showing no GUS expression. (g) Leaf of a 16-day-old seedling showing no GUS expression. (h) Flower showing GUS expression in the anthers. (i) Anther showing GUS expression in the pollen grains. (j) Silique showing no GUS expression.

Although the functions of profilin in *Arabidopsis* development remain to be elucidated the high level expression of *pfn* genes in vascular bundles and pollen grains strongly suggests a role of this actin binding in these two cell types. During differentiation of tracheary elements (Kobayashi *et al.*, 1987) the actin filaments dynamically rearrange from a longitudinal orientation to elliptical aggregates perpendicular to the long axis of the cell. The actin filaments guide the distribution pattern of microtubules in the developing tracheary elements and, thereby, control the position and orientation of the secondary walls (Kobayashi *et al.*, 1988). In the case of pollen tube growth, the pivotal role of actin filament has been established by the use of specific inhibitors (for reviews, see Mascarenhas, 1993; Pierson and Cresti, 1992). It is quite likely that profilin is involved in regulating actin filament dynamics in both systems. Analysis of transgenic *Arabidopsis* with increased or decreased amounts of profilin would help to define the functions of this protein in these as well as other cell types.

Experimental procedures

cDNA library screening

The coding region of the birch profilin cDNA from clone pKK322-3 (Valenta *et al.*, 1991) was amplified by PCR using AmpliTaq (Perkin Elmer) and random prime labelled with [α - ^{32} P]dCTP (Amersham Corp.). An *Arabidopsis thaliana*, ecotype Columbia, whole-plant cDNA library in Uni-ZAP XR vector (Stratagene Cloning system, La Jolla, CA) was screened by plaque lifts on nylon membranes (Hybond-N, Amersham Corp.). Hybridization was carried out in 100 mM sodium phosphate buffer, pH 7.2, 7% SDS, 15% formamide and 1 mM EDTA (Church and Gilbert, 1984) for 20 h at 50°C and the membranes were washed at 50°C with 100 mM sodium phosphate-buffer pH 7.2, 0.1% SDS and exposed to X-ray films (Kodak X-Omat) at -80°C for 24 h. Lambda phages recognized by the hybridization probe were purified. Bluescript SK- phagemids containing the clones of interest were excised according to the manufacturer's recommendations and the inserts sequenced as described below.

Genomic library screening

The isolated cDNAs encoding *Arabidopsis* profilin were used to screen an *A. thaliana* ecotype Columbia, genomic library in the Lambda ZAPII vector (Stratagene Cloning system, La Jolla, CA) as described above except that the washes were performed at room temperature. Lambda phages recognized by the hybridization probe were purified and excised as described above. The inserts were digested with the appropriate restriction enzymes and subcloned into Bluescript II phagemid vectors and sequenced as described below.

DNA sequencing

The complete sequence of both DNA strands was determined from an overlapping set of subclones obtained as described above and/or with the use of appropriate primers. Single-stranded DNA

from the Bluescript II phagemids was recovered and purified according to the manufacturer's recommendations. The single-stranded DNA was sequenced using Sequenase version 2 (United States Biochemical Corp.).

Preparation of gene-specific probes

The 3' untranslated regions of the four *pfn* genes were amplified by PCR (AmpliTaq, Perkin-Elmer) using the previously isolated clones as templates. The amplification products were purified by agarose gel electrophoresis and excised using Sephaglass (Pharmacia, Sweden).

RNA gel blot analysis

Total RNA was isolated from different organs of *A. thaliana* C24 tissues as previously described (Nagy *et al.*, 1988). Twenty micrograms of RNA per lane was separated on a 1% agarose gel and blotted on to Hybond-N (Amersham) filters. 3' gene-specific probes were labelled (Boehringer Mannheim) with [α - ^{32} P]dATP and [α - ^{32} P]dTTP using random primers. The labelled probes were purified on ChromaSpin TE-10 columns (Clontech). Hybridization was performed using ExpressHyb (Clontech) according to the manufacturer's recommendations using 15 ml of 2×10^6 c.p.m. ml $^{-1}$ hybridization solution. The blots were first washed in $2 \times$ SSC, 0.5% SDS at room temperature and then in $0.1 \times$ SSC, 0.1% SDS at 37°C before being exposed to X-ray films (Kodak X-Omat) at -80°C for the indicated period of time.

Cloning of *pfn3* cDNA

Based on the genomic sequence two primers with the appropriate enzyme restriction site flanking sequences were designed and the cDNA was amplified from the cDNA library by PCR as described below. The cDNA library phage-stock (5 μ l) was used directly as a template in a standard 100 μ l PCR reaction using AmpliTaq DNA polymerase (Perkin Elmer, Applied Biosystem Division, USA). After denaturation at 95°C for 5 min, 45 cycles of amplification were performed (each cycle: 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec) followed by a final extension at 72°C for 7 min using a GeneAmp PCR System 9600 (Perkin Elmer, Applied Biosystem Division, USA). The PCR products were analysed on a 1% agarose gel and a band of the expected size was isolated using a Sephaglass kit (Pharmacia, Sweden). The purified product was then cloned into Bluescript II SK+ T-vector prepared as previously described (Marchuk *et al.*, 1991) and sequenced.

Production of recombinant *Arabidopsis* profilins in *Escherichia coli*

pfn1 and *pfn3* cDNA coding regions were amplified by PCR using primers incorporating a *NdeI* restriction enzyme site at the ATG start codon and a *BamHI* restriction enzyme site immediately after the stop codon. The PCR products were cloned and the sequences verified as described above. The *Arabidopsis* profilin coding regions were then cloned into pET11a (Novagen) and the constructs transformed into the expression host BL21(DE3).

Profilin expression was induced by the addition of IPTG to 1 mM and continued incubation at 37°C for 3 h. The cells were harvested and washed with 50 mM Tris-HCl, 2 mM EDTA pH 8. The cells were then resuspended in 20 mM Tris-HCl, 30 mM sodiumpyrophosphate, 1 mM EDTA pH 8.1 and the proteins released by

sonication. The extract was centrifuged at 5000 *g* for 15 min at +4°C and to the supernatant was then added reduced glutathione and oxidized glutathione to a final concentration of 2 mM and 0.2 mM, respectively. Disulphide bond formation was allowed to occur for 2 h at +4°C. The extract was then cleared by centrifugation and filtration through 0.45 and 0.22 µm sterile filters. *Arabidopsis* profilin was then isolated on a HiTrap NHS-activated (Pharmacia) poly-L-proline (Sigma, P2254) column and eluted with 3 M urea in 20 mM Tris-HCl pH 7.5. Coupling of poly-L-proline to the column was carried out according to HiTrap column manufacturer's recommendations.

Complementation of *Saccharomyces cerevisiae* profilin deletion

Fusion genes comprising the yeast profilin promoter and *Arabidopsis* profilin coding sequences were generated as follows. The yeast profilin promoter (YPP) was obtained as a 500 bp *SalI*-*PvuI* fragment from the 1.7 kbp *Bam*HI-*Hind*III yeast profilin gene (Magdolen *et al.*, 1988). The *Arabidopsis* profilin coding regions were obtained by PCR as described above, using two sequence-specific primers incorporating the restriction sites *PvuI* and *SphI*. The purified product was then cloned into Bluescript II SK+ T-vector and sequenced as described above. Both the YPP and the DNA fragments encoding *Arabidopsis* PFN were isolated from plasmids propagated in the *E. coli* *dam*⁻ SCS110 strain. The YPP and PFN fragments were ligated and cloned into of the centromeric yeast shuttle vector pFL38 (Bonneaud *et al.*, 1991) cut with *SalI* and *SphI*. The YPP-yeast profilin gene fusion was also cloned into the same vector as control. The YPP-yeast profilin *Bam*HI-*Hind*III fragment was cloned directly using the *Bam*HI and *Hind*III sites in the vector.

Profilin deletion haploid strains containing only plasmid-borne profilin genes were generated by lithium acetate transformation of the appropriate plasmids into the *S. cerevisiae* diploid strain 22ABΔ1 (MATa/Mata, *lys*2-80(am)/*lys*2-80(am), *ura*3-52/*ura*3-52, *his*3-Δ200/*his*3-Δ200, *trp*1-1(am)/*trp*1-1(am), *leu*2-3,112/*leu*2-3,112, *PFY1*/p*fy*1Δ::LEU2), which carries a complete deletion in one of its two copies of the profilin genes (Haarer *et al.*, 1990), followed by sporulation on potassium acetate plates as described by Magdolen *et al.* (1988) and tetrad dissection. The haploid segregants of interest were selected as being Leu⁺ (marking the profilin deletion) and Ura⁺ (from the plasmid-borne URA3 gene). The plates were all incubated at 22°C unless otherwise stated.

Complementation of the *Schizosaccharomyces pombe* *cdc3*-profilin mutation

The *NdeI*-*Bam*HI coding region fragments of *pfn1* and *pfn3* used for production of the *Arabidopsis* profilins described above, were cloned downstream of the *nmt1* promoter in the *S. pombe* expression vector pREP1 (Maundrell, 1993). A construct pKG296, pREP3 carrying the *cdc3*⁺ cDNA (Balasubramanian *et al.*, 1994) was used as a positive control. The *S. pombe* profilin mutant strain *cdc3*-124 (Balasubramanian *et al.*, 1994) was transformed by electroporation (Prentice, 1992) and the transformants plated on SC-Leu plates and incubated at 22°C.

Construction of reporter gene fusions, plant transformation and histochemical analysis of β-glucuronidase activity in *Arabidopsis* and tobacco

DNA fragments containing 5' upstream sequences of the *pfn2* and *pfn4* genes were generated by PCR amplification as described

above using promoter-specific primers containing the appropriate restriction enzyme sites. The antisense primer sequences were chosen such that the relative distance between the cloning site and the ATG start codon of the β-glucuronidase (GUS) was maintained. The purified PCR amplified fragments were cloned into a SK+T-vector and the promoter fragments were then excised from this vector and cloned into the corresponding sites of the GUS expression vector VIP40, a derivative of the pMON721 vector (Van der Krol *et al.*, 1993).

The resulting chimeric constructs were introduced into *Agrobacterium tumefaciens*, strain ABI by electroporation (Nagel *et al.*, 1990; Singh *et al.*, 1993). Transformation of *A. thaliana* ecotype C24 was performed by the method of Valvekens *et al.* (1988) and of *Nicotiana tabacum* cv. SR1 by the leaf discs method described by Horsch *et al.* (1988). Transgenic *Arabidopsis* seedlings were germinated and selected on 1×MS medium (Sigma, M5524) supplemented with 3% sucrose, 2 g l⁻¹ phytagel (Sigma, P8169) and 50 µg ml⁻¹ kanamycin.

GUS assays were performed histochemically with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc, Jefferson, 1987) as the substrate using a procedure adapted from Toriyama *et al.* (1991). The organs or entire seedlings were emerged in the GUS-stain solution (2 mM X-Gluc, 0.1 M sodium phosphate buffer, pH 7.0, 0.5% Triton X-100, 2 mM K₃[Fe(CN)₆], 2 mM K₄[Fe(CN)₆], and 0.02% NaN₃) and vacuum infiltrated for about 10 min and then incubated at 37°C overnight or for 2 days depending on the degree of stain observed. The reaction was stopped and the plant material cleared by rinsing and incubation with 70% ethanol overnight at 37°C.

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