

Identification of Profilin As an Actin-binding Protein in Higher Plants*

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Profilin is a low molecular weight protein involved in the organization of the mammalian and protozoan cytoskeleton as well as in signal transduction. In this study, profilin is identified as an actin-binding protein in higher plants which is present in monocot and dicot angiosperms. Birch pollen profilin and actin can be co-purified as a complex, and purified recombinant birch pollen profilin can be used as an affinity matrix to obtain birch pollen actin. The binding of ¹²⁵I-labeled recombinant birch pollen profilin to plant and animal actins can be blocked by profilin-specific antibodies that react with different epitopes of birch profilin. One of the blocking antibodies was raised against the 25 COOH-terminal amino acids indicating the importance of this region in the profilactin complex formation.

Profilin is a low molecular weight protein that was described initially as an actin-binding protein in calf spleen (Carlsson *et al.*, 1977) and was shown to regulate polymerization of actin and interactions of actin filaments (Reichstein and Korn, 1979; Isenberg *et al.*, 1980). Profilin was later isolated and characterized in yeast (Oechsner *et al.*, 1987; Magdolen *et al.*, 1988; Haarer *et al.*, 1990), mouse (Widada *et al.*, 1989), cattle (Ampe *et al.*, 1988), man (Kwiatkowski *et al.*, 1988), *Tetrahymena* (Edamatsu *et al.*, 1991), and in slime mold (*Physarum*) (Takagi *et al.*, 1990). In these eukaryotic organisms, profilin interacts with actin by forming a profilactin complex that keeps actin in its monomeric form in the cell. Although it is proposed that by this mechanism profilin controls the assembly and reassembly of the eukaryotic cytoskeleton, only limited information is available regarding the interacting domains of profilin and actin and about the biological roles of profilin in the cell.

In *Thyone* sperm, profilin was proposed to initiate the acrosomal reaction, a key event in fertilization, by liberating monomeric actin which then polymerizes and forms protrusions that penetrate the egg (Tilney and Inoue, 1982; Tilney *et al.*, 1983). The functions of profilin, however, are probably more diverse than originally anticipated. Profilin also binds to polyphosphoinositides which reversibly seems to regulate the interaction of profilin with actin (Lassing and Lindberg, 1985; Goldschmidt-Clermont *et al.*, 1990, 1991). Profilin is, therefore, a component of a signal transduction cascade that is linked to

the cytoskeleton. Adenylylcyclase-associated protein, a component of the yeast adenylylcyclase complex, and profilin may represent a link between growth signals and remodeling of the cellular cytoskeleton (Vojtek *et al.*, 1991).

Despite these data concerning the properties and functions of profilin in eukaryotic organisms, nothing was known about an occurrence or function of profilin in higher plants. The potential roles of actin in shaping plant cells and forming the plant cytoskeleton have been described (Staiger and Lloyd, 1991). Actin was also identified as an important component of the pollen tube in germinating pollens of different plants (Heslop-Harrison and Heslop-Harrison, 1986, 1989). Little information is available concerning actin-binding proteins in plants.

Profilin was identified as an important birch pollen allergen (Valenta *et al.*, 1991a) which led to further investigation of the occurrence, properties, and functions of profilins in plants. In the present communication, actin-binding properties of plant profilins are investigated. It is shown that plant profilins bind to plant actins as well as to mammalian actins. The COOH-terminal portion of plant profilins is identified as an important actin-binding domain.

EXPERIMENTAL PROCEDURES

Materials—Pollens from birch (*Betula verrucosa*) and timothy grass (*Phleum pratense*) that were more than 95% pure as determined by light and electron microscopy were purchased from Allergon AB (Välinge, Sweden). Recombinant birch profilin was expressed in pKK223-3, *Escherichia coli* JM105 (Valenta *et al.*, 1991b), a source known to contain no actin, and was purified as described under *Purification of Profilins from Various Pollens*. A partial cDNA clone expressing the 25 COOH-terminal amino acids of birch profilin as a β -galactosidase fusion protein in λ gt11/*E. coli* Y1089 has been sequenced and characterized earlier (Valenta *et al.*, 1991a).

Aac8 is one of the two most frequently isolated actin cDNAs found in *Arabidopsis thaliana* leaf and root cDNA libraries. The ATG initiator codon is contained within a natural *Nco*I site, and a *Bam*HI site was engineered immediately adjacent to the most distal poly(A) processing site.¹ This 1.4-kilobase *Nco*I/*Bam*HI fragment was cloned into the corresponding replacement region of the expression vector pET15b (Novagene). *Aac8* actin protein was overexpressed in *E. coli* DE3 (Novagene) in the presence of 100 μ g/ml ampicillin as per the supplier's instructions with the exception that the cells were allowed to grow overnight after induction with isopropyl- β -D-thiogalactopyranoside. Actin-containing inclusion bodies were purified and actin protein was renatured from 6 M guanidine HCl as described (Lin *et al.*, 1991). This protein was centrifuged at 100,000 $\times g$ for 3 h. The supernatant fraction contained recombinant *Aac8* actin protein of about 95% purity based on Coomassie staining of SDS-PAGE² gels.

A peptide comprising the 25 COOH-terminal amino acids of birch profilin with the sequence EEPVTPGQCNMVVERLG DYLDQGL was

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¹ R. B. Meagher, unpublished data.

² The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

chemically synthesized at Sandoz Research Institute, Vienna, Austria, and a control peptide with a sequence which is not part of the profilin sequence, DSRTWLLRGWGTTLL, was synthesized at Cambridge Research Biochemicals, Northwich, UK

Characterization of Antibodies—IgE antibodies specific for plant profilins from sera of profilin-allergic patients were characterized as described (Valenta *et al.*, 1991a, 1992). Furthermore, a rabbit anti-celery profilin antibody designated RP1 with cross-reactivity to other plant profilins (Vallier *et al.*, 1992) was used. Two rabbit antibodies, RP2 and RP4, were raised against purified recombinant birch profilin that had been expressed in *E. coli* JM105/pKK223-3 (Valenta *et al.*, 1991b). One rabbit was immunized with the 25 COOH-terminal amino acid peptide of birch profilin conjugated to keyhole limpet hemocyanin using a conjugation kit (Pierce) to obtain antiserum RP3. All profilin antibodies were tested for reactivity with the profilin COOH terminus which in *Acanthamoeba* was shown to be involved in actin binding (Vandekerckhove *et al.*, 1989).

Antibodies were tested for binding to the 25 COOH-terminal amino acids of birch profilin expressed in λ gt11/*E. coli* Y1089. The rabbit anti-celery profilin antibody RP1, the rabbit anti-recombinant birch profilin antibody RP4, and the rabbit antibody raised against the COOH-terminal peptide RP3 bound to the COOH-terminal portion of birch profilin, whereas the second rabbit antibody raised against recombinant birch profilin, RP2, did not bind to this epitope.

Inhibition studies performed by preincubation of the rabbit antibodies with the COOH-terminal profilin peptide and a control peptide which is not part of the profilin sequence confirmed the above results. Table I summarizes the specificities of the anti-profilin antibodies.

Actin antibodies reacting with different regions of actin, Sac1 and Sac3, were described in detail (McLean *et al.*, 1990). Sac3:1 was raised against the 12 amino-terminal amino acids of soybean actin, whereas Sac1:257 was raised against an internal soybean actin peptide (FGCP-GVLYQPSMVGME). A mouse monoclonal antibody against rabbit striated muscle actin was purchased from DAKO, and a mouse monoclonal antibody against chicken gizzard actin was obtained from Chemicon, Temecula, CA.

Purification of Profilin from Various Pollens: Isolation of the Profilactin Complex from Birch Pollen—Pollen from birch and timothy grass was homogenized in PHEM-TX buffer (Lind *et al.*, 1987) using an ultraturrax homogenizer, and the lysate was centrifuged for 1 h at 65,000 $\times g$ at 4 °C. The supernatants were incubated overnight at 4 °C with poly-(L-proline) coupled to BrCN-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) (Lindberg *et al.*, 1988). After washing the matrix three times with TBS-ATP (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5 mM ATP), profilin was eluted with TBS-ATP-2 M urea and TBS-ATP containing 6 M urea. The eluted material was dialyzed against distilled water at 4 °C and lyophilized. To obtain the profilactin complex, this procedure had to be modified. Pollen was homogenized in ice-cold buffer G (2 mM Tris-HCl, pH 7.9, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM dithiothreitol, 1 $\times$ protease inhibitor mixture, Drubin *et al.*, 1988) and centrifuged at 12,000 $\times g$ at 4 °C for 60 min. The supernatant was applied to a poly-(L-proline)-Sepharose column which was washed with a solution containing 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 100 mM glycine, 0.1 mM dithiothreitol. The profilactin complex was eluted with buffer G containing 8 M urea, desalted on a Sephadex G-25 column (Pharmacia), equilibrated with buffer G, and concentrated by ultrafiltration (Centrisart I 1000, Sartorius, Germany).

Enrichment of Birch Pollen Actin Using Purified Recombinant Birch Profilin—Recombinant birch profilin was purified and coupled to CNBr-activated Sepharose 4B (Pharmacia). Birch pollen proteins were prepared as described for the affinity purification of profilin from birch pollen and incubated overnight at 4 °C with recombinant birch profilin-Sepharose. The Sepharose was washed and bound proteins were eluted as described for the poly-(L-proline) Sepharose. The proteins were dialyzed against distilled water and lyophilized. Proteins were then dissolved in water, subjected to SDS-PAGE, and blotted to nitrocellulose. To identify the eluted proteins, the blots were then probed with rabbit

anti-actin antibodies Sac1 and Sac3.

Blot Overlays—Purified chicken gizzard actin and rabbit skeletal muscle actin were obtained from Sigma, and purified *Arabidopsis* actin was prepared as described under "Materials." Bovine serum albumin as a negative control was purchased from Boehringer Mannheim. The proteins were dissolved in buffer G and subjected to SDS-PAGE. A section of each gel was stained with Coomassie Blue (Fig. 4A), and the rest of the gel was used for blotting to ensure that comparable amounts of each protein were transferred to nitrocellulose. Purified recombinant birch profilin was ¹²⁵I-labeled using the chloramine-T method. The iodinated protein was desalted by chromatography on Sephadex PD-10 columns (Pharmacia), and 50,000 cpm were subjected to SDS-PAGE. The gel was autoradiographed using Kodak X-Omat S films and intensifying screens to check the purity of the labeled protein. Before overlay, 150,000 cpm of ¹²⁵I-labeled profilin were preincubated in 1 ml of TBS Tween + 5% (w/v) powdered milk (TBS-Tween: 90 mM NaCl, 0.5% (v/v) Tween 20, 10 mM Tris-HCl, pH 7.5, Chia *et al.* (1991)) either with normal rabbit serum or the rabbit antibodies RP1 to RP4 in a 1:100 dilution. Nitrocellulose strips containing the different actins or bovine serum albumin as a negative control were washed twice for 5 min and once for 30 min in TBS-Tween-powdered milk before incubation with the preadsorbed ¹²⁵I-labeled recombinant birch profilin for 2 h. Strips were then washed three times for 5 min in TBS-Tween-powdered milk and once for 30 min. Strips were then dried and autoradiographed.

RESULTS AND DISCUSSION

The discovery of plant profilins as potent type I allergens (Valenta *et al.* 1991a, 1992; Vallier *et al.*, 1992) led to the hypothesis that the ubiquitous occurrence of profilins in plants and in particular pollen may be due to the biological function of profilins as plant actin-binding proteins. In eukaryotic cells, profilin is able to sequester actin monomers and by that controls actin polymerization. In resting pollen, actin was reported to occur as a complex (Heslop-Harrison and Heslop-Harrison, 1986) whereas during pollen germination actin polymerizes and forms filaments providing a backbone for the outgrowing pollen tube. It was therefore likely that profilins may form a complex with monomeric actin in resting plant pollen which represents a microfilament precursor for pollen germination.

In our study we attempted to characterize the occurrence, distribution, and properties of plant profilins. In a first step, profilins were purified to homogeneity from total protein extracts of birch pollen by affinity chromatography to poly-(L-proline)-Sepharose. Like profilins from other eukaryotes (Tanaka and Shibata, 1985; Lindberg *et al.*, 1988; Lind *et al.*, 1987), plant profilins bound specifically to this matrix. Fig. 1A shows that two different proteins of 14 kDa and approximately 13 kDa can be purified from birch pollen. Pollen of timothy

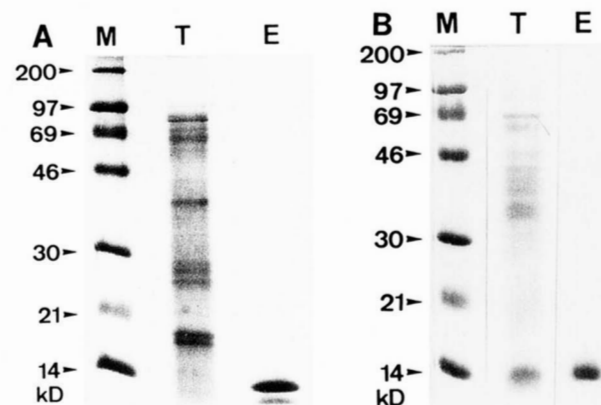


FIG. 1. Purification of birch and timothy grass profilin. Coomassie Blue-stained gels illustrating the purification of profilin from birch pollen (A) and from timothy grass pollen (B). Lane M, molecular weight marker; lane T, total pollen protein extracts before application to poly-(L-proline)-Sepharose. Lane E represents the material eluted from the matrix with 6 M urea.

TABLE I
Specificities of anti-profilin antibodies

Designation of antibody	Immunogen	Reactivity with the profilin COOH terminus
RP1	Celery profilin	+
RP2	Recombinant birch profilin	—
RP3	25 amino acids of profilin COOH terminus	+
RP4	Recombinant birch profilin	+

TABLE II
Sequence similarities of profilins

Profilin	Sequence identity of birch profilin	Sequence identity of timothy grass profilin
	%	%
<i>Saccharomyces</i>	31	33
<i>Acanthamoeba</i> I	38	40
<i>Acanthamoeba</i> II	40	40
<i>Physarum</i>	41	44
<i>Tetrahymena</i>	29	31
<i>Dictyostelium</i> I	45	49
<i>Dictyostelium</i> II	39	44
Sea urchin	28	29
Mouse	32	26
Calf	34	30
Man	33	27
Timothy grass	79	100
Birch	100	79

grass which is a monocot contained only one protein band of about 14 kDa (Fig. 1B). The proteins purified from birch and timothy grass reacted with each of the profilin-specific antibodies described under "Experimental Procedures."

By immunoscreening of birch and timothy grass pollen expression libraries, cDNA sequences and deduced amino acid sequences of birch (Valenta *et al.*, 1991a) and timothy grass pollen profilin³ were obtained. Table II summarizes the sequence similarities of profilins. Monocot and dicot profilins are highly homologous (79% amino acid sequence identity), whereas a comparable low identity of around 30% was found with other profilins.

Profilins had been described as major actin-binding proteins in eukaryotes, but no information was available whether typical properties of eukaryotic profilins are shared by the plant homologue. Several approaches were chosen to demonstrate the actin binding capacity of plant profilins.

In other eukaryotic organisms, it has been shown that profilin and actin form a profilactin complex that can be purified by its affinity to poly-(L-proline) (Lindberg *et al.*, 1988). To isolate profilin and the profilactin complex from birch pollen, conditions were chosen that maintain actin in a depolymerized form and thus prevent a dissociation of the profilactin complex (see "Experimental Procedures"). The products obtained by poly-(L-proline) affinity chromatography were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Fig. 2A shows in a Coomassie-stained gel the elution of bands with molecular masses of 43 kDa which is the size of actin, and of 13 to 14 kDa which is the size of profilin. The proteins were also blotted to nitrocellulose and identified as actin (43 kDa) and as profilin (13–14 kDa) using anti-profilin and anti-actin antibodies (Fig. 2B). Birch pollen actin could be detected with a monoclonal mouse anti-chicken gizzard actin antibody (lane 1) and with a mouse monoclonal antibody raised against rabbit α -sarcomeric muscle actin (lane 2) at around 43 kDa. Rabbit antibodies raised against celery profilin RP1 (lane 4) and recombinant birch profilin RP2 (lane 6) bound to both (14 kDa and 13 kDa) forms of birch pollen profilin. The same pattern was obtained with serum-IgE from a profilin-allergic patient (lane 7). No signals were obtained with the nonimmune sera or buffer controls without addition of a primary antibody (lanes 3, 5, and 8).

In an additional experiment, it is demonstrated that birch pollen actin can be enriched via affinity to Sepharose-coupled recombinant birch profilin. Using purified recombinant birch profilin bound to Sepharose as an affinity matrix, two proteins were purified from birch pollen with a molecular mass comparable to actin. Fig. 3 demonstrates that two rabbit anti-actin antibodies that were raised against peptides representing dif-

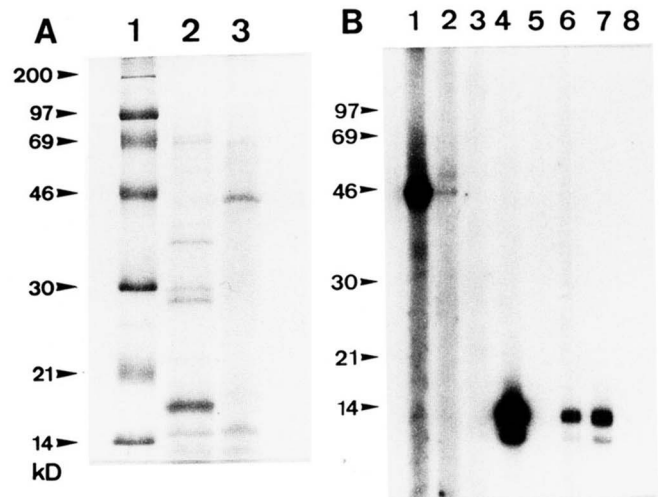


FIG. 2. Purification of the profilactin complex from birch pollen. A, Coomassie Blue-stained gel. Lane 1, molecular weight marker; lane 2, total birch pollen protein extract; lane 3, material bound to poly-(L-proline)-Sepharose. B, immunoblot identification of the products that had bound to poly-(L-proline)-Sepharose with different anti-actin and anti-profilin antibodies. Lane 1, mouse anti-chicken gizzard actin; lane 2, mouse anti-rabbit sarcomeric actin; lane 3, buffer control without addition of the primary antibody; lane 4, rabbit anti-celery profilin; lane 5, rabbit nonimmune serum; lane 6, rabbit anti-recombinant birch profilin; lane 7, serum IgE of a profilin-allergic patient; lane 8, buffer control without addition of the primary antibody.

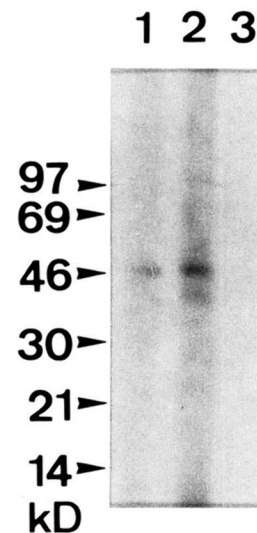


FIG. 3. Immunoblot of birch pollen actin purified by affinity to recombinant birch profilin. A birch pollen protein which had bound to recombinant birch profilin coupled to Sepharose was electrophoresed, blotted to nitrocellulose, and probed with rabbit anti-actin antibodies Sac1 (lane 1) and Sac3 (lane 2). Normal rabbit serum, lane 3.

ferent portions of actin (see "Materials") (McLean *et al.*, 1990) bind to the blotted proteins and thus identify the purified product as actin.

For detailed investigations of the actin-binding properties of recombinant birch profilin, the recombinant profilin was purified by affinity to poly-(L-proline)-Sepharose and used for binding to blotted actins from different species. Fig. 4A shows Coomassie-stained sections of the gels containing recombinant *Arabidopsis* actin, rabbit skeletal muscle actin, chicken gizzard actin, or bovine serum albumin as a control protein. The proteins were transferred to nitrocellulose and used in the subsequent overlay experiments.

¹²⁵I-labeled recombinant birch profilin was allowed to bind to filter-bound actins or bovine serum albumin (Fig. 4B) after

³ R. Valenta, unpublished data.

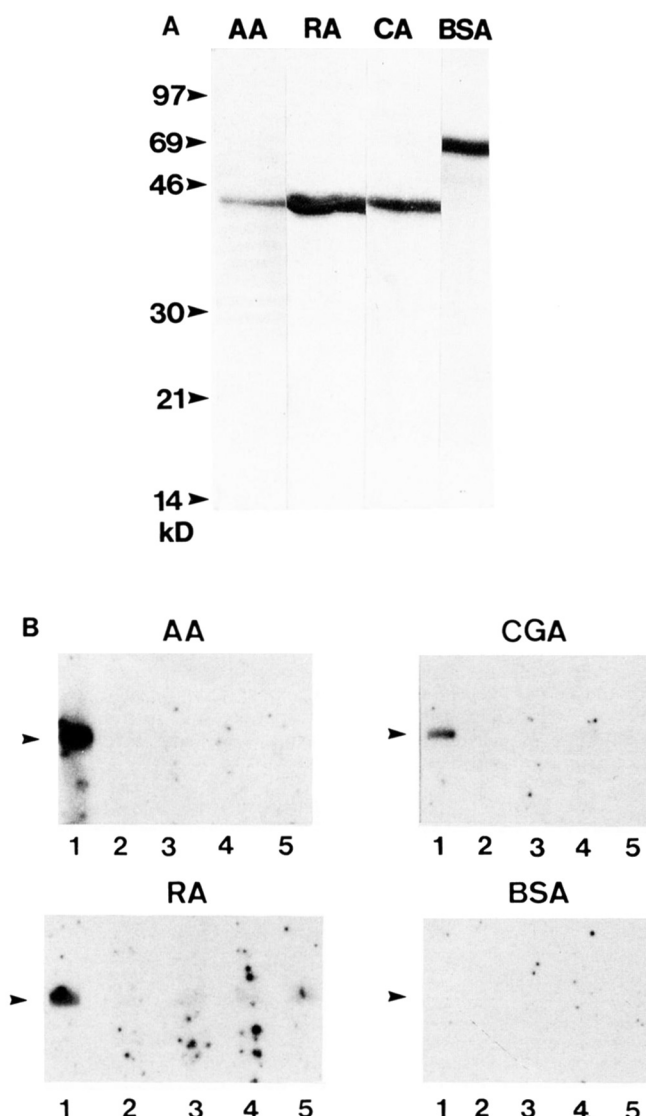


FIG. 4. Affinity of plant profilin to filter-bound actins. A, Coomassie Blue-stained gel sections containing the proteins used in the overlay experiments in B. AA, recombinant *Arabidopsis* actin (1 μ g/cm); RA, rabbit skeletal muscle actin (5 μ g/cm); CGA, chicken gizzard actin (3 μ g/cm); and BSA, bovine serum albumin (4 μ g/cm) as a control protein. B, blot overlay-purified recombinant birch profilin was ^{125} I-labeled and incubated with nitrocellulose strips containing recombinant *Arabidopsis* actin Aac8 (AA), rabbit skeletal muscle actin (RA), chicken gizzard actin (CGA), or bovine serum albumin (BSA). The ^{125} I-labeled profilin was preincubated either with normal rabbit serum (lane 1), with a rabbit anti-celery profilin antibody (lane 2), with rabbit anti-recombinant birch profilin antibodies (lanes 3 and 5) or a rabbit antibody raised against the 25 COOH-terminal amino acids of birch profilin (lane 4).

the profilin was preincubated with normal rabbit serum (lanes 1) or with the rabbit anti-profilin antibodies RP1 to RP4 (lanes 2–5). Recombinant birch profilin bound to all actins tested but not to the negative control. Preincubation of profilin with normal rabbit serum did not affect actin binding while preincubation with the profilin-specific antibodies led to almost complete inhibition of the profilactin interaction in the case of recombinant *Arabidopsis* actin or to a substantial reduction of the profilactin interaction in the case of rabbit skeletal muscle actin and chicken gizzard actin.

Binding of profilin to actin was inhibited by preincubation of profilin with an antibody reacting exclusively with the 25 COOH-terminal amino acids of profilin. This suggests that in plants the profilin COOH terminus is involved in the profilac-

tin interaction. Similar data were obtained in the case of *Acanthamoeba* profilin where the lysine 115 could be chemically cross-linked to glutamic acid 364 of actin (Vandekerckhove *et al.*, 1989). In the birch and timothy grass profilin sequences, arginine residues were found in a corresponding position.³

Despite the low degree of sequence similarity between plant and mammalian profilins (Table II), an interaction between plant profilins and mammalian actin could be demonstrated, suggesting that the actin binding domains in profilins are structurally well conserved. This is in agreement with first *in vivo* data showing that plant profilin cDNAs can be expressed in mammalian cells and formation of heterologous profilactin complexes can be observed (Rothkegel *et al.*, 1993).

It is known that profilactin acts as a microfilament precursor which may release polymerized actin upon activation (Markey *et al.*, 1981). Aggregated actin has already been described in different resting pollens complexed to an as yet unidentified protein and actin was reported to form microfilaments during pollen tube growth (Heslop-Harrison and Heslop-Harrison, 1986; Condeelis, 1974; Tang *et al.*, 1989). The identification of profilin as a plant actin-binding protein may now introduce a novel component in the plant cytoskeleton field. Profilin-actin interactions in plants may control processes involving a shift from a resting state to a dynamic state such as during pollen germination and tip growth. It is, however, likely that such processes are more likely accompanied by regulation of a pre-formed profilin pool than by *de novo* protein synthesis because a number of expression studies performed with different plant tissues and stimulated callus cultures showed constant levels of profilin isoforms (Swoboda *et al.*, 1992). Such a profilin pool could be regulated during growth and development of plants by differential interaction with proteins such as actin or with phospholipids.

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