

Characterization of maize (*Zea mays*) pollen profilin function *in vitro* and in live cells

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Profilin is a small, 12–15 kDa, actin-binding protein that interacts with at least three different ligands. The 1:1 interaction of profilin with globular actin (G-actin) was originally thought to provide a mechanism for sequestering actin monomers in the cytoplasm. It has recently become clear that the role of profilin in the cell is more complex, perhaps due to interactions with polyphosphoinositides and proline-rich proteins, or due to the ability to lower the critical concentration for actin assembly at the fast-growing barbed end of actin filaments. Because actin-binding proteins have been shown to behave differently with heterologous sources of actin, we characterized the interaction between maize pollen profilins and plant G-actin. The equilibrium dissociation constants measured by tryptophan fluorescence quenching were similar to those of other CaATP-G-actin–profilin

complexes ($K_d = 1.0\text{--}1.5\ \mu\text{M}$). The ability of maize profilin isoforms to bind poly-L-proline was analysed, and the K_d values for recombinant pollen and human profilins were similar when determined by two independent methods. However, the affinity of native maize pollen profilin for poly-L-proline was substantially lower than that of any of the recombinant proteins by one of these assays. The possibility of post-translational modification of profilin in the mature pollen grain is discussed. Finally, we quantified the effects of microinjection of each profilin isoform on the cytoarchitecture of *Tradescantia* stamen hair cells and show that the resultant disruption can be used to compare actin-binding proteins in living cells. The results are discussed in relation to a recent model of the interphase actin array in these plant cells.

INTRODUCTION

Profilin is a small protein (12–15 kDa) that was first identified in calf spleen extracts as a factor that co-purified in a 1:1 complex with globular actin (G-actin) [1]. It has since become clear that profilin occurs in many different cell types and organisms from every eukaryotic kingdom (for reviews see [2–4]). In plants, profilin was originally discovered as a major birch pollen allergen [5] and has subsequently been isolated or cloned from many plants [4], including maize [6,7]. Plant profilins are members of large multigene families with unique expression patterns and can be grouped into two major classes: those that are constitutively expressed in vegetative tissues; and those expressed primarily in reproductive tissues (reviewed in [4]). Multiple profilin isoforms can be expressed in individual tissues and cells, particularly in pollen (reviewed in [4]).

In addition to binding to G-actin, profilin also binds to proteins with proline-rich motifs and may be linked to signal-transduction cascades through binding to polyphosphoinositides [2,4,8]. The ability of profilin to bind to three different ligands may allow for complex regulation of profilin function. Indeed, there are conflicting data regarding the function of profilin in living cells. Microinjection of profilin directly into the cytoplasm led to decreased levels of filamentous actin (F-actin) in animal [9] and plant [10] cells, supporting a simple model where profilin serves to sequester actin monomers and promote actin depolymerization. Similarly, deletion of profilin genes from *Dictyostelium* resulted in an increased accumulation of F-actin that could be alleviated by transformation with either of the two endogenous profilin isoforms [11]. These results contrast mark-

edly with the effect of overexpressing profilin by transfection of mammalian cells, where a substantial increase in the proportion of F-actin was found but total actin levels remained unaltered [12]. The ability of profilin to promote actin-filament formation has been demonstrated *in vitro* by showing that profilin enhances polymerization from a pool of sequestered actin monomers [13].

A number of experiments that make use of heterologous systems suggest that profilin isoforms from different kingdoms are functionally similar. Plant profilin isoforms can complement profilin-deletion mutants of *Dictyostelium* [14] and yeast [15], demonstrating that widely divergent profilins are qualitatively similar *in vivo*. Overexpression of birch pollen profilin or human profilin in mammalian cells resulted in an increase in the stability of actin filaments, and birch profilin co-localized with the endogenous profilin [16]. These data led to the claim that profilin proteins are 'equivalent' in function [16]. However, any subtle roles of profilin in regulation of the actin cytoskeleton may not be detectable by such experiments. The profilin isoforms in vertebrates and the lower eukaryotes *Acanthamoeba* and *Dictyostelium* differ in their affinities for actin [17,18], PtdIns(4,5) P_2 [19,20] and poly-L-proline (PLP) [20]. Such contrasting characteristics suggest that the different isoforms may have distinct roles in the cell.

The presence of multiple profilin isoforms in maize pollen [6] led us to investigate whether they are functionally unique or functionally redundant. We characterized profilin function by microinjection of profilin into living cells and measuring the subsequent effect on actin-dependent nuclear position and by measuring the equilibrium dissociation constants for PLP and maize pollen G-actin. We report that maize pollen profilin

Abbreviations used: DTT, dithiothreitol; G-actin, globular actin; F-actin, filamentous actin; PLP, poly-L-proline; RSMA, rabbit skeletal muscle actin; VASP, vasodilator-stimulated phosphoprotein; ZmPRON, *Zea mays* profilin *n*.

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isoforms are significantly weaker actin-binding proteins than a vertebrate profilin.

Portions of this work have appeared in abstract form [20a].

MATERIALS AND METHODS

Reagents

PMSF, Tris, aprotinin, leupeptin, pepstatin A, benzamidine, phenanthroline, sodium fluoride, sodium pyrophosphate, PLP and electrophoretically pure DNase I (DN-EP) were obtained from Sigma (St. Louis, MO, U.S.A.). Dithiothreitol (DTT) and Na₂ATP were obtained from USB Specialty Biochemicals (Cleveland, OH, U.S.A.). A stock solution of 100 mM PMSF was prepared in isopropanol. The other protease inhibitors were mixed to form a stock solution (PI cocktail) in ethanol at the following concentrations: 1.6 mg/ml benzamidine, 0.1 mg/ml phenanthroline and 1 mg/ml each of aprotinin, leupeptin and pepstatin A. PLP was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ, U.S.A.) by the method of Rozycki et al. [21]. Rabbit skeletal muscle actin (RSMA) was prepared according to the methods of Spudich and Watt [22] with modifications as suggested by MacLean-Fletcher and Pollard [23].

Purification of native pollen and recombinant profilins

Profilin was isolated from *Zea mays* (maize, inbred line OH43) pollen collected in the field and stored at -75°C . Profilin was also isolated from bacterial cells expressing recombinant maize pollen profilin isoforms [14] or recombinant human profilin I [24]. Purification of profilin by PLP-affinity chromatography was performed essentially as described previously [25], except the extraction buffer contained 150 mM KCl, 20 mM Tris, 0.2 mM DTT, 1 mM PMSF and 1:200 PI cocktail, pH 7.5. The concentration of PI cocktail was reduced to 1:2000 for column elution buffers and omitted entirely from dialysis solutions. After dialysis against two changes of 1 litre, the protein solutions were aliquoted and stored at -75°C . Typical yields of recombinant protein were 5–10 mg/l culture for human, *Zea mays* profilin (ZmPRO)1 or ZmPRO3, and 1–2 mg/l culture for ZmPRO2. From 10 g of pollen, an average of 7.4 ± 1.5 (S.D.) mg ($n = 3$) of profilin was recovered, representing approx. 5% of total soluble protein. Protein eluted from PLP-Sepharose was separated by SDS/PAGE, and purity of profilins was determined by densitometry of Coomassie Blue-stained gels (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Purification of pollen actin

Maize pollen actin was purified by the method of Ren et al. [26]. A 10 g sample of frozen maize pollen was ground on ice for 20 min with a small amount of quartz sand and 20 ml of buffer A (10 mM Tris, 50 mM NaF, 30 mM NaPP_i, 0.5 mM CaCl₂, 0.5 mM DTT, 0.4 mM ATP, 0.5 mM PMSF and 1:200 PI cocktail, pH 8.5). An additional 30 ml of buffer A was added, and the suspension was sonicated (Sonic Dismembrator Model 300; Fisher Scientific, Pittsburgh, PA, U.S.A.) with five bursts of 20 s each. The suspension was centrifuged at 30000 g for 30 min at 4 °C, and the supernatant was transferred, avoiding the lipid-containing layer, into new tubes and centrifuged at 46000 g for 30 min. The clarified supernatant was removed, avoiding the lipid layer, and the pH was adjusted to 7.5 with NaOH. The extract was supplemented with 0.5 mM PMSF/1:200 PI cocktail/0.4 mM ATP/20 mg of human profilin, and centrifuged for 1 h at 100000 g. The supernatant was applied to a PLP-

Sepharose column pre-equilibrated with buffer B (5 mM Tris, 50 mM NaF, 30 mM NaPP_i, 0.4 mM ATP, 0.2 mM CaCl₂ and 0.01 % NaN₃, pH 7.5) at a flow rate < 1 ml/min. The matrix was washed with three column volumes of buffer B, and actin was eluted with 1 M KCl in buffer G (5 mM Tris, 0.2 mM CaCl₂, 0.01 % NaN₃, 0.5 mM DTT and 0.4 mM ATP, pH 8.0). All fractions that contained > 0.2 mg/ml actin were pooled and dialysed against two changes of 1 l of buffer G, pH 8.0, and 1 l of buffer G, pH 7.0. Actin polymerization was initiated by addition of 5 mM MgCl₂/100 mM KCl and incubated overnight at 4 °C. F-actin was collected by centrifugation at 100000 g for 3 h. The resulting pellet was resuspended in a small volume of buffer G (pH 8.0) and dialysed against three changes of buffer G (pH 8.0). The G-actin was centrifuged at 100000 g for 1 h before use.

Microinjection and microscopy

Stamen hair cells isolated from open flowers of *Tradescantia virginiana* were microinjected as described previously [10,14]. Needles were front-filled to a measured distance that was equivalent to a volume of 5 pl, estimated by injections of oil. Assuming that the vacuole occupies between 80 and 90% of the total cell volume, the injectate was approx. 10% of the cytoplasmic volume. Injections were recorded on video tape from a CCD camera (Javelin Electronics, Los Angeles, CA, U.S.A.) for subsequent analysis. Profilin was prepared by exchanging the extraction buffer with an injection buffer composed of 5 mM Tris/acetate and 0.2 mM DTT, pH 7.5, in a 10 kDa-cutoff ultrafiltration device (Schleicher & Schuell, Keene, NH, U.S.A.). DNase I was re-suspended in injection buffer and concentrated, as necessary, by ultrafiltration. The final concentration of all injection solutions was 100 μM, and at least two separate preparations of each protein were used for all data in this report. The *in vivo* effects of the proteins were compared by monitoring their disruptive effect on the actin-dependent position of the nucleus. Time zero was marked when the entire contents of the injection needle entered the cytoplasm, and each cell was monitored for a maximum of 20 min. Displacement of the nucleus was measured by monitoring the time it took the nucleus to move completely outside the perimeter, defined by its edge at time zero. If the nucleus did not exit the starting perimeter, the time recorded was 20 min. Cells in which the nucleus moved vertically within the cell, and those in which movement was hindered because the nucleus reached the cortex, were discarded. Injections into the vacuole were rare (< 2%) and were easily detected by the lack of a clear zone near the needle tip. At least 30 successful injections of each profilin were used for the statistical analysis.

G-actin binding

The affinities of recombinant or native profilins for CaATP-G-actin from pollen were determined by quenching of tryptophan fluorescence [27]. Quartz cuvettes (NSG Precision Cells, Farmingdale, NY, U.S.A.) were loaded with 0, 0.15, 0.30 or 0.45 μM pollen actin in buffer G and titrated with profilin from stock solutions of 250–300 μM in buffer G. The total increase in volume following profilin addition was always less than 1.5%. Fluorescence was recorded on a spectrofluorimeter (model 8000; SLM Instruments, Urbana, IL, U.S.A.) with excitation at 295 nm and emission at 330 nm. After each addition of profilin, fluorescence was averaged for approx. 1 min. The observed fluorescence minus fluorescence of actin alone was plotted against the concentration of profilin. To determine the K_d at each actin concentration, non-linear least squares

regression was performed using the program MacCurveFit (Raner Software, Mt. Waverly, Australia). Data were fit to the equation $F_{\text{obs}} - F_a = F_p + (F_{\text{pa}} - F_a - F_p)[\text{PA}]$, where F_{obs} is the measured fluorescence; F_a , F_p and F_{pa} are the intrinsic fluorescence coefficients for actin, profilin and the profilin-actin complex respectively; and [PA] is the concentration of profilin-actin complex [27]. Data for an experiment were discarded if one or more curve fits failed to converge on a value for K_d .

PLP binding

The K_d values of profilin isoforms for PLP were determined by monitoring the tryptophan fluorescence enhancement upon PLP binding [27,28] and by Scatchard plot analysis [29]. For fluorescence measurements, solutions of 5 μM profilin were titrated with PLP to a final concentration of approx. 2000 μM proline residues. Tryptophan fluorescence was monitored after each addition of PLP with excitation at 292 nm and emission at 335 nm, and the values were corrected for dilution. The fluorescence maximum was estimated by extrapolation of the regression line to the ordinate of double reciprocal plots of fluorescence change (ΔF) vs. [proline residues]. The data were plotted as $\Delta F/F_{\text{max}}$ versus [proline residues] and fitted to a hyperbolic function using MacCurveFit to derive K_d . For Scatchard plot analysis, serial dilutions of profilin were added to a slurry of PLP-Sepharose beads in profilin extraction buffer minus protease inhibitors and incubated at room temperature for 30 min. The beads were collected in a microcentrifuge, and the profilin concentration in the supernatant was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, U.S.A.) using BSA as a standard. Linear regression analysis on plots of [bound profilin]/[free profilin] versus [bound profilin] was used to derive a K_d , the slope of the regression line being equal to $-1/K_d$. For both assays, at least three determinations were performed with each profilin isoform.

RESULTS

Microinjection of profilin into living plant cells

To examine differences in the function of recombinant and native pollen profilin isoforms, we microinjected purified profilin into stamen hair cells of *Tradescantia*. Affinity purification of profilins from extracts of maize pollen or bacteria allowed recovery of

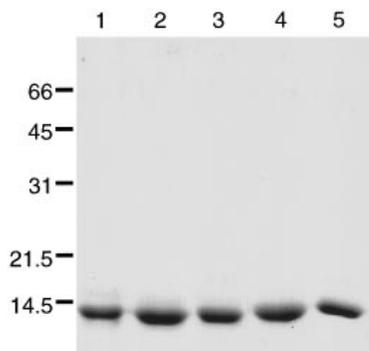


Figure 1 Profilin purified from maize pollen and bacterial cells

Coomassie Blue-stained SDS/PAGE gel with 2 μg of protein loaded per lane. Lanes 1–3, recombinant ZmPRO1–3; lane 4, native maize pollen profilin; lane 5, recombinant human profilin I. Densitometry indicates that all samples are > 95% pure.

large amounts of > 95% pure protein. As shown in Figure 1, the protein purified from pollen (lane 4) co-migrated with the three recombinant profilin isoforms (lanes 1, 2 and 3), and no lower M_r bands that would indicate degradation of the protein were detectable in any sample.

Equivalent doses of each protein were microinjected into *Tradescantia* stamen hair cells, and the time required for the nucleus to be displaced from a central position was measured (defined in the Materials and Methods section). The effects on cytoarchitecture and nuclear location for a representative injection of 100 μM native pollen profilin are shown in Figure 2. Before injection, the nucleus was positioned in the center of the vacuole and was supported by numerous transvacuolar cytoplasmic strands (Figure 2A). Within minutes after the injection of profilin, the number of strands had begun to decrease (Figure 2C), and the nucleus was displaced from its original position (Figure 2D). Eventually the nucleus moved to the cell cortex and very few transvacuolar strands remained (Figure 2E). The time of nuclear displacement for this cell was 5.6 min. The disruption of cytoplasmic architecture and streaming in *Tradescantia* stamen hair cells correlated with a reduction in F-actin (results not shown; see also [10,14]). Many injected cells were able to recover from the effects caused by this concentration of additional profilin. Within 1 h after injection, new transvacuolar strands appeared, vigorous streaming resumed and the nucleus returned to a central position (Figure 2F).

The effectiveness of each profilin isoform was determined by measuring nuclear displacement for at least 30 injected cells (Figure 3). Microinjection of buffer alone caused very little nuclear movement, resulting in an average nuclear displacement time of 18.0 ± 0.7 min (mean \pm S.E.M.; $n = 31$). The potent G-actin-binding protein DNase I caused a dramatic increase in the rate of nuclear movement and resulted in nuclear displacement within only 2.2 ± 0.3 min ($n = 30$). Human profilin required 4.6 ± 0.6 min ($n = 34$), consistent with the fact that its affinity for actin is substantially less than that of DNase I [30]. The pollen profilin isoforms were generally less effective than human profilin and there were significant differences. ZmPRO2 and native pollen profilin were nearly equivalent in their ability to cause nuclear displacement, requiring 6.1 ± 0.8 min ($n = 30$) and 6.3 ± 0.7 min ($n = 30$) respectively. ZmPRO1 and ZmPRO3 were similar and were also the least effective of the profilin isoforms, requiring 7.7 ± 0.6 min ($n = 34$) and 8.2 ± 0.9 min ($n = 30$) respectively. When these data were compared with human profilin by the t test, ZmPRO2 and native pollen profilin were not significantly different from human profilin ($P > 0.05$), whereas ZmPRO1 and ZmPRO3 were significantly different ($P = 0.0005$ and $P = 0.0014$ respectively).

PLP binding

To determine whether the differences in profilin function *in vivo* correlated with different biochemical properties for profilins *in vitro*, we measured the affinities of purified proteins for PLP. When the interaction was monitored by tryptophan fluorescence, only small differences were found between any of the profilins (Table 1). We measured a K_d for recombinant human profilin-PLP of 305 μM proline residues that was similar to the previously reported value of 359 μM [28]. When compared with recombinant human profilin, the maize pollen profilin isoforms all had slightly lower K_d values, ranging from 251 to 275 μM . However, native pollen profilin had a significantly lower K_d (249 μM) for PLP than did human profilin by the t test ($P = 0.02$).

The results from Scatchard plot analysis of profilin binding to PLP-Sepharose were somewhat different from those found with

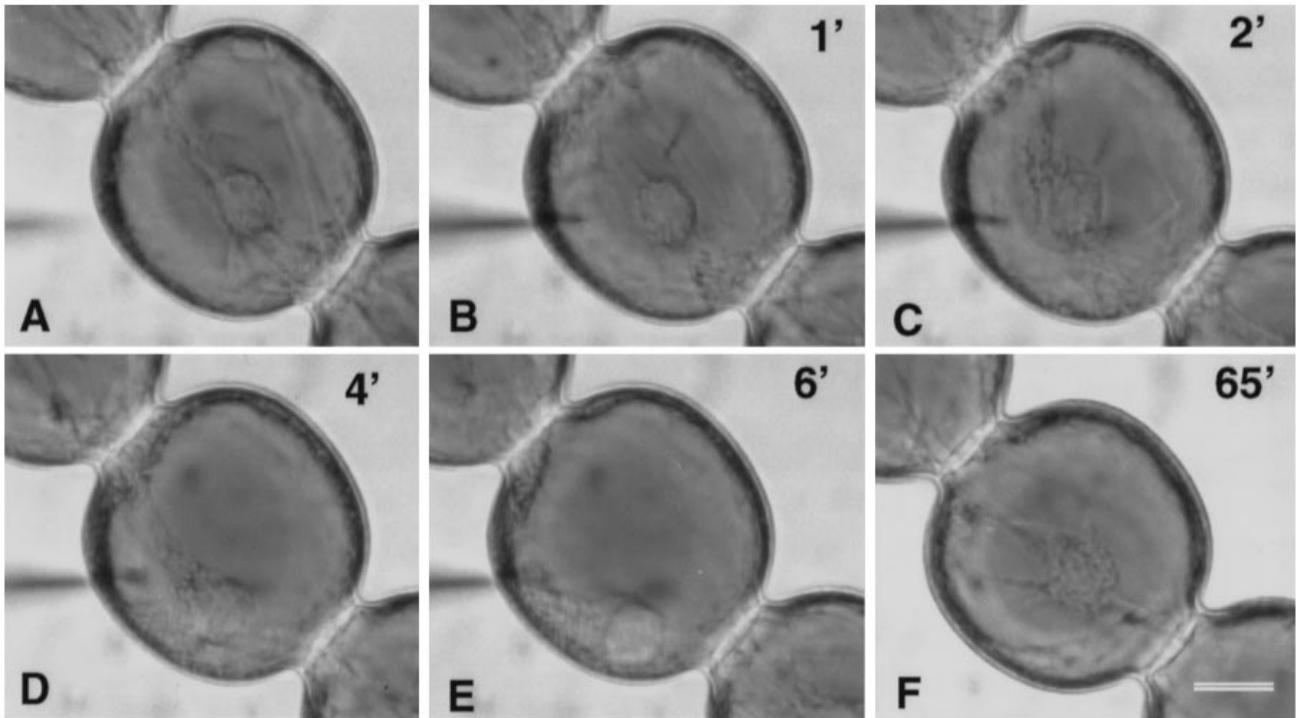


Figure 2 Microinjection of native pollen profilin causes disruption of cytoplasmic architecture

(A) *T. virginiana* stamen hair cell just before injection with 100 μM (needle concentration) native pollen profilin. (B–F) Micrographs of the same cell taken at the indicated times after microinjection. (B) By 1 min (') after injection, cytoplasmic strands begin to decrease in number. (C,D) Displacement of the nucleus from its original position. (E) Nucleus displaced fully to the cell periphery. (F) After 65 min, recovery of transvacuolar strands and central nuclear position were observed in this cell. Scale bar represents 20 μm .

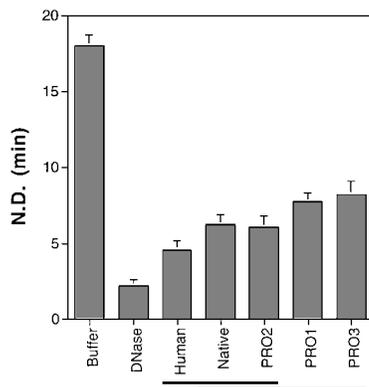


Figure 3 Actin-binding proteins differ substantially in their ability to cause nuclear displacement

Profilins and DNase I (100 μM needle concentration) were microinjected, and the time of nuclear displacement was recorded as described in the Materials and Methods section. Columns represent the average nuclear displacement (N.D.) time measured from at least 30 cells (mean \pm S.E.M.). Lines beneath the abscissa indicate samples where no significant differences could be discerned by the two-tailed *t* test.

fluorescence enhancement. Table 1 shows that the recombinant pollen profilins had similar K_d values, ranging from 0.8 μM for ZmPRO2 to 3.9 μM for ZmPRO3. These values were comparable with recombinant human profilin which had a K_d of 3.6 μM , similar to that reported previously [29]. No significant differences

Table 1 Affinity of profilin for PLP

The K_d values of profilin isoforms for PLP were determined by analysis of tryptophan fluorescence enhancement or by Scatchard plot analysis as described in the Materials and Methods section. Values are reported as mean $K_d \pm$ S.D., n = number of determinations. Fluorescence values are reported as μM proline residues.

Protein	Fluorescence K_d ($\mu\text{M} \pm$ S.D.)	n	PLP–Sepharose K_d ($\mu\text{M} \pm$ S.D.)	n
Human profilin I	305 \pm 31	4	3.6 \pm 2.0	4
ZmPRO1	275 \pm 15	4	2.7 \pm 1.2	3
ZmPRO2	262 \pm 17	3	0.8 \pm 0.4	3
ZmPRO3	251 \pm 35	4	3.9 \pm 2.7	4
Native pollen profilin	249 \pm 35	4	19.8 \pm 7.0	5

between any of the recombinant profilins were detected using the two-tailed *t* test. However, the K_d for native pollen profilin of 19.8 μM was substantially higher and was significantly different from all of the recombinant proteins ($P \leq 0.009$).

G-actin binding

To test whether the maize pollen profilin isoforms had different affinities for plant actin, we determined the K_d of each isoform for maize pollen actin. This was done by measuring the quenching

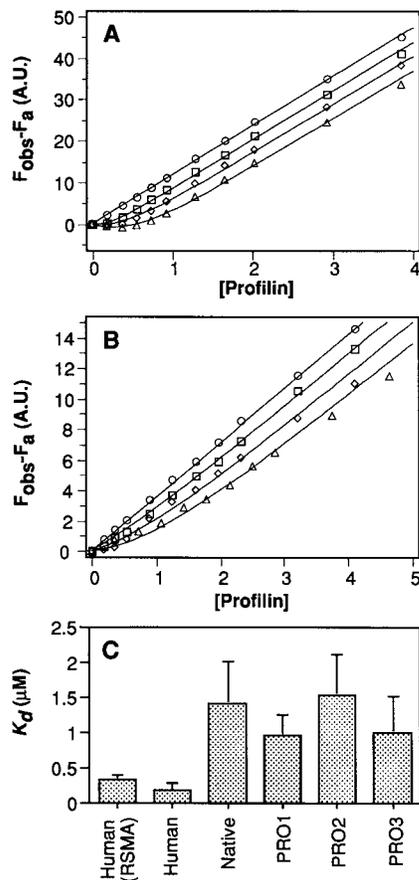


Figure 4 Analysis of profilin–G-actin-binding

The association of profilin with CaATP-G-actin from maize pollen results in quenching of tryptophan fluorescence. (A) Titration of 0 (○), 0.15 (□), 0.3 (◇) and 0.45 (△) μM pollen G-actin with human profilin. The K_d for this representative experiment was 0.15 μM. (B) Titration of 0 (○), 0.15 (□), 0.3 (◇) and 0.45 (△) μM pollen G-actin with ZmPRO1. The K_d for this experiment was 0.91 μM. (C) Histogram of the mean $K_d \pm$ S.D. from at least three experiments for each profilin isoform with pollen G-actin.

of tryptophan fluorescence that occurs when profilin binds to CaATP-G-actin [27]. The fluorescence emission at 330 nm for recombinant and native pollen profilins was approximately half that observed for the recombinant human profilin (results not shown). Addition of profilin to solutions of pollen actin resulted in quenching of the total fluorescence. Sequential addition of 0.1–3 μM human profilin to solutions of maize pollen actin produced plots (Figure 4A) that were similar to those reported for the interaction of calf spleen profilin and RSMA [27]. Analysis of the association by fitting the data to the equation described in the Materials and Methods section allowed the derivation of a $K_d = 0.2 \pm 0.1$ μM (mean \pm S.D.; $n = 5$) for human profilin–pollen G-actin. The K_d for the human profilin–RSMA complex was 0.3 ± 0.1 μM ($n = 3$), similar to the previously reported K_d of 0.2 μM for calf spleen profilin with RSMA that was determined using the same method [31]. Similar analyses for recombinant and native pollen profilins were performed, and Figure 4B shows a representative experiment for ZmPRO1. Although the total fluorescence change in the presence of maize pollen profilins was substantially less than was observed with human profilin, it was possible to determine K_d values for the profilin–actin complexes.

The K_d values for the native and recombinant maize pollen profilins were all similar to each other but were significantly higher than recombinant human profilin ($P < 0.005$) (Figure 4C). The K_d values of pollen actin for native pollen profilin, ZmPRO1, ZmPRO2 and ZmPRO3 were 1.4 ± 0.6 μM ($n = 4$), 1.0 ± 0.3 μM ($n = 4$), 1.5 ± 0.6 μM ($n = 3$) and 1.0 ± 0.5 μM ($n = 3$) respectively.

DISCUSSION

To understand how the dynamics and organization of the plant cytoskeleton are regulated, it is necessary to understand the properties of plant actin and actin-binding proteins. The presence of multiple isoforms of these proteins may reflect the need for tissue-specific or developmental regulation of gene expression, distinct functional roles for the isoforms or increased levels of gene expression (reviewed in [4,32]). At least three profilin genes are expressed in maize pollen [6], leading us to examine whether these are functionally distinct. Here, two major advances in the study of plant actin-binding proteins are reported. First, the disruption of cellular architecture in living cells is used to quantify differences in the properties of plant and non-plant profilins. Secondly, we report the first characterization of a plant actin-binding protein with actin isolated from the same tissue.

Relatively little is known about the biochemical properties of plant actin-binding proteins [4,33]. Typically, studies of the association between actin and plant profilins *in vitro* have used readily available sources of vertebrate actin [7,31,34]. The behaviour of actin-binding proteins with heterologous actin is not always representative of the interaction with the native binding partners. For example, RSMA added to extracts of *Acanthamoeba* behaved in a substantially different fashion than native *Acanthamoeba* actin [35]. Similarly, microinjection of RSMA into *Tradescantia* stamen hair cells results in the rapid polymerization of an aberrant aster-like array at the site of injection [26]. Actin-binding proteins can also behave differently in heterologous systems. For example, yeast profilin does not inhibit polymerization of RSMA but does inhibit polymerization of yeast actin [36]. The recent development of a rapid and convenient procedure to isolate polymerization-competent actin from pollen [26] allowed us to characterize the association of maize pollen profilin isoforms with a physiologically relevant source of actin.

The K_d values of profilin isoforms for G-actin typically range from 0.2 to 5 μM [8,27]. We found that the K_d values of maize pollen profilins for CaATP-G-actin from pollen, 1.0–1.5 μM, were within this range. However, vertebrate profilin bound to pollen actin and RSMA with a 5- to 7-fold lower K_d than the maize profilin isoforms. It is quite intriguing that human profilin has such a high affinity for plant actin. This observation formed the basis for a novel purification scheme for pollen actin that uses human profilin [26]. The K_d values for native and recombinant maize pollen profilin isoforms with plant actin were similar to those previously reported for the association between *Arabidopsis* profilins and RSMA using the same assay [31]. It should be noted that the ionic conditions required to observe tryptophan fluorescence quenching are not representative of those in the cell. Thus an analysis of the interaction between pollen profilin and actin under physiologically relevant conditions could provide further valuable insights. However, the values we obtained are similar to the K_d reported for the association of birch pollen profilin with RSMA in ionic conditions similar to those found in the cell [34].

The recent reports of crystal structures for birch pollen [37] and *Arabidopsis* [38] profilins have further defined the actin-binding surface of profilin isoforms. It appears that the residues

directly implicated in actin binding are not well conserved across kingdoms. In fact, only four residues involved in the primary co-crystal contact between bovine profilin and β -actin are chemically conserved [38]. The poor conservation of residues that compose the actin-binding surface may reflect the diversity in the actin isoforms that interact with the different profilins. The most notable conservation among profilins from different kingdoms appears to be the amino acids that comprise the PLP-binding site. Nearly half of the most highly conserved amino acids (8 of 18) have been implicated in PLP binding [38]. In the cell, binding to proteins with proline-rich sequences is likely to promote localization of profilin to specific sites and/or to modulate profilin activity. Several proteins containing proline-rich stretches interact with profilin *in vitro*; the best characterized is the vasodilator-stimulated phosphoprotein (VASP) from platelets. VASP contains several proline-rich repeats, interacts with both vertebrate and birch pollen profilin, and co-localizes with vertebrate profilin [39]. Profilin also interacts with proline-rich repeats on a number of other proteins, including *Drosophila* CAPPUCINO [40], two murine VASP-like proteins, Mena and Evl [41], and yeast Bni1p [42]. The presence of equivalent factors from plants has not yet been demonstrated.

Because the interaction between profilin and proline-rich proteins may have important consequences for the activity and targeting of profilin, we compared the affinity of maize pollen profilin isoforms for PLP. The recombinant plant and vertebrate profilins bound to PLP with a K_d in the hundreds of μM when determined by fluorescence and in the low μM range when determined by Scatchard plot analysis. These values are consistent with previous reports of non-plant profilins using the same assays [27–29] and confirms that plant profilins are functional PLP-binding proteins *in vitro*. The direct comparison of data obtained by the two different methods should be made with caution, because in the studies utilizing tryptophan fluorescence the values for K_d are reported as μM proline residues, and comparisons are typically made in μM Pro_{10–15}, since this was the shortest oligomer that bound with high affinity to profilin [27]. Surprisingly, we found a substantial difference in the ability to bind PLP between the native and recombinant pollen profilins by Scatchard plot analysis. Native pollen profilin had a 5–7-fold lower affinity for PLP than the recombinant proteins. However, this was not the case by fluorescence measurement, where native pollen profilin had the lowest K_d and was significantly better than human profilin. The reason for the difference in affinity of native pollen profilin for PLP in two different assays is not immediately obvious. The presence of denatured profilin, contaminating proteins or non-protein contaminants may have interfered with the Scatchard analysis. Because only the protein concentration in the supernatant is measured directly, the presence of small amounts of these factors could lead to an artificially high value for protein in the unbound fraction. This possibility seems unlikely, however, since the native pollen profilin does not appear to contain other protein species or degradation products by SDS/PAGE.

Microinjection of actin-binding proteins into live *Tradescantia* stamen hair cells provides a powerful tool to analyse the effect on actin organization and dynamics [10, 14]. This approach preserves the complexity of the biochemical interactions that occur among the injected protein, endogenous cytoplasmic factors and the actin cytoskeleton. The use of a live plant cell model is particularly important because the current knowledge of the types of actin-binding proteins that are present in plants is somewhat limited. For example, indirect interactions between actin-binding proteins could have dramatic effects on profilin function. The effect of profilin on F-actin levels varies depending on the state of the

barbed end of the filament; in the presence of barbed ends capped by accessory proteins, profilin sequesters actin monomers *in vitro* [13]. Alternatively, in the presence of a pool of sequestered actin monomers and free barbed ends, profilin can ‘desequester’ actin and promote polymerization [13].

The analysis of nuclear displacement provides a reliable measure to compare various actin-binding proteins. We postulate that the dynamics of the actin cytoskeleton in interphase *Tradescantia* stamen hair cells are somewhat static because the ends of actin filaments are capped by yet to be identified factors [4]. As outlined above, under these conditions the introduction of excess profilin would sequester actin monomers and promote depolymerization of F-actin. Because the nucleus is supported in the center of the cell by transvacuolar strands that are rich in F-actin and probably under tension, profilin-induced depolymerization causes the supporting strands to break and/or lose tension, resulting in displacement of the nucleus. The effects on nuclear position and cytoarchitecture correlated with the affinity of the proteins for G-actin *in vitro*. DNase I has a very high affinity for G-actin ($K_d = 0.05\text{--}2.0\text{ nM}$; [30]) and was the most effective at causing nuclear displacement. Human profilin was also quite effective at causing nuclear displacement, whereas all of the maize profilin isoforms were less effective. No statistically significant differences could be discerned between native pollen profilin and the three recombinant profilin isoforms, consistent with the findings for G-actin binding *in vitro*. Therefore the effects of profilin in the *Tradescantia* stamen hair cell suggest a role of sequestering actin monomers. This sequestering model is further supported by the fact that pollen actin can mitigate the effect when co-injected at 1:1 and 2:1 molar ratios with profilin [26].

Although these data are generally consistent with a sequestering activity, the finding that native pollen profilin and ZmPRO2 are nearly as potent and statistically indistinguishable from human profilin is quite surprising. Although the isoform composition of the native pollen profilin is not known, the results from the live cell assay may indicate that ZmPRO2, or another uncharacterized profilin isoform, is the major protein species. Alternatively, native profilin may be more potent in the nuclear displacement assay due to post-translational modifications that lead to altered actin-binding properties. In native pollen profilin preparations, at least two major species can be separated on two dimensional [6] or IEF slab gels. One of these has a unique pI when compared directly with the recombinant profilin isoforms (B. C. Gibbon and C. J. Staiger, unpublished work). This may represent evidence for a novel isoform and/or for post-translational modification of profilin in pollen.

Several other reasons for differences among the native and recombinant pollen profilins in the live cell assay can be considered. The affinity of maize pollen profilin isoforms for the actin isoform(s) in the *Tradescantia* stamen hair cell could be different than for the pollen actin used to measure K_d *in vitro*. Some of the profilin isoforms may be better able to promote polymerization on uncapped barbed ends *in vivo*. Nuclear displacement may not be due solely to depolymerization of F-actin but may depend on some uncharacterized secondary effect of profilin. Finally, interaction with endogenous factors, polyphosphoinositides, VASP-like proteins or kinases, for example, could alter the sequestering activity of profilin. Future experiments will be designed to test these possibilities.

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