The characterization of ligand-specific maize (*Zea mays*) profilin mutants

David R. KOVAR*, Bjørn K. DRØBAK†, David A. COLLINGS*¹ and Christopher J. STAIGER*²

*Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, U.S.A., and †Cell Signalling Group, Department of Disease and Stress Biology, John Innes Centre, Norwich NR4 7UH, U.K.

Profilins are low-molecular-mass (12–15 kDa) cytosolic proteins that are major regulators of actin assembly in all eukaryotic cells. In general, profilins from evolutionarily diverse organisms share the ability to bind to G-actin, poly-(L-proline) (PLP) and proline-rich proteins, and polyphosphoinositides. However, the functional importance of each of these interactions remains unclear and might differ between organisms. We investigated the importance of profilin's interaction with its various ligands in plant cells by characterizing four maize (*Zea mays*) profilin 5 (ZmPRO5) mutants that had single amino acid substitutions in the presumed sites of ligand interaction. Comparisons *in vitro* with wild-type ZmPRO5 showed that these mutations altered ligand association specifically. ZmPRO5-Y6F had a 3-fold increased affinity for PLP, ZmPRO5-Y6Q had a 5-fold decreased affinity for PLP, ZmPRO5-D8A had a 2-fold increased affinity for PtdIns(4,5)P₂ and ZmPRO5-K86A had a 35-fold decreased

affinity for G-actin. When the profilins were microinjected into *Tradescantia* stamen hair cells, ZmPRO5-Y6F increased the rate of nuclear displacement in stamen hairs, whereas ZmPRO5-K86A decreased the rate. Mutants with a decreased affinity for PLP (ZmPRO5-Y6Q) or an enhanced affinity for PtdIns(4,5)P₂ (ZmPRO5-D8A) were not significantly different from wild-type ZmPRO5 in affecting nuclear position. These results indicate that plant profilin's association with G-actin is extremely important and further substantiate the simple model that profilin acts primarily as a G-actin-sequestering protein in plant cells. Furthermore, interaction with proline-rich binding partners might also contribute to regulating profilin's effect on actin assembly in plant cells.

Key words: actin-binding protein, cytoskeleton, pollen, polyphosphoinositide, signal transduction.

INTRODUCTION

Plant cells often respond to intracellular and extracellular stimuli by reorganizing their microtubule and actin-filament cytoskeletons. Actin reorganization is necessary for, or coincident with, a variety of environmentally influenced processes, including cell division, cell elongation, responses to wounding or pathogen attack, plastid positioning, and pollen germination and extension of the pollen tube [1–4]. In all eukaryotic cells, the dynamic nature of the actin cytoskeleton depends on the unique constellation, the spatial distribution and the local activity of actin-binding proteins present in the cytoplasm. Actin-binding proteins control the size and activity of the actin subunit pool (G-actin), the nucleation of actin filament polymerization (F-actin), the rate of filament turnover by treadmilling and the assembly of actin filaments into higher-order structures (reviewed in [5–8]).

Profilins are low-molecular-mass (12–15 kDa), abundant, cytosolic actin-binding proteins that form a 1:1 complex with G-actin. Purified native and recombinant profilins have dual effects on actin assembly. Profilin can bind to monomeric actin and thereby prevent polymerization, yet under other conditions profilin stimulates polymerization [9,10]. Plant profilin was first discovered as a pollen allergen [11,12] and is structurally and biochemically similar to non-plant profilins (reviewed in [13,14]). In general, profilins from evolutionarily divergent organisms share the ability to bind to G-actin, poly-(L-proline) (PLP) and proline-rich proteins, and polyphosphoinositides. The functional importance of profilin's interaction with each of these three ligands has been investigated extensively and might differ between organisms [14,15].

We are interested in understanding the importance of these

interactions in the context of plant cells. Maize (*Zea mays*) expresses at least five profilin isoforms differing in their ability to alter cytoplasmic architecture and to disrupt actin arrays after microinjection into interphase plant cells (*Tradescantia* stamen hair cells) [16,17]. Characterization *in vitro* revealed that isoforms with an increased ability to disrupt actin arrays have increased affinities for both PLP and G-actin as well as a decreased affinity for PtdIns(4,5)P₂ [16,16a,17]. Furthermore, a single-amino-acid substitution mutant (ZmPRO1-Y6F) that selectively increases the affinity for PLP without affecting actin binding disrupts cellular architecture significantly faster than does wild-type profilin [16]. These results indicate that PLP binding is important for plant profilin function. However, plant profilin mutants with a decreased affinity for PLP, or with altered affinities for G-actin or PtdIns(4,5)P₂, have not yet been characterized.

To investigate the importance of profilin's interaction with different ligands in plant cells we engineered single-amino-acid replacement mutants that targeted the binding of G-actin, PLP and PtdIns(4,5)P₂ specifically. Each mutant was characterized *in vitro* in direct comparison with wild-type *Zea mays* profilin 5 (ZmPRO5), to be certain that it was altered for association with only one ligand. On microinjection into *Tradescantia* stamen hair cells, an increased ability to disrupt actin-based cellular architecture was correlated with an increased affinity for PLP, whereas a decreased ability to disrupt cytoarchitecture was correlated only with a decreased affinity for G-actin. Mutants with a decreased affinity for PLP, or an enhanced affinity for PtdIns(4,5)P₂, were not significantly different from wild-type ZmPRO5 in their ability to perturb cytoplasmic architecture. These results indicated that association with both G-actin and PLP is important for plant profilin function.

Abbreviations used: hPRO1, human profilin I; PIC, phosphoinositide-specific phospholipase C; PLC, phospholipase C; PLP, poly-(L-proline); SpPRO, *Schizosaccharomyces pombe* profilin; ZmPRO5, *Zea mays* profilin 5.

¹ Present address: School of Biological Sciences, Sydney University, NSW 2006, Australia.

² To whom correspondence should be addressed (e-mail cstaiger@bilbo.bio.purdue.edu).

MATERIALS AND METHODS

Site-directed mutagenesis and purification of maize profilin

Site-directed mutants of ZmPRO5 [17] were created by PCR amplification of wild-type ZmPRO5 cDNA coding region with Vent_R DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) and specific primer pairs. Three N-terminal mutants, ZmPRO5-Y6F (5'-CC CAT ATG TCG TGG CAG GCG TTC G-3'), ZmPRO5-Y6Q (5'-CC CAT ATG TCG TGG CAG GCG CAA GTC-3') and ZmPRO5-D8A (5'-CC CAT ATG TCG TGG CAG GCG TAC GTC GCT GAC-3'), were created by incorporating the desired mutation (bold) into the 5' primer along with an *NdeI* restriction site (underlined) for convenient cloning. The 3' primer (5'-GG GGA TCC TTA GAA GCC CTG TGC GAT CAG-3') for these three mutants mimics the last six codons, including the stop translation codon, and introduces a *BamHI* restriction site (underlined). The ZmPRO5-K86A mutant was created by overlap-extension PCR [18]. The outside primers were identical with those used to create an expression construct for wild-type ZmPRO5 [17], which incorporated a 5' *NdeI* site and a 3' *BamHI* site. The completely overlapping 30-mer internal primers (5'-GTT GTC ATC CGA GGA GCG AAG GGC ACT GGA-3' and 5'-TCC AGT GCC CTT CGC TCC TCG GAT GAC AAC-3') introduced the desired mutation (bold). After amplification by PCR, the 400-bp products for all four mutants were ligated into the expression plasmid pET-23a (Novagen, Madison, WI, U.S.A.) digested with the same enzymes. Confirmation of the desired mutations and conservation of the remainder of the sequence were verified by sequencing. The resulting mutant constructs were transformed into strain BL21(DE3) of *Escherichia coli*; protein expression was induced by the addition of 0.4 mM isopropyl β -D-thiogalactoside. Recombinant wild-type ZmPRO5 protein [17] and mutant proteins were purified on PLP-Sepharose [17,19], with the exception that the wash with 3 M urea was omitted when ZmPRO5-Y6Q was purified. Protein concentrations for wild-type and mutant profilins were determined with a molar absorption coefficient of 16000 M⁻¹·cm⁻¹ at 280 nm [17]. Typical yields of the purified profilins were 1–10 mg/l of bacterial culture. At least three independent batches of each profilin were used for each assay described below.

Maize pollen actin was purified as described previously and the concentration was determined with a molar absorption coefficient of 0.63 mg/ml at 290 nm [17,20,21].

Denaturation with urea

The stability of purified wild-type and mutant profilins was analysed by determining the concentration of urea required for their half-maximal denaturation [22]. Profilin (1 μ M) was incubated for 1 h at room temperature in buffer I [20 mM Tris/HCl (pH 7.5)/150 mM KCl/0.2 mM dithiothreitol] with 0–7 M urea; the tryptophan fluorescence of each sample was measured with excitation at 292 nm and emission at 370 nm. Normalized relative fluorescence was then plotted against urea concentration and fitted with a sigmoidal curve.

Binding to PLP and G-actin

An equilibrium dissociation constant (K_d) for the binding of profilin to PLP was determined by measuring the increase in intrinsic tryptophan fluorescence on complex formation [23,24], as described in detail previously [16,25].

The ability of 1 μ M profilin to decrease the concentration of filamentous actin in the presence of 55 μ M Ca²⁺, determined by monitoring a shift in the critical concentration (C_c) at steady

state, was used to measure profilin's apparent affinity for monomeric actin [17]. The mutant ZmPRO5-K86A at 1 μ M did not shift the C_c (results not shown); therefore 10 μ M ZmPRO5-K86A was assayed.

A decreased affinity of ZmPRO5-K86A for pollen G-actin was also demonstrated by affinity chromatography. Profilin-Sepharose affinity columns were prepared by coupling 4 mg/ml recombinant wild-type ZmPRO5 or ZmPRO5-K86A to CNBr-activated Sepharose 4B (Pharmacia) in accordance with the manufacturer's instructions. A maize pollen extract was prepared by grinding 10 g of pollen in 50 ml of extraction buffer [20 mM Tris/HCl (pH 8.5)/50 mM NaCl/5 mM MgCl₂/0.5% (v/v) Tween 20/0.2 mM dithiothreitol/0.2 mM ATP/0.5 mM PMSF/1:200 protease inhibitor cocktail] [26], followed by sonication (five times, 30 s each). The extract was then clarified by successive 30 min centrifugations at 30000 and 46000 g respectively. The supernatant was adjusted to pH 7.2, supplemented with 0.4 mM ATP and centrifuged at 100000 g for 1 h. The clarified supernatant was passed over a Sepharose CL-4B (Sigma) column to decrease non-specific binding to the profilin-Sepharose. The concentration of the flow-through was determined by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard; 50 mg of total protein (approx. 7 ml) was passed over either a wild-type ZmPRO5-Sepharose or a ZmPRO5-K86A-Sepharose column. The columns were washed and eluted successively with 20 ml of wash buffer [WB; 10 mM Tris/HCl (pH 7.2)/50 mM NaCl/5 mM MgCl₂/0.2 mM dithiothreitol/0.2 mM ATP], 10 ml WB plus 150 mM NaCl, 10 ml WB plus 950 mM NaCl, and 15 ml WB plus 7 M urea. Fractions (1.5 ml) were collected, pooled in groups of two, precipitated with 10% (w/v) trichloroacetic acid, separated by SDS/PAGE [10% (w/v) gel] and stained with Coomassie Brilliant Blue R (Sigma). The position of actin was verified by Western blots (results not shown).

Inhibition of phosphoinositide-specific phospholipase C (PIC) activity

The inhibition of bean (*Vicia faba*) plasma membrane PIC activity by the maize profilins was measured as described previously [27]. In brief, PIC activity was assayed by incubating bean plasma membranes at 25 °C in 50 μ l of buffer E [50 mM Tris/malate (pH 6.0)/10 μ M CaCl₂] with 50 μ M PtdIns(4,5)P₂ and 0.86 kBq [³H]PtdIns(4,5)P₂, in the presence of 5 μ M profilin. Reactions were stopped by the addition of 1 ml of chloroform/methanol (2:1, v/v). After incubation for 5 min on ice and the addition of 250 μ l of 0.6 M HCl, tubes were vortex-mixed and centrifuged at 14000 g for 2 min. A 400 μ l sample of the top phase was removed and radioactivity was determined by liquid-scintillation spectrometry after the addition of scintillation fluid (Hionic-Fluor; Hewlett-Packard).

Nuclear displacement assay

Freshly opened *Tradescantia virginiana* stamen hair cells were collected and microinjected with approx. 5–6 pl of protein solution [16,19,20,25]. At least 30 cells were injected for each profilin and the average times required for nuclear displacement were determined. To rule out the possibility that microinjection alone, or microinjection of any protein, affected the placement of the nucleus, we had previously injected equivalent concentrations of BSA and bovine γ -globulin [16,17,25,28]. These control injections did not cause a significant nuclear displacement in the 20 min assay.

RESULTS

Design of ligand-specific maize profilin mutants

We created four single-amino-acid substitution mutations in ZmPRO5 (ZmPRO5-Y6F, ZmPRO5-Y6Q, ZmPRO5-D8A and ZmPRO5-K86A) (Figure 1B) that, on the basis of co-crystals

and previous analysis of profilin mutants [29–31], were predicted to alter the association with only one ligand. The residues that are most highly conserved in profilins from different species are those implicated in PLP binding (Figure 1A); these form a hydrophobic patch between the N-terminal and C-terminal α -helices. Nine of the 20 amino acids that are conserved in more

Figure 1 Design of ligand-specific maize profilin mutants

(A) Alignment of the deduced amino acid sequences for *Arabidopsis thaliana* profilin 1 (AtPRF1; GenBank® accession number AAG10090), AtPRF4 (AAG10091), *Zea mays* profilin 1 (ZmPRO1; X73279), ZmPRO5 (AF201459), *S. pombe* (P39825) and hPRO1 (Human 1; A28622). The multiple sequence alignment was prepared by using the ClustalW algorithm of MacVector 7.0 software. Residues that are conserved in a majority of the displayed sequences are shaded grey. Gaps (–) were introduced to optimize the alignment. Amino acid residue numbering is based on AtPRF1. Conserved residues implicated in PLP binding are denoted by *, whereas those involved in actin binding are marked by #. The two regions of primary sequence that contribute to a plant-specific patch are overlined [32]. (B) Amino acid side group changes of four maize profilin mutants (ZmPRO5-Y6Q, ZmPRO5-Y6F, ZmPRO5-D8A and ZmPRO5-K86A) were modelled on the wild-type structure of AtPRF1 as determined by Thorn et al. [32]. The wild-type and corresponding mutated residues are shown as stick figures on a ribbon backbone of AtPRF1.

Table 1 Overview of the properties of maize profilin mutants

The stability of each mutant was measured by determining the concentration of urea required [mean $\pm$ S.D. (*n*)] to denature half the protein. The apparent K_d values [means $\pm$ S.D. (*n*)] for profilin binding to plant G-actin under polymerizing conditions, in the presence of 55 μ M Ca^{2+} , were determined by measuring the shift in C_c values at steady state. K_d values [means $\pm$ S.D. (*n*)] for binding to PLP are reported in μ M proline residues. Relative affinities for $\text{PtdIns}(4,5)\text{P}_2$ were determined by profilin's ability to inhibit plant phospholipase C. The column headed 'Live cell' shows the average time in min [mean $\pm$ S.E.M. (*n*)] required for nuclei to move outside the circumference of their original position and was measured after *Tradescantia* stamen hair cells were injected with 100 μ M (needle concentration) protein. Values for the ZmPRO5 mutants that were significantly different from the wild-type control by the two-tailed *t* test ($P < 0.002$) are shown in bold. Data for hPRO1 are taken from [16,16a,17] and D. R. Kovar, P. Wang, W. S. Sale, B. K. Drøbak and C. J. Staiger, unpublished work).

Mutant	[Urea] (M)	K_d (μ M)		Affinity for $\text{PtdIns}(4,5)\text{P}_2$	Live cell (min)
		Actin	PLP		
ZmPRO5	3.97 $\pm$ 0.07 (19)	0.31 $\pm$ 0.06 (9)	162 $\pm$ 17 (16)	—	5.1 $\pm$ 0.4 (85)*
ZmPRO5-Y6Q	2.85 $\pm$ 0.25 (12)	0.30 $\pm$ 0.06 (4)	864 $\pm$ 38 (11)	—	5.2 $\pm$ 0.5 (37)
ZmPRO5-Y6F	3.85 $\pm$ 0.08 (6)	0.29 $\pm$ 0.06 (5)	54 $\pm$ 06 (14)	—	3.0 $\pm$ 0.4 (41)
ZmPRO5-D8A	3.53 $\pm$ 0.09 (18)	0.28 $\pm$ 0.08 (4)	157 $\pm$ 09 (07)	+	5.6 $\pm$ 0.8 (30)
ZmPRO5-K86A	3.71 $\pm$ 0.05 (12)	11.7 $\pm$ 1.90 (6)	163 $\pm$ 05 (05)	—	12.1 $\pm$ 0.8 (30)
hPRO1	3.40 $\pm$ 0.05 (3)	0.14 $\pm$ 0.05 (4)	289 $\pm$ 32 (10)	++ +	4.6 $\pm$ 0.6 (34)

* A subset of these data was published previously [17].

than 80% of eukaryotic profilins are implicated in binding to PLP; 6 of these make direct contact with proline residues [29]. The conserved aromatic residue at position 6 (Y), contacts PLP directly [29] and has previously been mutated in both human profilin I (hPRO1-Y6F [30]) and *Schizosaccharomyces pombe* profilin (SpPRO-Y5A,D [31]). These profilin mutants have a decreased affinity for PLP. We previously tried to mimic hPRO1-Y6F with the same mutation on *Zea mays* profilin I (ZmPRO1-Y6F) but, interestingly, found it has a 2-fold increased affinity for PLP when compared with wild-type ZmPRO1 [16]. It was therefore possible that ZmPRO5-Y6F could have either an increased or a decreased affinity for PLP. ZmPRO5-Y6Q was predicted to have a decreased affinity for PLP on the basis of the replacement of an aromatic side group with an amide.

Residues thought to be involved in actin binding (Figure 1A) are less well conserved among profilins from different species [32]. A co-crystal of bovine profilin with β -actin showed that approx. 20 profilin residues contribute to a large interface with actin [33]. When a conserved positively charged lysine residue (K86 on AtPRF1; Figure 1A), which pairs with a glutamic acid residue on actin, was mutated in SpPRO-K81A,E,F,L,Y, the mutants had greatly diminished affinities for G-actin [31]. We predicted that a similar mutant on ZmPRO5 (ZmPRO5-K86A) would also have a decreased affinity for G-actin.

The phospholipid-binding site on the overall fold of eukaryotic profilins remains a matter of debate [14,15], although when a highly conserved aspartic acid residue (D8) on the N-terminal α -helix was changed to alanine in hPRO1-D8A [30], the mutant had an 8-fold higher affinity for $\text{PtdIns}(4,5)\text{P}_2$. The reason for the increased affinity of hPRO1-D8A for $\text{PtdIns}(4,5)\text{P}_2$ is not clear, but might relate to charge [30]. On the basis of those results we predicted that ZmPRO5-D8A would have an increased affinity for $\text{PtdIns}(4,5)\text{P}_2$.

Maize profilin mutants are stable

We wished to determine whether the maize profilin mutants were identical with wild-type profilin in all aspects except for their association with a single ligand. One effect that might change biochemical properties would be a loss of protein stability. Protein instability resulted in the failure of several fission-yeast mutants with single-amino-acid substitutions in profilin to complement for a loss of wild-type activity [31]. To test whether the maize profilin mutants had folding defects, their stability was

measured by denaturing them with increasing concentrations of urea. Protein unfolding causes intrinsic tryptophan fluorescence to increase. The concentration of urea required to denature half of wild-type ZmPRO5 was 3.97 M; several of the mutants were only slightly less stable, with ZmPRO5-Y6F, ZmPRO5-K86A and ZmPRO5-D8A having midpoints of 3.85, 3.71 and 3.53 M urea respectively (Table 1). The stability of ZmPRO5-Y6Q (2.85 M) was considerably different from that of the wild type. However, ZmPRO5-Y6Q was indistinguishable from the wild type in other biochemical characteristics (see below). Furthermore, a single-amino-acid substitution mutant in fission yeast that retained wild-type biochemical properties *in vitro* and was able to complement the loss of wild-type profilin *in vivo* was found to have a denaturation midpoint that was 1.3 M less than the wild type [31]. Although we cannot be certain of the extent to which ZmPRO5 tolerates instability, it seems that these four maize profilin mutants are folded reasonably well. We therefore conclude that any biochemical differences were due to alterations in the association with a specific ligand rather than to general folding defects.

ZmPRO5-Y6F and ZmPRO5-Y6Q have opposite effects on binding to PLP

The affinity of the maize profilin mutants for PLP was determined by measuring the increase in intrinsic tryptophan fluorescence when a profilin solution was titrated with PLP, as shown in Figure 2. ZmPRO5-Y6Q and ZmPRO5-Y6F had opposite effects on binding to PLP. In comparison with the wild type, less PLP was needed for saturation of complex formation with ZmPRO5-Y6F, whereas more PLP was needed for saturation of complex formation with ZmPRO5-Y6Q. Hyperbolic fits of the data permitted a determination of the average dissociation constants (K_d values) for the affinity of the different profilins for PLP. The K_d for wild-type ZmPRO5 was 162 μ M proline residues (Table 1), similar to our previous finding of 164 μ M [17]. The K_d for ZmPRO5-Y6F was 54 μ M and the K_d for ZmPRO5-Y6Q was 864 μ M proline residues. Previously we found that an identical mutation on *Zea mays* profilin I, ZmPRO1-Y6F, also had an increased affinity for PLP [16]. As expected, the other two mutants were not different from the wild type in their affinity for PLP. The K_d for ZmPRO5-D8A was 157 μ M and the K_d for ZmPRO5-K86A was 163 μ M proline residues (Table 1). Therefore, in comparison with the wild type, ZmPRO5-Y6F had a

Figure 2 ZmPRO5-Y6F and ZmPRO5-Y6Q have opposite effects on binding to PLP

The binding of 5 μ M wild-type ZmPRO5 ($\circ$), ZmPRO5-Y6F ($\diamond$) or ZmPRO5-Y6Q ($\square$) to PLP was assayed by measuring the increase in intrinsic profilin tryptophan fluorescence on the addition of increasing amounts of PLP. K_d values were determined by fitting the resulting curves with a hyperbolic function. In the representative experiment shown here the affinity for PLP was 165 μ M proline residues for ZmPRO5, 59 μ M for ZmPRO5-Y6F and 927 μ M for ZmPRO5-Y6Q.

Figure 3 ZmPRO5-D8A has an enhanced association with PtdIns(4,5) P_2

The hydrolysis of PtdIns(4,5) P_2 by phosphoinositide-specific phospholipase C (PIC) was measured in the absence or presence of 5 μ M wild-type (WT) or mutant maize profilins. Results are means $\pm$ S.D. for at least eight independent determinations. PIC activity in the absence of profilin ($\emptyset$ profilin) was set to 100%.

3-fold increased affinity for PLP, ZmPRO5-Y6Q had a 5-fold decreased affinity for PLP, and ZmPRO5-D8A or ZmPRO5-K86A had identical affinities for PLP.

ZmPRO5-D8A has an increased affinity for PtdIns(4,5) P_2

We investigated the association of the maize profilin mutants with membrane phospholipids by measuring their ability to inhibit the hydrolysis of PtdIns(4,5) P_2 by PIC from bean membrane [27], as shown in Figure 3. In comparison with a control without profilin, PIC activity was decreased to 77% in the presence of wild-type ZmPRO5 and to 58% in the presence of *Zea mays* profilin 1 (ZmPRO1). These results agree with our previous findings that ZmPRO5 does not bind well to PtdIns(4,5) P_2 (Table 1) when measured either by inhibition of PIC activity [17] or with a microfiltration assay ([34], and D. R.

Kovar, P. Wang, W. S. Sale, B. K. Drøbak and C. J. Staiger, unpublished work). In comparison, PIC activity was decreased to 69%, 70% and 82% in the presence of ZmPRO5-Y6Q, ZmPRO5-Y6F and ZmPRO5-K86A respectively. These values were not significantly different from that of wild-type ZmPRO5 ($P > 0.07$; Table 1). Conversely, PIC activity was decreased to 61% in the presence of ZmPRO5-D8A, which was significantly lower than in the wild-type ($P = 0.0004$; Table 1). Therefore, because wild-type ZmPRO5 decreased PIC activity by approx. 20% and ZmPRO5-D8A by 40%, the mutant can be assumed to have a 2-fold increased association with PtdIns(4,5) P_2 compared with wild-type ZmPRO5.

ZmPRO5-K86A has a decreased affinity for G-actin

To determine the apparent affinities of the maize profilin mutants for pollen G-actin, we measured the decrease in the amount of filamentous actin (F-actin) in the presence and in the absence of profilin. As shown in Figures 4(A) and 4(B), increasing concentrations of maize pollen G-actin alone or G-actin in the presence of either 1 μ M wild-type ZmPRO5, 1 μ M ZmPRO5-Y6Q or 10 μ M ZmPRO5-K86A were allowed to polymerize until steady state was reached. Subsequently, the relative amount of F-actin was determined by 90° light scattering and plotted against the starting concentration of G-actin. The point at which a linear fit of the data intercepts the x -axis is referred to as the critical concentration (C_c) for assembly and represents the minimum concentration of G-actin required for polymerization to occur. For the representative experiment shown in Figure 4(A), C_c for actin alone was 0.35 μ M. In the presence of profilin, C_c was 0.93 μ M for wild-type ZmPRO5 and 0.91 μ M for ZmPRO5-Y6Q. For the representative experiment shown in Figure 4(B), C_c for actin alone was 0.5 μ M. In the presence of profilin, C_c was 1.12 μ M for wild-type ZmPRO5 and 0.87 μ M for ZmPRO5-K86A.

The difference in C_c in the presence and in the absence of profilin represents the amount of profilin-actin complex formed under these conditions, assuming that profilin forms a 1:1 complex with G-actin and does not facilitate subunit addition at the barbed end. Using these measurements and the calculations stated previously [17], we determined the apparent affinity of profilin for actin. For the representative experiment shown in Figure 4(A), the apparent affinity for pollen G-actin was 0.25 μ M for wild-type ZmPRO5 and 0.28 μ M for ZmPRO5-Y6Q. For the representative experiment shown in Figure 4(B), the apparent affinity for pollen G-actin was 0.33 μ M for wild-type ZmPRO5 and 14.1 μ M for ZmPRO5-K86A. From several independent experiments, the average apparent affinity of wild-type ZmPRO5 was 0.31 μ M (Table 1), which is similar to our previous finding of 0.3 μ M [17]. As expected, ZmPRO5-Y6Q, ZmPRO5-Y6F and ZmPRO5-D8A, which had average apparent affinities of 0.30, 0.29 and 0.28 μ M respectively (Table 1), were not significantly different from that of the wild type ($P > 0.42$). Conversely, ZmPRO5-K86A had an average apparent affinity for G-actin of 11.7 μ M (Table 1), which was significantly different from that of wild-type ZmPRO5 ($P < 0.0001$). Therefore ZmPRO5-K86A had a 35-fold lower affinity than wild-type ZmPRO5 for G-actin.

The affinity of ZmPRO5-K86A for pollen actin was also investigated qualitatively by affinity chromatography. Equal amounts of a maize pollen extract were loaded simultaneously on wild-type ZmPRO5-Sepharose and ZmPRO5-K86A-Sepharose columns, as shown in Figure 4(C). After extensive washes with 50 mM NaCl, bound actin was eluted with stepwise washes of 200 mM NaCl, 1 M NaCl and 7 M urea. The resulting fractions were precipitated with 10% (w/v) trichloroacetic acid, separated

Figure 4 ZmPRO5-K86A has a decreased affinity for G-actin

(A) A representative experiment shows that wild-type ZmPRO5 or ZmPRO5-Y6Q shift the steady-state C_c values for actin assembly equally. Increasing concentrations of pollen G-actin were polymerized in the absence ($\square$) or presence of either $1 \mu\text{M}$ wild-type ZmPRO5 ($\diamond$) or $1 \mu\text{M}$ ZmPRO5-Y6Q ($\circ$). The X-intercept (C_c) of each regression line was $0.35 \mu\text{M}$ for actin alone and 0.93 or $0.91 \mu\text{M}$ in the presence of wild-type ZmPRO5 or ZmPRO5-Y6Q respectively. The resulting apparent K_d values were $0.25 \mu\text{M}$ for wild-type ZmPRO5 and $0.28 \mu\text{M}$ for ZmPRO5-Y6Q. (B) A representative experiment shows that $10 \mu\text{M}$ ZmPRO5-K86A ($\triangle$) shifts the steady-state C_c value for actin assembly less, when compared with actin alone ($\square$), than the shift caused by only $1 \mu\text{M}$ of wild-type ZmPRO5 ($\diamond$). The C_c was $0.5 \mu\text{M}$ for actin alone and 1.12 or $0.87 \mu\text{M}$ in the presence of wild-type ZmPRO5 or ZmPRO5-K86A respectively. The resulting apparent K_d values were $0.33 \mu\text{M}$ for wild-type ZmPRO5 and $14.1 \mu\text{M}$ for ZmPRO5-K86A. (C) The amount of native pollen actin binding to profilin-Sepharose affinity columns was examined. Maize pollen extracts were loaded on wild-type ZmPRO5-Sepharose or ZmPRO5-K86A-Sepharose columns. The amount of actin (A) in the extract (E) loaded on each column was approximately equal, but less actin was found in the flow-through fraction (FT) from the wild-type (WT) column. Furthermore, a much greater amount of actin was bound and eluted throughout the subsequent wash steps (200 mM NaCl , 1 M NaCl and 7 M urea) from the wild-type ZmPRO5-Sepharose column than from the ZmPRO5-K86A-Sepharose column. Abbreviation: AU, arbitrary units.

by SDS/PAGE and revealed with Coomassie Brilliant Blue. Considerably more actin was bound and eluted throughout each wash step from wild-type ZmPRO5-Sepharose than from ZmPRO5-K86A-Sepharose. Combined with the steady-state experiment above, these results indicated that ZmPRO5-K86A had a significantly lower affinity than wild-type ZmPRO5 for G-actin.

Figure 5 Maize profilin mutants differ in their ability to cause nuclear displacement

The average time (mean $\pm$ S.E.M.) required for nuclei to move outside the circumference of their original position was measured after *Tradescantia* stamen hair cells were injected with $100 \mu\text{M}$ (needle concentration) protein. Injected cells were monitored for a maximum of 20 min; at least 30 injections were performed for each protein. BSA at 1.4 mg/ml was injected previously to test the effects of introducing foreign protein into the cytoplasm [17]. The fold difference, compared with the wild type (WT), for the association of each mutant with a specific ligand is noted at the right.

Microinjection of profilin into living plant cells indicates that both PLP and G-actin are important ligands for plant profilins

We have developed a technique, involving the microinjection of *Tradescantia* stamen hair cells, to study the effects of increasing the cellular concentration of actin-binding proteins on actin-dependent processes ([16,17,20,25,35,36]). Organelles and vesicles contribute to rapid cytoplasmic streaming along numerous transvacuolar strands and the strands themselves are in constant motion. In cells near the tip of the hair, the nucleus often lies near the centre of the vacuole and is held in place by the transvacuolar strands. On microinjection of profilin into *Tradescantia* stamen hair cells, the transvacuolar strands become thinner and snap, leading to the displacement of the nucleus to the wall of cell [16,17,25,35]. The time required for the stamen hair nucleus to move one nuclear diameter is an extremely reliable method of measuring the ability of different profilins to disrupt the actin cytoskeleton in the live-cell assay. Previously we have detected differences in the rate of nuclear displacement after injection of the two biochemically dissimilar classes of maize profilin isoforms [16,17].

In the present study, when wild-type ZmPRO5 was microinjected into the stamen hair cell cytoplasm the average time for nuclear displacement was 5.1 min (Figure 5, Table 1). When either ZmPRO5-Y6Q (5-fold decreased affinity for PLP) or ZmPRO5-D8A [increased affinity for $\text{PtdIns}(4,5)\text{P}_2$] were microinjected, the time required for nuclear displacement was not significantly different from that for wild-type ZmPRO5 ($P > 0.46$), with average times of 5.2 and 5.6 min respectively (Figure 5, Table 1). When ZmPRO5-Y6F (3-fold increased affinity for PLP) was microinjected, the nuclear displacement time was significantly faster than that for wild-type ZmPRO5 ($P = 0.001$), with an average time of 3.0 min (Figure 5, Table 1). Conversely, when ZmPRO5-K86A (35-fold decreased affinity for G-actin) was microinjected, the nuclear displacement time was significantly slower than that for wild-type ZmPRO5 ($P < 0.0001$), with an average time of 12.1 min (Figure 5, Table 1).

DISCUSSION

Profilin is a small (12–15 kDa) cytosolic protein that has been implicated in the regulation of actin assembly in all eukaryotic cells. In general, evolutionarily diverse organisms contain profilins that share the ability to bind to G-actin, PLP and proline-rich proteins, and polyphosphoinositides [14,15,37]. The functional importance of these three interactions remains unclear and might differ between organisms. To increase our understanding of the significance of plant profilin's interaction with its different ligands we characterized four single-amino-acid substitution mutants of ZmPRO5 that have altered associations with a specific ligand.

Compared with wild-type ZmPRO5, ZmPRO5-Y6F had a 3-fold increased affinity for PLP, ZmPRO5-Y6Q had a 5-fold decreased affinity for PLP, ZmPRO5-D8A had a 2-fold increased association with PtdIns(4,5) P_2 and ZmPRO5-K86A had a 35-fold decreased affinity for G-actin. When microinjected into interphase *Tradescantia* stamen hair cells, an increased rate of displacing the nucleus was correlated with an increased affinity for PLP (ZmPRO5-Y6F), whereas a decreased rate was correlated with a decreased affinity for G-actin (ZmPRO5-K86A). A decreased affinity for PLP (ZmPRO5-Y6Q) or an enhanced affinity for PtdIns(4,5) P_2 (ZmPRO5-D8A) did not significantly change profilin's ability to displace the nucleus.

Profilin function in a live-cell assay correlates with actin-binding properties

Profilin isoforms from angiosperm plants, vertebrates, yeasts and vaccinia virus have been characterized extensively (reviewed in [14,15,37]). The formation of a 1:1 complex between profilin and G-actin can have complex effects on actin assembly. Because profilins inhibit the addition of monomers to the slow-growing (pointed) end of actin filaments but not to the fast-growing (barbed) end [38], profilins can have opposite effects on the assembly of actin *in vitro*, depending on the availability of barbed ends [9,10]. In the presence of barbed ends that are capped, profilin functions as a simple sequestering protein, preventing actin polymerization. However, when the barbed end of filaments are not capped and a large pool of actin monomers exists, the profilin-actin complex can assemble on F-actin. Furthermore, most profilins are able to function as a nucleotide exchange factor for monomeric actin; this has led to models in which profilin contributes to actin assembly by 'recharging' subunits with ATP [39,40]. Interestingly, plant profilins do not stimulate nucleotide exchange with either animal [22,41] or plant actin [17], yet are still able to promote assembly under appropriate conditions [41].

The ability of profilins to promote both polymerization and depolymerization has been confirmed genetically. In budding yeast and *Dictyostelium*, mutational analyses suggest that profilin has a sequestering function [42,43], whereas *Drosophila* and fission-yeast loss-of-function mutants indicate that profilin is necessary for actin polymerization [44,45].

Most available evidence suggests that profilins act primarily as actin-sequestering proteins in plant cells [4,14]. When *Arabidopsis* profilin is overexpressed in *S. pombe*, the yeast actin cytoskeleton is perturbed [46]; maize profilins complement the increased levels of F-actin present in *Dictyostelium* profilin-deficient cells [19]. Measurements of profilin and actin concentrations in plant cells [47,48], along with the high affinity of native plant profilin for plant actin [17,25], indicate that profilin can account for the bulk of the sequestered actin pool. Finally, when the concentration of profilin in plant cells is increased by microinjection, the abun-

dance of actin filaments is decreased and actin-dependent processes such as cell-plate formation during cytokinesis, cell growth and cytoplasmic streaming are inhibited [35,49,50]. All of these effects could be explained by an increase in cellular profilin concentration causing the depolymerization and sequestering of actin [13,14].

The results reported here provide further evidence that plant profilins primarily depolymerize and sequester actin. Previously we found that maize contains two functionally distinct classes of profilins differing in their ability to displace the nucleus after microinjection into *Tradescantia* stamen hair cells. Isoforms with an increased ability to displace the nucleus have a 4-fold increased affinity for G-actin [16,16a,17]. Conversely, ZmPRO5-K86A, which had a 35-fold decreased affinity for G-actin, was significantly slower at displacing the nucleus when compared with wild-type ZmPRO5. This result was not similar to that with a mammalian profilin mutant that was also defective in actin binding but had a dominant effect (suppressed actin polymerization) in live cells [51].

We conclude that, on microinjection into *Tradescantia* stamen hair cells, the most significant contribution to nuclear displacement is probably the ability of profilin to bind to G-actin. However, it was interesting to find that a 35-fold decrease in affinity for G-actin did not abolish profilin's effect on nuclear displacement. The time required for nuclear displacement when ZmPRO5-K86A was microinjected into stamen hair cells was only double the time required for wild-type ZmPRO5. This suggested that other profilin properties are probably also important in living cells.

Enhanced association with PLP results in a dominant effect for profilin in the live cell

The story might be complicated by profilin's association with PLP (and proline-rich proteins) and PtdIns(4,5) P_2 . In addition to differences in affinity for actin, the maize profilin classes also differ in their affinity for PLP and PtdIns(4,5) P_2 . Isoforms with an increased ability to displace the nucleus also have an increased affinity for PLP [16,17]. It is possible that differences in affinity for PLP, as well as for actin, contribute to differences between the maize profilins' ability to displace the nucleus. We found that ZmPRO5-Y6F, which had a 3-fold enhanced affinity for PLP, displaced the nucleus significantly faster (in almost half the time) than did wild-type ZmPRO5. This result was in complete agreement with our previous finding that an identical mutation of *Zea mays* profilin 1 (ZmPRO1-Y6F) also has an increased affinity for PLP and displaces the nucleus significantly faster than does wild-type ZmPRO1 [16]. However, it was surprising that the complementary mutant ZmPRO5-Y6Q, with a 5-fold decreased affinity for PLP, did not displace the nucleus significantly more slowly than did wild-type ZmPRO5. Additionally, a mutant that does not bind to PLP at all (ZmPRO5-W3L) was also indistinguishable from wild-type ZmPRO5 in causing nuclear displacement (results not shown).

Proline-rich binding partners of profilin include the vasodilator-stimulated phosphoprotein ('VASP') and related proteins, several formin-like proteins, aczonin, drebrin and gephyrin [52–56]. These interactions are proposed to link profilin to signal transduction cascades that result in a reorganization of the actin cytoskeleton, through regulating either the subcellular distribution or the activity of profilin [37,57–59]. Evidence for plant proline-rich profilin-binding proteins is sparse. However, members of the formin-homology ('FH')-domain family of proteins from *Arabidopsis* have been identified [60], and at least one of these interacts with profilin in the yeast two-hybrid assay [61].

The dominant effect on nuclear displacement of the plant profilin-Y6F mutation is not currently understood, but several possibilities can be considered. For instance, profilin-Y6F might be able to interact with some cellular component that wild-type profilin does not normally bind. Another possibility is that measured affinities for PLP do not accurately reflect a given profilin's affinity for natural proline-rich ligands. For example, mammalian profilin I binds more strongly to the proline-rich protein N-WASP than does mammalian profilin II, even though profilin II has a higher affinity for PLP [51]. Sequestration in the nucleus might also contribute to differences between wild-type and Y6F mutant profilins [62]. Wild-type profilin might be sequestered into the stamen hair cell nucleus, where it cannot interact with and depolymerize cytoplasmic actin filaments, more efficiently than the plant profilin-Y6F mutant. A more intriguing possibility is that plant profilin-Y6F interacts more strongly with natural proline-rich binding partners; perhaps this interaction enhances the actin-binding properties of profilin. A proline-rich peptide from vasodilator-stimulated phosphoprotein alters the effect of profilin on actin dynamics [63]. Finally, the higher affinity of plant profilin-Y6F for PLP could displace endogenous *Tradescantia* profilin from intracellular sites where it is sequestered from interacting with actin. If *Tradescantia* profilin has a higher affinity than the maize profilins for the endogenous actin, the nucleus might be displaced more quickly on the release of *Tradescantia* profilin from sequestration. Unfortunately it is still not clear why ZmPRO5-Y6Q, with a 5-fold decreased affinity for PLP, was not different from wild-type ZmPRO5 in the live-cell assay.

Profilin's interaction with membrane polyphosphoinositides has been suggested to link profilin to intracellular signalling events [57,64]. The association of profilin with PtdIns(4,5) P_2 inhibits hydrolysis by phospholipase C (PLC), unless PLC is phosphorylated [57,65]. On the activation of PLC by tyrosine phosphorylation, profilin bound to PtdIns(4,5) P_2 could be released into the cytoplasm and cause the reorganization of actin [37,58]. ZmPRO5-D8A, which had a 2-fold increased association with PtdIns(4,5) P_2 compared with wild-type ZmPRO5, was indistinguishable from wild-type ZmPRO5 in the time required for nuclear displacement. However, it is possible that this assay was not sensitive enough to detect a difference between wild-type ZmPRO5 and a mutant with only a modest difference in association with PtdIns(4,5) P_2 .

Summary

Not surprisingly, our results indicate that affinity for G-actin might be the most significant property of plant profilin's function; they also further substantiate the simple model that profilin acts primarily as a G-actin-sequestering protein in plant cells [13,14]. Presumably, interactions between plant profilins and proline-rich proteins or phosphoinositides are important, but it is not at present clear how profilins are regulated by these interactions. However, we do know that plant profilins exist in large gene families consisting of two differentially expressed and functionally distinct classes, at least in maize, that probably contribute to the regulation of profilin's role in F-actin organization [16,16a,17]. Additionally, Ca^{2+} concentration affects the ability of plant profilins to prevent actin polymerization *in vitro* [17,66,67] and phosphorylation might also contribute to the regulation of plant profilin [68]. Furthermore, we should not rule out the possibility that plant-specific profilin ligands will be identified that are involved in regulating profilin activity in plant cells.

Elegant studies of profilin mutants in the genetically tractable yeast systems have provided interesting clues to the importance

of profilin's interaction with G-actin and proline-rich proteins *in vivo* [31,69]. Ligand-specific point mutations were made and tested for their ability to complement for loss of wild-type activity. It was found that profilin's interaction with both G-actin and proline-rich proteins, as well as profilin's ability to enhance nucleotide exchange on actin, are essential [31,69]. Studies of transgenic plants overexpressing ligand-specific plant profilin mutants should be equally informative. It will be interesting to investigate the effect on actin polymer levels, actin filament organization and actin-dependent cellular processes (namely cytoplasmic streaming, cell division, pollen tube germination and growth) when the mutants with either enhanced PLP binding (Y6F) or decreased G-actin binding (K86A) are overexpressed in various plant tissues.

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