<u>Final Technical Report</u> for DE-FG02-99ER20337A01 ($291,000; 3 yrs)
Identification of Actin-Binding Proteins from Maize Pollen
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Specific Aims—The goal of this project was to gain an understanding of how actin filament organization and dynamics are controlled in flowering plants. Specifically, we proposed to identify unique proteins with novel functions by investigating biochemical strategies for the isolation and characterization of actin-binding proteins (ABPs). In particular, our hunt was designed to identify capping proteins and nucleation factors. The <u>specific aims</u> included:

1) to use F-actin affinity chromatography (FAAC) as a general strategy to isolate pollen ABPs
2) to produce polyclonal antisera and perform subcellular localization in pollen tubes
3) to isolate cDNA clones for the most promising ABPs
4) to further purify and characterize ABP interactions with actin *in vitro*.

Summary of Progress
By employing affinity chromatography on F-actin or DNase I columns, we have identified at least two novel ABPs from pollen, PrABP80 (gelsolin-like) and ZmABP30, We have also cloned and expressed recombinant protein, as well as generated polyclonal antisera, for 6 interesting ABPs from *Arabidopsis* (fimbrin AtFIM1, capping protein α/β (AtCP), adenylyl cyclase-associated protein (AtCAP), AtCapG & AtVLN1). We performed quantitative analyses of the biochemical properties for two of these previously uncharacterized ABPs (fimbrin and capping protein). Our studies provide the first evidence for fimbrin activity in plants, demonstrate the existence of barbed-end capping factors and a gelsolin-like severing activity, and provide the quantitative data necessary to establish and test models of F-actin organization and dynamics in plant cells.

i. Identification of ABPs from pollen

<u>F-actin affinity chromatography (FAAC)</u> was our initial choice for identification of pollen ABPs. At least a dozen experiments were performed in which high-speed supernatants were passed over columns of phalloidin-stabilized F-actin or BSA (as a control for non-specific binding). After extensive buffer washes, bound proteins were eluted with a step-gradient of increasing salt and analyzed by SDS-PAGE. These analyses consistently yielded bands of ~120 kDa and 70 kDa which were specific to the high salt eluates from FAAC. These polypeptides were identified as maize orthologs of 135-ABP and heat shock protein (HSP) 70, respectively, by MALDI-TOF peptide mass fingerprinting and by Western immunoblotting. 135-ABP was originally identified by Shimmen and colleagues as an F-actin crosslinking protein from lily pollen that belongs to the villin family. HSP70 from several organisms binds and co-sediments with F-actin. Thus, the FAAC strategy is successful at identifying known ABPs. Attempts to identify 110-kDa and the higher M_r (150–200 kDa) polypeptides have met with less success. In part, this is because these proteins are present in variable amounts from different experiments, but is more likely due to the paucity of sequence information for maize in current databases. For this work (see below for PrABP80), as well as other funded projects in the lab, we have had much greater success with MS identification of unknown proteins from dicot pollen. We will, however, continue an effort to obtain sequence information for the unknown maize pollen ABPs by ESI-MS/MS, and pursue these during the renewal period should they turn out to be novel ABPs. Otherwise, we have switched our focus to the fractionation of pollen extracts by DNase I chromatography.

During the purification of native actin from *Papaver rhoeas* (field poppy) pollen, we discovered that an abundant 80-kDa polypeptide bound to DNase I columns with high affinity. Because numerous ABPs have been identified from other systems by <u>DNase I chromatography</u>, and because the yields and reproducibility were significantly better than for FAAC, we concentrated on this approach for the

identification of novel ABPs from pollen. Extracts from maize and poppy pollen have been fractionated using the high salt extraction and binding conditions of Schafer et al. (1998). With this approach we identified at least two novel or previously uncharacterized ABPs and several others await identification by MS. Here, we describe the preliminary findings for the poppy 80-kDa ABP (PrABP80).

We have a long-standing, extremely productive collaboration with Dr. Noni Franklin-Tong (Univ. Birmingham, UK) to study the role of actin and stimulus-responsive ABPs during the SI response of poppy pollen. **PrABP80** eluted from DNase I columns with EGTA and was enriched further by chromatography on Q-Sepharose. The major polypeptide contained small amounts (10–20%) of a 42/40-kDa doublet, but otherwise was quite pure. Because the 80-kDa polypeptide from poppy could bind and co-sediment with F-actin in a calcium-dependent manner at high but not low centrifugal forces, we pursued a molecular identification by mass spectrometry. Q-TOF peptide mass fingerprinting and ESI-MS/MS sequence analysis identified the same 'hit(s)' in the databases, from several different purifications; therefore, we are quite confident that this protein is a gelsolin-like polypeptide. Indeed, sequences for 9 unique fragments shared 44–92% amino acid sequence identity with 135-ABP or AtVLN3. A similar protein isolated from maize pollen (ZmABP80) also has peptide sequences related to gelsolins, but typically had more contaminants than the poppy ABP fraction. PrABP80 and ZmABP80 both cross-react with antibodies against lily 135-ABP (villin-like) and AtVILLIN1. Villin is related to gelsolin, but includes a second, C-terminal actin-binding module of ~ 8 kDa called the villin-head piece (VHP). Intriguingly, all gelsolin-related genes in current plant databases are predicted to contain a VHP. Gelsolins are calcium-regulated actin filament severing and capping proteins, whereas villins are predominantly crosslinking proteins. At elevated calcium, some villins cap or sever filaments, but others do not. Our analysis of AtVLN1 demonstrates unambiguously that a plant villin lacks both activities. Moreover, 135-ABP and 115-ABP from lily pollen do not have severing activity. The molecular mass, the lack of sequence information for a putative VHP, and the absence of actin filament cross-linking activity all suggest that PrABP80 is a gelsolin-like molecule. We were therefore quite interested to determine the properties of PrABP80 *in vitro*.

Biochemical analysis, with a variety of kinetic assays and pyrene-labeled actin, demonstrated that PrABP80 has actin filament severing, nucleating and barbed-end capping activities. At steady-state, the 80-kDa ABP reduced the low shear viscosity of F-actin solutions, with saturation of the effect occuring at a molar ratio of 1:1000 PrABP80 to actin. This is consistent with shortening of the average filament length, which could occur by depolymerization, capping of filament ends, or severing. Saturation at low molar ratios of ABP to actin suggests that this does not result from depolymerization through monomer binding. During polymerization from monomers, substoichiometric amounts of ABP80 reduced the lag period required for actin assembly and enhanced spontaneous filament formation. This nucleation activity is consistent for either severing or capping proteins. PrABP80 also inhibited addition of profilin–actin complexes to the barbed-end of filaments in a seeded elongation assay, similar to *Arabidopsis* capping protein, and had an apparent K_d for filament ends of 7 nM. Unlike capping protein, however, PrABP80 enhanced dilution-mediated depolymerization of pre-formed filaments. The latter result is consistent only with the presence of a severing protein or a depolymerizing factor. Again, because this effect occured at substoichiometric amounts of ABP80, severing is the more likely explanation.

Fluorescence microscope analysis, with the resolution to visualize single actin filaments, provided striking confirmation of the severing and capping activities. When co-polymerized with actin, PrABP80 reduced mean filament lengths in a dose-dependent manner. Again, this is consistent with either capping or severing proteins. When pre-formed rhodamine-labeled (red) filament seeds were allowed to elongate Alexa 488 (green) actin filaments in the absence of ABP80, extension from both ends of the seed was observed. The extent of growth on opposite ends of a given seed was markedly different, reflecting the different binding constants at the two ends of actin filaments. However, when the seeds were

preincubated with PrABP80, a limited amount of polymerization occured from only one end. In addition to limiting actin polymerization, PrABP80 reduced the mean length of phalloidin-stabilized actin filaments, an effect not observed with capping proteins. Finally, direct visualization of individual filaments over time revealed that PrABP80 causes numerous breaks along the backbone of phalloidin-labeled filaments. Hence, PrABP80 is a potent actin filament severing and barbed-end capping protein. In none of these fluorescence microscope assays, were bundles or cross-linked networks of actin filaments observed, consistent with the lack of villin-like activity. Although plant ADF/cofilins have weak severing activity, this is the first direct demonstration of a calcium-regulated severing activity from plant cells, and the first convincing evidence for the existence of plant gelsolin. This work has been submitted to *Journal of Biological Chemistry*, and was provisionally accepted in Dec. 2003 pending some minor corrections. Additional characterization of the biochemical, cytological and genetic properties of PrABP80 will be pursued as part of the renewal proposal submitted to DOE in June 2003. We propose that ABP80 is a major player in the signal-mediated destruction of the actin cytoskeleton during the SI response of pollen. Specifically, ABP80 is likely to cooperate with the calcium-stimulated sequestering activity of profilin by blocking addition of abundant profilin–actin onto the ends of existing filaments (capping) and creating new pointed ends for actin disassembly (severing).

ii. Characterization of *Arabidopsis* FIMBRIN1 (AtFIM1)

To develop methods for analysis of ABPs biochemically and cytologically, we turned to the *Arabidopsis* genome database.   A family of 5 fimbrin-like genes was identified, and detailed analysis of AtFIM1 was pursued in collaboration with Dr. David McCurdy (Newcastle University, Australia). A Ph.D. student in my lab, David Kovar, discovered that AtFIM1 is a *bona fide* cross-linking protein responsible for generating higher-order structures from actin filaments. Moreover, the cross-linking activity appears to stabilize actin filaments against depolymerization by profilin. Recombinant AtFIM1 was purified to homogeneity as a GST-fusion protein, and its interactions with plant and muscle actin analyzed biochemically. In low-speed sedimentation assays F-actin does not pellet on its own, whereas in the presence of AtFIM1 actin filaments can be pelleted, consistent with the formation of bundles or cross-linked meshworks. At high centrifugal forces, a titration series of AtFIM1 with a constant amount of F-actin can be used to determine the stoichiometry and apparent affinity for binding. For AtFIM1, saturation of filaments occurs at a 1:4 stoichiometry and a biphasic binding interaction is observed. AtFIM1 has a high affinity ($K_d \sim 0.5~\mu$M) and a low affinity ($K_d = 1.9~\mu$M) binding site, which we speculate relates to the presence of two tandem actin-binding domains with different affinities for F-actin. Unlike other eukaryotic fimbrins, AtFIM1 activity is insensitive to calcium and does not show a preference for actin isoform. The ability to generate crosslinked networks was confirmed by fluorescence microscopy. This contrasts with most members of the fimbrin family, which bundle actin filaments. When the cellular concentration of fimbrin is increased by microinjection, cytoplasmic streaming and transvacuolar strand dynamics are inhibited. This is likely due to the alteration of actin filament dynamics. Biochemically, we show that fimbrin-coated actin filaments are stabilized against profilin-mediated depolymerization *in vitro*. Finally, we used AtFIM1 as a marker for filament organization and dynamics *in vitro* by generating fluorescent analogs that are introduced into plant cells by microinjection. Unlike fluorescent phalloidin, fimbrin marks a fine dynamic network of cortical actin filaments in stamen hair cells. These findings were published as two peer-reviewed publications in high-quality journals. Further characterization of the FIMBRIN gene family from *Arabidopsis* is now supported by the NSF (0130576-MCB).

iii. Biochemical Analysis of *Arabidopsis* CAPPING PROTEIN (AtCP)

Proteins which cap, sever, nucleate, or dynamize filaments have been isolated from many organisms and shown to be important regulators of actin dynamics by modulating reactions at the ends of actin filaments. In plants, there is little or no evidence for the existence of capping proteins. We identified subunits for the heterodimeric capping protein complex from *Arabidopsis* (AtCP) in the sequence databases, cloned each subunit and co-expressed them in bacteria. Antibodies generated against each subunit recognize

appropriate molecular weight polypeptides from *Arabidopsis* extracts and confirm the expression of AtCP in various tissues. The purified, recombinant protein (α/β heterodimer of 39- and 31-kDa subunits) binds to the barbed ends of actin filaments with K_d values of 12–24 nM, as assayed both kinetically and at steady state. Substoichiometric amounts of AtCP prevent addition of profilin–actin complexes to barbed ends during a seeded elongation reaction and suppress dilution-mediated actin depolymerization. AtCP does not, however, sever actin filaments and does not have a preference for the source of actin isoform. During assembly from actin monomers, AtCP eliminates the initial lag period for actin polymerization and enhances spontaneous assembly. Indeed, the efficiency of actin nucleation is quite high, with a maximum of 1 new pointed-end created for each 24 CP polypeptides. This compares favorably with the activity of mouse CapZ, which has a maximal nucleation of 1 filament per 6 CapZ polypeptides. The activity of AtCP is not affected by calcium, but is sensitive to PtdIns(4,5)P$_2$, indicating that it may transduce certain extracellular and intrinsic signals into changes in cytoskeletal architecture. Surprisingly, AtCP is also binds and is regulated by phosphatidic acid, which is gaining acceptance as a major signaling lipid in plant cell wound and stress responses. The ability of AtCP to prevent addition of new actin subunits as the barbed-end of filaments is confirmed by fluoresence microscopy and further reveals that capping protein prevents end-to-end annealing of filaments. We propose that AtCP is a major regulator of actin dynamics in plant cells that, together with abundant profilin, is responsible for maintaining a large pool of actin subunits and a surprisingly small population of F-actin. This work was published in the *Journal of Biological Chemistry*, and further analysis of AtCP is a central theme of the renewal proposal submitted to DOE in June 2003.

Publications Resulting from DOE Funding (DE-FG02-99ER20337A01)

   a. Peer-reviewed publications
   1. Kovar, D.R., C.J. Staiger, E.A. Weaver, and D.W. McCurdy. 2000. AtFim1 is an actin filament cross-linking protein from *Arabidopsis thaliana*. *Plant J*. 24: 625-636
   2. Kovar, D.R., B.C. Gibbon, D.W. McCurdy, and C.J. Staiger. 2001. Fluorescently-labeled fimbrin decorates a dynamic actin filament network in live plant cells. *Planta* 213: 390-395
   3. Huang, S., L. Blanchoin, D.R. Kovar, and C.J. Staiger. 2003. *Arabidopsis* capping protein (AtCP) is a heterodimer that regulates assembly at the barbed ends of actin filaments. *J. Biol. Chem*., 278: 44832-44842
   4. Huang, S., L. Blanchoin, F. Chaudhry, V.E. Franklin-Tong, and C.J. Staiger. A gelsolin-like protein from *Papaver rhoeas* pollen (PrABP80) stimulates calcium-regulated severing and depolymerization of actin filaments. *J. Biol. Chem., provisionally accepted 12/03*

   b. Invited book chapters, review articles and books
   5. Kovar, D.R. and C.J. Staiger. 2000. Actin depolymerizing factor. *In* Actin: A Dynamic Framework for Multiple Plant Cell Functions. C.J. Staiger, F. Baluska, D. Volkmann, and P. Barlow (eds) Kluwer: Dordrecht, pp. 67-85
   6. McCurdy, D.W. and C.J. Staiger. 2000. Fimbrin. *In* Actin: A Dynamic Framework for Multiple Plant Cell Functions. C.J. Staiger, F. Baluska, D. Volkmann, and P. Barlow (eds) Kluwer; Dordrecht, pp. 87-102
   7. Staiger, C.J., F. Baluska, D. Volkmann, and P. Barlow (editors). 2000. Actin: A dynamic framework for multiple plant cell functions. Kluwer: Dordrecht, The Netherlands, 659 pp.
   8. Staiger, C.J. 2000. Signaling to the actin cytoskeleton in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 51: 257-288
   9. McCurdy, D.W., D.R., Kovar, and C.J. Staiger. 2001. Actin and actin-binding proteins in higher plants. *Protoplasma* 215: 89-104
   10. Staiger, C.J., and P.J. Hussey. 2003. Actin and actin-modulating proteins. *In* P.J. Hussey (ed.) The Plant Cytoskeleton in Cell Differentiation and Development. Blackwell: Sheffield UK, *in press*.
   11. McGough, A., C. J. Staiger, S. Huang, J.-K. Min, L. Nollah, and K. Simonetti. 2003. The gelsolin superfamily of actin regulatory proteins: Modular structures, versatile functions. *FEBS Lett*. 552: 75-81.
   12. Drøbak, B.K., Franklin-Tong, V.E., and C.J. Staiger. 2004. Tansley Review: The role of the actin cytoskeleton in plant cell signaling. *New Phytol., in pres*