BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 61–65 (1998)
ARTICLE NO. RC989088

Purification of an Actin-binding Protein Composed of 115-kDa Polypeptide from Pollen Tubes of Lily

Tsuyoshi Nakayasu, Etsuo Yokota,[1] and Teruo Shimmen
Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Park City, Hyogo 678-12, Japan

Received June 29, 1998

From lily pollen tubes, an actin-binding protein composed of 115-kDa polypeptide was purified sequentially by co-precipitation method with F-actin, hydroxylapatite column, gel filtration column and DE-52 ion exchange column chromatography. This component displayed a tendency to aggregate in solutions of low ionic strength, indicating a hydrophilic characteristic. Under physiological ionic conditions, this component bound to F-actin in an actin-concentration-dependent was saturable manner. Binding of this component to F-actin was independent of ATP and Ca^{2+}-concentrations. Fluorescent microscopy revealed that F-actin labeled with rhodamine-phalloidin showed bundling in the presence of this component. Judging from the lack of antibody cross-reactivity, this component does not seem to be related to α-actinin of skeletal muscle and plant 135-kDa actin-bundling protein. Therefore, this component is the F-actin binding protein, which has not been identified thus far in plant cells.

Key Words: **actin; actin-binding protein; lily; pollen tubes.**

The actin-binding proteins, including monomer-sequestering, end-capping, cross-linking, severing and side-binding proteins, regulate the polymerizing states or architectures of actin filaments (1-3). They are also believed to mediate the organization of the actin cytoskeleton in plant cells where remarkable or dramatic changes occur during the cell cycle or developmental process (4, 5). The biochemical or functional properties of profilin or actin-depolymerizing factor (ADF), low molecular weight actin-binding and depolymerizing protein, have been established to some extent (6). However, few biochemical and molecular biochemical studies have been performed on other classes of actin-binding proteins in plant cells.

In the germinating pollen, strands and bundles of actin filaments parallel to the long axis of pollen tube are present and responsible for cytoplasmic streaming and transport of vegetative nuclei and generative cells (7, 8). Recently, we purified a 170-kDa component and a 135-kDa component (P-135-ABP) from lily pollen tubes and identified those as a myosin heavy chain and an actin-bundling protein, respectively (9, 10). We further reproted that 115-kDa polypeptides from a crude extract of lily pollens bound specifically to exogeneously added F-actin, beside of 170-kDa myosin heavy chain and P-135-ABP (9; in this report, we referred to the 115-kDa polypeptide as the 110-kDa polypeptide). In the present study, we purified the 115-kDa component from a crude extract of lily pollen and showed that it is F-actin binding protein, which has not been identified in plant cells.

MATERIALS AND METHODS

Purification of a 115-kDa component from lily pollen tubes. Pollen of lily (*Lilium longiflorum*) was allowed to germinate in a culture medium (7% sucrose, 1.27 mM $Ca(NO_3)_2$, 0.162 mM boric acid, 0.99 mM KNO_3, and 3 mM KH_2PO_4) at 28°C for 1.5 to 2 hr. Unless otherwise noted, each procedure was performed at 0 to 4°C. In each column step described below, the fractions including the 115-kDa component were determined by SDS-PAGE.

Preparation of a crude extract from the germinating pollen and subsequent co-precipitation with F-actin prepared from chicken breast muscle were carried out according to Yokota and Shimmen (9). The extract of the co-precipitant with F-actin in a solution (0.2 M KCl, 10 mM EGTA, 6 mM $MgCl_2$, 5 mM ATP, 5 mM potassium phosphate buffer (pH 7.0), 100 μg/ml leupeptin, 1 mM PMSF, 2 mM DTT and 30 mM PIPES-KOH (pH7.0)) was chromatographed on a hydroxylapatite column pre-equilibrated with a solution containing 5 mM potassium phosphate buffer (pH 7.0), 0.2 M KCl, 10 mM EGTA, 6 mM $MgCl_2$, 50 μg/ml leupeptin, 0.5 mM PMSF, 1 mM DTT and 30 mM PIPES-KOH (pH 7.0). The adsorbed materials were eluted with a linear concentration gradient of potassium phosphate buffer

[1] Corresponding author. Fax: 81-7915-8-0175. E-mail: yokota@sci.himeji-tech.ac.jp.

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; P-135-ABP, plant 135-kDa actin-bundling protein; PIPES, piperazine-N,N′- bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-291X/98 $25.00

(pH 7.0) from 5 to 400 mM. Fractions containing the 115-kDa component were pooled and then concentrated by ultrafiltration using Amicon PM 10 membrane (Amicon Co., Lexington, MA). This concentrated sample was chromatographed on Sephacryl S-300 gel filtration column (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) pre-equilibrated with a solution of 5 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 50 μg/ml leupeptin, 0.5 mM PMSF, 1 mM DTT and 30 mM PIPES-KOH (pH 7.0). Fractions containing 115-kDa component were pooled and then directly applied to DE-52 ion exchange column (Whatman Biosystems Ltd., Kent, UK) pre-equilibrated with the same solution used for the gel filtration. The adsorbed materials were eluted with a linear concentration gradient of 5 to 100 mM KCl in pre-equilibrated solution. After fractionation, KCl concentration of each fraction was adjusted to 0.1 M by adding 3 M KCl solution.

Cosedimentation analysis of the 115-kDa component with F-actin. The 115-kDa component fraction (final concentration of 1.7 μg/ml) was mixed with F-actin in 100 μl of an assay solution containing 0.1 M KCl, 1 mM EGTA, 2 mM $MgCl_2$, 50 μg/ml leupeptin, 0.5 mM PMSF, 1 mM DTT and 30 mM PIPES-KOH (pH 7.0), and kept standing for 20 min at 20°C. As a control, the 115-kDa component fraction or F-actin alone was treated in the same manner. To examine the effect of Ca^{2+} or ATP on the co-sedimentation of 115-kDa component with F-actin, $CaCl_2$ (final concentration of 1.5 mM) or ATP (final concentration of 4 mM) was added to the assay solution, respectively. The samples were centrifuged at 200,000×g for 20 min at 20°C. Pellets were suspended in 100 μl of the assay solution. Both the supernatants and pellets were electrophoresed on 7.5% polyacrylamide gel. After staining of the gels, the amounts of actin and 115-kDa polypeptide in the supernatants and pellets were measured by densitometry (Densito-Pattern Analyzer EPA-3000; Cosmo Bio. Co., Ltd., Tokyo) as described in the preceding paper (10).

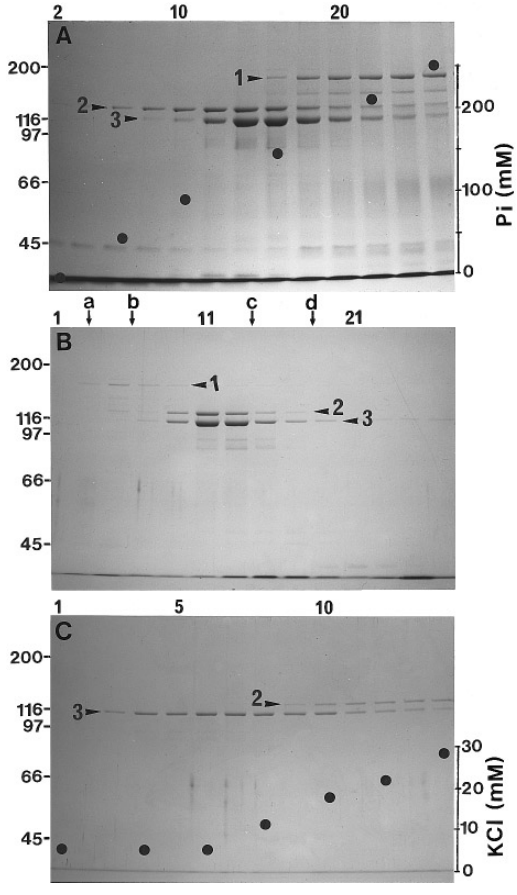

FIG. 1. Hydroxylapatite (A), Sephacryl S-300 gel filtration (B) and DE-52 ion exchange (C) column chromatographies. Closed circles in A and C indicate the concentration of potassium phosphate and KCl, respectively. Arrows in B indicate the void volume (a), elution position of 670-kDa thyroglobulin (b), 158-kDa bovine γ-globulin (c) and 44-kDa ovalbumin (d). Arrowheads indicate the 170-kDa mysoin heavy chain (1), 135-kDa polypeptide of P-135-ABP (2) and 115-kDa polypeptide (3). The molecular weight ($\times 10^{-3}$) of standard proteins are indicated on the left. Fraction number is shown on the top of figures.

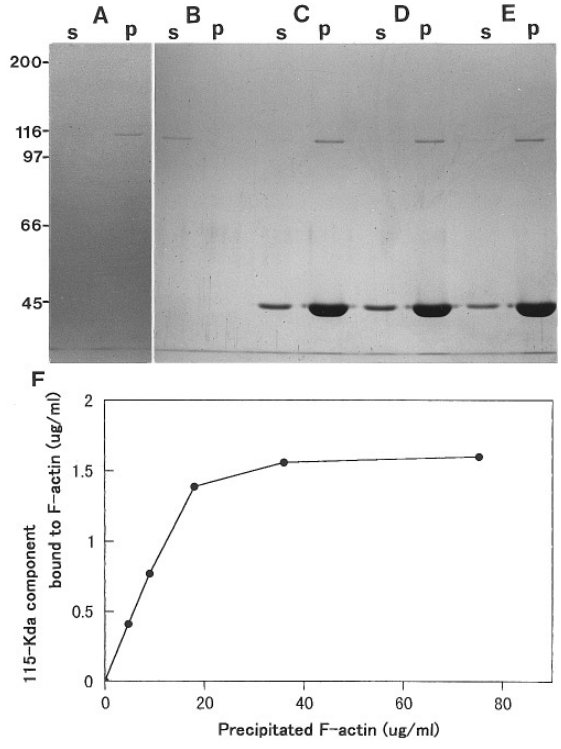

FIG. 2. Co-sedimentation of 115-kDa component with F-actin. The 115-kDa component alone was centrifuged without (A) or with the addition of 0.1 M KCl (B). The mixture of 115-kDa component (final concentration at 1.7 μg/ml) and F-actin (final concentration at 80 μg/ml) was centrifuged in -ATP, -Ca^{2+} (C), +Ca^{2+} (D), or +ATP (E). F, plots of the amount of 115-kDa component bound to F-actin versus precipitated F-actin. s, supernatant; p, pellet. Samples of C-D contained 0.1 M KCl.

Interaction of rhodamine-phalloidin labeled F-actin with the 115-kDa component. F-actin was labeled with rhodamine-phalloidin (Molecular Probes, Inc., OR) according to Kohno et al. (11). It was then added to the 115-kDa component fraction in the assay medium used for the co-sedimentation procedure. The concentrations of 115-kDa component and rhodamine-phelloidin labeled F-actin were adjusted to 1.6 μg/ml and 3 μg/ml, respectively. After incubation for 20 min at 20°C, the sample was observed under a microscope (BH2; Olympus Co., Tokyo) equipped with epifluorescence optics (BH2-RFC; Olympus Co.). Images were recorded on video tapes with a video tape recorder (National NV-FS65; National Co., Ltd, Tokyo) through a high sensitivity television camera (C2400-08 SIT camera, Hamamatsu Photonics K., K., Tokyo), and were processed with image ΣIII (Nippon Avionics, Co., Ltd., Tokyo). AS a control, rhodamine-phalloidin labeled F-actin alone was treated in the same manner. To examine the effect of Ca^{2+} or ATP, Ca^{2+} (final concentration of 1.5

mM) or ATP (final concentration of 4 mM) was added to the assay solution.

Other methods. α-Actinin was purified partially from chicken gizzard according to the method of Feramisco and Burridge (12), and F-actin from chicken breast muscle according to the method of Kohama (13). SDS-PAGE was performed according to the method of Laemmli (14). Unless otherwise mentioned, 7.5% polyacrylamide gel was used as the separating gel. The gels were stained with Coomassie brilliant blue. Immunoblotting was performed as described previously (15). Anti-serum against P-135-ABP (10) or against α-actinin of chicken gizzard (Sigma Chem., Co., St. Louis, MO) was diluted 2,000- or 1,000-fold, respectively. Protein concentration was determined by the method of Bradford (16).

RESULTS AND DISCUSSION

Purification of the 115-kDa Component from Lily Pollen Tubes

The 115-kDa component in the crude extract was coprecipitated with exogeneously added F-actin (data not shown), as reported previously (9, 10). This precipitant was extracted with the 0.2 M KCl solution described in Materials and Methods. The extract was applied to the hydroxylapatite column, and the adsorbed materials were eluted with a linear concentration gradient of potassium phosphate. As reported previously (9), the 115-kDa component was eluted at about 0.1 M potassium phosphate (Fig. 1A). Fractions 15 to 19 in Fig. 1A were concentrated and then applied to Sephacryl S-300 gel filtration column. Both the 115-kDa component and P-135-ABP were eluted at similar positions around 260 kDa (Fig. 1B). The 170-kDa myosin was eluted in the fraction near the void volume. Fractions 10 to 13 in Fig. 1B were pooled and directly applied to DE-52 ion exchange column. The 115-kDa component was present in the flow-through and 5 mM to 20 mM KCl-eluted fractions, while P-135-ABP was eluted by KCl at concentration higher than 20 mM. After addition of KCl at a final concentration of 0.1 M, fractions

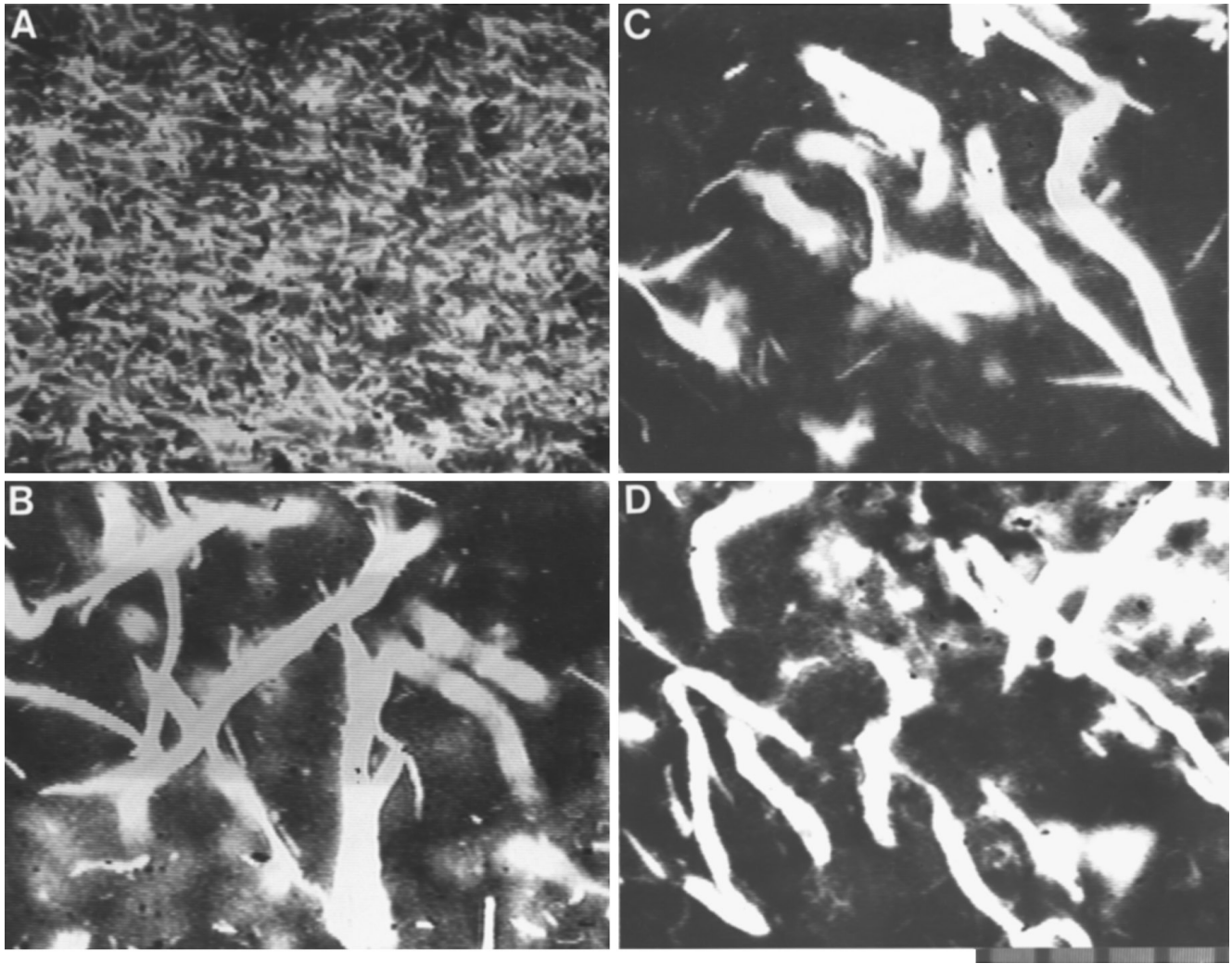

FIG. 3. Fluorescence micrographs of mixtures of 115-kDa component and rhodamine-phalloidin labeled F-actin in -ATP, -Ca^{2+} (B), +Ca^{2+} (C) or +ATP (D). A, rhodamine-phalloidin labeled F-actin alone. The scale represents 10 μm.

4 to 7 in Fig. 1C were pooled and used as the purified 115-kDa component.

When the 115-kDa component fraction was left without addition of 0.1 M KCl, the component sedimented on centrifugation at 150,000×g for 10 min (Fig. 2A), indicating that it forms an aggregate under the low ionic strength condition. This suggests that the component is hydrophilic.

Binding Properties of 115-kDa Component to F-actin

Under the centrifugation conditions described in Materials and Methods, the 115-kDa component remained in the supernatant in the absence of F-actin (Fig. 2B), but sedimented in its presence (Fig. 2C). While the binding of the 115-kDa component to F-actin was not affected by Ca^{2+} (Fig. 2D) and ATP (Fig. 2E), it was dependent on actin concentration (Fig. 2F). The amount of 115-kDa component bond to F-actin became saturated at concentrations higher than 20 μg/ml.

To visualize the interaction of the 115-kDa component with F-actin, rhodamine-phalloidin labeled F-actin was added to the 115-kDa component, and the mixture was observed by fluorescence microscopy. In the absence of the 115-kDa component, rhodamine-phalloidin labeled F-actin because dispersed in a disorder manner on the glass surface (Fig. 3A) and displayed Brownian motion. However, in the presence of the 115-kDa component, numerous bundles of rhodamine-phalloidin labeled F-actin were observed (Fig. 3B). These bundles with the 115-kDa component were also seen in the presence of Ca^{2+} (Fig. 3C) and ATP (Fig. 3D).

Antigenicity of the 115-kDa Component

The 115-kDa polypeptide was not recognized by the antiserum against P-135-ABP (Fig. 4B). Our preceding paper (10) reported that this antiserum crossreacted specifically with only the 135-kDa polypeptide in a crude protein sample of lily pollen. These results indicate that the 115-kDa component is not proteolytic fragments of P-135-ABP formed during purification steps. The molecular mass of the 115-kDa polypeptide on SDS-PAGE is close to that of α-actinin, an F-actin cross-linking protein, which is composed of subunits of 90 to 110 kDa (1,2). The component recognized by monoclonal antibodies against chicken α-actinin have been found around nuclei in *Acetabularia* cells (17). However, molecular mass of this component has not been shown by immunoblotting (17). The polyclonal antiserum against chicken α-actinin recognized neither the 115-kDa polypeptide nor the 135-kDa polypeptide of P-135-ABP (Fig. 4C). Therefore, we concluded that the 115-kDa component is distinct from α-actinin-like component identified immunocytochemically in *Acetabularia* cells. In immunoblotting assay, no polypeptide crossreacted specifically with the polyclonal antiserum against chicken α-actinin in a crude protein sample

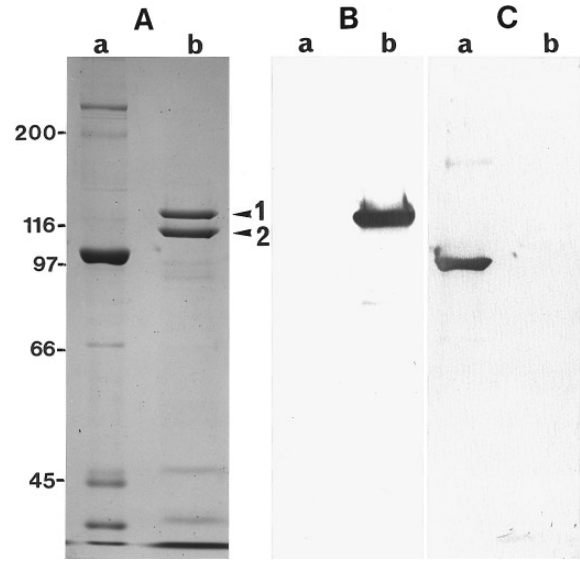

FIG. 4. Immunoblots of partially purified chicken α-actinin (a) and hydroxylapatite column fraction containing both P-135-ABP and 115-kDa component (b). A, Coomassie brilliant blue staining of the gel. B and C, immunoblot with antiserum against 135-kDa polypeptide of P-135-ABP and with antiserum against chicken α-actinin, respectively. Arrowheads indicate the 135-kDa polypeptide (1) and 115-kDa polypeptide (2).

from lily pollen tubes (data not shown). Hence, it is doubtful whether the component processing similar antigenicity to animal α-actinin really exists in plant cells. The other F-actin-binding proteins that have been identified in plants cells so far are spectrin-like polypeptides (14, 15) and elongation factor-1α (16, 17). However, judging from molecular mass data, the 115-kDa component is obviously different from these F-actin-binding proteins.

Grolig et al. (22) suggested that 110-kDa polypeptide of *Chara* is a myosin, based on the crossreactivity with monoclonal antibodies against myosin II heavy chain and binding to F-actin in ATP-dependent manner. Following results exclude a possibility that 115-kDa polypeptide is a higher plant analogue to the 110-kDa-myosin like polypeptide reported in *Chara* cells. The binding of isolated 115-kDa component to F-actin was not affected by ATP (Fig. 2 and 3). In the present study, the co-precipitant of a crude extract with F-actin was treated with a solution containing ATP to release the 115-kDa component from F-actin. However, we have shown that the 115-kDa component co-precipitated with F-actin in the crude extract was also dissociated from F-actin by the treatment with a high ionic strength solution lacking ATP. On the other hand, the 170-kDa myosin remains to be associated with F-actin under the same condition (10). These results indicate that the 115-kDa component is a novel F-actin-binding protein in plant cells.

The 115-kDa component appears to bundle F-actin

in the presence of 0.1 M KCl (Fig. 3), a physiological concentration of protoplasm in plant cells (23, 24). It is therefore considered to be a bundling factor like P-135-ABP. On the other hand, the 115-kDa component shows a tendency to aggregate, especially in low ionic strength solution (Fig. 2). Hence, the possibility can not be ruled out that the F-actin bundling was due to some small artificial aggregate of the 115-kDa component, which did not sediment under centrifugation even in the presence of 0.1 M KCl. Further biochemical and ultrastructural studies are needed to establish the identify of this 115-kDa component from lily pollen tubes.

ACKNOWLEDGMENT

We thank the National Live Stock Breeding Center Hyogo Station for the gift of chicken breast muscle.

REFERENCES

1. Stossel, T. P., Chaponnier, C., Ezzell, R. M., Hartwig, J. H., Janmey, P. A., Kwiatkowski, D. J., Lind, S. E., Smith, D. B., Southwick, F. S., Yin, H. L., and Zaner, K. S. (1985) *Ann. Rev. Cell Biol.* **1,** 353–402.
2. Pollard, T. D., and Cooper, J. A. (1986) *Ann. Rev. Biochem.* **55,** 987–1035.
3. Vanderkerckhove, J., and Vancompernolle, K. (1992) *Curr. Opin. Cell Biol.* **4,** 36–42.
4. Staiger, C. J., and Lloyd, C. W. (1991) *Curr. Opin. Cell Biol.* **3,** 33–42.
5. Shibaoka, H., and Nagai, R. (1994) *Curr. Opin. Cell Biol.* **6,** 10–15.
6. Staiger, C. J., Gibbon, B. C., Kovar, D. R., and Zonia, L. E. (1997) *Trends Plant Sci.* **7,** 275–281.
7. Pierson, E. S., and Cresti, M. (1992) *Int. Rew. Cytol.* **140,** 73–125.
8. Shimmen, T., and Yokota, E. (1994) *Int. Rew. Cytol.* **155,** 97–139.
9. Yokota, E., and Shimmen, T. (1994) *Protoplasma* **177,** 153–162.
10. Yokota, E., Takahara, K-I., and Shimmen, T. (1998) *Plant Physiol.* **116,** 1421–1429.
11. Kohno, T., Okagaki, T., Kohama, K., and Shimmen, T. (1991) *Protoplasma* **161,** 75–77.
12. Feramisco, J. R. and Burridge, K. (1980). *J. Biol. Chem.* **255,** 1194–1199.
13. Kohama, K. (1981) *J. Biochem.* **90,** 497–501.
14. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
15. Yokota, E., McDonald, A. R., Liu, B., Shimmen, T., and Palevitz, B. A. (1995) *Protoplasma* **185,** 178–187.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–284.
17. Tischendorf, G., Sawitzky, D., and Werz, G. (1987) *Cell Motil. Cytoskel.* **7,** 78–86.
18. Michaud, D., Guillet, G., Rogers, P. A., and Charest, P. M. (1991) *FEBS Lett.* **294,** 77–80.
19. Faraday, C. D., and Spanswick, R. M. (1993) *FEBS Lett.* **318,** 313–316.
20. Yang, W., Burkhart, W., Cavallius, J., Merrick, W. C., and Boss, W. F. (1993) *J. Biol. Chem.* **268,** 392–398.
21. Collings, D. A., Wasteneys, G. O., Miyazaki, M., and Williamson, R. E. (1994) *Cell Biol. Int.* **18,** 1019–1024.
22. Grolig, F., Schroder, J., Sawitzky, H., and Lange, U. (1996) *Cell Biol. Int.* **20,** 365–373.
23. Tazawa, M., Kishimoto, U., and Kikuyama, M. (1974) *Plant Cell Physiol.* **15,** 103–110.
24. Shimmen, T., and Tazawa, M. (1983) *Plant Cell Physiol.* **24,** 1511–1524.