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Immuno-Reactant to RhoA Antibody Co-Localizes with Cortical Actin Filaments in Guard Cells of Open Stomata in *Commelina communis* L.

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Stomatal regulation is essential for the growth of land plants. Pairs of guard cells that delineate the stomata perceive stimuli and respond to acquire the optimum aperture. The actin cytoskeleton participates in signaling pathways of the guard cell (Kim et al., 1995; Eun and Lee, 1997; Hwang et al., 1997). To identify the upstream molecules that regulate actin dynamics in plant cells, we immunoblotted proteins extracted from leaves of *Commelina communis* L. with the RhoA antibody, and identified one band of 26KD from the epidermis. Using immunofluorescence microscopy, we examined the subcellular distribution of the immuno-reactant(s) in guard cells. When stomata were open under light, the organization of the immuno-reactant(s) resembled the radial arrangement of cortical actin filaments of guard cells. Double-labeling of the guard cells, using the RhoA and actin antibodies as primary antibodies, showed that the immuno-reactant(s) of the RhoA antibody and actin filaments co-localized in the cortex of illuminated guard cells. However, the pattern was not found in guard cells when stomata were closed under darkness or by ABA, conditions under which cortical actin proteins are disassembled in guard cells. From these observations, we can suggest the possible presence of a RhoA-like protein and its involvement in the organization of the actin cytoskeleton in guard cells.

Keywords: ABA, Abscisic acid, Actin cytoskeleton, Guard cell, RhoA

Regulation of stomatal aperture is fundamental for the growth and development of land plants; Insufficiently opened stomata limit carbon fixation, while those that are excessively opened cause withering. The optimal aperture depends on precise signaling pathways in the guard cells. Actin filaments in those cells participate in the signal transduction processes, with physiological stimuli inducing dynamic changes in actin organization (Eun and Lee, 1997) and perturbation of the process interfering with normal stomatal responses (Kim et al., 1995; Hwang et al., 1997). Although the regulators of actin dynamics have been studied in plant cells (Valster et al., 1997; Smertenko et al., 1998), the switch molecules for actin assembly in guard cells are not well understood.

RHO is a class of small GTP-binding proteins whose activities are switched on by binding with GTP and turned off by hydrolysis of GTP to GDP. Each of the three distinctive subclasses of RHO -- Rho, Rac, and Cdc42 -- profoundly affects the organization and remodeling of actin filaments in animal cells (Machesky and Hall, 1996; Tapon and Hall, 1997; Aspenström, 1999). In plants, numerous genes have been identified as Rac homologues (Borg et al., 1997; Winge et al., 1997; Li et al., 1998; Valster et al., 2000), but no gene has yet been discovered that can be categorized into a Rho subclass. In the present study, we used a commercially available antibody to human RhoA to test whether guard cells contain Rho-like proteins, and to examine their distribution with respect to the changing organization of actin filaments in those cells. One of our objectives was to determine whether the immuno-reactant(s) to the RhoA antibody were closely associated with cortical actin filaments (as in animal and yeast cells), thereby suggesting a role in actin polymerization in guard cells.

MATERIALS AND METHODS

Plant Material

Plants of *Commelina communis* L. were grown from seeds in a greenhouse at 22 to 28°C. The light period was 13 to 16 h, with the highest photon flux density of 1000 μmol m^{-2} s^{-1} at noon. We used fully expanded leaves from four- to five-week-old plants.

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Abbreviations: FITC, fluorescein; TRITC, tetramethyl rhodamine isothiocyanate.

Protein Extraction

Whole leaves and the epidermis peeled from the undersides of leaves were frozen separately in liquid nitrogen, then ground in a mortar. The resulting powders were dissolved in 20 mM Tris buffer (pH 7.7) that contained 2 mM DTT, 1 mM EDTA, and 1 mM phenylmethylsulfonic acid, 2 μM pepstatin, 0.8 μM aprotinin, 10 μM chymostatin, and 20 μM leupetin. Homogenates were centrifuged at 20,000*g* for 20 min. Protein concentration of each supernatant was determined through a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Protease inhibitors were purchased from Boeringer Mannheim (Mannheim, Germany) and all chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Western Blot

Each extracted supernatant was separated on a 15% gel by SDS-PAGE and transferred to a nitrocellulose membrane according to the manufacturer's instructions (Novex, San Diego, CA, USA). The membrane was blocked for 1 h in 3.5% BSA that was dissolved in Tris-buffered saline containing 0.05% (v/v) Tween 20. It was then incubated for 3 h with a monoclonal antibody produced against a peptide corresponding to amino acid 120 - 150 of human RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Another incubation was then conducted with the alkaline phosphatase-conjugated anti mouse IgG (Promega, Madison, WI, USA) for 2 h. Primary and the secondary antibodies were diluted with the blocking solution to 1:100 and 1:1000, respectively.

Stomatal Opening and Closing

To induce stomatal opening, leaves were floated on water with their abaxial surfaces facing cool-white fluorescent lamps, and were illuminated for 2 to 3 h in the morning at a photon flux density of 130 to150 μmol m^{-2} s^{-1}. For stomatal closure, plants were either kept in the dark or else their abaxial epidermises were peeled off and treated for 1 h with a medium containing 10 μM ABA and 30 mM KCl/10 mM K^+-Mes buffer (pH 6.1). Stomatal opening and closing were confirmed under a microscope before fixation.

Immunofluorescence Microscopy

Immunolocalization in the guard cells followed procedures described by Eun and Lee (1997), with the following modifications. ABA-treated epidermal pieces and those peeled from the abaxial side of light- or dark-treated leaves were fixed for 1 h in 50 mM phosphate buffer (pH 6.8) that contained 200 μM *m*-maleimidobenzoyl N-hydroxysuccinimide ester, 5 mM EGTA, 0.05% (v/v) Triton X-100, 0.3 mM phenylmethylsulfonic acid, 1.5 μM aprotinin, 2 μM pepstatin, and 20 μM leupetin. The fixed samples were frozen in liquid nitrogen and fractured with a prechilled metal block, then extracted in 1% (v/v) Triton X-100 for 2 h. This extraction process removed cytosol and improved contrast of the detergent-resistant cytoskeletal elements. Epidermal pieces were labeled in one of three ways. For localization of actin, the pieces were incubated with the IgM-type actin antibody (Amersham, Buckinghamshire, UK), followed by incubation with the fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgM. To localize the immuno-reactant to the RhoA antibody, they were incubated with the IgG type RhoA antibody, followed by incubation with FITC-conjugated goat anti mouse IgG. For double-labeling of actin and the immuno-reactant to the RhoA antibody in the same cells, epidermal pieces were incubated with a mixture of the two primary antibodies, then with a mixture of FITC-conjugated goat anti-mouse IgM and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti mouse IgG. The actin antibody, RhoA antibody, FITC-conjugated goat anti mouse IgM, and TRITC-conjugated goat anti mouse IgG were diluted to 1:40, 1:100, 1:100, and 1:60 (v/v), respectively, using a phosphate-buffered saline that contained 0.05% (v/v) Triton X-100. Following each incubation, the epidermis was washed three times with a phosphate-buffered saline containing 0.05% (v/v) Triton X-100. Labeled guard cells were observed under a fluorescence microscope (Optiphot-2, Nikon) that was equipped with filter blocks of a narrow band pass (for FITC: excitation 465 to 495 nm and barrier 515 to 555 nm; for TRITC: excitation 540/25 nm and barrier 605/55 nm). Images were recorded on T-Max 400 film (Kodak, NY, USA) using a photographic attachment (Microflex UFX-DX, Nikon).

RESULTS

Western Blot of Leaf Proteins with RhoA Antibody

To test the presence and the size of the protein that reacts with the RhoA antibody in plants, proteins

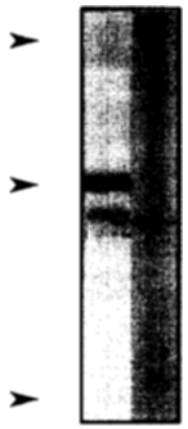

Figure 1. Western blot of *C. communis* leaf extracts using RhoA antibody. Proteins extracted from whole leaves and the leaf epidermis were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Two bands of 28 and 26 KD from whole leaves (left lane) and a single band of 26 KD from the leaf epidermis (right lane) were labeled. Arrowheads indicate the positions of molecular weight standards of 44, 28, and 18 KD from the top.

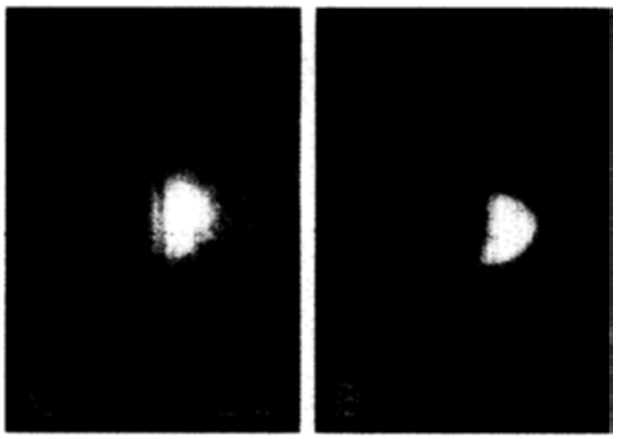

Figure 2. Localization of the immuno-reactant to RhoA antibody in the guard cell of a stoma open under light. Epidermal pieces obtained from illuminated leaves were labeled with RhoA antibody. The stoma appears closed due to the loss of guard cell turgor during chemical fixation. For the pair of guard cells, only the one on the right was labeled. The immuno-reactant was localized as radial arrays at the cortex of the cell. Images are focused at the cortex (A) and at the plane of the nucleus (B). Bar = 10 μm.

extracted from *C. communis* were incubated with that antibody. From whole leaves and from the epidermis, two bands (28 and 26 KD) and a single band of 26 KD reacted with the RhoA antibody, respectively (Fig. 1). The molecular weights of the immuno-reactants did not vary much from that of human Rho A, which is 21 KD.

Localization of the Immuno-Reactant of RhoA Antibody in Guard Cells

Eun and Lee (1997) have documented the stimulus-dependent reorganizations of actin in the guard cell system. A regulator for the actin dynamics is expected to have a close spatial relationship with actin in the cell. Thus we investigated the localization of the 26-KD immuno-reactant to RhoA antibody in guard cells. When illuminated leaves with open stomata were incubated with the RhoA antibody, the immuno-reactant was localized at the cortex of guard cells in a radially arranged filamentous form (Fig. 2A). Fluorescence was also associated with unidentified aggregates in the cortex (Fig. 2A) and throughout the subcortical region of the cell (Fig. 2B). In addition, the entire nucleus was brightly labeled. Unlike mammalian cells, whose cytosol also retains diffuse fluorescence as an indicator of the cytosolic location of Rho, such labeling was not clear in guard

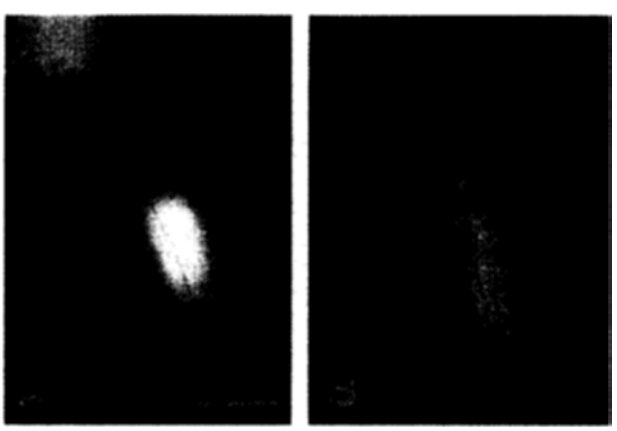

Figure 3. Double-labeling of the guard cell of a stoma open under light. The epidermal fragments of illuminated leaves were double-labeled with RhoA antibody (A) and actin antibody (B). The stoma appears closed due to the loss of guard cell turgor during chemical fixation. A guard cell positioned at the edge of an epidermal piece was focused at the cortex. Radial arrays of immuno-reactant to RhoA antibody appear to co-localize with cortical actin filaments. Bar = 10 μm.

cells. This may have been due to our particular labeling methods; i.e., after fixation, we used an extraction step to remove the cytosol to contrast weakly labeled structures. Controls incubated with a normal mouse serum contained no distinct fluorescence (data not shown).

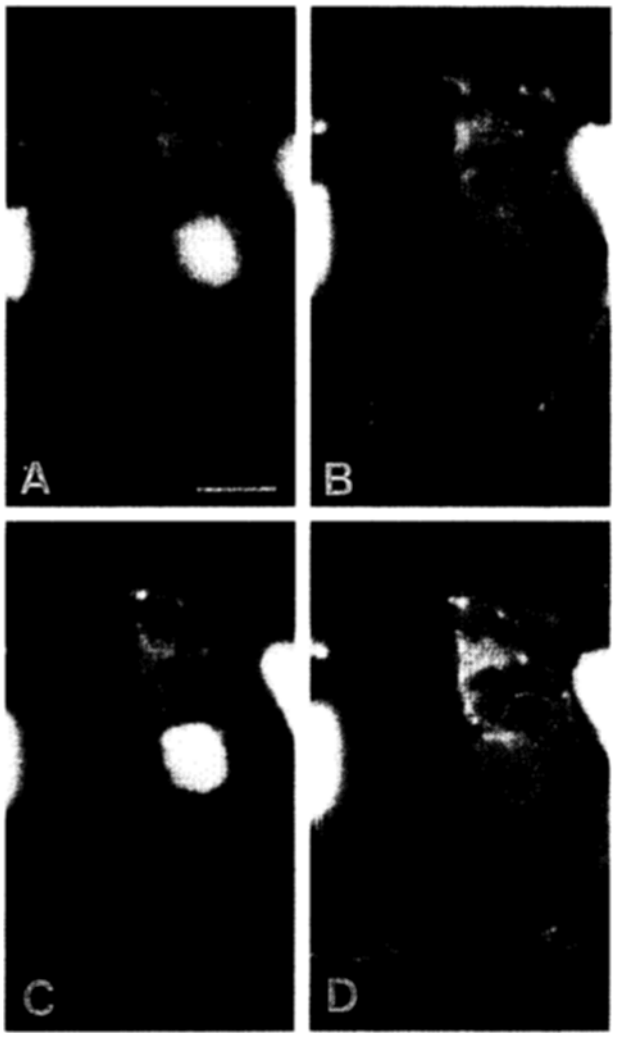

Figure 4. Double-labeling of a guard cell from a dark-closed stoma. Epidermal pieces peeled from dark-kept leaves were labeled with RhoA antibody (A, C) and actin antibody (B, D). The labeled guard cell on the right is focused at the cortex (A, B) and at the plane of the nucleus (C, D). The immuno-reactant to RhoA antibody, being scattered throughout the cell, lacks filamentous orientation (A, C). Radial actin filaments were mostly depolymerized at the cortex (B), and randomly oriented actin filaments were sparsely arranged at the cortex and around the nucleus (D). Bar = 10 μm.

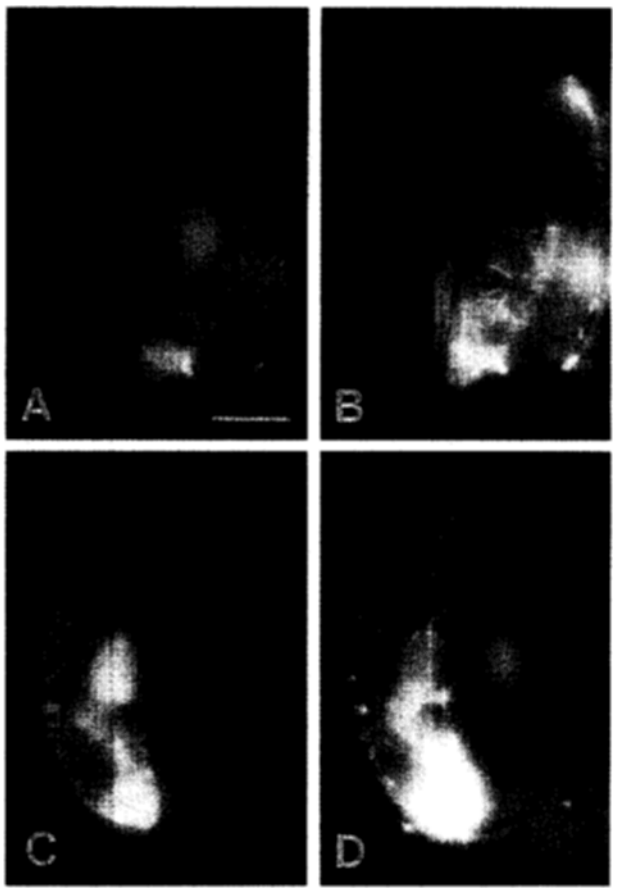

Figure 5. Double-labeling of guard cells of ABA-closed stomata. The epidermis of an ABA-treated leaf was labeled with RhoA antibody (A, C) or actin antibody (B, D). The guard cell on the right is focused at the cell cortex (A, B). A guard cell on the left of another stomatal complex is focused at the plane of the nucleus (C, D). The immuno-reactant to RhoA antibody lacks the filamentous orientation (A, C), as was found in dark-kept leaves. Actin was localized as short fragments in the cortex (B) and as random filaments in the subcortical region (D). Bar = 10 μm.

Double-Labeling of Guard Cells with RhoA and Actin Antibodies

Radial arrangement of the RhoA immuno-reactant at the cortex of the guard cells resembles that of actin filaments in illuminated guard cells of open stomata. Therefore, we examined the organization of both actin and the immuno-reactant of the RhoA antibody in the same cells. When guard cells of illuminated leaves were double-labeled, the RhoA immuno-reactant was found to co-localize with cortical actin arrays in the guard cells (compare Fig. 3, A and B).

This filamentous arrangement in the guard cells of light-opened stomata was not observed in guard cells of dark-closed stomata (Fig. 4, A and C) or in those from ABA-exposed epidermal pieces. (Fig. 5, A and C). Under these conditions, the majority of the radial actin filaments were depolymerized, and were replaced by a few random filaments in the cortical (Figs. 4B and 5B) and subcortical regions (Figs. 4D and 5D). The fact that distribution of the RhoA immuno-reactant did not match the random actin

arrays in these cells eliminates the possibility that fluorescence obtained with the RhoA antibody was from non-specific cross activities of antibodies used for the double-labeling.

DISCUSSION

RHO GTPases are involved in a number of cellular processes. One of their prominent roles is associated with cell shaping and motility via control of the actin cytoskeleton. In mammals, Rho, Rac, and Cdc42 -- all three subfamilies of the RHO GTPases -- regulate distinct arrays of actin. In plants, the Rac group dominates the RHO family. For example, *Arabidopsis* has at least 13 different genes, but neither Cdc42 nor a 'true' Rho has been discovered (Valster et al., 2000). However, the C3 exoenzyme, a specific inhibitor of the Rho subfamily, alters plant cell functions such as ABA-induced stomatal closing (Eun and Lee, 1996), as well as cytoplasmic streaming in pollen tubes (Lin and Yang, 1997) and stamen hairs (Valster et al., 2000). These effects suggest that Rho-like proteins do function in plant cells.

RhoA, the most frequently studied isoform of the subfamily, is expressed ubiquitously in mammalian cells, and regulates actin organization. Its activity has been revealed in experiments using the C3 exoenzyme, both active and inactive forms of the RhoA protein (Takai et al., 1995; Aspenström, 1999). Localization studies have also demonstrated the role of RhoA in actin assembly. In transformed cells expressing RhoA, the protein was found where actin filaments were clustered, including membrane ruffling areas and cell-cell adhesion sites (Takaishi et al., 1995). The endogenous RhoA was also located in the actin-rich domain of the plasma membrane (Michaely et al., 1999) and at the focal contacts (Menager et al., 1999). In addition, homologues of RhoA were associated with the cleavage furrow of sea urchin eggs and with actin patches at growing sites in yeast (Yamochi et al., 1994; Arellano et al., 1997). In this context, co-localization of the immuno-reactant to the RhoA antibody and the cortical actin arrays found in guard cells suggests a role for an RhoA-like protein in the organization of actin in plant systems.

Although Rho subfamily members have been difficult to detect molecularly in plants, our data suggest that a protein exists whose cellular localization and three-dimensional structure in an antigenic site are similar to RhoA. Further studies, including affinity purification of the immuno-reactant to the RhoA antibody and characterization of the protein, are needed to determine whether the protein is an authentic Rho in plant cells.

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