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Profilin and Rop GTPases are localized at infection sites of plant cells

Short communication

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Summary. We have found 5 profilin cDNAs in cultured parsley cells, representing a small gene family of about 5 members in parsley. Specific antibodies were produced using heterologously expressed parsley profilin as antigen. Western blot analysis revealed the occurrence of similar amounts of profilin in roots and green parts of parsley plants. Immunocytochemical staining of parsley cells infected with the oomycetous plant pathogen *Phytophthora infestans* clearly revealed that profilin accumulates at the site on the plasma membrane subtending the oomycetous appressorium, where the actin cables focus. We also observed the accumulation of Rop GTPases around this site, which might point to a potential function in signaling to the cytoskeleton.

Keywords: Fungal infection; Plant defense; Cell polarization; Actin filament reorganization.

Introduction

In plants, the actin cytoskeleton performs essential tasks in many cellular processes, including morphogenesis, cell division, and cytoplasmic streaming (Volkmann and Baluska 1999, Mathur and Hülskamp 2002). Since plant cells are immotile and encased by a rigid cell wall, dynamic actin filament organization appears to be very important for the targeted transportation of organelles and vesicles, and the site-specific delivery of materials.

The remodeling of actin filament architecture is known to be regulated by associated proteins that bind either to monomeric or polymeric actin. One of the best characterized examples of such proteins in plants is profilin (Staiger et al. 1997). This abundant, low-molecular-mass (12–15 kDa) cytoplasmic protein has been identified in all eukaryotic organisms studied. Several isoforms have been found, also in plants, that appear to be differentially expressed and are encoded by small multigene families (Kandasamy et al. 2002). Profilin was originally characterized by its ability to bind in a 1 : 1 complex to monomeric actin (Carlsson et al. 1977, Sun et al. 1995). However, its role in regulating the organization of the actin cytoskeleton is rather complex and not yet fully understood. It can either promote or prevent actin polymerization depending on the size of the globular-actin (G-actin) pool and the ratio of this pool to profilin, as well as the differential cooperation with a number of other actin-binding proteins, for instance, actin-depolymerizing factor, β-thymosin and formin homology proteins (Ballweber et al. 1998). Profilins also interact with other ligands in addition to actin: membrane polyphosphoinositides and stretches of poly- or oligo-L-proline in proline-rich proteins (Drobak et al. 2004). These properties, particularly the binding to phosphatidylinositol 4,5-bisphosphate (PIP_2) resulting in dissociation of profilactin, hint at a role for profilin in signal transduction; indications for this have been found in animal and fungal organisms (Schlüter et al. 1998). From all these data, one might expect profilin to be concentrated at sites of highly dynamic actin filaments.

Animal and fungal GTPases of the Rho family, comprising Cdc42, Rac and Rho subfamilies, are well known for their role as key regulators of the actin cytoskeleton (Hall 1998). Besides mediating alterations in actin dynamics

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and intracellular transport, they control, via activation of protein kinase cascades, a broad range of physiological changes, including gene transcription, cell cycle progression and production of reactive oxygen species (Ridley 2001). Recent data imply that plant Rho homologues may have similar cellular functions (Valster et al. 2000, Zheng and Yang 2000, Vernoud et al. 2003). Pollen tubes elongate by tip growth, which is dependent on actin-filament-mediated transport of secretory vesicles along the longitudinal axis to the tip. Genetic evidence clearly shows that Rac-type proteins have an essential function in polar growth and act by controlling actin filament assembly (Kost et al. 1999). Furthermore, pollen tube Rac localizes to the plasma membrane at the tip, where it physically associates with a phosphatidylinositol monophosphate kinase activity and colocalizes with PIP_2, the specific product of this enzyme reaction. Similarly, polar localization of Rop (Rac of plants) GTPases was also found in *Arabidopsis thaliana* root hair trichoblasts at sites of outgrowing root hairs, even before budding (Molendijk et al. 2001), indicating involvement in polar cytoskeletal reorganization. These data suggest that small GTPases act by regulating site-directed actin filament polymerization. This view was recently reinforced by the finding that ROPs activate two counteracting pathways controlled by the ROP targets RIC3 and RIC4 (Fu et al. 2005, Gu et al. 2005). These pathways regulate each other to control actin dynamics and cell morphogenesis, for example, tip growth in pollen tubes and interdigitating growth in pavement cells of the leaf epidermis. Furthermore, small GTPases may function as molecular switches mediating between membrane-receptor-based perception of exogenous signals and their transduction to the cytoskeleton in cooperation with the polyphosphate inositol pathway and PIP_2-actin-binding proteins, such as profilin and actin-depolymerizing factor.

An excellent example of exogenously triggered induction of cytoskeleton polarization is the plant cell defense response to fungal infection. An important component of plant resistance to fungal pathogens is the formation of localized wall thickenings at penetration sites, so-called papilla, to prevent pathogen ingress (Schmelzer 2002). This process comprises site-directed transport and secretion of various materials which are deposited at the plant cell wall beneath and around the fungal infection structures and involves reorientation of the actin filament and microtubule architecture towards the penetration site (Schmelzer 2002, Takemoto et al. 2003). We have made intensive use of cultured parsley cells infected with *Phytophthora infestans* as a model system to study the plant defense response at the level of individual cells (Gross et al. 1993, Naton et al. 1996). Microscopic examination revealed that major features of the defense response in this model system largely resemble the in planta situation, including cytoplasmic and cytoskeletal reorganization. Here we have studied the occurrence and expression of profilins in parsley and the accumulation of profilin and Rop GTPases together with the formation of a new actin focus at the penetration site.

Material and methods

Cultivation of plant cells and oomycetes

Suspension-cultured *Petroselinum crispum* cells were grown in HA medium in constant darkness at 26 °C as described earlier (Kombrink and Hahlbrock 1986). *Phytophthora infestans* mycelium was grown on vegetable juice agar at 18 °C in the dark. Production of sporangia and maturation and release of zoospores on rye agar was performed according to the procedure described by Gross et al. (1993).

Cloning of parsley (P. crispum) profilins

Degenerate primers were derived from two profilin regions conserved among plants (Christensen et al. 1996): primer 1, AAR TAY ATG GTI ATI CAR GGI GA (amino acid sequence KYMVIQGE); primer 2 (reverse), TCI ACI ACC ATR TTR CAY TGI CC (amino acid sequence GQCNMVVE). By using these primers for PCR with cDNA from cultured parsley cells as template, a respective profilin fragment was amplified. The missing 3′ and 5′ ends were completed by rapid amplification of cDNA ends. The resulting clone was named PcPRF1. The sequence was used to screen the cDNA library of cultured parsley cells for other profilin-like sequences. In total, 30 profilin-like sequences were found corresponding to 5 different profilin cDNAs named PcPRF1 to PcPRF5.

DNA sequencing

Sequencing was performed by the in-house DNA sequencing facility on Applied Biosystems (Weiterstadt, Federal Republic of Germany) Abi Prism 377 and 3700 sequencers using BigDye-terminator chemistry. Premixed reagents were from Applied Biosystems.

Generation and purification of antibodies

PcPRF1 was cloned into the pQE31 vector via *Bam*HI/*Pst*I and transformed into *Escherichia coli* to generate PcPRF1-6 × His. Expression and purification of the fusion protein was performed as described by Robatzek and Somssich (2001). Briefly, heterologous expression was induced by addition of IPTG to a 500 ml liquid culture of *E. coli* during exponential growth. At an OD_{600} of 2.0, cells were harvested by centrifugation and lysed. PcPRF1-6 × His was purified from the lysate using Ni-agarose affinity chromatography. The eluates from the Ni-agarose column were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were cut out from the polyacrylamide gels, frozen in liquid nitrogen and ground with a mortar and pestle to a fine powder. Immunization and generation of antisera in rabbits was performed by Biogenes Inc., Berlin, Federal Republic of Germany. Antisera were tested with protein extracts from parsley. The antiserum was affinity-purified using heterologously expressed PcPRF1 bound to Ni-agarose following the protocol of Gu et al. (1994).

Western blot analysis

Parsley organs and tissues and suspension-cultured parsley cells were ground in liquid nitrogen using a mortar and pestle. The powder was resuspended in extraction buffer (sterile solution of 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 10 mM EDTA, 0.1% SDS; just before use, 20 µl of 200 mM permethylene sulfoxide [PMSF] in acetone and 100 µl of 20% Triton-X 100 were added to 10 ml of this buffer). Cell debris was removed by centrifugation at 10000 **g** for 30 min at 4 °C. Aliquots of the supernatant or microsomal preparations were separated by PAGE (10–15%) along with prestained molecular-weight markers (SeeBlue; Invitrogen, Karlsruhe, Federal Republic of Germany). For microsomal preparations, the tissue was ground at 4 °C with sand in 50 mM HEPES, pH 5, containing 0.5 M sucrose, 6 mg of polyvinyl polypyrrolidine per ml, 5 mM ascorbic acid, and 1 mM dithiothreitol. The homogenate was centrifuged at 7000 **g**. The supernatant was centrifuged at 32000 **g** and 4 °C, and the resulting pellet was resuspended in 1 ml of 5 mM K_2H–H_2PO_4 buffer, pH 7.8, containing 0.33 M sucrose, and stored at −20 °C. Protein bands were transferred to nitrocellulose membrane by electrophoretic blotting (Electro Eluter; Bio-Rad, Munich, Federal Republic of Germany). Membranes were blocked with 2% (w/v) milk powder (Neuform, Hamburg, Federal Republic of Germany) in TBS (8% NaCl, 0.2% KCl, 3% Tris-HCl, pH 7.4) for 60 min at room temperature and then incubated overnight at 4 °C with appropriate concentrations of primary antibodies (1 : 500 to 1 : 2000). After rinsing 3 times in milk powder–TBS, secondary antibodies labeled with alkaline phosphatase (Sigma-Aldrich, Munich, Federal Republic of Germany) were added (1 : 1000 dilution) and membranes were incubated for 2 h at room temperature. After rinsing 3 times with TBS-T (phosphate-buffered saline [PBS] with 0.1% Tween 20) and TBS, and once with water, the blots were stained with 5-bromo-4-chloro-3-indolylphosphate toluidine salt–nitro-blue tetrazolium chloride (Sigma-Aldrich) following the manufacturer's instructions. After the appearance of bands, the reaction was stopped by the addition of deionised water.

Immunocytochemistry

Cultured parsley cells were infected with *P. infestans* on microscope slides and subsequently immunocytochemically stained according to the method of Gross et al. (1993). Affinity-purified anti-profilin antibodies and affinity-purified antibodies against Rop4 from *Arabidopsis thaliana* were used as primary antibodies (dilutions of 1 : 10 to 1 : 100 in bovine serum albumin–PBS). In competition experiments, 1–10 µg of purified, heterologously expressed PcPRF1-6 × His was added to the primary anti-profilin antibodies. The anti-Rop4 antibodies were kindly provided by A. Molendijk. Secondary antibodies were diluted 1 : 200 in bovine serum albumin–PBS for CY3-conjugated antibodies and 1 : 150 for fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies (Sigma-Aldrich). Slides were incubated with the antibody solution for 1 h at 37 °C and then rinsed 3 times with PBS and 3 times with microtubule-stabilizing buffer. The stained cells were inspected and photographed with a Zeiss Axiophot light microscope equipped with epifluorescence and a digital imaging system (JVC KY-F70 camera, Diskus imaging software; Technisches Büro Hilgers, Königswinter, Federal Republic of Germany).

Results

Characterization and expression of profilins from P. crispum

Using PCR technology with *P. crispum* cDNA as template and degenerate primers, a cDNA fragment of profilin was amplified. The fragment was subjected to rapid amplification of cDNA ends and a full-length cDNA, PcPRF1, was obtained.

Using this cDNA as a probe to screen a cDNA library of suspension-cultured *P. crispum* cells, four additional profilin cDNAs with very similar deduced amino acid sequences (GenBank accession nr. AY900012–AY900016) were found. Employing DNA from cultured parsley cells in Southern blot experiments with PcPRF1 as the probe, 3 to 5 major bands were detected under moderate stringency. Thus, the parsley profilins constitute a small gene family of about 5 members.

Specific antibodies were generated against heterologously expressed PcPRF1 and affinity purified. These antibodies recognized purified, heterologously expressed PcPRF1 on Western blots (Fig. 1, lane 2) and a band corresponding to the molecular mass of profilin, about 14 kDa, was detected by Western blot analysis of extracts from cultured parsley cells, as well as from leaves and roots of parsley plants (Fig. 1, lanes 3–5). The preimmune serum control displayed no signals (Fig. 1, lane 1). The parsley profilin isoforms show high homology among themselves (87–98%) and with the profilins from *Arabidopsis thaliana* (85–91%). There is also considerable homology (44–48%) with the mouse and human profilins. Thus, the antibodies against PcPRF1 most probably do not discriminate between isoforms. Irrespective of isoforms, profilins were found to be expressed at similar levels in terrestrial and aerial parts of parsley plants. Considerably higher levels were found in maturing pollen by immunohistochemical staining of flower bud sections (data not shown).

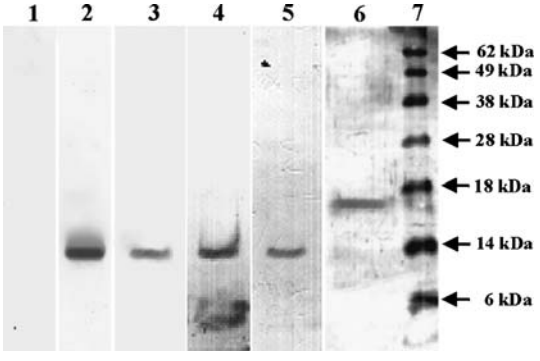

Fig. 1. Western blot analysis using preimmune serum and anti-profilin and anti-Rop-GTPase antibodies. Protein extracts from cultured parsley cells were incubated with preimmune serum (*1*), purified recombinant profilin (*2*), and protein extracts from cultured parsley cells (*3*), parsley leaves (*4*), and roots (*5*) were incubated with the anti-profilin antibodies, and a microsomal preparation from cultured parsley cells was incubated with the anti-Rop4 (*A. thaliana*) antibodies (*6*). Prestained protein markers for the indicated molecular masses were coseparated and blotted (*7*)

Cellular redistribution of actin, profilin, and Rop-GTPase upon pathogen infection

Cultured parsley cells were infected with the oomycetous plant pathogen *P. infestans* to study the induced changes in cellular organization of the actin cytoskeleton and its binding protein profilin, as well as the potential signaling component Rop GTPase, by immunocytochemistry. During cocultivation of *P. infestans* germlings and cultured parsley cells on microscopic slides, the growing germ tube of the pathogen contacts and attaches to cells through the formation of an appressorium and subsequently penetrates the cell wall. Upon attachment and penetration, the plant cell reorients actin filaments towards the area of the plasma membrane beneath the appressorium of the pathogen (Fig. 2A, B). As seen in Fig. 2C and D, strong accumulation of profilin was found both at the infection site and in the plant cell nucleus. To verify the specificity of the immunocytochemical staining and the redistribution of profilin, we performed a number of control experiments (Fig. 3). When infected cultured parsley cells were incubated with Cy3 fluorescently labeled secondary antibodies alone, no fluorescence was detectable (Fig. 3A, B) and only weak, unspecific fluorescent labeling was observed after staining with the preimmune serum (Fig. 3E, F). The strong staining of nuclei was also seen in uninfected cells incubated with the anti-profilin antibodies (Fig. 3C, D). Addition of purified, heterologously expressed PcPRF1 to the infected cells drastically decreased the immunostaining at the infection site and of the nucleus by competing with the cellular profilin for the antibody (Fig. 3G, H). Thus, the accumulation of profilin at the infection site appeared to be specific and correlated with the reorientation of actin filaments towards this site.

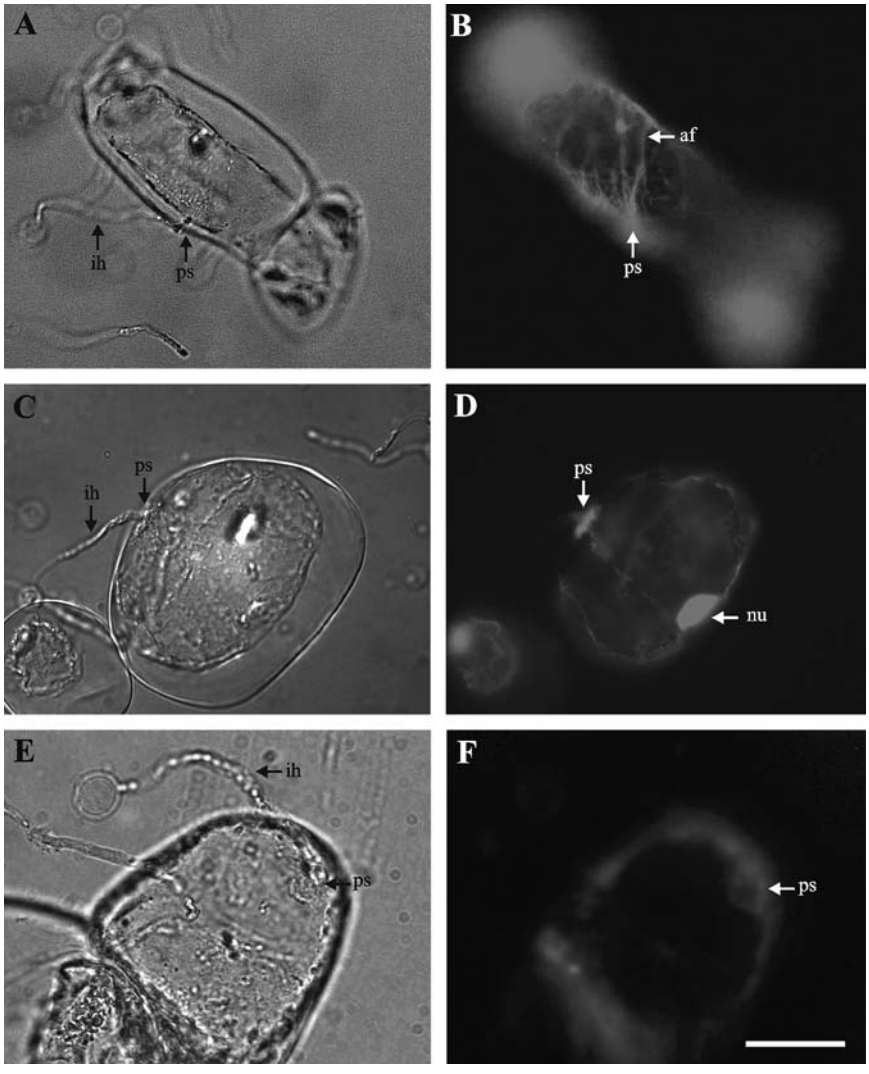

Fig. 2 A–F. Immunocytological localization of actin, profilin, and Rop GTPase in infected cultured parsley cells. Cultured parsley cells were infected with *P. infestans* on microscope slides and then immunocytochemically stained with primary antibodies specific for actin (**A** and **B**), profilin (**C** and **D**) and *A. thaliana* Rop4 (**E** and **F**), and Cy3 fluorescently labeled secondary antibodies. Panels A, C, and E are bright-field images of the respective fluorescence images B, D, and F. *af* Actin filament; *ih* infection hypha; *nu* nucleus; *ps* penetration site. Bar: 20 μm

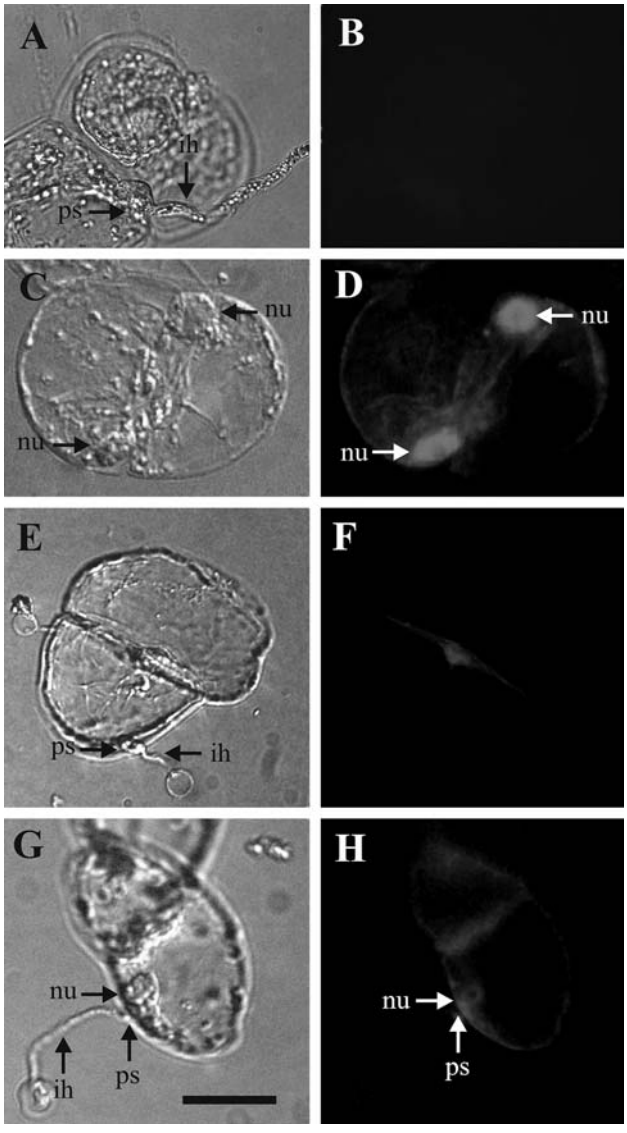

Fig. 3 A–H. Immunocytochemical control experiments with cultured parsley cells. **A** and **B** Infected cells incubated with secondary Cy3 fluorescently labeled antibodies only; **C** and **D** noninfected cells stained with anti-profilin antibodies and Cy3 fluorescently labeled secondary antibodies; **E** and **F** infected cells stained with preimmune serum and Cy3 fluorescently labeled secondary antibodies; **G** and **H** infected cells stained with anti-profilin antibodies in the presence of 10 μg of purified recombinant profilin and Cy3 fluorescently labeled secondary antibodies. Panels A, C, E, and G are bright-field images of the respective fluorescence images B, D, F, and H. *ih* Infection hypha; *nu* nucleus; *ps* penetration site. Bar: 20 μm

The potential involvement of small GTPases in the defense mechanism was investigated using antibodies against Rop4 from *A. thaliana*. First, the cross-reactivity of these antibodies with parsley proteins was tested in Western blots. In protein extracts of parsley microsomal fractions, one single band at a molecular mass of about 18 kDa, corresponding to the molecular mass of monomeric Rop, was detected (Fig. 1). Immunocytochemical staining of infected parsley cells with the anti-Rop4 antibodies showed weak but significant staining at the plasma membrane, especially at the infection site, where a halo of antibody fluorescence was observed around the invading hyphae (Fig. 2 E, F).

Discussion

Profilins have been found in all eukaryotic organisms and appear to be ubiquitously expressed, indicating essential functions. In plants, profilins are encoded by small gene families of about 5 members (Staiger et al. 1993, Mittermann et al. 1995, Huang et al. 1996). Similarly, our screening of a cDNA library and Southern blot analysis indicated the presence of up to 5 profilin genes in parsley. We found profilins throughout the whole plant with increased concentrations in maturing pollen of flowers, which is also typical for other plants (Christensen et al. 1996). Plant profilins were first identified as the major allergen in pollen (Valenta et al. 1991).

With respect to intracellular localization in plants, profilins are normally equally distributed within the cytoplasm, but they are often also found within the nucleus (Braun et al. 1999, Holzinger et al. 2000, Valster et al. 2003). When we overexpressed profilin (PcPRF1) as a GFP-fusion protein (C-terminal or N-terminal) in parsley protoplasts, the cytoplasm and nucleus displayed intense GFP fluorescence; however, cytoplasmic strands could no longer be observed. Coexpression of profilin-RFP and talin-GFP for fluorescent tagging of actin filaments showed the lack of transversal actin filaments and cytoplasmic strands in protoplasts (data not shown). These results confirm earlier reports that microinjection of profilin into plant cells results in the disappearance of cytoplasmic strands and transversal actin filament bundles (Staiger et al. 1994, Valster et al. 1997). Thus, the artificial elevation of the apparent cytoplasmic concentration of profilin clearly leads to the depolymerization of actin filaments and drastic disturbance of cytoplasmic morphology and dynamics, as a consequence of its sequestering function. The function of nuclear profilin is unknown, although it has been speculated that it might be involved in signal transduction cascades between the nucleus and cytoplasm (Baluska et al. 2001). Our results show that, upon fungal or oomycete infection and the consequent induction of actin filament polarization, profilin localizes to the actin focus at the plasma membrane beneath the site of cell wall penetration. In quite a number of publications, similar concentrations of profilin were found in cytoplasmic areas with high actin filament dynamics, such as the cell cortex, filopodia and focal adhesions in *Acanthamoeba castellani*,

Schizosaccharomyces pombe, and mammalian cells (reviewed in Schlüter et al. 1998). In plants, profilin is codistributed with the apical actin cap in growing root hairs and is specifically localized in the bulge during root hair initiation and subsequently forms a weak tip-to-base gradient in the elongating root hairs (Baluska et al. 2000). However, in another tip-growing plant cell, the pollen tube, profilin was found to be rather homogeneously distributed and not concentrated at the tip (Vidali and Hepler 1997), and no specific localization of profilin was found in the phragmoplast of dividing plant cells, which contains a dense array of actin filaments (Valster et al. 2003). Similarly, in other studies employing microinjection of fluorescently labeled profilin into *Acanthamoeba castellanii* and mammalian tissue culture cells, profilin was not found to colocalize with actin filaments (Tarachandani and Wang 1996, Kaiser et al. 1999). The source of these conflicting results is presently unclear. From our control experiments, in which only secondary antibodies or preimmune serum were applied, we can rule out that the fluorescence labeling at the penetration site is simply caused by autofluorescence of cytoplasmic aggregates or unspecific antibody staining. In an earlier publication, we showed that cultured parsley cells exhibit a bright blue autofluorescence at infection sites upon UV-light excitation (330–390 nm), while other excitation wavelengths, for instance, the green light used here for Cy3 excitation, have no effect (Naton et al. 1996). The staining of nuclei in noninfected cells with the anti-profilin antibodies indicates a constitutive localization of profilin in this organelle. As we were able to substantially reduce the staining at the penetration site and in the nucleus by adding purified, heterologously expressed profilin to the anti-profilin antibodies, the labeling at these sites must be regarded as true accumulation of profilin.

In outgrowing root hair bulges, elevated levels of PIP_2, a potential ligand of profilin, were found using specific anti-PIP_2 antibodies for immunocytochemical localization (Braun et al. 1999). In experiments with similar commercially available antibodies, we observed staining of nuclei in parsley cells but not around the penetration site (data not shown). However, it was unclear whether this was due to experimental differences, such as the fixation conditions for optimal membrane preservation. Nuclear localization of PIP_2 has been clearly shown for mammalian cells (Martelli et al. 1995, Boronenkov et al. 1998, Osborne et al. 2001). The process of root hair initiation shows striking similarities in various aspects to fungal-infection-induced cellular reorganization (Schmelzer 2002). As in the cellular defense response to fungal invasion, the cell creates a special microcompartment at the cell wall, which involves site-directed reorientation of actin filaments and microfilament-dependent transport processes for cell wall construction and fortification. These similarities suggest that various exogenous or endogenous triggers might induce a similar program leading to cellular polarization. Our observations of Rop GTPases accumulating around the penetration site, i.e., the site of polarization, as in the process of root hair initiation (Molendijk et al. 2001), are in line with this assumption. The activity of Rop GTPases in plant cell polarization could probably be linked to actin cytoskeleton reorganization; however, this has yet to be proven. It is already clear that small GTPases play a crucial role in plant defense responses, such as the generation of reactive oxygen species (oxidative burst), expression of defense-related genes, and production of phytoalexins (Ono et al. 2001, Wong et al. 2004). In a recent study of the defense response of barley to the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, it was shown that actin cytoskeleton polarization to sites of attempted fungal penetration is modulated by the receptor-like transmembrane protein MLO and the RAC/ROP family G protein RACB (Opalski et al. 2005). Interestingly, in susceptible interactions of barley and *B. graminis* f. sp. *hordei*, members of the ROP G protein family were found to be important for pathogen access and, hence, establishment of disease (Schultheiss et al. 2003). Apparently, in cases of nonrecognition and impaired defense, pathogenic fungi take advantage of GTPase functions for the establishment of biotrophy. Thus, components of the signaling pathway to the actin cytoskeleton might be involved in a complex network concerned with the sensing of exogenous triggers and resulting in a variety of potential physiological answers and dynamic morphological changes.

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