Mastoparan Alters Subcellular Distribution of Profilin and Remodels F-Actin Cytoskeleton in Cells of Maize Root Apices

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Indirect immunofluorescence localization of profilin in cells of maize root apices revealed that this abundant protein was present both in the cytoplasm and within nuclei. Nucleo-cytoplasmic partitioning of profilin exhibits tissue-specific and developmental features. Mastoparan-mediated activation of heterotrimeric G-proteins, presumably through triggering a phosphoinositide-signaling pathway based on phosphatidylinositol-4,5-bisphosphate (PIP2), induced recolocalization of profilin from nuclei into the cytoplasm of root apex cells. In contrast, PIP2 accumulated within nuclei of mastoparan-treated root cells. Intriguingly, cytoplasmic accumulation of profilin was associated with remodeling of F-actin arrays in root apex cells. Specifically, dense F-actin networks were dismantled and distinct actin patches became associated with the periphery of small vacuoles. On the other hand, disruption of F-actin with the G-actin sequestering agent latrunculin B does not affect the subcellular distribution of profilin or PIP2. These data suggest that nuclear profilin can mediate a stimulus-response action on the actin cytoskeleton which is somehow linked to a phosphoinositide-signaling cascade.

Key words: Actin — Maize root — Nucleus — Phosphoinositide signaling — Profilin.

Abbreviations: AFs, Actin filaments; PA, Phosphatidic acid; PI, Phosphoinositide; PIP2, Phosphatidylinositol-4,5-bisphosphate; PLP, Poly-l-proline; PRA, Profilin rabbit antibody; ZmPRO3, Zea mays profilin isoform 3.

Introduction

Profilins are small, ubiquitous and abundant soluble proteins that affect the dynamic behavior of actin filaments (AFs) in cells of all eukaryotes (e.g. Schlüter et al. 1997, Staiger et al. 1997, Gibbon and Staiger 2000, Staiger 2000). Two contrasting activities of profilin result from its ability to both promote or inhibit the assembly of AFs depending on the ratio of profilin to G-actin, ionic conditions and on presence/activities of other actin-binding proteins (Pantaloni and Carlier 1993, Staiger et al. 1997, Gibbon and Staiger 2000). Profilin is enriched at peripheral cytoplasmic domains of animal cells which have dynamic AFs that rapidly respond to diverse signals (Buß et al. 1992, Mayboroda et al. 1997). Moreover, birch pollen profilin co-localizes with dynamic AFs when expressed in animal cells (Mayboroda et al. 1997). This suggests that plant and animal profilins obey the same principles responsible for their subcellular localizations (for animal cells see, e.g., Hartwig et al. 1989, Buß et al. 1992, Bubb et al. 1998). Importantly, recent genetic studies have revealed that profilin plays critical roles for basic processes of plant development (Ramachandran et al. 2000, McKinney et al. 2001).

Profilins from non-plant systems are well known to interact with several classes of proteins that contain long continuous stretches of proline residues (Reinhard et al. 1995, Kay et al. 2000). These interactions are implicated in allowing profilins to be effectively targeted to distinct subcellular domains (Kang et al. 1997). In addition, data from animal cells show that profilin can be sequestered from the G-actin-associated cytoplasmic pool via interactions with phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) (e.g. Lassing and Lindberg 1985). For yeast cells, it was shown that metabolism of PIP2 plays a crucial role in subcellular localization of profilin (Ostrander et al. 1995). Since profilin inhibits the diesteractive cleavage of PIP2 by phospholipase C (for plant cells see Drsba et al. 1994), profilin–PIP2 interactions are relevant for signal transduction cascades implicated in regulation of intracellular cytoplasmic calcium levels (Berridge and Irvine 1989, for plant cells see Munnik et al. 1998). Interestingly in this respect, both PI 4-kinase and PI 3-kinase can associate with the actin cytoskeleton (Xu et al. 1992, Dve et al. 1994, Stevenson et al. 1998). On the other hand, profilin can stimulate PI 3-kinase activity (Singh et al. 1996).

In the present study, we have investigated subcellular distributions of profilin and PIP2 in cells of maize root apices. Roots are suitable for such a study as their cells are, in contrast to other plant organs, rich in transcripts for phosphatidylinositol-4-phosphate 5-kinase (Mikami et al. 1998), an enzyme which phosphorylates phosphatidylinositol-4-phosphate to produce PIP2. We report that maize profilins loosely co-distribute with PIP2 at cell peripheries as well as in the cytoplasm. Mastoparan, a potent stimulator of PIP2 hydrolysis (for plants see
Western blots

Western blot analysis (Fig. 1) was accomplished with two different profilin antibodies: one antibody was raised against recombinant maize isoform ZmPRO3 (Fig. 1, lanes 1–4) while another one, called here PRA, was raised against native maize pollen profilin (Fig. 1, lanes 5–8). Both these profilin antibodies recognized profilins as distinct bands at about 14 kDa in the soluble protein fraction (Fig. 1, lanes 1, 3, 5 and 7). The respective pre-immune sera did not react with these 14 kDa species but did faintly recognize several minor protein species of higher M<sub>r</sub> (data not shown). Competitive blocking of both antibodies, using a mixture of recombinant profilins, efficiently depleted the 14 kDa bands (Fig. 1, lanes 2, 4, 6 and 8) while the faint non-specific bands were still visible (data not shown). Mastoparan-treated roots invariably showed stronger profilin signal than control roots (Fig. 1, compare lanes 1 and 5 for control roots with lanes 3 and 7 for mastoparan-treated roots).

Immunofluorescence of profilin in control and mastoparan-treated root apices

Polyclonal rabbit antibodies PRA and anti-ZmPRO3 showed diffuse and punctate labeling of the cytoplasm which was typically complemented by distinct enrichment of profilin within nuclei and at the cell periphery (for PRA see Fig. 2A–L, for anti-ZmPRO3 see Fig. 2M–P). Importantly, using PRA pre-immune serum (not shown), as well as immunodepletion of both PRA (Fig. 2G) and anti-ZmPRO3 (Fig. 4C) antibodies, resulted in no visible signals when the same exposure times were used. The cell periphery-associated labeling is most prominent in epidermal cells located in the distal part of meristem (Fig. 2A) and in the transition zone (Fig. 2C), as well as metaxylem cells in the transition zone (Fig. 2H). Epidermis cells in the basal part of meristem accumulate profilins in nuclei and only faint signal remains associated with the cell periphery (see arrow in Fig. 2B). In the cortex, nuclear labeling predominated both in the basal (Fig. 2B) and apical (Fig. 2I, M) parts of meristem. But in the transition zone, some profilin-depleted nuclei (about 15% in the cortex and epidermis) occurred (white star in Fig. 2F).

Nuclei of epidermal cells near the root cap junction are only faintly stained (Fig. 2A) when compared with nuclei of developmentally more advanced epidermis cells (Fig. 2C). The unique staining of the epidermis, when compared with other root tissues, was more evident in the elongation region (Fig. 2D, E). Here, specialized epidermal cells transform from diffusely growing cells into root hair initiating trichoblasts with growth (exocytosis) restricted to distinct domains (open stars in Fig. 2D, E). These domains ultimately transform into tips of emerging root hairs (not shown). Accumulation of profilin within the growing bulge is typically associated with progressive depletion of nuclear profilins (black star in Fig. 2D) and profilin-positive nuclei were never found in trichoblasts with profilin “caps” at bulging domains. Similarly, about 10% of nuclei in the root cortex are depleted in profilins (for PRA antibody see white star in Fig. 2F; the same is true for the ZmPRO3 antibody, data not shown). This subcellular distribution pattern of PRA-positive profilin was confirmed using a polyclonal antibody raised against recombinant maize profilin ZmPRO3 (not shown). Generally, we can conclude that anti-ZmPRO3 labeling patterns are almost identical to those obtained with the PRA antibody.

Mastoparan proved to be very effective in altering the subcellular distributions of profilin. In mastoparan treated root apices, intranuclear profilin labelings were depleted and both PRA (Fig. 2J–L) and anti-ZmPRO3 (Fig. 2N–P) reactive profilin accumulated in the cytoplasm. This feature was more prominent in cortical cells of the transition zone (Fig. 2K, O) and the apical part of the elongation region (Fig. 2L, P) than in cortical cells of the meristem (Fig. 2I, M; for appropriate control cortex cells see Fig. 2I, M). Mastoparan analog M17 was also effective in this respect (not shown) suggesting that its biological activity is high for maize roots (for soybean suspension cells see Schroeder-Taylor and Low 1997).
Mastoparan redistributes nuclear profilin
**Immunofluorescence localization of PIP2**

A commercially available monospecific PIP2 antibody proved to be highly reactive in maize root cells. The strongest signal was in meristematic cells having the whole cytoplasm labeled while nuclei showed almost no signal (Fig. 3A). Whereas the addition of PIP2 to the antibody inhibited its immunofluorescence reactivity almost completely (Fig. 3B), the addition of PA was without any discernible effect (Fig. 3C).

In cortex cells of the transition zone (Fig. 3D) and elongation region (Fig. 3E, F), the most prominent signal was associated with cellular peripheries in a pattern that strongly resembled the plasma membrane-associated profilin labelings. To substantiate the plasma membrane association of PIP2, we have applied a monoclonal antibody raised against the maize plasma membrane H+-ATPase (Jahn et al. 1998). This labeled cellular peripheries (Fig. 3J) in a fashion similar to the PIP2 antibody (compare Fig. 3E, F). The nuclear envelopes with associated cytoplasmic strands (Fig. 3E), as well as nucleoplasm of some (10%) cortical cells in the transition zone (not shown), were also PIP2-positive. In order to further assess the punctate cell periphery labelings, presumably associated with the plasma membrane, we inspected paradermal sections encompassing the whole plasma membranes within root sections. Such images showed that plasma membrane-associated PIP2 is organized in the form of dots and patches (Fig. 3G, H). In mastoparan treated root apices, anti-PIP2 labeling was depleted in the cytoplasm and increased within nuclei of transition zone cells of the root cortex (Fig. 3I).

**Mastoparan alters F-actin arrangements**

While latrunculin B-mediated disintegration of AFs (Fig. 4F, compare with Fig. 4D, E) had no discernible effects on either profilin (Fig. 4A) or PIP2 distributions (Fig. 4B), 2 h of mastoparan treatment altered F-actin distributions in most root apex cells. In cells of control maize roots, AFs are organized in the form of networks radiating from nuclear envelopes towards cellular peripheries (Fig. 4E shows interphase cells of the cortex) or F-actin cables organized between actin-enriched cross-walls (Fig. 4D shows post-mitotic cells of the outer stele and periphery; for more details on F-actin organization in cells of maize root apices see Baluška et al. 1997a). Mastoparan dismantled both F-actin networks and bundles in cortical cells of maize root apices (Fig. 4G corresponds to Fig. 4D) which transformed into distinct patches associated with peripheries of small vacuoles (Fig. 4H–I). On the other hand, latrunculin B-induced depolymerization of F-actin was associated with accumulation of actin fluorescence within nuclei (black stars in Fig. 4F).

**Discussion**

Our study documents that maize root apices are suitable model object to study basic principles of phosphoinositide (PI) signaling pathways in plants (for other suitable plant systems see Munnik et al. 1998, Drøbak et al. 1996) and their putative links with the actin cytoskeleton (Volkmann and Baluška 1999, Staiger 2000). Our immunofluorescence data document that, in addition to the diffuse cytoplasmic labeling, profilin localizes to distinct subcellular domains distributed at cellular peripheries, cytoplasmic strands and within nuclei of maize root cells. In this respect, our findings are fundamentally different from previous profilin immunolocalization studies in plant cells which, however, were done almost exclusively on in vitro germinating pollen grains and on tip-growing pollen tubes (Grote et al. 1995, Mittermann et al. 1996, Hess and Valenta 1997, Vidali and Hepler 1997). The only study on subcellular distribution of plant profilin in non-pollen cells is that of Vidali et al. (1995) in which tissue-specific distributions of profilin are described in control and Rhizobium-infected roots of common bean. As profilin interacts specifically with PIP2 which can sequester profilin from its cytoplasmic pool (for plant cells see Drøbak et al. 1994), intracellular distributions of PIP2 are of particular interest. From earlier biochemical work it is known that a large portion of cellular PIP2 associates with plant plasma membrane (Wheeler and Boss 1987, Irvine et al. 1989). Recently we have shown localization of maize profilin at the plasma membrane of developing pollen of birch and maize (von Witsch et al. 1998).

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**Fig. 2** Profilin distributions in cells of control and pharmacologically treated maize root apices as revealed with two antibodies raised against maize profilins, PRA (A–L) and anti-ZmPRO3 (M–P). (A) Young meristematic cells of epidermis showed abundant profilin labeling with PRA antibody, especially at cellular peripheries facing the root surface (thick arrow). Note that nuclei (stars) were only faintly labeled at this developmental stage of the epidermis. (B) Depletion of peripheral labeling (thick arrow) and increase in nuclear labeling just a few epidermal cells further, in the proximal part of the meristem. (C) In the transition zone, epidermal peripheries facing root surface showed again profilin accumulations (thick arrow) and nuclei were strongly labeled with the PRA antibody which also recognized cytoplasmic strands. (D) In the elongation region, root hair initiation in epidermis was accompanied with massive accumulation of profilins within outgrowing bulge (indicated with open star) and a slight reduction of the nuclear labeling (black star). (E) Finally, the bulge (open star) is the most dominant profilin domain while nuclei are often not recognizable at all. (F) Similarly in the root cortex, 10% of nuclei in transition zone cells showed no profilin staining (white star) while the adjacent ones were typically strongly labeled (black star). (G) Blocking of PRA antibody with profilins resulted in no labeling at all. (H) Prominent cell periphery-associated aggregates (see arrow pointing at a cross wall) were often labeled in prospective metaxylem elements with the PRA antibody. Metaxylem nucleus is also profilin-positive (star). (I–P) After mastoparan treatment, both PRA (J–L) and ZmPRO3 (N–P) signal decreased in nuclei and accumulated under side walls in cells of the basal part of cortex meristem (J; for appropriate control cells see LM). Nuclei in post-mitotic cells of the cortex (K, O) and metaxylem (L, P) did not retain any profilin signal. Nuclei are indicated by stars. Bar = 7 µm.
Here, we have applied well-characterized monoclonal antibody raised against PIP$_2$ (Fukami et al. 1988, Hay et al. 1995, Gilmore and Burridge 1996, Bubb et al. 1998, Huang et al. 1998, Kaznacheyeva et al. 2000, Mayer et al. 2000) on Steedman’s wax sections taken from maize root apices. This antibody labels distinct domains at cellular peripheries, cytoplasmic strands and nuclear envelopes. Strong cytoplasmic labeling in meristematic cells might be attributed to the fact...
Mastoparan redistributes nuclear profilin

that, besides the plasma membrane, cytoplasmic leaflets of all endocellular membranes are also associated with PIP$_2$ (e.g. Helms et al. 1991, Hay et al. 1995, Chung et al. 1998, Godi et al. 1999). PIP$_2$ localizes also to apices of tip growing root hairs (Braun et al. 1999, Baluška et al. 2000b, Baluška et al. 2000c) and pollen tubes where it apparently regulate their actin-based polarized growth in a common pathway with Rho GTPases (Kost et al. 1999, Molendijk et al. 2001).

The general consensus is that PI signaling cascades also exist in higher plant cells (reviewed by Drobak 1993, Drobak et al. 1996, Munnik et al. 1998). Several pharmacological agents are used to dissect PI signaling cascades in plant cells.

Fig. 4  (A, B) Double labeling with anti-ZmPRO3 (A) and anti-PIP$_2$ (B) antibodies in elongating cortex cells of latrunculin-treated root apices. (C) Blocking of anti-ZmPRO3 with profilins resulted in no labeling. (D, E) AFs in cells of the stele-cortex interface (D), and in cortex cells (E). (F) Latrunculin B treatment depolymerizes all AFs resulting in diffuse fluorescence which accumulates within nuclei (stars). (G–J) Mastoparan (10 μM for 2 h) induced dramatic reorganization of the actin cytoskeleton in cells of maize root apices. Note that all AF networks and bundles were effectively dismantled (G) and that actin-positive patches were associated preferentially with vacuolar peripheries (arrowheads in H and asterisks in I, J). (G) Cortex cells at the middle part of meristem; (H) endodermis/pericycle interface at the apical part of transition zone; (I) cortex at the apical part of transition zone; (J) cortex at the apical part of meristem. Nuclei are indicated by stars. Bar = 7 μm (A–I) and 12 μm (J).
Mastoparan redistributes nuclear profilin

among which mastoparan, a tetracapeptide isolated from wasp venom, represents one of the best understood agents (van Himbergen et al. 1999). Mastoparan is a potent stimulator of phospholipase A₂, C- and D-activities in plants (Drobnak and Watkins 1994, Scherer 1995, Cho et al. 1995, Tucker and Boss 1996, Franklin-Tong et al. 1996, van Himbergen et al. 1999, den Hartog et al. 2001). Importantly, our data reveal that subcellular distributions of profilin and PIP₂ are altered profoundly by mastoparan. In particular, mastoparan redistributed profilin from nuclei into the cytoplasm. In this respect it is interesting to note that mastoparan, when imposed on protoplasts isolated from transgenic tobacco plants expressing calcium reporter aequorin, stimulated transient increases of nuclear free calcium levels independently from those induced in the cytoplasm (Pauly et al. 2000).

We have recorded similar depletion of nuclear profilin also using mastoparan analog M17 (van Himbergen et al. 1999), which is known to exert rather lower activities in other plant systems (e.g. van Himbergen et al. 1999, Frank et al. 2000, den Hartog et al. 2001). Our data, however, indicate that biological activities of this analog can be high in maize root apices, and we strongly recommend not to take for granted its alleged inactivity (see also van Himbergen et al. 1999). High biological activity of the mastoparan analog M17 was recorded also for soybean cell suspension cultures (Schroeder-Taylor and Low 1997).

From other organisms, it is well known that PIP₂ is enriched at specific plasma membrane domains in a tissue- and development-specific fashion (e.g. Hope and Pike 1996, Glaser et al. 1996). These latter domains may act as putative F-actin organizing centers (Baluška et al. 2000b) where, under the action of external stimuli, PIP₂ hydrolysis might lead to dissociation of profilin and other PIP₂-actin binding proteins which then mediate changes in organization of the actin cytoskeleton (Hartwig et al. 1995). Lipid rafts are preferred platforms for PIP₂-activated actin polymerization (Rozelle et al. 2000). Moreover, PIP₂-based lipid rafts appear to play a central role in signaling across the plasma membrane (Lisanti et al. 1994, Kurzchalia and Parton 1999). Numerous data implicate PIP₂ and diverse actin-binding proteins as effectors of extracellular signals (e.g. Machesky and Pollard 1993, Lisco-vitch and Cantley 1995, Clarke et al. 1998, Stauffer et al. 1998, Toker 1998), allowing effective perception of extracellular signals at the plasma membrane and its further transduction via cytoskeletal complexes (Janmey 1998, Machesky and Insall 1999, for plant cells see Volkmann and Baluška 1999, Staiger 2000). Such a scenario was recently reported for the tip growing pollen tubes where compartmentalized PIP₂ acts in a common pathway with Rho-family GTP-binding proteins to support the actin-dependent polar tube growth (Kost et al. 1999). This may also be true for actively growing root hair tips where apical enrichments of PIP₂ colocalize with accumulations of profilin, dense F-actin meshworks (Braun et al. 1999, Baluška et al. 2000a, Baluška et al. 2000b, Baluška et al. 2000c) and Rop GTPases (Molenijik et al. 2001).

Other authors also reported nuclear localization of plant profilin using either chemical fixation (Vidal et al. 1995) or high-pressure freeze fixation technique (Hess and Valenta 1997, Holzinger et al. 1997, Holzinger et al. 2000). Similarly, nuclear profilin has occasionally been reported for some animal cells (e.g. Mayboroda et al. 1997). Maize profilin localizes to root cell nuclei in a constitutive fashion. Importantly, however, few post-mitotic cortical nuclei (about 10%) are depleted in profilin in control cells indicating that this small and soluble molecule might shuttle between the nuclear and cytoplasmic compartments, similarly as it was shown for G-actin (Wada et al. 1998, Rando et al. 2000). Plant cells seem to be different from other eukaryotic systems, not only because they express larger number of profilin isoforms (Gibbon and Staiger 2000) but also because a significant portion of plant profilin constitutively localizes to nuclei. This unique feature of plant profilin might prove to be relevant for signal transduction cascades between the plasma membrane and the nucleus as these are known to be mediated, at least in part, via nuclear shuttling proteins (Goldfarb 1991, Laskey and Dingwall 1993, Mahanty et al. 1999). In support of the nucleus–cytoplasm shuttling concept, we show that mastoparan induces redistributions of profilin from nuclei to the cytoplasm, especially in cortical cells of the transition zone (Baluška et al. 2001c). Intriguingly, this sudden increase of cytoplasmic profilin was accompanied by a re-organization of F-actin arrays throughout maize root apices. A sudden increase of profilin levels within the cytoplasm, after microinjection of profilin, was shown to rapidly depolymerize AFs in vivo in living Tradescantia stamen hair cells (Staiger et al. 1994, Karakesisoglou et al. 1996, Gibbon et al. 1997, Gibbon et al. 1998). Therefore, putative stimulus-responsive relocations of nuclear profilin into the cytoplasm would impinge on organization of actin cytoskeleton around the nucleus. In fact, our study documents that AF arrays disintegrate and the actin signal accumulates in the form of distinct actin patches at peripheries of small vacuoles.

Plant profilin might turn out to be a shuttling protein, acting perhaps as a second messenger within the framework of PI-based signaling pathways (Clarke et al. 1998). Similar second messenger-like behavior was proposed for another actin binding protein, coflin/ADF, in both animal and plant cells (Samstag et al. 1994, Jiang et al. 1997). By efficient sequestration of plant profilin within the nuclear compartment and its stimulus-responsive release into the cytoplasm, plant nuclei could directly remodel the actin cytoskeleton within the cytoplasm. This feature fits well to our plant “cell body” concept (Baluška et al. 1997b, Baluška et al. 1998, Baluška et al. 2000a, Baluška et al. 2001b) which provides a conceptual framework for the capability of plant nuclei to actively remodel the cytoplasmic architecture.
Materials and methods

Materials

Maize grains (Zea mays L. cv. Alarik), obtained from Force Limagrain (Darmstadt, Germany), were soaked for 6 h and left to germinate in well-moistened rolls of filter paper for 4 d in darkness at 20°C. Young seedlings with straight primary roots, 50–70 mm long, were selected and growing root apices were excised for fixation. For experimental treatments, some seedlings were transferred to containers with latrunculin B (10 μM, 1 h), mastoparan (10 μM, 2 h) and mastoparan analog M17 (10 μM, 2 h). With the exception of latrunculin B (Calbiochem, Bad Soden, Germany) and mastoparan analog M17 (Bachem AG, Heidelberg, Germany), all chemicals were obtained from Sigma Chemicals.

Sample preparation

Maize root tips were processed for indirect immunofluorescence as previously described in detail (Baluška et al. 1992, Baluška et al. 1997a). In brief, apical root segments (6–8 mm long) were fixed in 3.7% formaldehyde prepared in stabilizing buffer (SB: 50 mM PIPES buffer, 5 mM MgSO4, 5 mM EGTA, pH 6.9), for 1 h. Following a rinse in SB, they were dehydrated in a graded ethanol series diluted with phosphate-buffered saline (PBS). Subsequently, segments were infiltrated with Steedman’s wax (a mixture of PEG 400 diestearate and 1-hexadecanol, 9:1, v/v) by an over-night incubation in absolute ethanol/wax (1:1, v/v). This was followed by incubation (6 h) in pure wax to remove residual ethanol from root tissues. Root segments were then embedded by allowing the wax to polymerize at room temperature.

Immunoblotting of protein extracts from maize roots

Root apices (8 mm long) of 4-day-old Zea mays seedlings, grown on wet filter paper and submerged in water (2 h) and in mastoparan-containing solution (10 μM, 2 h), were excised and collected into buffer containing 50 mM Tris-HCl pH 7.4, 300 mM sucrose, 5 mM KCl, NaCl and EDTA, 2 mM ascorbic acid, 10 mM freshly added DTT and a cocktail of protease inhibitors (10 μg ml−1 pepstatin A, leupeptin, aprotinin, benzamidin, 1 μg ml−1 phenanthroline). Afterwars, all segments were mechanically homogenized using mortar on dry ice. The homogenate was centrifuged at 23,000 g for 20 min at 4°C to remove cellular debris, nuclei and other large organelles. The pellet was discarded and the supernatant containing soluble and microsomal proteins was subjected to discontinuous SDS-PAGE using 15% mini slab gels at 15 μg of protein per lane. Gels were wet-blotted onto nitrocellulose and membranes were then used for immunoblotting. This was accomplished with rabbit polyclonal profilin antibodies raised against recombinant ZmPRO3 maize profilin isoform (anti-ZmPRO3; Karakesioglou et al. 1996) and maize native pollen profilins (PRA; von Witsch et al. 1998), as well as with corresponding pre-immune sera, all in a dilution of 1:200 in TTBS. Preincubations of anti-ZmPRO3 and PRA with recombinant profilins were carried out by incubating 1 ml of diluted antibody with 60 μg of ZmPRO3 and 20 μg of ZmPRO1, 2 and 4 (Gibbon et al. 1997, Gibbon et al. 1998) for 1 h at room temperature. For protein visualization, alkaline phosphatase-coupled secondary antibodies (Promega, WI, U.S.A.) were employed and visualized with Fast-Red kit of Sigma Chemicals.

Indirect immunofluorescence

Medium longitudinal 7 μm thick Steedman’s wax sections were placed on slides coated with glycerol-albumen (Serva, Heidelberg, Germany), allowing them to expand on drops of distilled water. In order to facilitate penetration of antibodies, the sections were dewaxed in ethanol, rehydrated in an ethanol/PBS series, and allowed to stand in SB for 45 min. After a 10 min rinse with absolute methanol at –20°C, they were transferred to SB for 30 min at room temperature. They were then incubated with rabbit polyclonal profilin antibodies PRA and anti-ZmPRO3, raised against native maize pollen profilins and recombinant maize profilin isoform ZmPRO3, respectively. Both antibodies and their respective pre-immune sera were applied at 1:100 dilution, made up in PBS, for 90 min at room temperature. Antibodies raised against maize H’-ATPase (provided by W. Michalk, University of Freiburg, Germany), actin (monoclonal, clone C4, ICN Biomedicals, Costa Mesa, CA, U.S.A.) and PIP2 (PerSeptive Biosystems, Inc., Framingham, MA, U.S.A.) were applied on sections in 1:200 dilutions. After another rinse in SB, the root sections were stained with FITC-conjugated anti-rabbit (PRA, ZmPRO3) and anti-mouse IgG (PIP2, actin, H’-ATPase) raised in goat (Sigma Chemical Co., St. Louis, MO, U.S.A.) diluted 1:200 in PBS for 90 min at room temperature. For double labelings, Alexa dyes (Alexa 488-FITC and Alexa 546-TRITC) were used (Molecular Probes Europe, Leiden, The Netherlands). A further rinse in PBS (10 min) preceded 10 min in 0.01% Toluidine Blue (in PBS), which diminished the natural autofluorescence of root tissues (Baluška et al. 1992).

Using anti-fade mounting containing p-phenylenediamine, root sections were mounted under a coverslip. Fluorescence was examined with an Axiovert 405M inverted light microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard FITC exciter and barrier filters (BP 450–490, LP 520). Photographs were taken on Kodak T-Max films.

Competitive inhibition and other controls for profilin and PIP2 antibodies

Immunoblocking of profilin antibodies was accomplished with a mixture of recombinant maize pollen profilins. In particular, 1 ml of diluted PRA or anti-ZmPRO3 was incubated with 60 μg of ZmPRO3 and 20 μg of ZmPRO1, 2 and 4 (Karakesioglou et al. 1996, Gibbon et al. 1997, Gibbon et al. 1998). PIP2 antibody used in the present study (PerSeptive Biosystems, Inc., Framingham, MA, U.S.A.) is in use for several years now (Fukami et al. 1988, Hay et al. 1995, Gilmore and Burridge 1996, Bubb et al. 1998, Huang et al. 1998, Kaznacheyeva et al. 2000, Mayer et al. 2000). Importantly, its specificity has been confirmed here for maize roots: 10 μl of PIP2 antibody was incubated with 20 μg of native PIP2 and this immuno-coupling negated any signal in immunofluorescence. According to the manufacturer (PerSeptive Biosystems), the PIP2 antibody may also recognize (<5%) phosphatidic acid (PA). We have tested this for maize roots using native PA (Sigma Chemicals). In contrast to PIP2, addition of 20 μg of PA to 10 μl of PIP2 antibody did not influence the labeling pattern confirming the specificity of this antibody for PIP2 in maize roots.

Additional controls included omitting the first antibody or using the appropriate pre-immune sera. No labeling was detected in these cases. Moreover, applying another rabbit polyclonal antibody raised against maize calreticulin using the same labeling procedure, resulted in completely different labeling patterns (Baluška et al. 1999).

Acknowledgements

We would like to thank C.J. Staiger (Purdue University, U.S.A.) for critical reading of our manuscript and for providing us with recombinant profilin and polyclonal ZmPRO3 antibody. Financial support by Deutsches Zentrum für Luft- und Raumfahrt (DLR, Köln) is gratefully acknowledged. F.B. is partially supported by the Grant Agency VEGA (Slovak Academy of Sciences, project No. 6030).
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Vidali, L., Pérez, H.E., López, V.V., Noguez, R., Zamudio, F. and Sánchez, F.
Mastoparan redistributes nuclear profilin


(Received September 29, 2000; Accepted June 11, 2001)