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Biochemical and immunohistochemical characterization of *Mimosa* annexin

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Abstract To characterize the biochemical properties of plant annexin, we isolated annexin from *Mimosa pudica* L. and analyzed the biochemical properties conserved between *Mimosa* annexin and animal annexins, e.g. the ability to bind phospholipid and F-actin in the presence of calcium. We show that *Mimosa* annexin is distributed in a wide variety of tissues. Immunoblot analysis also revealed that the amount of annexin is developmentally regulated. To identify novel functions of *Mimosa* annexin, we examined the pattern of distribution and the regulation of its expression in the pulvinus. The amount of annexin in the pulvinus increased at night and was sensitive to abscisic acid; however, there was no detectable induction of annexin by cold or mechanical stimulus. Annexin distribution in the cell periphery during the daytime was changed to a cytoplasmic distribution at night, indicating that *Mimosa* annexin may contribute to the nyctinastic movement in the pulvinus.

Keywords Abscisic acid · Actin · Annexin · *Mimosa* · Seismonasty

Abbreviations ABA: Abscisic acid · PC: Phosphatidylcholine · PS: Phosphatidylserine

Introduction

Annexins comprise a diverse, multigene family of calcium-dependent membrane-binding proteins that serve as targets for calcium in most eukaryotic cells (Clark and Roux 1995; Gerke and Moss 1997, 2002). Annexins have

been studied extensively in animal cells and appear to be multifunctional because they play roles in essential cellular processes such as vesicle trafficking (Emans et al. 1993; Futter et al. 1993; Seemann et al. 1996), membrane trafficking, ion transport, mitotic signaling and DNA replication (Gerke and Moss 1997; Seaton and Dedman 1998). Plant annexins share biochemical and structural properties with their animal homologs (Clark and Roux 1995; Clark et al. 2001b; Delmer and Potikha 1997). Plant and animal annexins possess a structurally similar 60- to 70-amino acid motif that is repeated four or eight times. Recent analysis has shown that *Arabidopsis* annexins are composed of seven isoforms. Comparison of the structural repeats of these seven isoforms shows that there are important differences in the repeat regions, and these differences appear to be correlated with the unique function of each isoform (Clark et al. 2001b). Annexin from *Medicago truncatula* is reported to play a role in nodulation signaling, which is a phenomenon peculiar to legumes (de Carvalho-Niebel et al. 1998, 2002). Developmentally regulated tissue distributions of *Arabidopsis* annexins (AnnAt1 and AnnAt2) have been well analyzed by in situ RNA hybridization (Clark et al. 2001a). AnnAt1 is expressed throughout the root and root hairs except at the root tips, where its expression is confined to outer cells of the root cap. Maize annexin is also known to distribute to outer cells of the root cap, and it modulates exocytosis in a calcium-dependent manner (Carroll et al. 1998). Expression of plant annexin is induced by environmental signals. Expression of alfalfa annexin, for example, is increased in response to osmotic stress, abscisic acid (ABA) or water deficiency (Kovács et al. 1998). A recent report has shown that wheat annexin accumulates in the plasma membrane in response to cold treatment and binds to the plasma membrane in a calcium-independent manner, thereby acting as an intrinsic membrane protein, similar to a calcium channel (Breton et al. 2000; Clark et al. 2001b; Kovács et al. 1998). Bell pepper annexin has been shown to have a calcium channel activity in vitro, and this activity is higher than that of any animal annexins tested to date (Hofmann

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et al. 2000). These reports indicate that plant annexins are multifunctional proteins, similar to animal annexins. Although several studies have elucidated the roles of plant annexins in the root, less is known about the physiological functions of plant annexin in other tissues.

In this study, we investigated the expression and localization of annexin in *Mimosa pudica*. To identify novel functions of *Mimosa* annexin, we focused on seismonastic and nyctinastic movements. Plant and animal annexins bind filamentous actin (F-actin) in vitro (Burgoyne and Geisow 1989; Calvert et al. 1996); however, this ability has not been elucidated in vivo. Dynamic rearrangement of actin filaments during seismonastic movement has been reported (Fleurat-Lessard et al. 1988; Morillon et al. 2001). In seismonastic and nyctinastic movements, calcium influx occurs from the apoplast through the plasma membrane (Campbell and Thomson 1977). Therefore, we examined whether plant annexin is involved in the reorganization of the actin cytoskeleton.

Materials and methods

Proteins

Actin was prepared from an acetone powder of rabbit skeletal muscle according to the method of Pardee and Spudich (1982). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Purification of annexin from *M. pudica*

Mimosa pudica L. was grown in a greenhouse under a cycle of 16 h light and 8 h darkness. Approximately 1-week-old plants were used. Annexin was purified by an established protocol (Hoshino et al. 1995) with slight modifications. Briefly, whole tissues from *M. pudica* were homogenized at 4°C in a motor-driven Teflon glass homogenizer with 4 vol. of 0.2 M Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol (2-ME), 0.4 M sorbitol, 10% glycerol, 32 mg ml⁻¹ polyvinylpyrrolidone, 5 µg ml⁻¹ leupeptin, 5 µg ml⁻¹ pepstatin-A, 50 µg ml⁻¹ *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (*p*-AP-MSF) and 50 mg ml⁻¹ BSA. The homogenate was centrifuged at 12,000 *g* for 20 min. The supernatant was precipitated in 40–70% saturated ammonium sulfate. The precipitate was dissolved in 25 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2-ME and 20 mM NaCl (Buffer A). After centrifugation at 12,000 *g* for 10 min, appropriate volumes of CaCl₂ and MgCl₂ stock solutions were added to the supernatant (Buffer B) to give final concentrations of 10 mM. Following incubation for 1 h and centrifugation at 12,000 *g* for 10 min, pellets were washed three times with Buffer B and were re-dis-

solved in Buffer A containing 5 mM EGTA. Purified annexin was obtained by centrifugation at 150,000 *g* for 1 h and was used in the experiments.

Amino acid sequence analysis

Internal sequences from *Mimosa* annexin were determined by digestion of the purified protein with trypsin. Purification of fragments and determination of amino-terminal sequences of the purified peptides were carried out at The University of British Columbia's Nucleic Acid Protein Service (NAPS) Unit.

Phospholipid-binding assay

Phospholipid-binding assays were performed by the method of Kojima et al. (1992) with slight modifications. Phosphatidylcholine (PC) alone, or a 1:1 mixture of PC and phosphatidylserine (PS; Sigma) was suspended in Tris-buffered saline (TBS; pH 7.6) and sonicated for 15 min to prepare phospholipid vesicles. Solutions (20 µl) containing phospholipid vesicles (5 µg) and annexin (1 µg) were incubated at 37°C for 1 h in the presence of 2 mM CaCl₂ or 2 mM EGTA. The mixture was centrifuged at 12,000 *g* for 10 min, and the pellets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sedimentation analysis of annexin with F-actin

Purified annexin was dialyzed against sedimentation buffer (15 mM Tris-HCl (pH 8.0), 0.1 M KCl, 0.1 mM CaCl₂, 0.1 mM ATP, 2 mM MgCl₂, 0.01% NaN₃). The dialysate was concentrated with a Centricon 10 filter (Amicon, Beverly, MA, USA) to appropriate concentration (approx. 10 µg ml⁻¹). CaCl₂ or EGTA (to a final concentration of 2 mM) was added to each sample. Rabbit skeletal muscle actin was polymerized by adding KCl to a final concentration of 0.1 M and incubation for more than 1 h at room temperature. The F-actin formed was added to each annexin-containing sample to a final concentration of 0.1 mg ml⁻¹. After incubation for 30 min at room temperature, each mixture was centrifuged at 13,000 *g* for 15 min, and supernatants and precipitates were analyzed by SDS-PAGE.

Electron microscopy

Samples prepared as previously described in the sedimentation analysis with F-actin were mounted on Formvar-coated grids and negatively stained with 1% uranyl acetate. Each sample was examined and photographed with a LEO 906 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

SDS-PAGE and immunoblot analysis

Purified *Mimosa* annexin was resolved by SDS-PAGE (Laemmli 1970) followed by electrophoretic elution of annexin peptide from the gels. Annexin peptide was dialyzed in PBS and used as antigen. Rabbits were immunized with the peptide in Freund's complete adjuvant and were boosted twice with peptide in Freund's incomplete adjuvant. Serum containing anti-*Mimosa* annexin antibody was purified by incubating the serum with nitrocellulose-bound antigen and then eluting it at low pH (Sambrook et al. 1989). For immunoblot analysis, proteins were subjected to SDS-PAGE on 12.5% acrylamide gels and were electrophoretically transferred to nitrocellulose membranes (0.45- μ m pore size; Schleicher & Schüll, Dassel, Germany) at 100 mA for 1 h. The membranes were incubated in 5% skimmed milk (Snow Brand Milk Products Co., Tokyo, Japan)/TPBS (0.05% Tween 20 in PBS). After washing with TPBS (three times for 5 min each), the membranes were incubated with an affinity purified anti-*Mimosa* annexin antibody (diluted 1:150), and/or anti-actin monoclonal antibody (diluted 1:2,000, clone C4; ICN Pharmaceuticals, Costa Mesa, CA, USA), followed by incubation with secondary antibodies: horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted 1:2,000; DAKO, Kyoto, Japan) for anti-*Mimosa* annexin antibody or HRP-conjugated anti-mouse IgG₁ (diluted 1:2,000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for anti-actin antibody. Detection was performed with an ECL detection kit (Amersham Pharmacia Biotech).

Injection of ABA into the pulvinus

To expose cells in the pulvinus to ABA, stems with one pulvinus were cut down, and the lower terminal was connected to a tube filled with 3% ethanol containing ABA. The other end of the tube was connected to a syringe including the same solution. The solution was injected until several drops came out from the top end of the cut petiole at 4°C. The stems were maintained in this manner for 1 h, and the pulvinus was cut off and used for immunoblot analysis.

Immunocytochemistry

For immunofluorescence, the pulvini from 1-month-old plants were cut out and fixed overnight in 3% paraformaldehyde, frozen in Tissue-Tek OCT compound (Miles, Elkhart, IN, USA), and cut into 20- μ m sections using a cryostat (model 505E; Leica Microsystems, Wetzlar, Germany). The sections were placed onto glass microscope slides, air-dried, fixed again in -20°C methanol for 5 min, and permeabilized at room temperature in 0.1% Triton X-100 for 5 min. After washing with PBS, nonspecific binding was blocked with 10%

normal goat serum and 5% BSA in TPBS for 30 min. Affinity-purified anti-*Mimosa* annexin antibody was used as the primary antibody (diluted 1:50), and the secondary antibody was Texas red-conjugated anti-rabbit IgG antibody (diluted 1:150; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Specimens were examined with a confocal microscope (LSM410; Carl Zeiss).

Partial purification of cytosol and membrane fractions

Cytosol and membrane fractions were prepared according to an established method (Larsson et al. 1987). Briefly, main pulvini of *Mimosa* (1.5 g) were harvested at 9:00 a.m. or 9:00 p.m. and homogenized in 9 ml 0.2 M Tris-HCl buffer (pH 8.0) containing 0.5 M sucrose, 8 mg ml⁻¹ polyvinylpyrrolidone, 5 mM 2-ME, 10% glycerol, 10 μ g ml⁻¹ *p*-APMSF, 5 μ g ml⁻¹ leupeptin and 5 μ g ml⁻¹ pepstatin. The homogenate was centrifuged at 10,000 *g* for 10 min to remove cell debris. Cytosol and membrane fractions were separated by further centrifugation at 50,000 *g* for 30 min. The precipitated membrane fraction was dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS and 0.72 M 2-ME. Both cytosol and crude membrane fractions were used to detect the distribution of annexin by immunoblot analysis. Tubulin, a cytosol marker, was detected with anti- α -tubulin monoclonal antibody, (diluted 1:10,000; clone DM 1A; Sigma) and HRP-conjugated secondary antibody against mouse IgG₁.

Results

Purification of annexin from *M. pudica*

Accumulating molecular biological evidence has revealed that plant annexins are distributed in a wide variety of tissues in which each isoform has a unique function (Kovács et al. 1998). Less has been reported about the biochemical properties of plant annexin. To analyze the biochemical properties of annexin, we purified annexin from *M. pudica* (Fig. 1). Annexins are known to form oligomeric complexes in the presence of high concentrations of calcium. Thus, repeated centrifugation in the presence or absence of calcium effectively concentrated a 35-kDa protein (p35) from crude plant extracts (Fig. 1, lane 3). Isolated p35 exists as a monomer, as determined by comparing the elution time of annexin to that of molecular weight standards from high-performance gel chromatography (HPLC; TSK-gel G2000SW; Tosoh, Tokyo, Japan—data not shown).

To characterize p35, we analyzed partial amino acid sequences of p35 and obtained three internal peptide sequences (Fig. 2). All of these peptide sequences showed high homology with other plant annexins, suggesting that p35 belongs to the annexin family.

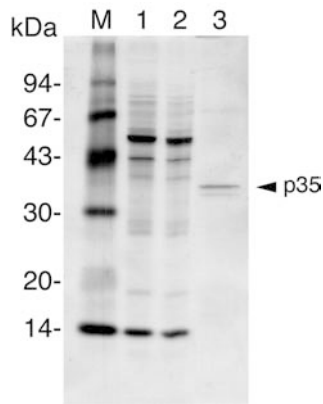


Fig. 1 Purification of p35 (annexin) from *Mimosa pudica*. Total extract (lane 1) of whole tissues of *M. pudica* was precipitated in 40–70% saturated ammonium sulfate (lane 2). The precipitate was dissolved in Buffer A. After clarification by centrifugation, annexin was concentrated by addition of 10 mM CaCl_2 and MgCl_2 to the supernatant. Precipitate containing annexin was dissolved in solution without calcium and was further purified by centrifugation. A 35-kDa protein (*p35*) was obtained in the supernatant (lane 3). Samples were resolved by SDS-PAGE (12.5% gels) and stained with Coomassie brilliant blue. *M* Molecular weight markers

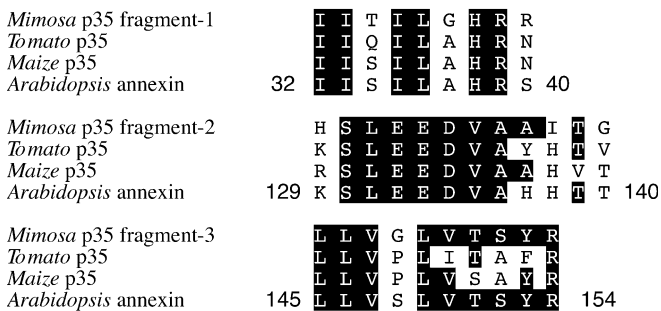


Fig. 2 Alignment of internal amino acid sequences of p35 from *M. pudica* with plant annexins. Internal amino acid sequences of p35 (fragments 1, 2 and 3) were aligned with partial sequences of tomato (*Lycopersicon esculentum*) p35 (Ac# T06322), maize (*Zea mays*) p35 (Ac# T02975) and *Arabidopsis thaliana* annexin (Ac# CAA67608). Numbers denote amino acid position of *Arabidopsis* annexin. *Highlighted residues* indicate identical residue to *Mimosa p35*

Phospholipid-binding properties

Annexin family members bind to phospholipid in the presence of calcium. To confirm that p35 from *M. pudica* possesses phospholipid-binding properties, binding to phospholipids such as phosphatidylcholine (PC) and a 1:1 mixture of PC and phosphatidylserine (PS) was examined. After incubation of p35 with phospholipids, lipid complexes were precipitated by centrifugation. Aliquots of supernatants and precipitated proteins were analyzed by SDS-PAGE. p35 bound to PS but not to PC, as shown in Fig. 3a. Addition of EGTA at the same concentration as calcium reduced the phospholipid-

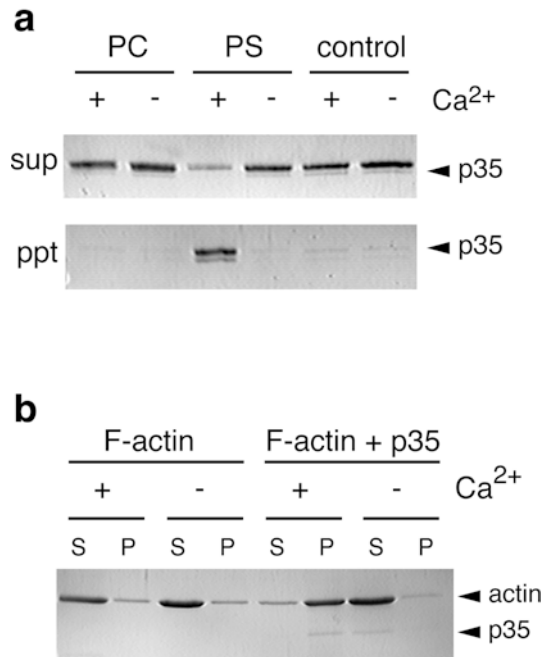


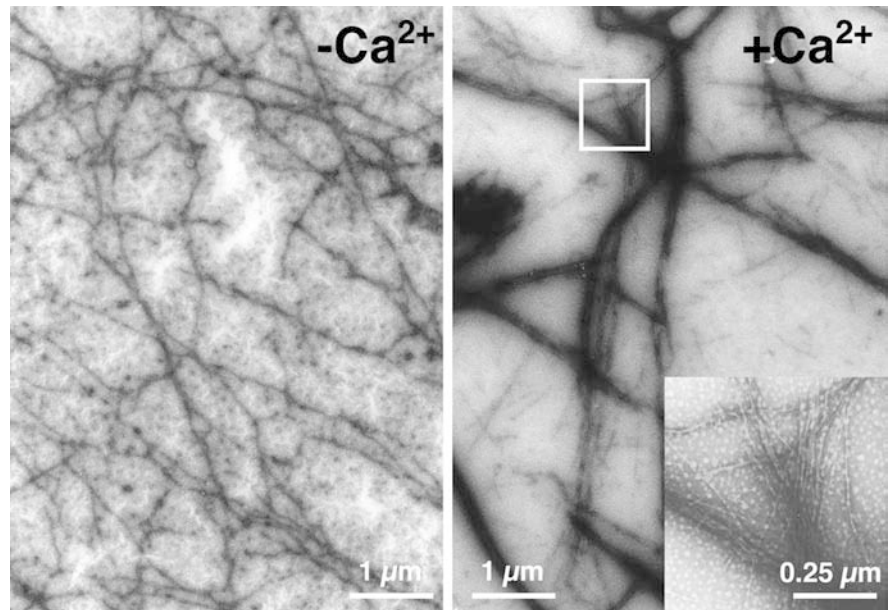
Fig. 3a, b Biochemical characterization of p35. **a** Ability of *Mimosa* annexin to bind to phosphatidylcholine (PC) alone or a 1:1 mixture of PC and phosphatidylserine (PS). Aliquots (20 μl) containing phospholipid vesicles (5 μg) and *Mimosa* annexin (1 μg) were incubated at 37°C for 1 h in the presence of 2 mM CaCl_2 ($+\text{Ca}^{2+}$) or 2 mM EGTA ($-\text{Ca}^{2+}$). The mixture was centrifuged, and the supernatant (*sup*) and pellet (*ppt*) were subjected to SDS-PAGE. *Control* Without phospholipid. **b** Sedimentation analysis of p35 with F-actin. Samples of F-actin alone or F-actin plus p35 in the presence of 2 mM CaCl_2 ($+\text{Ca}^{2+}$) or 2 mM EGTA ($-\text{Ca}^{2+}$) were centrifuged. The resulting supernatants (*S*) and precipitates (*P*) were subjected to SDS-PAGE

binding activity of p35 to PS, suggesting that the binding is sensitive to calcium.

Interaction of *Mimosa* annexin with F-actin in vitro

Tomato annexin binds to actin in a calcium- and pH-dependent manner, and the binding is specific for F-actin (filamentous), not G-actin (globular and monomeric) (Calvert et al. 1996). To examine the binding ability of p35 to F-actin, we performed a sedimentation assay. F-actin was incubated with or without p35 in the presence or absence of calcium for 30 min at room temperature. After centrifugation at 130,000 g for 15 min, the supernatants and pellets were subjected to SDS-PAGE (Fig. 3b). In the presence of F-actin alone or F-actin plus p35 in the absence of calcium, actin was detected in the supernatant. However, a large amount of actin was precipitated in the presence of p35 and calcium. These results further indicate that p35 is a *Mimosa* annexin. The p35 (*Mimosa* annexin)-F-actin complex was then examined by electron microscopy. Actin filaments in the presence of *Mimosa* annexin formed thick bundles in a calcium-dependent manner (Fig. 4). To our

Fig. 4 Electron micrographs of negatively stained F-actin. *Mimosa* annexin was added to F-actin solution in the presence of 2 mM EGTA ($-Ca^{2+}$) or 2 mM $CaCl_2$ ($+Ca^{2+}$). *Right panel* The *insert* is a high-power view of the *boxed area*, showing actin bundles in the presence of Ca^{2+} and *Mimosa* annexin



knowledge, this is the first report that plant annexin forms actin filament bundles in vitro.

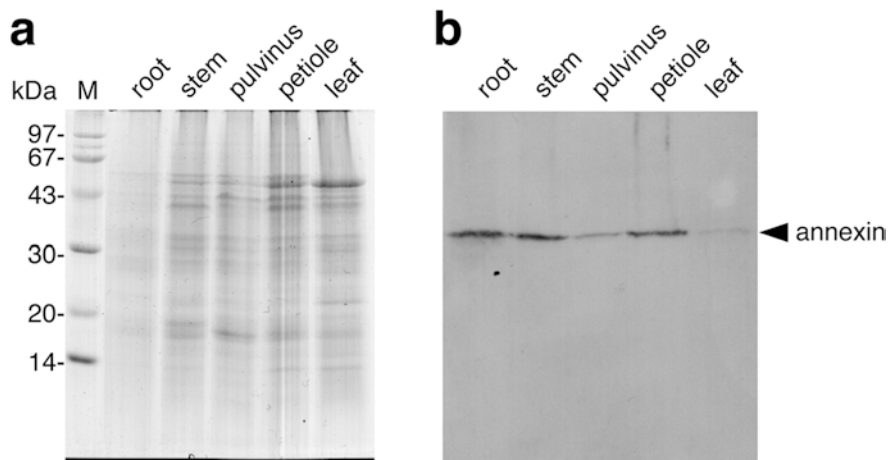
Analysis of annexin distribution

Northern blot analysis of annexins from various plant species has shown that most annexins are widely expressed (Clark et al. 2001a; Gidrol et al. 1996; Proust et al. 1999), with the exception of annexin from *Medicago truncatula*, which was detected only in the root and is involved in nodulation signaling (de Carvalho-Niebel

et al. 1998). Plant annexins in the root have been studied extensively. In this study, a polyclonal antibody against *Mimosa* annexin was used to investigate annexin distribution. Immunoblot analysis of several tissue extracts was performed (Fig. 5). *Mimosa* annexin was detected in all tissues, with the highest level in the root. Less annexin was detected in leaf extracts.

To identify the function of annexin, the change in the amount of daytime (9:00 a.m.) annexin during development was examined by immunoblot analysis (Fig. 6a), using actin as an internal standard. The amount of annexin from whole plants increased significantly and peaked at 5 days after seeding (DAS), and then decreased (Fig. 6b). The cotyledon has extended by 5 DAS, and the first leaf with a pulvinus responds to various stimuli by 10 DAS. Our results indicate that expression of *Mimosa* annexin is developmentally regulated and may play a role in developmental events, although the exact contribution of annexin is not known.

Fig. 5a, b Immunoblot analysis of tissue extracts. Protein staining (a) and immunoblot analysis (b) of extracts from roots (*root*), stems (*stem*), pulvini (*pulvinus*), petioles (*petiole*) and leaves (*leaf*). *M* Molecular weight markers. Each tissue extract was homogenized in Laemmli's SDS sample solution (Laemmli 1970), and clarified by centrifugation at 12,000 *g* for 20 min. Approximately 10 μ g of each extract was subjected to SDS-PAGE. Affinity-purified polyclonal antibody raised against *Mimosa* annexin was used for immunoblotting



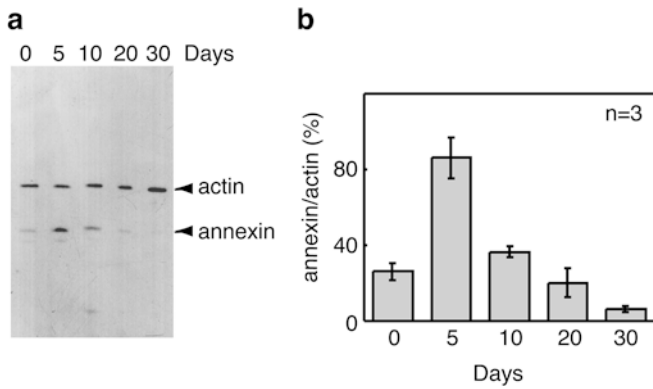


Fig. 6a, b The change in the amount of annexin is developmentally regulated. **a** Representative immunoblot showing developmentally regulated change in the amount of *Mimosa* annexin. Samples (approximately 10 μ g) were prepared from plants harvested at appropriate days after seedling, as described in the legend for Fig. 5. Amounts of annexin and actin were analyzed by double immunoblotting using anti-*Mimosa* annexin antibody and anti-actin antibody (C4), respectively. **b** The amount of annexin relative to an actin internal standard was quantified from three separate experiments. Annexin increased significantly from 0 to 5 days after seedling and decreased significantly thereafter. Data are means \pm SD; a one-way factorial analysis of variance (ANOVA, $P < 0.001$) was used to analyze the results

Annexin levels in the pulvinus change in response to ABA

Annexin is a multifunctional protein with potentially unique roles in each tissue. Expression of annexin is activated by several stresses and by the addition of exogenous ABA (Kovács et al. 1998). Thus, we analyzed the level of annexin in the pulvinus under various conditions (Fig. 7). Figure 7a shows no appreciable changes in the amount of annexin in response to cold stress, maintenance in the dark, or mechanical stimulation. Interestingly, the amount of annexin at night (9:00 p.m.) was much higher than that in the daytime (9:00 a.m.). To examine whether ABA can affect the amount of annexin in the pulvinus, we injected the indicated concentrations of ABA directly into the pulvinus during the daytime. As shown in Fig. 7b, the amount of annexin increased in an ABA-dependent manner, with the highest level at 75 μ M ABA.

Localization of annexin in the pulvinus of *M. pudica*

Affinity-purified rabbit polyclonal antibody against *Mimosa* annexin was used to examine the localization of *Mimosa* annexin in the pulvinus (Fig. 8). *Mimosa* annexin was localized in the outermost periphery of motor cells of the pulvinus during the daytime, and it was detected in the cytoplasm at night. There was no obvious difference in localization before and after seismonastic movement (data not shown). To confirm the day/night change in localization, annexin in the membrane and cytosolic fractions was examined by immunoblot anal-

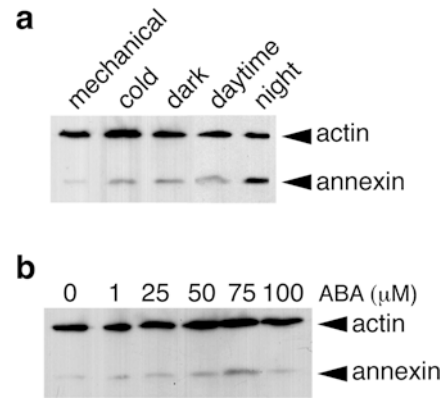


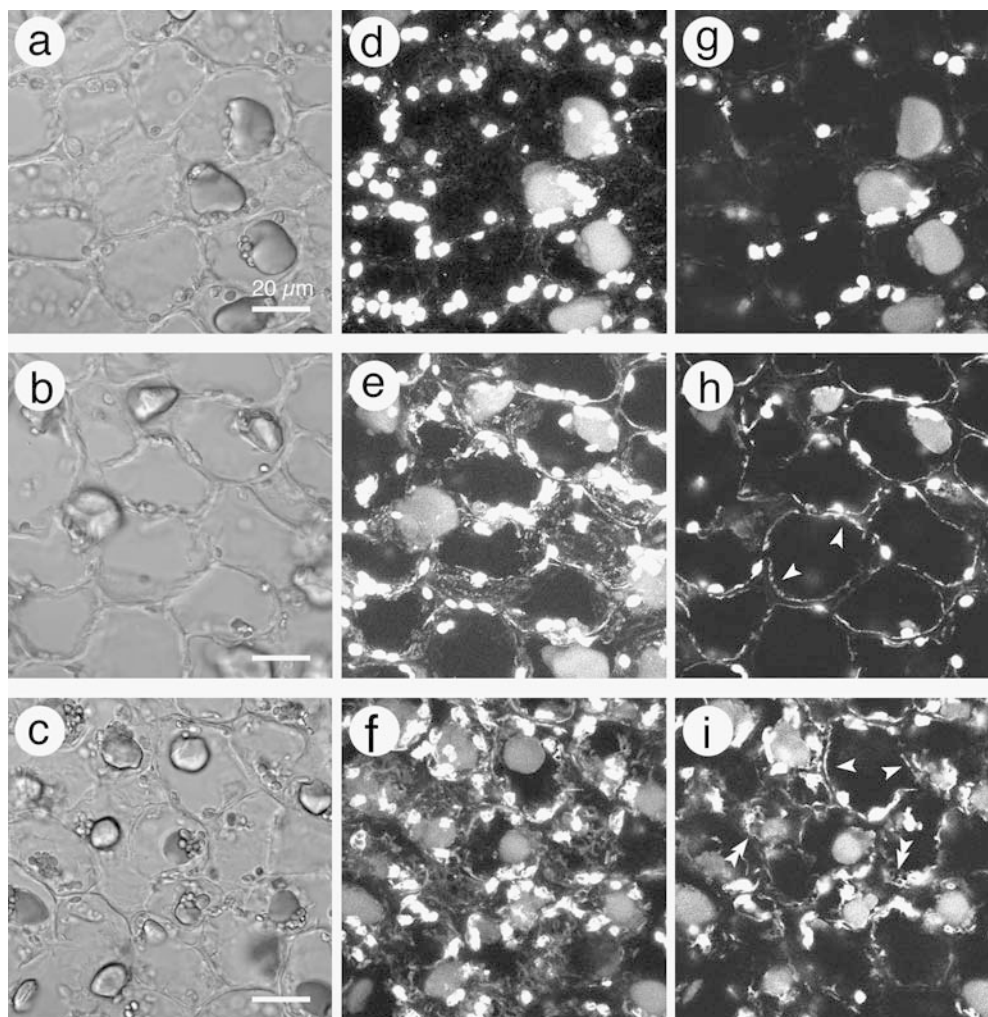
Fig. 7a, b Induction of *Mimosa* annexin in the pulvinus under various conditions. Induction of *Mimosa* annexin in the pulvinus was detected by double immunoblotting. Samples (approximately 10 μ g) were prepared as described in the legend for Fig. 5. **a** Plants were harvested just after touching (*mechanical*), after maintaining at 4°C for 2 h under approx. 8,000 lux of light (*cold*), after maintaining at 4°C for 2 h in the dark (*dark*) during the daytime, or harvested at 9:00 a.m. (*daytime*) or 9:00 p.m. (*night*). **b** The effect of ABA on induction of *Mimosa* annexin was examined 1 h after injection of ABA at the indicated concentrations (see Materials and methods)

ysis (Fig. 9). Annexin was recovered from the membrane fraction but not from the cytosolic fraction, regardless of the sampling time, suggesting the association of annexin with cytoplasmic organelles and membranes.

Discussion

In this study, we purified a 35-kDa protein from the higher plant *M. pudica* to elucidate the biochemical properties of plant annexin. Analysis of the partial amino acid sequence of the isolated 35-kDa protein revealed that it has high homology with other plant annexins. Annexins comprise a multigene family (Clark et al. 2001a), and several isoforms of annexin have been shown by biochemical and molecular biological studies to be expressed in plants. Annexins from tomato and maize have different molecular weights because they are derived from different gene products (Smallwood et al. 1990; Battey et al. 1996). However, in cotton fibers, the 34-kDa annexin band is made up of three isoforms with isoelectric points ranging from 6.1 to 6.5 (Andrawis et al. 1993). These different isoforms may represent a single gene product that has undergone posttranslational modification, such as phosphorylation. In this work, we identified at least one isoform of annexin in *Mimosa*. However, a minor band is present just below that of p35 annexin in Fig. 1, lane 3. Purified anti-*Mimosa* annexin antibody reacted specifically with p35 annexin (Fig. 5) but not with the lower peptide (data not shown), suggesting that the lower peptide is not a degradation product but may be another isoform of *Mimosa* annexin. In the present study, we could not determine the amino acid sequence of the peptide because of the low

Fig. 8a–i Immunolocalization of *Mimosa* annexin in the pulvinus. Twenty-micron sections were prepared from the pulvinus. The figure shows bright-field images (a–c), confocal images (d–f) and single-plane confocal images (g–i) of motor cell in the pulvinus. **d,g** Nonspecific staining by secondary antibody. Large and small spherical patches are autofluorescence of tannin vacuoles and chloroplasts, respectively. The pulvinus was cut off during the daytime (b,e,h) or at night (a,c,d,f,g,i), and fixed quickly with paraformaldehyde. Localization of *Mimosa* annexin was examined with anti-*Mimosa* annexin antibody (d–i). *Mimosa* annexin is localized to the periphery of motor cells during the daytime (h, arrowheads), and it was detected predominantly in the cytoplasm (i, double-arrowheads) and the periphery (i, arrowheads) of motor cells at night. Bars = 20 μ m



amounts of the peptide. Annexin family members exert a calcium-dependent phospholipid-binding activity. In the present study, we showed that *Mimosa* annexin exhibits calcium-dependent binding to acidic phospholipid. We also investigated the effect of other metal ions on phospholipid-binding activity. As determined by sedimentation analysis (data not shown), in the presence of nickel or zinc, *Mimosa* annexin bound to a 1:1 mixture of PC and PS but not to PC, similar to animal annexin (Kojima et al. 1992). Vertebrate annexins localize to the submembranous region at cytoskeletal anchoring sites, and interact with actin or cytoskeletal binding proteins, indicating a possible role for annexin in cytoskeletal rearrangement (Gerke and Moss 1997; Hawkins et al. 2000). Plant annexins also interact with F-actin (Calvert et al. 1996; Hu et al. 2000). *Medicago* annexin is thought to play a role in the rearrangement of the cytoskeleton during the early stage of nodulation (de Carvalho-Niebel et al. 2002). In our study, we showed that *Mimosa* annexin binds to F-actin in the presence of calcium and also forms bundles of F-actin in the presence of calcium in vitro. However, the distribution of *Mimosa* annexin is not identical to that of actin, which forms fibrous net-

works in the cytoplasm of the motor cells (Fleurat-Lessard 1990). To identify physiological functions of *Mimosa* annexin, we examined the distribution of annexin by immunoblot analysis. *Mimosa* annexin was distributed in all tissues examined, with the highest level in the root, similar to *Arabidopsis* annexin (Clark et al. 2001a). Annexin is known to show diverse functions in different tissues. *Mimosa* annexin may have specific roles in nyctinastic and/or seismonastic movements.

The pulvinus is located at the base of the petioles and is involved in two interesting movements of the *Mimosa* plant, which responds to external stimuli. One is a nyctinastic movement, similar to that of other legumes. The other is a seismonastic movement, in which petioles bend rapidly in response to mechanical, thermal, electrical or chemical stimuli. In both cases, loss of osmotic pressure from motor cells in the pulvini is thought to cause these movements. To determine if *Mimosa* annexin is involved in these movements, the protein levels of annexin in the pulvinus were examined under several conditions. We showed that much more annexin was detected at night than that during daytime. Expression of annexin is also regulated by environmental signals.

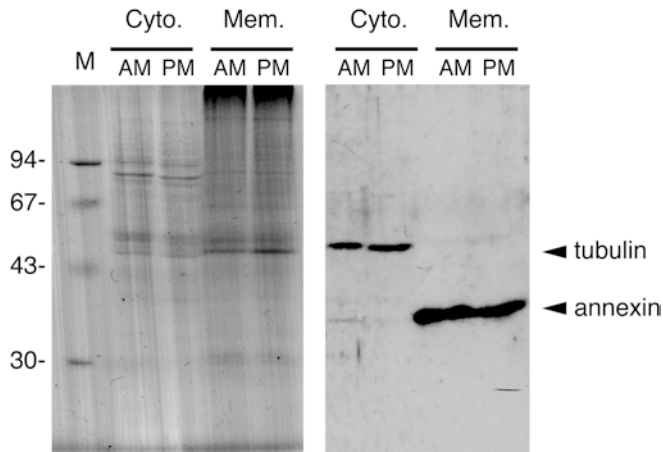


Fig. 9 Immunoblot analysis of cytoplasm and membrane fractions from *Mimosa pudica*. Cytoplasm (Cyto.) and membrane (Mem.) fractions were prepared from main pulvini harvested at 9:00 a.m. (AM) or 9:00 p.m. (PM). Samples (15 µg/lane) were separated on 10% acrylamide gels and stained with Coomassie brilliant blue (left panel) or blotted onto nitrocellulose membranes and detected with specific antibodies (right panel). *Mimosa* annexin and tubulin were detected by double immunoblotting with anti-*Mimosa* annexin polyclonal antibody and anti- α -tubulin monoclonal antibody. M Molecular weight markers

For example, alfalfa annexin mRNA is increased in response to osmotic stress and ABA (Kovács et al. 1998). Therefore, we examined the effect of ABA on the expression of annexin by injecting ABA directly into the pulvinus, and found that the level of *Mimosa* annexin protein is increased by ABA. Study of the *Mimosa* and *Cassia* pulvini indicates that ABA induces leaflet closing (Bonnemain et al. 1978; Bourbouloux et al. 1994; Everat-Bourbouloux et al. 1990). In *Phaseolus*, shrinkage of pulvinar protoplasts in response to 1 µM ABA has also been reported (Iino et al. 2001). Treatment of epidermal peels of day flower (*Commelina communis*) with 10 µM ABA affects the actin cytoskeleton of guard cells and results in stomatal closure (Eun and Lee 1997). This led us to speculate that *Mimosa* annexin participates in ABA signaling during nyctinastic movements.

Involvement of annexin in nyctinastic movements was further analyzed by immunohistochemical techniques. In most cases, plant annexin is localized to intracellular membranes or to the cytoplasm (Blackbourn et al. 1992; Clark et al. 1992, 1994; Clark and Roux 1995; Seals and Randall 1997; Thonat et al. 1997), with the exception of alfalfa annexin, which is localized to the nucleus (Kovács et al. 1998). Annexin from *Bryonia dioica* is detected throughout the cytoplasm in parenchymal cells from untreated internodes and accumulates near the plasma membrane 30 min after rubbing (Thonat et al. 1997). *Mimosa* annexin is localized to the periphery of pulvinar cells during the daytime. However, we observed a drastic change in distribution at night. As shown in Fig. 8, strong signals were detected predominantly in the cytoplasm at night. Whether this result reflects a change in the distribution of annexin or an

increase in expression levels in the cytoplasm could not be determined by our indirect immunofluorescence analysis. The distribution was examined by immunoblot analysis of partially purified cytosol and membrane fractions. Tubulin immunolabeling was used as a cytosolic marker. Annexin was recovered not in the cytosol but in the membrane fraction, regardless of sampling time. Because the membrane fraction contained plasma membrane as well as Golgi, endoplasmic reticulum and thylakoid membranes, further investigations are needed to determine which part of the membrane fraction is associated with annexin. Since osmotically regulated recycling of the plasma membrane has been reported in plant cells (Homann 1998; Zorec and Tester 1993), one possible function of *Mimosa* annexin may be in membrane recycling during nyctinastic movements, which are caused by a loss of turgor pressure of motor cells in the pulvini. Taken together, *Mimosa* annexin appears to contribute to the nyctinastic movement; however, further analysis is necessary to prove this.

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