Plant Physiol. (1997) 114: 981–988

Effects of a Mechanical Stimulation on Localization of Annexin-Like Proteins in *Bryonia dioica* Internodes

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Mechanical stimulation exerted by rubbing a young internode of *Bryonia dioica* plants inhibits its growth. Previous cellular and biochemical studies showed that this growth inhibition is associated with Ca^{2+} redistribution and profound modifications of plasma membrane characteristics. We extracted and purified Ca^{2+}-dependent phospholipid-binding proteins from *B. dioica* internodes. Two main proteins, p33 and p35, and other minor bands were isolated and identified as annexin-like proteins because of their biochemical properties and their cross-reactions with antibodies against maize (*Zea mays* L.) annexins. Rabbit antiserum was obtained by injection of *B. dioica* p35. This antiserum was used for the immunocytolocalization of annexin-like proteins in internode parenchyma cells. It appeared that the distribution of annexin-like proteins was different before and 30 min after the mechanical stimulation. Western analysis of proteins in membrane fractions after separation by free-flow electrophoresis showed that p35 was present in most fractions, whereas p33 appeared mainly in plasmalemma-enriched fractions after the mechanical stimulation. It is hypothesized that a subcellular redistribution of these proteins might be involved in growth inhibition by mechanical stress.

Ca^{2+} is involved in the regulation of several physiological processes in higher plants and is generally regarded as a second messenger (Hepler and Wayne, 1985; Bush, 1995). Many external or endogenous stimuli can induce an increase in the cytoplasmic free Ca^{2+} concentration, which triggers a variety of cellular responses by activating Ca^{2+}-dependent proteins (Hepler and Wayne, 1985). The best-characterized Ca^{2+}-modulated proteins from plants are calmodulin and Ca^{2+}-dependent, calmodulin-independent protein kinases (Poovaiha and Reddy, 1993). Annexins are among the other Ca^{2+}-binding proteins known in animals (Geisow and Walker, 1986; Crumpton and Dedman, 1990) and in a few plants (Smallwood et al., 1990; Blackbourn et al., 1992; Clark and Roux, 1995).

Annexins belong to a family of structurally related proteins that bind phospholipids in a Ca^{2+}-dependent manner (Haigler et al., 1989). All members of the annexin family share a conserved 70-amino acid repeating unit that contains the endonexin fold (a highly conserved consensus sequence). The different annexins are distinguished by their highly variable N-terminal region. This region, which varies in both sequence and length (Barton et al., 1991), is believed to determine the function of each particular annexin type. Several potential functions have been assigned to annexins based on in vitro properties. Various members of the family have been shown to be involved in membrane-cytoskeleton interactions (Huber et al., 1990), membrane-fusion events in exocytosis (Clark et al., 1992; Creutz, 1992) and endocytosis (Lin et al., 1992; Blackbourn and Battey, 1993), regulation of cell-surface receptors, and intracellular signaling as substrates for Tyr kinase and protein kinase C (Glenney, 1986; Rojas et al.,1990; Sarafian et al., 1991).

Mechanical stimulation exerted on young internodes of *Bryonia dioica* reduces their elongation and increases their radial expansion (Boyer et al., 1979). This process is known as thigmomorphogenesis, a developmental response in plants to a mechanical stress (Jaffé, 1973). Previous studies have shown that this process involves a rapid increase of peroxidase activity, an acceleration of lignification (De Jaegher et al., 1985; De Jaegher and Boyer, 1987), and a transient increase of ethylene production (Boyer et al., 1983, 1986). The external stimulation affects the plasma membrane H^+-ATPase activity and induces an increase of plasma membrane fluidity with modifications of some membrane components (Bourgeade and Boyer, 1994; Mathieu et al., 1995). A rapid leakage of Ca^{2+} from the plasma membrane surface, followed by an influx of the ion into the cytoplasm (Thonat et al., 1993), as well as changes in Ca^{2+}-ATPase activity have also been observed after rubbing of *B. dioica* internodes. This suggests a role of Ca^{2+} in thigmomorphogenic signal transduction (Bourgeade et al., 1991). Moreover, the mechanical stimulation induces the expression of calmodulin-encoding mRNA (Galaud et al., 1993). The obvious involvement of Ca^{2+} and of calmodulin in the early responses of *B. dioica* to wounding prompted us to look for other Ca^{2+}-dependent proteins that could participate in the thigmomorphogenic process. We describe here the extraction and purification of two annexin-like proteins and their localization in stem cells before and after mechanical stimulation of *B. dioica* inter-

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Abbreviation: FFE, free-flow electrophoresis.

nodes. The data obtained show that the subcellular distribution of these proteins was modified after internode rubbing.

MATERIALS AND METHODS

Bryonia dioica plants were grown from roots placed in 50-L containers with compost. They were kept for several weeks under a 16-h photoperiod supplied by fluorescent tubes (LJL TF65, Mazda, Mazda-Eclairage Chalons, Saône, France) giving an irradiation of 60 μmol m^{-2} s^{-1} with a temperature regime of 25°C (light)/20°C (dark) and 60 to 80% RH. Mechanical stimulation (rubbing) was performed on the youngest internodes by holding them between the thumb and the forefinger while gently rubbing up and down for several seconds. Rubbed internodes were used for protein or membrane extraction and for microscopic observation. Similar untreated internodes were used as controls.

Purification of Annexin-Like Proteins

Annexins were purified as described by Boustead et al. (1989) with the following modifications: *B. dioica* internodes were homogenized in a mortar in a 20 mM Hepes buffer brought to pH 7 with NaOH and containing 0.15 M NaCl (3 mL g^{-1} fresh weight). The homogenate was filtered through a 100-μm nylon cloth. EGTA (0.2 M) was added to the filtrate to give a final concentration of 10 mM before centrifugation at 7,000*g* for 45 min at 4°C. $CaCl_2$ (2 M) was added to the resulting supernatant to give a final concentration of 15 mM. A fresh suspension of phospholipids, prepared by homogenization of bovine brain extract (type VII, Sigma) in 1.8 mL of distilled water in a glass homogenizer, was added to the supernatant. After 30 min on ice, the suspension was centrifuged for 30 min at 18,000*g*. The pellet was resuspended and washed twice in 20 mM Hepes buffer, pH 7, containing 3 mM $CaCl_2$. Each wash was followed by centrifugation for 30 min at 18,000*g*. The final pellet was resuspended in 20 mM Hepes buffer, pH 7, containing 30 mM EGTA. After 30 min on ice, the suspension was centrifuged for 1.5 h at 100,000*g*. The supernatant was then loaded on a column of DEAE-Sephacel (Pharmacia) and equilibrated in Hepes buffer, pH 7, containing 5 mM EGTA. The column was eluted with a linear NaCl gradient (0–300 mM). The A_{280} was determined in each 2-mL fraction.

SDS-PAGE and Western Analysis

Analysis by SDS-PAGE was performed on 12.5% polyacrylamide gels as described by Laemmli (1970) with molecular weight standards from Sigma and from Bio-Rad (prestained standards). Proteins separated by SDS-PAGE were electrotransferred onto 0.45-μm nitrocellulose membrane (Sigma) as described by Towbin et al. (1979) or stained with silver nitrate according to the method of Blum et al. (1967).

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was carried out as described by O'Farrel (1975) modified by Hochstrasser (1988). After DEAE-Sephacel chromatography, fractions containing the p33 and p35 annexin-like proteins were concentrated and made free of salt by using a microconcentrator (Centricon 10, Amicon, Beverly, MA). Urea up to a final concentration of 8 M, 2% Triton X-100, 2% resolytes (pH 4–8; BDH, Poole, UK), and 0.5% dithiotreitol were added to the sample. pI markers (Pharmacia) were used to determine the pH gradient in first-dimension gels. Gels were stained with Coomassie blue.

Preparation of Antisera

Proteins were recovered from gels with a Biotrap (Schleicher & Schuell). Electroelution was performed at 100 V overnight in buffer (25 mM Tris and 129 mM Gly, pH 8.3). The protein sample was mixed with Freund's adjuvant (complete for the first injection and incomplete for the last two) and 60 μg of proteins was injected into a New Zealand White rabbit three times at weekly intervals. Antiserum was collected 10 d after the last injection.

Western Analysis

Proteins from different fractions obtained after elution of a DEAE-Sephacel column were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated in TBS (20 mM Tris and 137 mM NaCl, pH 7.6, with HCl) containing 0.075% Tween 20 and 0.1% BSA for 2 h, and then washed three times in TBS containing 0.05% Tween 20 before a 3-h incubation in antiserum diluted (1:800) in TBS containing 0.075% Tween 20 and 0.1% BSA at room temperature. After three washes with TBS-0.05% Tween 20, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, 1:8000 dilution) for 2 h at room temperature. Three washes were performed, two with TBS/0.05% Tween 20 and the last one with TBS alone, before staining the membrane with the alkaline phosphatase substrate kit (Bio-Rad).

Immunocytolocalization

Tissue Preparation

B. dioica internode segments (3 mm long) were cut and fixed in 100 mM phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde and 500 mM Suc. After 2 h of fixation the internodes were thoroughly washed in the same buffer and kept overnight at 4°C in 100 mM phosphate buffer containing 1 M Suc. The samples were then frozen at −60°C using an ethanol-CO_2 freezing mixture and embedded in Reten (Henkel, Boulogne Billancourt, France) according to the method of Gahan et al. (1967).

Sectioning

Serial transverse sections 10 μm thick were cut on a motor-driven cryostat (model 5030, Bright Instruments Co.,

Huntingdon, UK) and placed onto gelatin-coated glass slides.

Immunofluorescence

Sections were removed from the cryostat cabinet and dried for 1 h at room temperature. They were then incubated for 20 min in TBS supplemented with 0.5% Triton X-100, and washed three times for 10 min each time in 10 mM Gly in TBS, then for 20 min in undiluted goat serum to block the nonspecific-binding sites of the tissue. Sections were incubated overnight at 4°C in a moist chamber in antiserum diluted 1:40 in TBS containing 0.02% Triton X-100 and 10% goat serum. After removal of the antiserum the sections were washed several times with 10 mM Gly in TBS and treated with undiluted goat serum. They were incubated for 2 h at room temperature in fluorescein isothyocyanate-conjugated goat anti-rabbit IgG (Sigma). The sections were washed again three to four times for 10 min each time with TBS. To cancel the autofluorescence of the tissue, the sections were transferred into 0.001% Evans blue diluted in TBS for 10 min. After several rinses, they were finally mounted in polyvinyl alcohol medium as described by Lenette (1978). The specificity of immunolabeling was assessed by omission in the procedure described above of the first antibody. The sections were observed with a photomicroscope (Laborlux S, Leicam, Wetzlar, Germany) equipped with a 50-W mercury vapor lamp and an I 2/3 block filter (excitation filter BP 450–490, suppression filter LP 520, Leitz). Photographs were taken with a Mikrophot Wild MP0–5 camera (Leica) using Tri-X Pan film (ASA 400, Kodak).

Membrane Extraction

A microsome preparation was obtained by the method described by Canut et al. (1988), with some modifications. Internodes (25 g) were homogenized in an extraction buffer containing 100 mM Hepes, 500 mM Suc, 10 mM KCl, 1 mM $MgCl_2$, 1 mM ascorbate, 1 mM PMSF, and 2 mM DTT, pH 7.5, at a ratio of 1 mL g^{-1} fresh weight. After a first centrifugation at low speed (4,000*g*), microsomes were collected by centrifugation at 30,000*g* for 30 min. The pellet was resuspended in an FFE chamber buffer (0.015 M triethanolamine, 0.04 M potassium acetate, 0.01 M Glc, 0.25 M Gly, and 0.03 M Suc brought to pH 7.5 with acetic acid). After centrifugation at 30,000*g* for 30 min the pellet was resuspended in 2 mL of chamber buffer and injected into the electrophosesis chamber.

FFE

The FFE separation of the crude membrane extract was performed according to the method of Sandelius et al. (1986) using an Elphor-VAP 21 apparatus (Bender and Hobein, München, Germany). The separation was run at a constant current of 130 mA (about 1800 V) and a temperature of 8°C. The distribution of membranes after separation was monitored from the A_{280}. The membranes were then collected from pooled fractions by centrifugation at 30,000*g* for 30 min. Protein content was determined by the method of Bradford (1976). The protein pattern of membranes in pooled fractions was analyzed by gel electrophoresis. Membranes were treated with 20% SDS and boiled for 10 min at 100°C before being loaded on 12.5% acrylamide gels.

Enzyme Markers

Plasma membrane distribution was estimated by measuring glucan synthase activity and the inhibition of H^+-ATPase by vanadate, tonoplast with pyrophosphatase activity, and ER with antimycine A-insensitive, NADPH-dependent Cyt *c* reductase according to procedures described by Malatialy et al. (1988).

RESULTS

The addition of phospholipids to an extract from *B. dioica* internodes in the presence of Ca^{2+}, followed by the solubilization of the proteins remaining bound to phospholipids by EGTA, allowed the selection of a small number of proteins, as shown by the electrophoretic pattern presented in Figure 1A. An additional separation of these proteins was obtained using chromatography through DEAE-Sephacel. The proteins that did not bind to the resin were discarded and the bound proteins were eluted by a NaCl gradient (Fig. 1B). The fractions absorbing at 280 nm were kept and the proteins they contained were resolved by SDS-PAGE. (Fig. 1C). Two main bands with molecular masses of 33 and 35 kD were present in several fractions. Other minor bands also appeared in some fractions. All of these bands corresponded to proteins that exhibited the capacity of binding to phospholipids if Ca^{2+} was present.

After transfer onto a nitrocellulose membrane, the proteins were recognized by polyclonal antibodies raised against p33 and p35 annexins from maize (Blackbourn et al., 1991); according to their molecular weight, they were called either p33 or p35 (Fig. 2). A purified sample of these two proteins was subjected to two-dimensional electrophoresis (Fig. 3). After staining of the gel, each protein appeared to consist of two isoforms with pIs of 7.2 and 7.8. Preparative electrophoresis was performed using pooled fractions from DEAE-Sephacel chromatography. The gel portion containing the p35 band was excised and the recovered protein injected into a rabbit to obtain antibodies. Figure 4A shows the electrophoretic separation of the preparation used for rabbit immunization. As shown in Figure 4, B and C, the antiserum by this procedure recognized p35 and p33.

The rabbit antiserum raised against p35 was used for immunocytochemical detection of annexin-like proteins in *B. dioica* internode sections. A comparison between parenchyma cells from control and treated (rubbed) internodes showed that the former exhibited a bright fluorescence (Fig. 5A), indicating that p33 and/or p35 were present throughout the cytoplasm. Thirty minutes after mechanical stimulation fluorescence was restricted to the periphery of cells (Fig. 5B). There was no fluorescence at all in control sections incubated without rabbit antiserum (Fig. 5C). These observations indicate that upon rubbing, the cellular

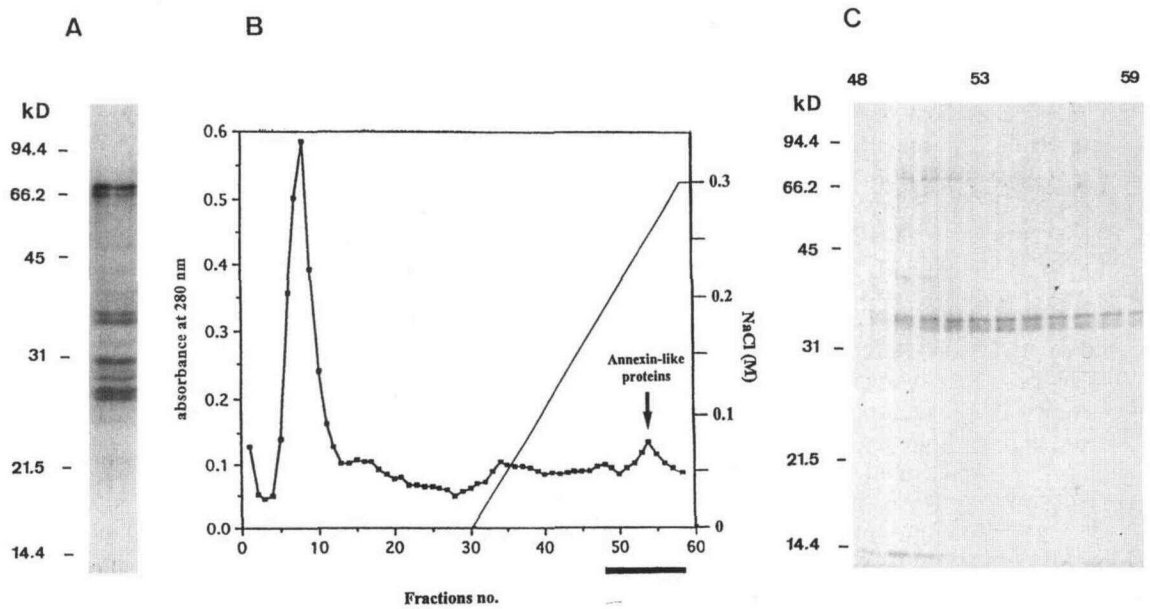

Figure 1. Separation of Ca^{2+}-dependent phospholipid-binding proteins by SDS-PAGE. A, Crude preparation after the release of proteins from phospholipids by EGTA. B, Elution profile of proteins present in the last supernatant of extraction from a DEAE-Sephacel column. The inset shows silver nitrate-stained SDS-PAGE gels. C, Successive fractions collected during the elution of a DEAE-Sephacel column by a NaCl gradient.

distribution of the annexin-like proteins p33 and p35 was modified.

FFE, which allows the rough separation of several cell membranes in one step, was used to study further the subcellular redistribution of p35 and related proteins after rubbing. As shown in Figure 6, membranes from a crude microsome preparation from *B. dioica* internodes were distributed among about 20 fractions after FFE. These were pooled into seven fractions (A–G), which were used for marker enzyme assays and immunodetection of p33 and

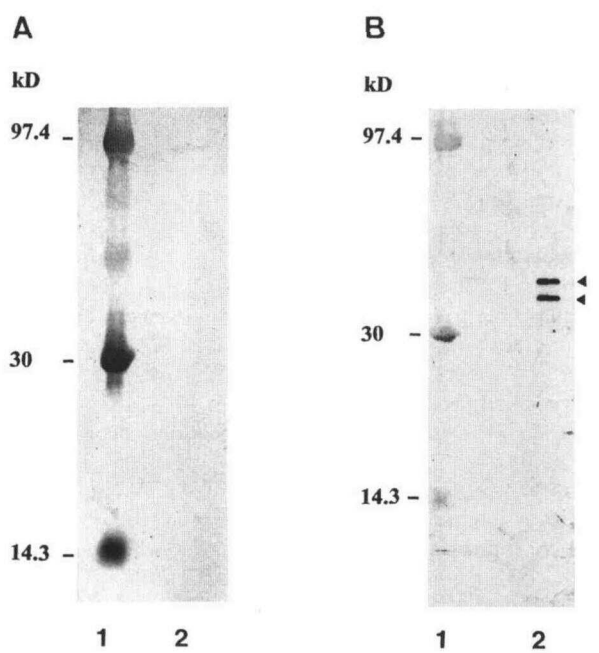

Figure 2. Immunostaining of proteins eluted from DEAE Sephacel (compare with Fig. 1C) after their transfer onto a nitrocellulose membrane. A, Immunoblots incubated with preimmune serum. B, Immunoblots incubated with a rabbit antibody raised against maize annexins.

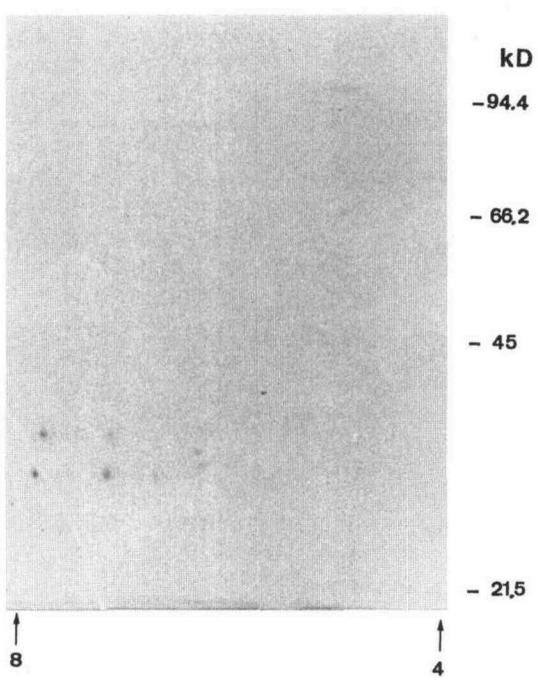

Figure 3. Two-dimensional gel electrophoresis separation of p33 and p35 purified by chromatography through a DEAE-Sephacel column.

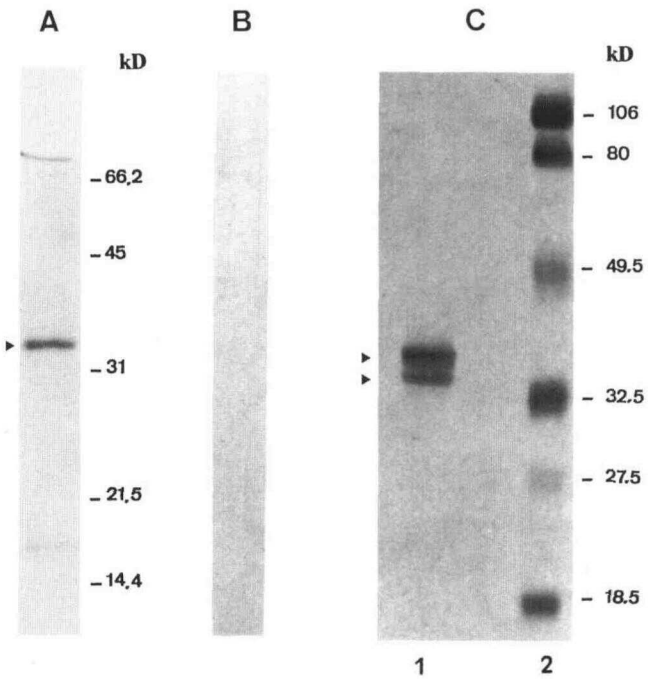

Figure 4. A, Purified p35 recovered from preparative SDS-PAGE. B, Immunoblots incubated with preimmune serum. C, Immunostaining of proteins eluted from a DEAE-Sephacel column and separated by SDS-PAGE after their transfer onto a nitrocellulose membrane.

p35. The repartitioning of the glucan synthase-specific activity and of the sensitivity of H^+-ATPase to vanadate (Fig. 6A) indicated that the F and G fractions were enriched in plasma membrane. This localization was consistent with those already reported for other plant materials (Sandelius et al., 1986; Canut et al., 1988; Malatialy et al., 1988). Measurements of NADH-Cyt *c* reductase and pyrophosphatase gave the position of the ER and tonoplast (Fig. 6B).

The apparent distribution shown in Figure 6 for membrane from control internodes was not modified in rubbed internodes (data not shown). Two FFE separations were performed the same day with untreated internodes (Fig. 7A) and internodes extracted 30 min after mechanical stimulation (Fig. 7B). The FFE fractions were pooled according to the scheme shown in Figure 7. Membrane proteins from pooled fractions (A–G) were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Annexin-like proteins were then detected using anti-p35 antiserum. As shown in Figure 7, only a very small number of bands were stained by the antiserum. In both cases, fraction E exhibited the strongest labeling of p35. Fractions F and G from rubbed internodes seemed to contain more p35 than the corresponding fractions from control internodes. A slight p33 band was detected in E and G fractions from rubbed internodes but not from control internodes. In addition, a band of 23 kD, which did not exist in soluble proteins, was visible in many fractions. Neither p33 nor p35 were detected in fractions A and B. According to the localization of the various marker enzymes (Fig. 6), it was concluded that plasma membrane and possibly ER were the membranes to which p33 and p35 bound. Mechanical stimulation of the internodes seemed to enhance the amount of the annexin-like proteins found associated with the plasma membrane.

DISCUSSION

Several papers have been published concerning the mechanism that could explain the modification of *B. dioica* internode growth induced by a mechanical stimulus (Boyer et al., 1979, 1983, 1986; De Jaegher et al., 1985; De Jaegher and Boyer, 1987; Bourgeade et al., 1991; Galaud et al., 1993; Thonat et al., 1993; Bourgeade and Boyer, 1994; Mathieu et al., 1995). Among the processes that seem to be rapidly affected by this stimulus are the modifications of plasma membrane lipids (Mathieu et al., 1995), Ca^{2+} redistribution (Thonat et al., 1993), and the expression of a calmodulin gene (Galaud et al., 1993). All of these data point to a central role for Ca^{2+} in the thigmomorphogenic response. Changes in the distribution and the concentration of cellular Ca^{2+} have been thought to modulate the activity of

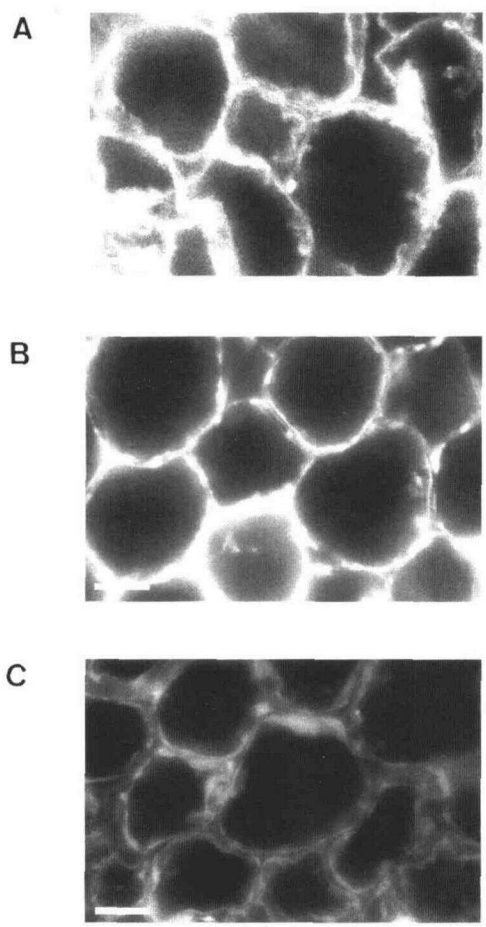

Figure 5. Immunofluorescence localization of annexins in cross-sections of *B. dioica* internodes. Cryosections were treated with the antiserum raised against *B. dioica* as the primary antibody, followed by fluorescein isothiocyanate-labeled secondary antibody. A, Parenchyma cells of an untreated internode. B, Parenchyma cells of an internode 30 min after rubbing. C, Parenchyma cells of an untreated internode that had been incubated without the first antibody. Bars indicate 20 μm.

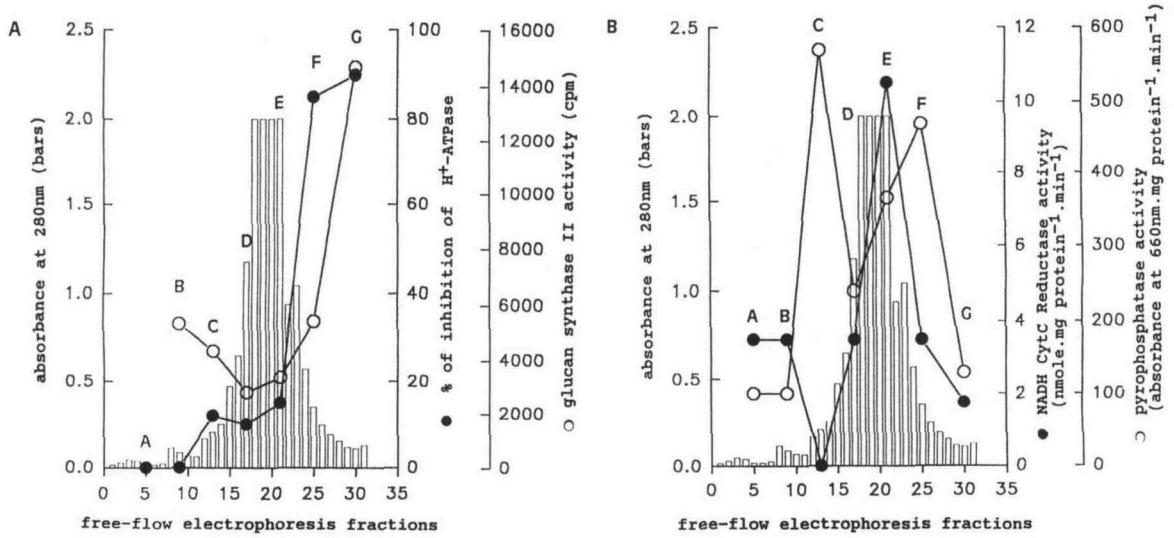

Figure 6. FFE separation of membranes from *B. dioica* internodes. Membranes were quantified by reading A_{280}. A, Levels of glucan synthase activity and of the inhibition of H^+-ATPase by vanadate in seven pooled fractions (A–G). B, Levels of pyrophosphatase and NADH Cyt *c* reductase activities in the pooled fractions.

many proteins (Hepler and Wayne, 1985), including annexins, a family characterized by increased lipophilicity upon Ca^{2+} addition.

The data obtained in this study showed the existence of at least two main annexin-like proteins in *B. dioica*. These proteins, p33 and p35, were identified by their Ca^{2+}-dependent ability to bind phospholipids and by their positive reaction with antibodies raised against maize annexins (Blackbourn et al., 1991). Their molecular weight appeared to be quite similar to those of annexins. In addition, a minor 23-kD band present in the western analysis of some FFE fractions has also been found in maize (Blackbourn et al., 1991), and a 68-kD band that copurified with p33 and p35 (Fig. 1C) might also belong to the annexin family. Two-dimensional electrophoresis separation showed that p33 and p35 exist under two isoforms (Fig. 3). Such isoforms have also been reported for other plant annexins (Clark et al., 1992), and it has been proposed that they could result from a phosphorylation process (Kaetzel et al., 1989). The above data showed that a single plant organ can contain several different annexin-like proteins.

Because of their particular binding properties, it was hypothesized that the subcellular distribution of annexins could be modified when internodes were rubbed, since this treatment affected Ca^{2+} localization in the cells (Thonat et al., 1993). The use of immunocytochemical detection indicated that annexins, which were spread throughout the cytoplasm in parenchyma cells from untreated internodes, accumulated near the plasma membrane 30 min after the treatment (Fig. 5). Such a redistribution was also suggested

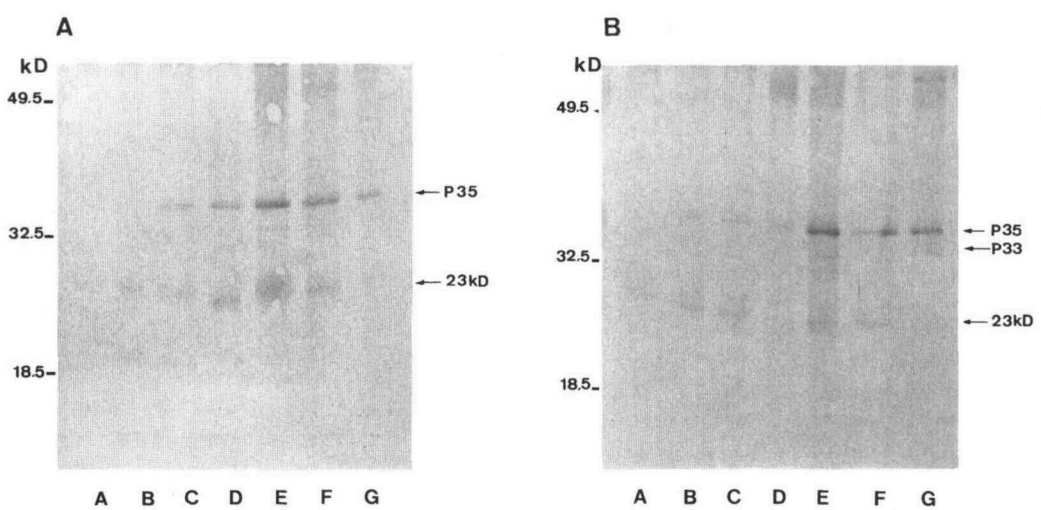

Figure 7. Immunostaining of annexin-like proteins in the pooled fractions obtained after membrane separation by FFE. Twenty micrograms of membrane proteins was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. A, Membranes from untreated internodes. B, Membranes from rubbed internodes.

by the increased immunodetection of annexins in fractions enriched in plasma membrane after membrane separation by FFE (Fig. 7). Two processes could be responsible for this observed change in annexin localization, an increase in the Ca^{2+} concentration (Bourgeade et al., 1991; Thonat et al., 1993) or a change in phospholipid composition (Mathieu et al., 1995), both of which could affect the plasma membrane after wounding, increasing the affinity of annexins for this membrane. The reversible binding of annexins to plasma membrane in cotton has already been described (Andrawis et al., 1993). The present work showed that annexin-like proteins were present in *B. dioica* and could be involved in the thigmomorphological response of this particular plant. Further work is necessary to understand the function of these Ca^{2+}-dependent proteins.

ACKNOWLEDGMENTS

We thank Dr. N. Battey (School of Plant Sciences, Reading, UK) for providing antiserum to maize p33 and p35, Dr. P. Crespi (University of Geneva) for his help during FFE experiments, and Dr. J.L. Julien for useful suggestions on the manuscript.

Received February 13, 1997; accepted March 22, 1997.
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