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Localization of a calcium channel-like protein in the sieve element plasma membrane

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Abstract. There is evidence that calcium (Ca^{2+}) activity in sieve elements is much higher than in adjacent cells, which raises questions about Ca^{2+} exchange across the sieve element plasma membrane. We looked for the presence of Ca^{2+} channels in tobacco (*Nicotiana tabacum* L.) and *Pistia stratiotes* (L.) using a monoclonal antibody to a dihydropyridine (DHP)-type Ca channel (MAB427). Immunolabeling at the light microscope level gave strong signals along the sieve element of both species, and with the transmission electron microscope this label was found associated with the plasma membrane. Western blot analysis using the MAB427 antibody detects a protein between 175 and 220 kDa (approximately the size expected of the α-1 subunit of the Ca^{2+} channel protein in mammals) in blots containing either *Pistia* or tobacco leaf microsomal membrane protein extracts. As a further test for Ca^{2+} channels, hand-sectioned young *Pistia* leaves were treated with the fluorescent-tagged Ca^{2+} channel blocker 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-3-(indacene)propionic acid (DM-BODIPY)-DHP. The phloem, as well as calcium oxalate idioblast cells, exhibited strong fluorescence compared to general parenchyma cells. The binding of DM-BODIPY-DHP was inhibited by pre-treatment of sections with nifedipine, a competitive inhibitor of DHP-type Ca^{2+} channels in animal systems. The results indicate that sieve elements may be enriched with Ca^{2+} channels which might be partly responsible for the high Ca^{2+} activities observed in sieve sap, and also for rapid phloem responses, such as Ca^{2+} activated callose deposition.

Keywords: calcium, calcium channels, phloem, sieve elements, transport.

Introduction

Calcium (Ca^{2+}) concentrations are kept at low levels within plant cells to increase the efficacy of small concentration changes in the regulation of signal transduction cascades. High concentrations of Ca^{2+} also precipitate inorganic phosphate, a key compound in metabolic pathways. In the mesophyll cytosol, total Ca^{2+} levels may be between 1 and 10 mM; however, much of this Ca^{2+} is in a bound form, thus maintaining free Ca^{2+} concentrations between 30 and 400 nM (White *et al.* 1992; Muir and Sanders 1997). Ca^{2+} ATPases and Ca^{2+}/H^+ antiporters present in the endoplasmic reticulum (ER), tonoplast and plasma membrane pump Ca^{2+} from the cytosol into the apoplast and cellular compartments as part of the mechanism of regulation of steady-state Ca^{2+} activity in the cytosol (Piñeros and Tester 1997*a*).

In phloem sieve elements, vacuoles and 'normal' ER are absent, so regulation of Ca^{2+} activity is thought to be by sequestration within the sieve element reticulum or pumping into the apoplast by Ca^{2+}-ATPases (Sjolund and Shih 1983; Arsanto 1986; Behnke and Sjolund 1990). Ca^{2+} is generally considered to be phloem-immobile, even though total calcium (ionic and chelated) concentrations of 10–3000 μM have been measured in phloem sap, and Ca^{2+} activity of up to 10–30 μM has been measured in aphid stylet exudates (D. B. Fisher, pers. comm.; Brauer *et al.* 1998). Ca^{2+}-dependent protein kinases have been identified in the phloem sap of rice and cucumber, indicating a possible role of Ca^{2+} in signaling within sieve tubes (Nakamura *et al.* 1995; Avdiushko *et al.* 1997). Reports of high Ca^{2+} activity in sieve tube sap relative to adjacent cells suggests the possible involvement of Ca^{2+} channels, which have relatively high capacity, in Ca^{2+} regulation in sieve tubes. However, general information on presence and distribution of Ca^{2+} channels in various plant cells and tissues is sparse.

Abbreviations used: CTC, chlortetracycline; DHP, dihydropyridine; DM-BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-3-(indacene)-propionic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; IP_3, 1,4,5-inositol triphosphate; PAA, phenylalkylamine; PVPP, polyvinylpolypyrrolidone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Recent studies have contributed greatly to our understanding of Ca^{2+} channel function in a few plant systems. With respect to internal fluxes, it has been shown that Ca^{2+} can be released from storage compartments by Ca^{2+} channel activation (Piñeros and Tester 1997*a*). For example, a vacuolar 1,4,5-inositol triphosphate (IP_3)-inducible Ca^{2+} channel is activated in response to osmotic, light or fungal elicitors, allowing Ca^{2+} to flow down its concentration gradient into the cytoplasm (Bush *et al.* 1989; Canut *et al.* 1993; Piñeros and Tester 1995). This channel can be partially inhibited by the phenylalkylamine (PAA) channel blocker, verapamil. Other vacuolar Ca^{2+} channels are insensitive to IP_3, yet inhibited by verapamil and nifedipine (Piñeros and Tester 1995).

There is also considerable information available on the properties of root plasma membrane Ca^{2+} channels. Although all known root Ca^{2+} channels are activated by membrane depolarizations, they differ in their sensitivities to La^{3+}, Gd^{3+} and verapamil (White 1998*a*). One root plasma membrane channel, *rca*, is thought to remain closed unless activated by a membrane depolarization. Abscisic acid, Ca^{2+} and IP_3 have no effect on electrophysiological channel activity. When the extracellular side of this channel is exposed to 0.5 μM verapamil, Ca^{2+} influx can be blocked in a reversible manner (Piñeros and Tester 1997*b*). The DHP Ca^{2+} channel blocker, nifedipine, (10–100 μM) had no effect on Ca^{2+} flux through the *rca* channel. Most other electrophysiologically characterized Ca^{2+} channels in roots are not sensitive to either PAAs or DHPs.

Ca^{2+} channel studies in plants have focused primarily on electrophysiological and $^{45}Ca^{2+}$ flux experiments. In contrast, in animals, Ca^{2+} channels have been cloned and subsequently characterized to a much greater extent. In animal systems, the L-type Ca^{2+} channel is composed of five subunits, encoded by four genes. Ca^{2+} pass though a pore formed by the largest subunit (α-1) in response to membrane depolarizations. The α-1 subunit may be weakly glycosylated and it contains binding sites for DHP (isradipine and nifedipine) and PAA (verapamil and methoxyverapamil) inhibitors on the extracellular side of the plasma membrane (Catterall *et al.* 1989; Knaus *et al.* 1992*a*). Significantly, α-1 gene homologs are expressed in a tissue and developmentally specific manner (Diebold *et al.* 1992).

Much of the pharmacological results of use of Ca^{2+} channel blockers in plant systems indicate presence of L-type Ca^{2+} channels, which are sensitive to DHP and/or PAA channel blockers. A monoclonal antibody against the α-1 subunit (pore-forming subunit) of an L-type Ca^{2+} channel in mouse is commercially available. This subunit, which has some very highly conserved regions, also contains a DHP binding site. Thus, we decided to test this antibody as a probe for the presence of L-type channels in phloem cells. Fluorescent tagged DHP and PAA inhibitors, which bind L-type Ca^{2+} channels, can also be used in cellular localization studies. We have used the probes to determine if Ca^{2+} channels are involved in sieve element physiology. We provide evidence in support of the existence of L-type Ca^{2+} channels in the sieve element plasma membranes of *Pistia stratiotes* L. and tobacco (*Nicotiana tabacum* L.). *Pistia* is an aquatic plant that rapidly accumulates Ca^{2+} as calcium oxalate crystals, a feature that makes *Pistia* a particularly good model system to study Ca^{2+} compartmentation in plants (Tarlyn *et al.* 1998). Preliminary data also indicated DHP/PAA-sensitive Ca^{2+} channels are present in this plant (Li *et al.* 1992).

Materials and methods

Plant material

Pistia plants were grown hydroponically in a dilute solution of Peters 20-20-20. Temperatures were maintained at 26/20°C during the 16/8 h light/dark cycle with high pressure sodium and metal halide lighting conditions within the growth chamber. Tobacco (cultivar SR2) was grown in the chamber maintained at 25/20°C during the 14/10 light/dark cycle at 200 μmol photons m^{-2} s^{-1}.

Antibody

Immunolabeling and immunoblotting studies were performed using a monoclonal antibody MAB427 [Mouse anti-dihydropyridine binding complex (α-1 subunit)] purchased from Chemicon International, Inc. (Temecula CA, USA).

Gel electrophoresis and western immunoblotting

Dissected *Pistia* shoot apices and tobacco leaves approximately 30% expanded were sampled for soluble and membrane protein extractions. For soluble protein extractions, plant material was ground in liquid nitrogen and suspended in extraction buffer (25 mM Tricine, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1% PVPP, pH 7.5). Samples were centrifuged at 15 000 *g* for 10 min and the supernatant was collected for protein quantification and electrophoresis. For plant membrane proteins, tissue was homogenized in sucrose extraction buffer (10 mM Tris and 250 mM sucrose, pH 7.5) and the homogenate was centrifuged at 3000 *g* for 5 min. Supernatant was collected and centrifuged at 10 000 *g* for 10 min and the pellet was discarded. This supernatant was centrifuged at 100 000 *g* for 2 h. The resulting microsomal pellet was resuspended in soluble protein extraction buffer. All proteins were quantified using a Pierce Coomassie Protein Assay Reagent according to the manufacturer's directions (Rockford IL, USA). Loading buffer (final concentration 25 mM Tris, pH 7.3, 1% SDS, 5% glycerol, 10% 2-mercaptoethanol and 0.2% Triton X-100) and bromophenol blue were added to each sample prior to boiling for 10 min immediately before electrophoresis.

Samples (100 μg) of membrane and soluble *Pistia* proteins and 50-μg samples of membrane and soluble tobacco proteins were loaded on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and electrophoresis was performed according to Laemmli (1970). Bio-Rad prestained SDS–PAGE broad range standards (Bio-Rad, Hercules CA, USA) were included on each gel. Representative gels were stained with Coomassie blue overnight and destained according to the Hoefer Scientific Instruments (1994) electrophoresis guide.

Proteins were electroblotted from unstained gels onto pre-wetted polyvinylidene difluoride membranes (Bio-Rad) using a Towbin transfer buffer with 5% methanol (Hoefer Scientific Instruments 1994). Membranes were rinsed with water and allowed to air-dry. Blots were wetted in methanol and blocked with Western Blocking Reagent (Roche Molecular Biochemicals, Indianapolis IN, USA) for 1 h and then incubated in MAB427 (1:500 dilution in 0.5% blocking solution) overnight at room temperature. Blots were washed for 3 × 15 min with TBST (50 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.5) and incubated for 1 h in anti-mouse-alkaline phosphatase-tagged antibody (Sigma, St. Louis MO, USA) using a 1:500 dilution in TBST. Alkaline phosphatase color reactions were performed according to the manufacturer's instructions (Roche Molecular Biochemicals). Blot images were digitized using a Bio-Rad Gel Doc 1000.

Immunocytochemistry

Tobacco leaf samples (one-third expanded) and immature *Pistia* leaves (1 cm in length) were fixed for 16 h in 1.25% glutaraldehyde, 2% paraformaldehyde and 50 mM Pipes, pH 7.2. Tissue pieces were washed in the same buffer and dehydrated in an ethanol series prior to infiltration with LR White embedding resin. Following dehydration in ethanol, infiltration with LR White resin was performed at 4°C prior to polymerization overnight at 50°C. Sections (1 µm) were mounted on silanized slides (Digene, Beltsville MD, USA) in water and allowed to dry at 60°C. Sections were blocked for 1 h using TBST–BSA (10 mM Tris, 150 mM NaCl, 0.1% Tween and 1% BSA, pH 7.2) and incubated at room temperature for 15 h with a 1:25 dilution of MAB427 in TBST–BSA. To limit bacterial contamination, 0.1% sodium azide was included in the antibody solution.

Following 4 × 15 min washes with TBST–BSA, slides were treated with Protein A/G 15 nm tagged gold (Goldmark, Ted Pella, Redding CA, USA) for 1 h. Washes (15 min) were performed with TBST–BSA, TBST, and water. After 15 min of silver enhancement treatment (Amersham-Pharmacia Biotech Inc., Piscataway NJ, USA), sections were counterstained with 0.5% Safranin O, coverslipped, and observed using a Bio-Rad MRC–1024 laser scanning confocal microscope from which transmitted and reflected images were collected. Thin sections (silver-gold interference color) were mounted on formvar coated nickel grids and treated as described for thick sections. Sections on grids were counterstained for 5 min using a solution comprised of 3 parts aqueous uranyl acetate (4%) and 1 part potassium permanganate (1%) solution. Sections were observed on a JEOL1200EX transmission electron microscope using an accelerating voltage of 80 kV.

Fluorescence microscopy

Hand-cut sections of 1-cm-long *Pistia* leaves were incubated in a 0.5 µM solution of DM-BODIPY-DHP (Molecular Probes, Eugene OR, USA) in sucrose buffer (0.4 M sucrose, 5 mM HEPES and 2 mM $CaCl_2$, pH 7). Some tissue sections were pre-treated for 4 h with nifedipine (400 µM in sucrose buffer) before incubation in DM-BODIPY-DHP solutions. Images were collected using the Bio-Rad MRC 1024 confocal microscope.

Frozen sections (20 µm) of tobacco were cut using a Cryocut 1800 cryomicrotome (Leica Microsystems, Deerfield IL, USA) and mounted on gelatin-coated slides. Sections were washed with sucrose buffer for 2 × 10 min and treated with 0.5 µM DM-BODIPY-PAA in sucrose buffer (Molecular Probes) for 1 h. Following a 10-min buffer wash, they were incubated in ethidium bromide (1:100 000 dilution) for 30 min prior to observation with the confocal microscope.

Pistia hand-cut sections were treated with 10 µM chlortetracycline (CTC, Calbiochem, San Diego CA, USA) in sucrose buffer without Ca^{2+}, observed using a Leitz Ortholux 2 epifluorescence microscope with a xenon-bulb, and images captured using a DKC500 digital video camera (Sony Corporation of America, New York City NY, USA). Some control sections were pre-treated with 10 mM ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in sucrose buffer (Sigma) to chelate Ca^{2+} from the cells.

Results

Electrophysiological and patch clamp studies have provided evidence supporting the existence of Ca^{2+} channels in plant plasma membranes (Thuleau *et al.* 1994*a*, *b*). In addition, the use of Ca^{2+} channel inhibitors has demonstrated the importance of the flux of Ca^{2+} across the plasma membrane via channels for the induction of Ca^{2+}-dependent responses within cells. Recently, Vallee *et al.* (1997) used fluorescently tagged dyes to localize putative Ca^{2+} channels in the plasma membrane of sunflower protoplasts. Our results with the monoclonal antibody made against a mammalian α-1 subunit of the L-type Ca^{2+} channel, and fluorescently tagged Ca^{2+} channel inhibitor probes have provided two lines of evidence in support of the existence of L-type Ca^{2+} channels in the phloem.

Protein analysis

Western blot analysis using the MAB427 antibody detected a single protein band in *Pistia* and two bands in tobacco samples of microsomal membrane protein extracts (Fig. 1). Due to the gel concentration used, the high molecular weight proteins are not well separated and in a very narrow zone of the gel, but based on R_f values using standards, the labeled proteins are between 175 and 220 kDa. The band identified in plant membrane preparations corresponds to the approximate size of cloned α-1 subunit Ca^{2+} channel proteins in animal systems (187–210 kDa) (Grabner *et al.* 1991; Hui *et al.* 1991). The MAB427 antibody did not bind to total soluble protein extracts from either species (Fig. 1; tobacco not shown). These data support the possibility that MAB427 is recognizing a membrane-bound plant Ca^{2+} channel protein.

In tobacco, a second band of higher molecular weight than the *Pistia* band was also identified by the MAB427 antibody. Hui *et al.* (1991) showed that rat brain Ca^{2+} channel cDNAs contain putative extracellular glycosylation sites. Perhaps the higher molecular weight band in tobacco may be a glycosylated form of the protein, but this is purely speculation at this time.

Localization of MAB427 binding

In both *Pistia* and tobacco, MAB427 immunolabeling at the light microscope level was specific to mature sieve elements, as indicated by the localization of the yellow coloration of the reflected silver-enhanced gold particles (Figs 2*A*, *C*, *E*). Very low non-specific binding was occasionally present in mesophyll cells or other cells of the veins. No label was found in *Pistia* or tobacco controls treated only with secondary antibody (Figs 2*B* and D).

Ultrastructural observations in the transmission electron microscope revealed that the MAB427 localized to the plasma membrane of sieve elements but not adjacent cell types in both *Pistia* and tobacco leaf sections (Figs 3*A*, *B*, *D*). Controls without primary antibody exhibited no label (Figs 3*C* and *E*). Label was confined to the plasma membrane and was not identified in sieve element reticulum or other sieve element organelles. The density of gold particles on plasmodesmata between companion cells and sieve elements was not any higher than the general non-specific background label (Figs 3*F* and *G*).

Localization of Ca^{2+} and channel blocker binding sites

When young *Pistia* leaves were treated with the fluorescent-tagged Ca^{2+} channel blocker DM-BODIPY-DHP (0.5 μM) in sucrose buffer, the phloem, as well as calcium oxalate crystal idioblast cells, exhibited much more intense fluorescence than surrounding cell types (Figs 4*A* and *B*). This was true for large (Fig. 4*A*) and minor veins (Fig. 4*B*). The binding of DM-BODIPY-DHP was inhibited by a 4-h pre-treatment of sections with 400 μM nifedipine in sucrose buffer, a competitive inhibitor of L-type Ca^{2+} channels in animal systems (Fig. 4*C*). Lower concentrations of nifedipine did not completely block the binding of the DM-BODIPY-DHP dye. Similar labeling patterns were seen when DM-BODIPY-PAA was used on *Pistia* (not shown).

When tobacco leaf sections were treated with DM-BODIPY-PAA and incubated in ethidium bromide to decrease yellow–green autofluorescence of the tissue (particularly xylem), phloem fluoresced green, whereas parenchyma cells and mesophyll cells were red (Fig. 4*D*). Xylem exhibited a combination of non-specific binding of DM-BODIPY-PAA and autofluorescence, resulting in a yellow coloration. Control tissue treated with buffer followed by ethidium bromide fluoresced red under the same conditions (Fig. 4*E*). DM-BODIPY-DHP also resulted in labeling of phloem in tobacco, but the intensity of labeling was much less than seen in *Pistia* and much less than with DM-BODIPY-PAA (not shown). For both species, the sectioning resulted in fairly thick sections and some collapse of the protoplasm due to loss of turgor. This is probably partly responsible for the labeling pattern seen with these probes being dense along some cells and having a patchy appearance in others.

The fluorescent indicator CTC, which recognizes bound calcium, gave strong fluorescence of the phloem in *Pistia*, suggesting the presence of increased Ca^{2+} within the cells of the phloem (Fig. 4*F*). CTC fluorescence was significantly reduced when the fresh hand-cut sections were pre-treated

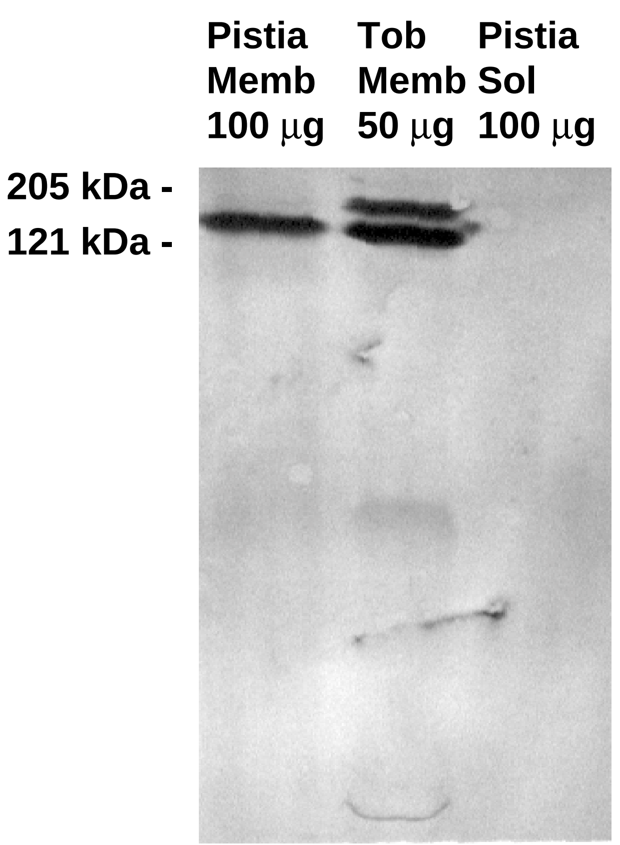

Fig. 1. Western immunoblot of *Pistia* and tobacco (Tob) microsomal membrane (Memb) and soluble (Sol) protein extracts using MAB427 antibody to α-1 subunit of the Ca^{2+} channel. Proteins of the expected size are labeled in the microsomal membrane samples but not the soluble protein sample. *Pistia* shows a single high molecular weight protein while tobacco has two proteins of slightly different molecular weight that are recognized by MAB427.

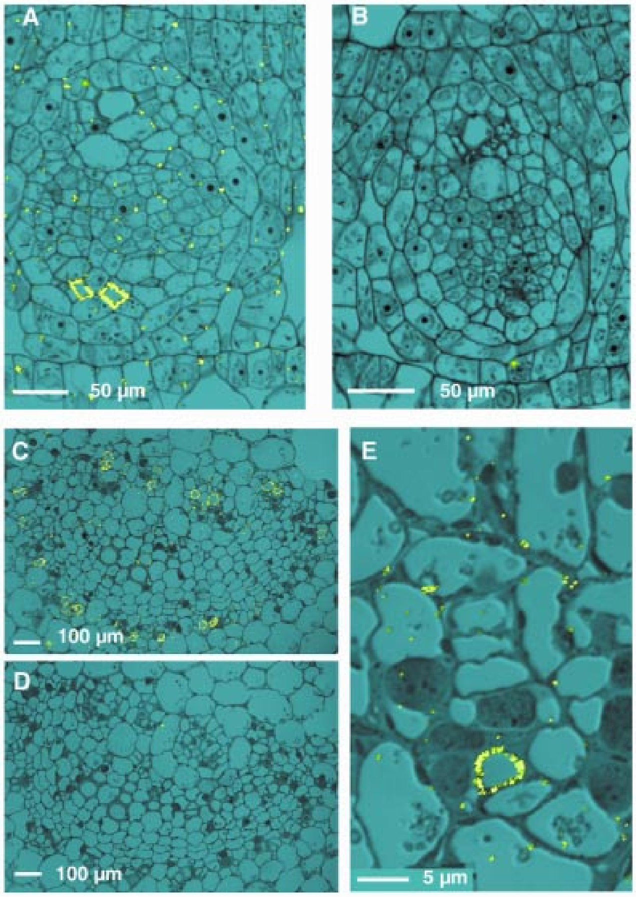

Fig. 2. Reflected/transmitted overlays of silver-enhanced gold labeling for MAB427 in tissue sections. Label appears as yellow particles. In *Pistia* (*A*) and tobacco (*C*, *E*), label is primarily associated with mature sieve elements in both major and minor veins. Controls treated with only secondary antibody in *Pistia* (*B*) and tobacco (*D*) had no label.

with EGTA, a Ca^{2+} chelator (Fig. 4*G*). Since EGTA reduces CTC fluorescence, the relative fluorescence of CTC within various cell types may provide a reliable qualitative indicator of relative Ca^{2+} concentration.

Discussion

Electrophysiological evidence of voltage-dependent Ca^{2+} channels has previously been demonstrated in the plasma membranes of plants (Graziana *et al.* 1988; Thuleau *et al.* 1994*a*; White 1998*a*, *b*), but the cell-specific location and quantitative differences between cell types has not been carefully examined in complex tissues. The cell biology and biochemical studies presented here indicate that sieve element plasma membranes are enriched in a Ca^{2+} channel-like protein relative to surrounding cells. The activity of Ca^{2+} channels has been characterized in membranes of a number of plant species using a range of physiological techniques, but to our knowledge, this is the first study that indicates their presence in the phloem. The monoclonal antibody used for this study is made against mouse T-tubule dihydropyridine binding complex, which is the α-1 subunit of a L-type Ca^{2+} channel.

The western blot analysis shows that the antibody specifically recognizes a plasma membrane protein of approximately the correct molecular weight for the channel subunit to which the antibody was raised. While non-specific binding to wall polymers or glycosyl groups of membrane glycoproteins is always a possibility, there is not labeling of multiple bands on the western blots, which might be

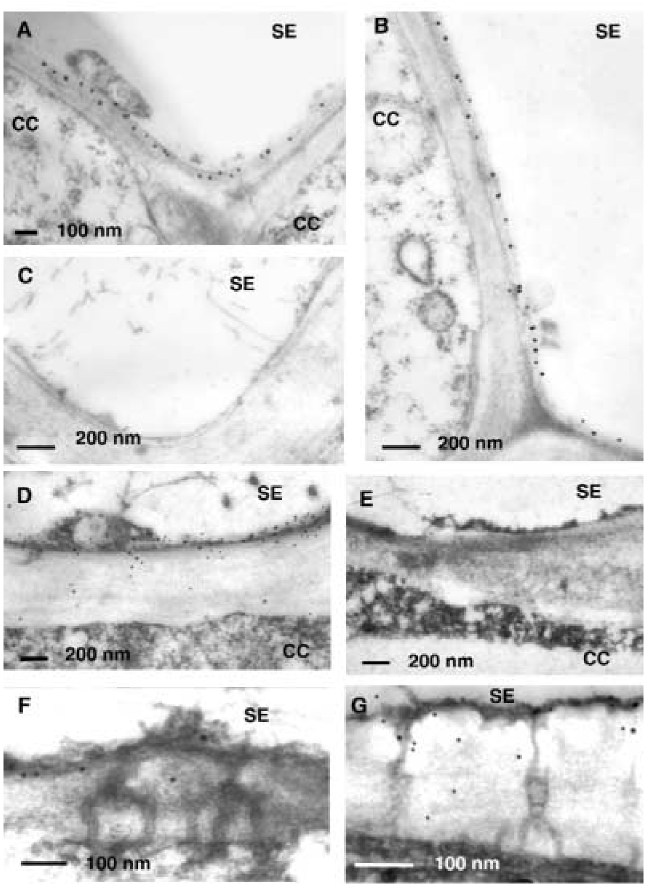

Fig. 3. Transmission electron microscopy of immunogold localization of MAB427 binding. MAB427 labels the plasma membrane of sieve elements (SE) but not adjacent cells in both *Pistia* (*A*, *B*) and tobacco (*D*) leaf sections. Controls for both species treated with secondary antibody only had no labeling (*C*, *E*). Plasmodesmata between companion cells and sieve elements in *Pistia* (*F*) and tobacco (*G*) had little or no label associated with them.

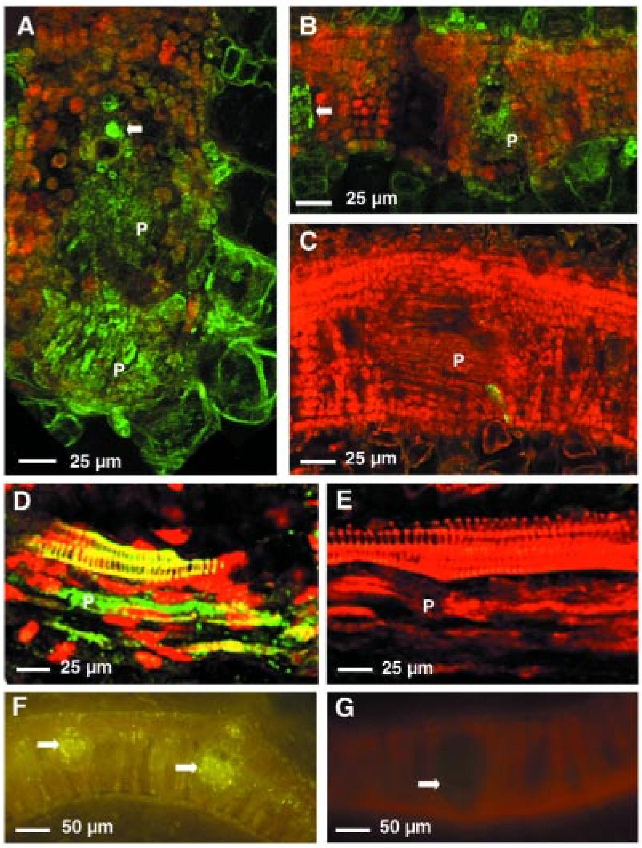

Fig. 4. Use of fluorescent-tagged Ca^{2+} channel blockers and CTC to probe for localization of Ca^{2+} channels and Ca^{2+} in leaf tissues. (*A–C*) *Pistia* leaf. Phloem (P) as well as calcium oxalate crystal idioblast cells (arrows), exhibited intense fluorescence (*A*, *B*) when treated with the tagged Ca^{2+} channel blocker DM-BODIPY-DHP. Binding of the DM-BODIPY-DHP is inhibited by a 4-h pretreatment with the competitive inhibitor, nifedipine (*C*). (*D*, *E*) Tobacco leaf. Phloem in sections treated with DM-BODIPY-PAA fluoresces bright green while surrounding parenchyma and mesophyll fluoresce red (*D*). Non-specific binding of DM-BODIPY-PAA to xylem combined with autofluorescence results in yellow coloration. Control tissues treated only with ethidium bromide fluoresced red under the same conditions (*E*). (*F*, *G*) *Pistia* leaf tissue treated with CTC before (*F*) or after (*G*) treatment with the Ca^{2+} chelator EGTA. CTC gives rise to bright fluorescence of the phloem relative to surrounding tissues, indicating relatively higher Ca^{2+} concentration. This fluorescence does not occur if the sections are pretreated with EGTA to remove Ca^{2+}.

expected if the antibody was recognizing a carbohydrate epitope (common in peripheral and transmembrane proteins). The fairly specific labeling in the sieve tubes indicates that the antibody recognizes a channel-like protein that is specific to the sieve elements, or that the protein is in much higher concentration in sieve elements and thus more easily detected with this technique. We cannot say for certain that the protein recognized on blots and in sections is a Ca^{2+} channel without further isolation and biochemical characterization of the actual protein. However, the data on Ca^{2+} channel blocker localization also suggests the sieve element is enriched in Ca^{2+} channels.

The channel blocker probes label sieve elements as well as other vascular cells, while MAB427 labels primarily sieve elements, although there is slight labeling of other vascular cells (Figs 2*A* *vs* 4*A*). The antibody apparently recognizes epitopes that are either specific to the sieve element plasma membrane channel or are present in proteins most highly expressed in this cell type. In contrast, the Ca^{2+} channel blockers are less selective and evidently interact with a range of L-type channels, giving rise to label throughout the vascular bundle as well as in other cell types as mentioned above.

Ca^{2+} channel blockers have been used extensively in plant systems to study channel presence and activity. For example, the maxi cation channel (permitting K^+ efflux and Ca^{2+} influx) in the plasma membrane of rye roots is inhibited by verapamil, but not nifedipine (White 1996). PAA-type Ca^{2+} channel inhibitors prevent the influx of Ca^{2+} in carrot cell protoplasts and maize shoots (Graziana *et al.* 1988; Thuleau *et al.* 1993, 1994*a*; White 1998*b*). In moss protonema, Ca^{2+} influx is sensitive to both nifedipine and verapamil inhibitors (Schumaker and Gizinski 1993). In fact, in moss, 10 μM nifedipine reduced Ca^{2+}-dependent bud formation by more than 50%. These electrophysiological studies with blockers provide one line of evidence supporting the presence of L-type Ca^{2+} channels in plant plasma membranes. Our results with fluorescent-tagged Ca^{2+} channel blockers support the MAB427 antibody data indicating Ca^{2+} channels are present in sieve elements.

The validity of the use of the fluorescent Ca^{2+} channel probes in plant and animal systems has been demonstrated in a number of recent studies. Fluorescent tagged DHP and PAAs bind reversibly to the α-1 subunit of L-type Ca^{2+} channels (Knaus *et al.* 1992*a*, *b*) and these compounds also block Ca^{2+} uptake in animal cells (Knaus *et al.* 1992*a*). Shaw and Quatrano (1996) demonstrated that DM-BODIPY-DHP fluorescence coincides with an asymmetric distribution of Ca^{2+} ions in the *Fucus* zygote cell periphery. DM-BODIPY-DHP binding could be competitively blocked by unlabeled DHP, and extended incubation in DHP resulted in an inhibition of rhizoid growth. Vallee *et al.* (1997) performed similar experiments using DM-BODIPY-PAA and DM-BODIPY-DHP probes in sunflower protoplasts. In their chase experiments, protoplasts were incubated for 30 min with a fluorescent probe (1 μM) and then verapamil, bepridil (a PAA), or nifedipine at concentrations between 5 and 500 μM. DM-BODIPY-PAA fluorescence in protoplasts was significantly reduced when samples incubated in probe were subsequently treated with verapamil or bepridil; similarly, DM-BODIPY-DHP fluorescence was inhibited by a nifedipine treatment. The phloem DM-BODIPY-DHP studies illustrated here demonstrate a similar phenomenon. The putative Ca^{2+} channels in the sieve element plasma membrane appear to have pharmacological properties with respect to blockers that are like those described for *Fucus* and sunflower (Shaw and Quatrano 1996; Vallee *et al.* 1997).

The observation of generally higher bound calcium in phloem using CTC fluorescence, while not directly related to Ca^{2+} channels, is consistent with a role of sieve elements in Ca^{2+} sequestration and regulation. CTC is a UV-excitable compound that accumulates in compartments where free Ca^{2+} is associated with membranes. Observations allow for qualitative hints as to the extent of Ca^{2+} sequestration in organelles (Tsien 1989). CTC fluorescence has been used to monitor the Ca^{2+} distribution within developing *Fucus* zygotes (Kropf and Quatrano 1987). In *Pistia* leaves, CTC fluorescence reveals a higher concentration of Ca^{2+} in phloem than in mesophyll cells. This is in agreement with observations in *Ricinus*, where the phloem Ca^{2+} concentration was shown to be up to 1000-fold higher than is usually found in the cytoplasm of parenchyma cells (White *et al.* 1992; Muir and Sanders 1997; Brauer *et al.* 1998).

The role of the putative Ca^{2+} channels in sieve elements is unknown at this time, although a function in rapid signaling or other types of fast response is probable. It appears that in plants the DHP- and PAA-sensitive Ca^{2+} channels may remain in the closed configuration until the plasma membrane undergoes a depolarization event (Thuleau at al. 1994*b*). When Ca^{2+} channels open, there is a flux of Ca^{2+} from the apoplast into the cytoplasm. Depolarization can be triggered by stimuli such as low temperature, phytotoxins, auxin, abscisic acid, light or pathogen attack (Thuleau *et al.* 1994*a*, *b*; White 1998*b*). Messiaen at al. (1993) demonstrated that oligogalacturonides induce an increase of Ca^{2+} in the periphery of carrot protoplasts within 20 min of induction. Since exogenously applied verapamil blocked the Ca^{2+} accumulation in that study, the transport of exogenous Ca^{2+} through Ca^{2+} channels may be the source of increased levels of Ca^{2+} within the protoplasts.

Ca^{2+} influx can trigger multiple types of signal transduction pathways, in a manner dependent upon the nature of the stimulus. Of relevance to our findings is the relationship between Ca^{2+} and callose formation, since callose is known to be involved in wound response in sieve tubes, and increases in cytoplasmic Ca^{2+} can induce callose formation (Kauss *et al.* 1991; Messiaen *et al.* 1995). Callose formation can be blocked by complexing external Ca^{2+} (Kauss *et al.*

1991), and callose deposition in suspension cultures and pollen tubes can be partially inhibited by nifedipine, indicating participation of Ca^{2+} channels (Waldmann *et al.* 1988; Heslop-Harrison and Heslop-Harrison 1992). The synthesis of callose is catalysed by 1,3-β-glucan synthase (callose synthase) on the plasma membrane. Even when UDPG is available, callose synthase is inactive when cytosolic free Ca^{2+} is in the 100–300 nM range, and callose formation is not induced unless activated by cytosolic Ca^{2+} concentrations in the micromolar level (Fink *et al.* 1987). We propose that the putative Ca^{2+} channels along the sieve element may become activated during wounding or pathogen attack, facilitating Ca^{2+} influx from the specialized wall region immediately adjacent to the plasma membrane (Lucas and Franceschi 1982). This influx could stimulate callose synthesis, leading to sealing of wounds, plasmodesmata and sieve plates. While putative channels were not seen along the sieve plates, Ca^{2+} may quickly accumulate in the sieve plate region due to Ca^{2+} transport during pressure surges from wounds at some distance.

The existence of putative Ca^{2+} channels in plant systems is well known but these channels are only poorly characterized compared to what is known about channels in animal systems. The temporal, spatial and pharmacological distribution of Ca^{2+} channels in plant systems require considerable study to further our understanding of their roles in development, defense and other cellular functions. The evidence presented here demonstrates that putative DHP-type Ca^{2+} channels are enriched on the sieve element plasma membrane, indicating that tissue-specific expression of pharmacologically distinct forms is possible in plants, and may add to the diversity of Ca^{2+} signaling responses to developmental and environmental stimuli.

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References

Arsanto JP (1986) Ca^{2+}-binding sites and phosphatase activities in sieve element reticulum and P-protein of chick pea phloem. A cytochemical and *X*-ray microanalysis survey. *Protoplasma* **132**, 160–171.

Avdiushko SA, Ye XS, Croft KP, Kuc J (1997) Phosphorylation of proteins in cucumber exudates and evidence for protein kinase activity. *Journal of Plant Physiology* **150**, 552–559.

Behnke H-D, Sjolund RD (1990) (Eds) 'Sieve elements: comparative structure, induction and development.' (Springer-Verlag: New York)

Brauer M, Zhong W-J, Jelitto T, Schobert C, Sanders D, Komor E (1998) Free calcium ion concentration in the sieve-tube sap of *Ricinus communis* L. seedlings. *Planta* **206**, 103–107.

Bush DS, Biswas AK, Jones RL (1989) Gibberellic-acid-stimulated Ca^{2+} accumulation in endoplasmic reticulum of barley aleurone: Ca^{2+} transport and steady-state levels. *Planta* **178**, 411–420.

Canut H, Carrasco A, Rossignol M, Ranjeva R (1993) Is vacuole the richest store of IP_3-mobilizable calcium in plant cells? *Plant Science* **90**, 135–143.

Catterall WA, Seagar MJ, Takahashi M, Curtis BM (1989) Molecular properties of dihydropyridine-sensitive calcium channels. In 'Cell calcium metabolism'. (Ed. G Fiskum) pp. 33–45. (Plenum Press: New York)

Diebold RJ, Koch WJ, Ellinor PT, Wang J-J, Muthuchamy M, Wieczorek DF, Schwartz A (1992) Mutually exclusive exon splicing of the cardiac calcium channel α1 subunit gene generates developmentally regulated isoforms in the rat heart. *Proceedings of the National Academy of Sciences USA* **89**, 1497–1501.

Fink J, Jeblick W, Blascheck W, Kauss H (1987) Calcium ions and polyamines activate the plasma-membrane-located 1,3 β-glucan synthase. *Planta* **171**, 130–135.

Grabner M, Friedrich K, Knaus H-G, Striessnig J, Scheffauer F, Staudinger R, Koch WJ, Schwartz A, Glossmann H (1991) Calcium channels from *Cyprinus carpio* skeletal muscle. *Proceedings of the National Academy of Sciences USA* **88**, 727–731.

Graziana A, Fosset M, Ranjeva R, Hetherington AM, Lazdunski M (1988) Ca^{2+} channel inhibitors that bind to plant cell membranes block Ca^{2+} entry into protoplasts. *Biochemistry* **27**, 764–768.

Heslop-Harrison J, Heslop-Harrison Y (1992) Germination of monocolpate angiosperm pollen: effects of inhibitory factors and the Ca^{2+}-channel blocker, nifedipine. *Annals of Botany* **69**, 395–403.

Hoefer Scientific Instruments (1994) 'Hoefer protein electrophoresis applications guide.' (Hoefer Scientific Instruments: San Francisco)

Hui A, Ellinor PT, Krizanova O, Wang J-J, Diebold RJ, Schwartz A (1991) Molecular cloning of multiple subtypes of a novel rat brain isoform of the α1 subunit of the voltage-dependent calcium channel. *Neuron* **7**, 35–44.

Kauss H, Waldmann T, Jeblick W, Takemoto JY (1991) The phytotoxin syringomycin elicits Ca^{2+}-dependent callose synthesis in suspension-cultured cells of *Catharanthus roseus*. *Physiologia Plantarum* **81**, 134–138.

Knaus H-G, Moshammer T, Friedrich K, Kang HC, Haugland RP, Glossmann H (1992*a*) *In vivo* labeling of L-type Ca^{2+} channels by fluorescent dihydropyridines: evidence for a functional, extracellular heparin-binding site. *Proceedings of the National Academy of Sciences USA* **89**, 3586–3590.

Knaus H-G, Moshammer T, Kang HC, Haugland RP, Glossmann H (1992*b*) A unique fluorescent phenylalkylamine probe for L-type Ca^{2+} channels. *Journal of Biological Chemistry* **267**, 2179–2189.

Kropf DL, Quatrano RS (1987) Localization of membrane-associated calcium during development of fucoid algae using chlorotetracycline. *Planta* **171**, 158–170.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li X, Goss J, Franceschi VR (1992) Evidence for calcium channel involvement in Ca oxalate formation. *Plant Physiology* **99** (Supplement), 69.

Lucas WJ, Franceschi VR (1982) Organization of the sieve-element walls of leaf minor veins. *Journal of Ultrastructure Research* **81**, 209–221.

Messiaen J, Read ND, Van Cutsem P, Trewavas AJ (1993) Cell wall oligogalacturonides increase cytosolic free calcium in carrot protoplasts. *Journal of Cell Science* **104**, 365–371.

Messiaen J, Nerinckz F, Van Cutsem P (1995) Callose synthesis in spirostanol treated carrot cells is not triggered by cytosolic calcium, cytosolic pH or membrane potential changes. *Plant and Cell Physiology* **36**, 1213–1220.

Muir SR, Sanders D (1997) Inositol 1,4,5-triphosphate-sensitive Ca^{2+} release across nonvacuolar membranes in cauliflower. *Plant Physiology* **114**, 1511–1521.

Nakamura S, Hayashi H, Mori S, Chino M (1995) Detection and characterization of protein kinases in rice phloem sap. *Plant and Cell Physiology* **36**, 19–27.

Piñeros M, Tester M (1995) Characterization of a voltage-dependent Ca^{2+}-selective channel from wheat roots. *Planta* **195**, 478–488.

Piñeros M, Tester M (1997*a*) Calcium channels in higher plant cells: selectivity, regulation and pharmacology. *Journal of Experimental Botany* **48**, 551–577.

Piñeros M, Tester M (1997*b*) Characterization of the high-affinity verapamil binding site in a plant plasma membrane Ca^{2+}-selective channel. *Journal of Membrane Biology* **157**, 139–145.

Schumaker KS, Gizinski MJ (1993) Cytokinin stimulates dihydropyridine-sensitive calcium uptake in moss protoplasts. *Proceedings of the National Academy of Sciences USA* **90**, 10 937–10 941.

Shaw SL, Quatrano RS (1996) Polar localization of a dihydropyridine receptor on living *Fucus* zygotes. *Journal of Cell Science* **109**, 335–342.

Sjolund RD, Shih CY (1983) Freeze-fracture analysis of phloem structure in plant tissue cultures. *Journal of Ultrastructure Research* **82**, 111–121.

Tarlyn NM, Kostman TA, Nakata PA, Keates SE, Franceschi VR (1998) Axenic culture of *Pistia stratiotes* for use in plant biochemical studies. *Aquatic Botany* **60**, 161–168.

Tsien RY (1989) Fluorescent indicators of ion concentrations. *Methods in Cell Biology* **30**, 127–156.

Thuleau P, Graziana A, Ranjeva R, Schroeder JI (1993) Solubilized proteins from carrot (*Daucus carota* L.) membranes bind calcium channel blockers and form calcium-permeable ion channels. *Proceedings of the National Academy of Sciences USA* **90**, 765–769.

Thuleau P, Moreau M, Schroeder JI, Ranjeva R (1994*a*) Recruitment of plasma membrane voltage-dependent calcium-permeable channels in carrot cells. *EMBO Journal* **13**, 5843–5847.

Thuleau P, Ward JM, Ranjeva R, Schroeder JI (1994*b*) Voltage-dependent calcium-permeable channels in the plasma membrane of a higher plant cell. *Proceedings of the National Academy of Sciences USA* **13**, 2970–2975.

Vallee N, Briere C, Petitprez M, Barthou H, Souvre A, Alibert G (1997) Studies on ion channel antagonist-binding sites in sunflower protoplasts. *FEBS Letters* **411**, 115–118.

Waldmann T, Jeblick W, Kauss H (1988) Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. *Planta* **173**, 88–95.

White PJ (1996) Specificity of ion channel inhibitors for the maxi cation channel in rye root plasma membranes. *Journal of Experimental Botany* **47**, 713–716.

White PJ (1998*a*) Calcium channels in the plasma membrane of root cells. *Annals of Botany* **81**, 173–183.

White PJ (1998*b*) Voltage-dependent Ca^{2+} uptake by right-side-out plasma membrane vesicles derived from maize shoots. *Journal of Plant Physiology* **152**, 17–24.

White PJ, Banfield J, Diaz M (1992) Unidirectional Ca^{2+} fluxes in roots of rye (*Secale cereale* L.). A comparison of excised roots with roots of intact plants. *Journal of Experimental Botany* **43**, 1061–1074.

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