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Monoclonal antibody TeM 106 reacts with a tonoplast intrinsic protein of 106 kDa from *Brassica oleracea* L.

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SUMMARY

A monoclonal antibody, designated TeM 106, that recognizes an intrinsic protein from the vacuole membrane (tonoplast) of cauliflower (*Brassica oleracea* L. var. *botrytis*) is described. Mice were immunized with a tonoplast fraction that had been purified from differentiating meristematic cells from the cauliflower head. Hybridomas were generated and screened by means of Enzyme Linked Immuno Sorbent Assays for differential reactivity to tonoplast over non-related proteins (bovine serum albumin). One out of 14 reactive murine clones was selected on the basis of its stability, secretory efficiency, and high affinity of the secreted antibodies. TeM 106 is an IgM which was shown by indirect immunofluorescence microscopy of frozen thin sections to bind specifically to the tonoplast of highly vacuolated cells as well as to the tonoplast of small vacuoles in meristematic cells. The molecular specificities of TeM 106 were preliminarily determined using electrophoretic transfer procedures (immunoblotting). TeM 106 reacted with a single protein band of 106,000 M_r from the tonoplast of cauliflower. Using two-dimensional gel electrophoresis, it was shown that the epitope is borne by a single polypeptide. The antigen is a glycopeptide containing mannose and/or glucose residues in the oligosaccharide side chain but the epitope, resistant to the metaperiodate oxidation, is contained in the polypeptide backbone. Salt elution experiments indicated that the antigen, unlike several proteins from the tonoplast, is not eluted from the membrane by KCl treatments and is, therefore, tentatively considered as a tonoplast intrinsic protein, designated TIP 106.

Key words: monoclonal antibody, Brassica, tonoplast

INTRODUCTION

The vacuole membrane (tonoplast) separates the vacuolar sap and the cytosol in plant cells and is responsible for the regulated transport of ions, primary and secondary metabolites (Boller and Wiemken, 1986) and water (Maurel et al., 1993) between the two compartments. The vacuole also acts as a repository for deleterious compounds, such as heavy metals and xenobiotics (Martinoïa et al., 1993), ensuring they do not disrupt cytoplasmic metabolism, while other reserve materials may be stored transiently or permanently in the organelle (Taiz, 1992).

The detailed characterization of the molecular complexes involved in these activities is still in its infancy. Some components, including several ion channels and a few transporters, are only known on the basis of their activities (Hedrich and Schroeder, 1989; Johannes et al., 1992) but they have not yet been isolated and biochemically characterized. Many others probably remain to be discovered. At present, the only well-characterized tonoplast proteins are the V-type H^+-ATPase (Parry et al., 1989; Sze, 1985; Ward et al., 1992), the H^+-PPase (Rea and Poole, 1993; Rea et al., 1992; Sarafian et al., 1992; Walker and Leigh, 1981), and members of the TIP family which probably function in water transport (Chrispeels and Maurel, 1994; Höfte et al., 1992; Maurel et al., 1993; Marty-Mazars et al., 1995). Isoforms, specific for plant species, developmental stages, tissues or intracellular compartments may exist for all of these proteins (Berkelman et al., 1994; Chrispeels and Maurel, 1994; Gogarten et al., 1992; Höfte et al., 1992).

Monoclonal antibodies prepared against a complete set of tonoplast components would be useful tools to recognize these known proteins in a wide variety of plants, to discriminate between isoforms differing in discrete domains or to screen for yet unknown tonoplast-specific proteins. In this report, we have attempted to do this by immunizing mice with a complete tonoplast fraction. Fourteen murine clones of hybridoma cells secreting antibodies against tonoplast epitopes have been obtained. We have selected a stable, well-growing clone that secretes large quantities of antibodies which bind avidly to a tonoplast intrinsic protein with a relative molecular mass of 106,000.

MATERIALS AND METHODS

Materials

All chemicals were obtained from the Sigma Chemical Co Ltd unless otherwise stated. Cell culture media were from Gibco (BRL, Life Technologies).

Sp2/0-Ag14 myeloma cells were a gift from Dr A. Tournefier (Immunologie Comparée, CNRS UA 674, Université de Bourgogne, Dijon). All cells and hybridomas were grown in a humidified 5% CO_2 atmosphere at 37°C in 260 ml tissue culture flasks (Nunclon™, NUNC).

Cauliflower (*Brassica oleracea* L. var. *botrytis*) heads were purchased from local producers and used immediately.

Balb/c female mice, 2-4 weeks old, were obtained from the Laboratory of the Immunotechnologies at the medical school (Dr P. Pothier, Université de Bourgogne, Dijon).

Preparation of tonoplast-enriched fraction

Intact vacuoles and tonoplast (vacuole membrane) fragments from cauliflower heads were prepared as previously described for red beetroot (Marty, 1982). Briefly, the tissue was minced and then sliced three times. Pellets from the 20,000 ***g*** minute centrifugation (crude vacuole homogenate) containing intact vacuoles, nuclei, mitochondria, plastids and tissue fragments were obtained. Tonoplast fragments were prepared directly from the 20,000 ***g*** minute pellets using a procedure which by-passes the need for extensive vacuole purification and yields purified membranes (Leigh et al., 1979; Marty, 1982; Marty and Branton, 1980; Marty et al., 1980; Marty-Mazars et al., 1995). All steps were carried out at 4°C in the presence of proteases inhibitors: 1 mM PMSF (phenyl methyl sulfonyl fluoride), 1 mM *o*-phenanthroline, 20 μg ml^{-1} aprotinin.

Whole cell lysate

Five grams of meristematic tissue from the head of a freshly harvested cauliflower were ground in liquid nitrogen with a pestle and mortar. The powdered brei was resuspended in 25 ml of buffer containing 1 mM EDTA (ethylenediamine-tetraacetic acid), 1 mM DTT (dithiothreitol), 50 mM Hepes-HCl, pH 8, 10 μg ml^{-1} aprotinin, 0.5 mM *o*-phenanthroline and 0.5 mM PMSF. After 2 centrifugations (6,500 ***g*** minute), the supernatant was collected and stored at −70°C until use.

Protein

Protein was estimated routinely by the dye-binding method of Bradford (1976) using BSA (bovine serum albumin) as a standard.

Immunization

Monoclonal antibodies were generated essentially according to the procedure of Köhler and Milstein (1975). Tonoplast-enriched suspensions (50 μg protein) were washed, resuspended in 250 μl PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) mixed with an equal volume of Freund's incomplete adjuvant and injected intraperitoneally (i.p.) into mice on day 1.

At 2 week intervals, mice were injected i.p. three times with the same amount of the above-mentioned immunogen. Ten days after the fourth injection, sera were collected and screened by the whole tonoplast Enzyme Linked Immuno Sorbent Assay (ELISA) (see below) for reactivity towards tonoplast. Twenty days later, mice received an i.p. boost of tonoplast suspensions (50 μg protein) in 250 μl PBS without adjuvant. Six days after i.p. boosting, the mouse with polyclonal serum reacting with most of the tonoplast polypeptides was killed for hybridomas production.

Serum collection

Before immunization of the mouse, drops of blood (100 μl maximum) were collected in a narrow glass tube by bleeding the tail. Following exsanguination, a maximum amount of blood was obtained from the thoracic cavity of immunized animals. The blood was allowed to clot for 1 hour at room temperature and subsequently stored in the refrigerator overnight for retraction. The polyclonal sera were collected after centrifugation at 2,500 ***g*** for 30 minutes and finally stored with 0.01% sodium azide at 4°C.

Production of hybridomas

Hybridomas were produced by standard methods (Galfre et al., 1977). Briefly, the spleen was removed, washed, and fused (1:1 splenocytes to myeloma cells; 135×10^6 cells total) with the mouse myeloma cells (line Sp2/0-Ag14) deficient in HGPRT (hypoxanthine-guanine-phosphoribosyl transferase) (Schulman et al., 1978) using 50% PEG, M_r 4,000 (polyethylene glycol) (Gibco). The cells were resuspended in RPMI 1640 culture medium containing 20% (v/v) FCS (foetal calf serum), 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotics (200 units ml^{-1} penicillin G, 200 μg ml^{-1} streptomycin), 50 mM β-mercaptoethanol and 1 mM HAT (hypoxanthine-aminopterine-thymidine). Cells were plated (13.5×10^4 cells/0.1 ml HAT medium/well) in tissue culture plates (Nunclon™, NUNC) with 96 wells already containing 50 μl HAT medium/well with macrophages as feeder cells.

After 2 weeks, hybridoma cells were transferred to RPMI 1640 culture medium containing 10% (v/v) FCS and 1 mM HT (hypoxanthine-thymidine).

Culture supernatants were screened by ELISA and positive hybridomas were cloned twice by limiting dilution (Goding, 1980).

Monoclonal antibody production

Two methods were used to obtain large quantities of monoclonal antibodies. In the first method, hybridomas were expanded slowly in vitro by transfer to 24-well plates, then to 6-well plates and finally to 260 ml flasks. The cells were maintained in exponential growth for propagation until large amounts of antibodies were obtained. After centrifugation (200 ***g*** for 10 minutes) the cells were finally frozen down to −70°C (10^6 cells in 1 ml of RPMI 1640 culture medium containing 10% (v/v) DMSO (dimethylsulfoxide) and 20% (v/v) FCS). When required, the cells were thawed at 4°C and grown again either in vitro or in vivo. The supernatants were kept with 0.01% sodium azide at 4°C.

In the second method, hybridomas were grown in ascitic fluids according to Hoogenraad and Wraight (1986) with minor modifications. Briefly, mice were primed by i.p. injection of 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane) 5-10 days before i.p. inoculation with 10^6 hybridoma cells. Seven to fourteen days later, the ascitic fluid was drained. The mouse was sacrificed and the fluid (4-6 ml) was drawn slowly using a Pasteur pipette and collected into a 15 ml tube. The ascitic fluid was cleared by centrifugation at 2,000 ***g*** for 10 minutes, then the cellular debris were spun down at 10,000 ***g*** for 30 minutes. 100 μl aliquots were frozen at −20°C. When necessary, the ascitic fluid was thawed and kept with 0.01% sodium azide at 4°C.

Isotyping of monoclonal antibodies

Isotyping of the monoclonal antibodies raised was made using ELISA kits obtained commercially (ISO-2 from Sigma).

Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA was carried out according to Engvall and Perlmann (1971). Washed tonoplast membranes (50 μg protein) were resuspended in 5 ml PBS. Tonoplast suspension (T^+, 0.5 μg per well) was added to 96-well ELISA plates (NUNC, Maxisorp) and plates were incubated for 15 hours at 4°C in a humidified atmosphere. The plates were washed 3 times with TBS (Tris-buffered saline: 137 mM NaCl, 20 mM Tris-HCl, pH 7.6)-Tween-20 (0.05%, w/v) (TBST), blocked with 1% (w/v) BSA in TBST (200 μl/well) for 1 hour at room temperature, and washed 3 times with TBST before immediate use.

Polyclonal sera (1:6,000 in TBST), mAbs from hybridoma cell culture supernatants (undiluted), or from ascitic fluid (diluted 1:100 in TBST) were added (100 μl/well) to the washed, tonoplast-coated wells and incubated for 3 hours at room temperature. ELISA plates were subsequently washed 4 times with TBST.

Alkaline phosphatase-labeled goat anti-mouse IgG (H + L, Biosys)

(100 µl per well) was added (1:4,000 in TBST) and incubated for 2 hours at room temperature. The plates were washed 3 times with 200 µl TBST and once with 100 µl of diethanolamine buffer (10%, v/v, diethanol-amine, 50 mM $MgCl_2$, adjusted to pH 9.6 with HCl). The substrate *p*-nitrophenyl phosphate disodium (1 mg ml^{-1} in diethanol-amine buffer) was added (100 µl/well) and incubated for 20 minutes at room temperature in the dark. The ELISA plates were read at 405 nm on a Titer Trek Multiscan plate reader.

BSA (1%, w/v, in PBS)-coated wells were used as controls (T^-) in all experiments. Nonspecific binding was determined using controls made with preimmune serum or in the absence of primary antibodies. ELISA ratios were calculated as follows: T^+_{A405}/T^-_{A405}.

One-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

SDS-PAGE was done according to the method of Laemmli (1970). The tonoplast pellet was treated as described by Marty-Mazars et al. (1995). The polyacrylamide gels were prepared as previously described (Marty-Mazars et al., 1995), except that the slab gels were 0.75 mm thick and consisted of a 7-15% (w/v) acrylamide resolving gel (65×85 mm) and a 3% (w/v) acrylamide stacking gel (15×85 mm) with either 10 wells for analytical work or a single well for preparative purposes.

Polypeptides in the resolving gels were either fixed by 25% (v/v) isopropanol and 10% (v/v) acetic acid for Coomassie Blue staining (Fairbanks et al., 1971) or fixed by 40% (v/v) ethanol and 10% (v/v) acetic acid for silver staining (Damerval et al., 1987). Apparent molecular masses of tonoplast polypeptides were calculated based on the mobilities of protein standards. Marker proteins used were: rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa (Bio-Rad).

Two-dimensional PAGE

Two-dimensional gel electrophoresis was optimized according to Damerval et al. (1986) as described in Marty-Mazars et al. (1995) with the following modifications. The second dimension SDS gels were 1.5 mm thick and consisted of a 7-15% (w/v) acrylamide resolving gel (140×110 mm) overlaid with a 140×25 mm stacking gel of 3% (w/v) acrylamide.

Two dimensional gels were fixed and silver stained (Damerval et al., 1987) or electrophoretically transferred to an Immobilon-P^{TM} membrane (see below).

Western blot analysis

Proteins were transferred from the polyacrylamide gel by electroblotting onto Immobilon-P^{TM} (Millipore) membranes using a Millipore semi-dry transfer apparatus (80 V, 2.5 mA cm^{-2} for 40 minutes at room temperature) according to the manufacturer's instructions. Nonspecific protein binding sites were blocked with 2% (w/v) Tween-20 in TBS for 15 minutes at room temperature. The membrane was incubated for 1 hour with either monoclonal antibodies (undiluted cell culture supernatants or ascitic fluids diluted 1:100 in TBST), polyclonal mouse antibodies (1:2,000) or preimmune serum (1:1,000) as a control, and washed 4 times in TBST for 10 minutes each. Alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) was added as a secondary antibody (1:1,000 in TBST) for 1 hour at room temperature. The immunoblots were washed in TBST as before and then in 100 mM Tris-HCl, pH 8.6. Blots were finally developed using 5 mg α-naphtyl acid phosphate, 0.04% (w/v) fast blue RR salt (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi[zinc chloride] salt), and 10 mM $MgCl_2$ in 80 mM Tris-HCl, pH 8.6.

KCl elution

Tonoplast-enriched suspensions (100 µg protein) were treated for 30 minutes at 37°C in a reciprocal shaking bath with KCl (0-1.5 M) in 40 µl of 10 mM Tris-HCl, pH 6.5, containing 1 mM EDTA and 0.1 mM PMSF.

The extracted membranes were recovered by sedimentation at 16,000 ***g*** for 40 minutes in an Eppendorf centrifuge. The pellets were directly solubilized for SDS-PAGE. Supernatants were evaporated in Speedvac (Jouan) and the residual materials were solubilized for SDS-PAGE.

Periodate oxidation

The periodate oxidation of glycoproteins was carried out according to Woodward et al. (1985). Polypeptides from the tonoplast-enriched fraction that had been subjected to electrophoresis were electroblotted onto a nitrocellulose membrane (0.2 µm pore size) (Schleicher & Schuell). Nonspecific binding sites were blocked with TBS-2% (w/v) Tween-20 for 15 minutes at room temperature. Blots were cut into strips and used in experimental and control conditions. Strips were washed with 60 mM sodium acetate, pH 4.8, for 10 minutes at room temperature, and then incubated with sodium metaperiodate (0-30 mM) in sodium acetate buffer for 1 hour in the dark at room temperature. Control and experimental strips were then rinsed for 10 minutes with sodium acetate buffer and incubated for 30 minutes with 1% (w/v) glycine in TBS. Following 5 washes with TBST, the strips were then treated for immunodetection as described before (see under western blot analysis).

Con A binding

Affinity blotting was performed according to the method of Faye and Chrispeels (1985). Tonoplast polypeptides were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in TBSCT (15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% (w/v) Tween-20) for 60 minutes at room temperature. Peroxidase-conjugated Con A was applied at a concentration of 10 µg ml^{-1} in TBSCT for 1 hour at room temperature. After 4 washes in TBSCT for 5 minutes each, the nitrocellulose membrane was incubated in a solution containing 10 mg DAB (diaminobenzidine.4 HCl) in 20 ml of 100 mM citrate-phosphate buffer pH 5. The reaction was started by addition of 60 µl H_2O_2 (30%, v/v).

Immunofluorescence microscopy

Florets from the head of *Brassica oleracea* (ca. 1 mm^3) were fixed with 2% (v/v) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1 hour at 4°C. The specimens were then washed 3 times for 10 minutes each with the same buffer and impregnated with 2.3 M sucrose in PBS for approximately 3 hours at room temperature. Each tissue fragment was mounted on a specimen holder and frozen in liquid nitrogen. Frozen thin sections (0.5 µm to 1 µm) were cut with a Reichert-Jung ultracut FC 4E microtome (Leica SARL) at –80°C to –90°C using glass knives according to Tokuyasu (1973, 1980). The sections attached to glass slides were placed in a humid chamber and treated for immunofluorescence (Coudrier et al., 1981; Griffiths et al., 1983). They were first blocked with 10% (v/v) NBCS (new born calf serum) (FlowBio) in PBS for 10 minutes to prevent nonspecific binding, washed again 5 minutes with free buffer, and then incubated for 3 hours at 20°C with ascitic liquid at dilutions ranging from 1:10 to 1:1,000. Sections were washed 3 times with PBS for 5 minutes each. Biotinylated goat anti-mouse IgM (Amersham) was added at a 1:200 dilution for 1 hour at 20°C. Streptavidin conjugated to fluorescein solution (Amersham) was added at a dilution of 1:50 for 30 minutes at 4°C in the dark. The preparations were mounted in Citifluor liquid (Agar Scientific Ltd) and observed in a Zeiss axiophot photomicroscope equiped with Nomarski optics and epifluorescence illumination with barrier filters set for fluorescein (BP 546-12/FT 580/LP 590) and 63× or 100× oil immersion lens.

RESULTS

Mice were immunized with the tonoplast-enriched fraction collected at the 1.045 g cm^{-3}/1.085 g cm^{-3} interface of the discontinuous sucrose gradient. Hybridomas were generated using splenocytes from the PD-3 mouse that exhibited the highest immune response when its polyclonal serum was used to probe the tonoplast proteins by western blotting. Conditioned media from all the hybridoma clones (total clones) were screened in ELISA for binding to whole tonoplast fraction. A summary of these results is shown in Fig. 1. The PD-3 mouse yielded 8 hybridoma clones with T^+/T^- ELISA ratios of 24 or greater and 6 hybridomas with T^+/T^- ELISA ratios between 1.38 and 2.34 with undiluted culture supernatants. The immunization protocol thus resulted in the production of two classes of clones that reacted differentially with molecules on the tonoplast vesicles.

The hybridomas that gave the highest response were evaluated by whole-tonoplast ELISA for their avidity for tonoplast antigens (Fig. 2). The hybridoma G6D2 which reacted the quickest was selected. The antibodies secreted by G6D2 were called TeM 106 (TeM: Monoclonal antibodies against an epitope from the Tonoplast) and were used for all subsequent studies reported here. TeM was an IgM type.

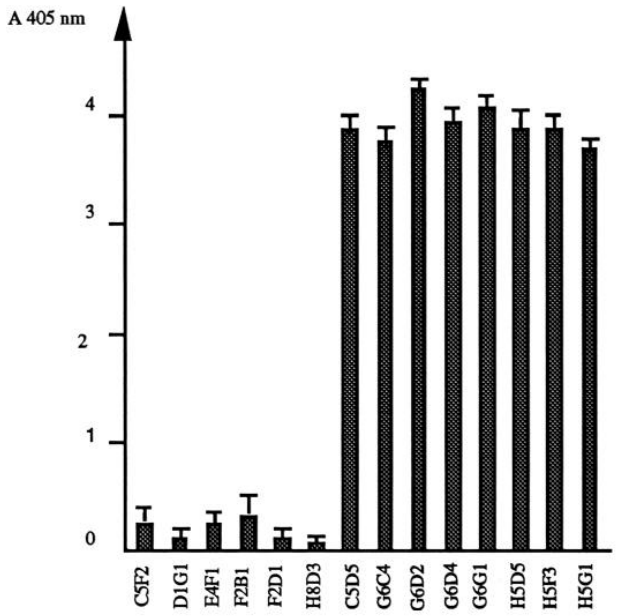

Fig. 1. Differential immune reactivity of antibodies. On culture day 4, conditioned media from hybridoma clones prepared from tonoplast-inoculated mice were screened in the whole tonoplast ELISA assay for reactivity. Medium (100 µl) from each culture well was collected, used undiluted, and tested in ELISA as described in Materials and Methods. Plates were coated with either whole tonoplast vesicles (T^+, 0.5 µg protein per well) or BSA as a control (T^-, 0.5 mg protein per well). The ELISA plates were read at 405 nm. Nonspecific binding was determined using BSA-coated plates and subtracted from the experimental samples. Data were derived from triplicate experiments. Only hybridoma clones (100 µl of medium alone) having ELISA T^+ values of greater than 5 times the background were included in the calculation.

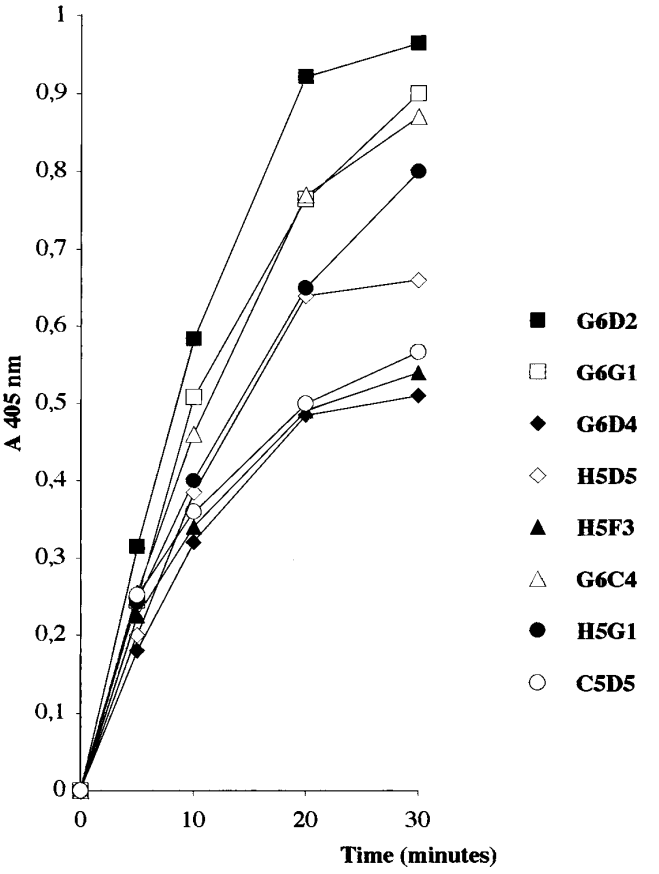

Fig. 2. Time-course of the formation of the immunocomplex. Conditioned media from hybridoma clones having whole tonoplast ELISA ratios (T^+_{A405}/T^-_{A405}) greater than 24 were screened in a comparative time-course assay to select for the clone secreting mAbs of highest reactivity to whole tonoplast. Tonoplast vesicles (0.5 µg protein per well) were added to wells and allowed to attach for 15 hours at 4°C. The plates were treated with 1% (w/v) BSA in TBST to block nonspecific binding sites, then washed 3 times with TBST and 100 µl of undiluted conditioned media containing mAbs was added. Incubations were carried out at 20°C for 5, 10, 20 and 30 minutes. At each time point the media were removed and an ELISA assay was carried out and A values were read at 405 nm. Nonspecific binding was determined using BSA-coated plates and subtracted from the experimental samples.

Immunofluorescence localization of the antigen

To ascertain the localization of the cellular determinants recognized by the monoclonal antibody secreted by the G6D2 clone that we have selected, intact meristematic cells were analyzed by indirect immunofluorescence microscopy. Figure 3A shows an immunofluorescence micrograph illustrating the reactivity of the mAb. Cells from the same field were observed under Nomarski (Fig. 3B) and phase contrast (Fig. 3C) optics. The staining pattern indicates that the mAb recognizes epitopes localized on the tonoplast while the cell wall, cytoplasm and the nucleus are not labeled. The immunofluorescent labeling was occasionally seen as a punctate pattern. No distinct staining pattern was observed with the preimmune serum of the mouse (data not shown) indicating that the localization of the molecules recognized by the mAb at the periphery of the

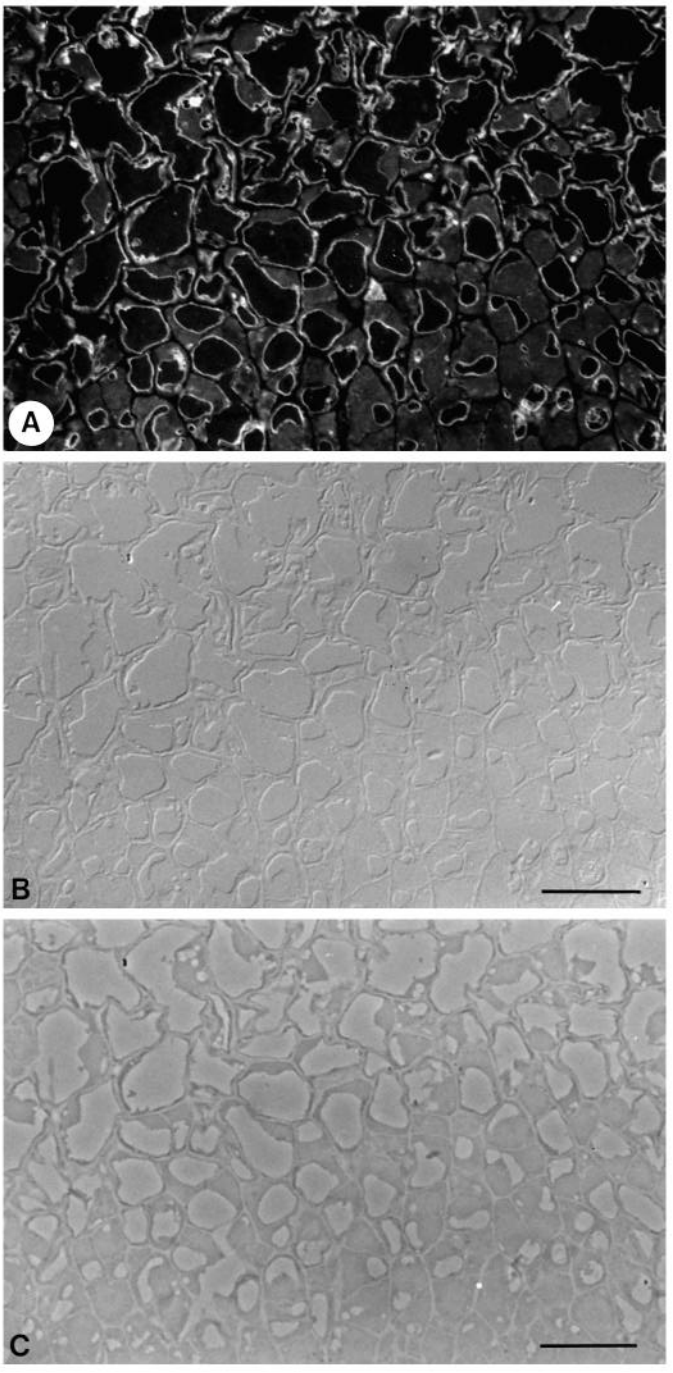

Fig. 3. Indirect immunofluorescence localization of the 106 kDa polypeptide recognized by mAb TeM 106. Cells from the shoot meristem of cauliflower were fixed as described in Materials and Methods. Frozen thin sections (1-2 μm) were incubated with the ascitic fluid derived from the clone G6D2 (dilution 1:100). The sections were then treated with biotinylated goat anti-mouse IgM followed by fluorescein-conjugated streptavidin at a dilution recommended by the manufacturer (A). The same field is shown in Nomarski (B) and in phase-contrast (C) optics. Bar, 10 μm.

vacuole is specific and not due to nonspecific binding of immunoglobulins.

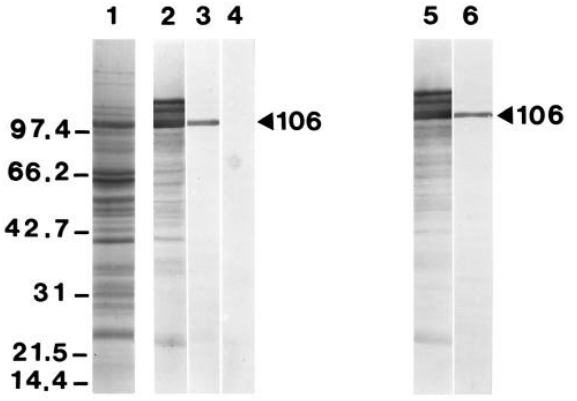

Fig. 4. Coomassie Blue staining (lane 1) and immunoblot analysis (lanes 2-6) of polypeptides solubilized from purified tonoplast (lanes 1-4) and total cell lysates (lanes 5 and 6) of cauliflower. Lane 1, Coomassie Blue staining of polypeptides purified from the tonoplast and separated by SDS-PAGE under reducing conditions. Lanes 2-4, immunoblot analysis of tonoplast proteins separated by SDS-PAGE under reducing conditions, electroblotted onto Immobilon-P^{TM} membrane, and probed with polyclonal serum of the PD-3 mouse (dilution 1:2,000 in TBST, lane 2), mAb TeM 106 secreted by the clone G6D2 (ascitic fluid, dilution 1:100 in TBST, lane 3), and preimmune serum (dilution 1:1,000 in TBST, lane 4). Lanes 5 and 6, immunoblot analysis of proteins solubilized from a total cell lysate, electroblotted onto Immobilon-P^{TM} membrane, and probed with polyclonal serum of the PD-3 mouse (dilution 1:2,000 in TBST, lane 5), and mAb TeM 106 secreted by the clone G6D2 (ascitic fluid, dilution 1:100 in TBST, lane 6). The same amount of protein (15 μg) was loaded on each lane. Molecular mass standards (kDa) are shown at the left.

Western blotting

To determine the nature and specificity of the antigens recognized by mAb TeM 106, tonoplast-enriched fractions and whole cell lysates were subjected to electrophoresis in reducing SDS-PAGE gels and probed after western blotting with the polyclonal serum (Fig. 4, lanes 2 and 5, respectively), mAb TeM 106 (Fig. 4, lanes 3 and 6, respectively), and the preimmune serum (Fig. 4, lane 4). The polyclonal serum clearly recognized a triplet of major polypeptides with relative molecular masses about 100,000 in the total cell lysates and in the tonoplast fractions. Several minor immunoreactive bands could also be detected. Selected mAb specifically recognized a single polypeptide band migrating at about 106 kDa in the tonoplast-enriched fractions and in whole cell lysates (Fig. 4, lanes 3 and 6, respectively). No polypeptides were detected in whole cell lysates (data not shown) and in the tonoplast-enriched fractions (Fig. 4, lane 4) when probed with the preimmune serum, indicating that this mAb specifically recognized the major 106 kDa polypeptide from the tonoplast.

2-D gel electrophoresis

Proteins solubilized from the tonoplast-enriched fractions can be separated with better resolution by 2-D gel electrophoresis. In order to determine whether mAb TeM 106 binds to a single polypeptide or to several isoforms which comigrate in one band on one-dimensional gel electrophoresis, tonoplast polypeptides were separated by two-dimensional gel electrophoresis, electroblotted onto Immobilon-P^{TM} membrane, and probed with the mAb. Only one single polypeptide with a

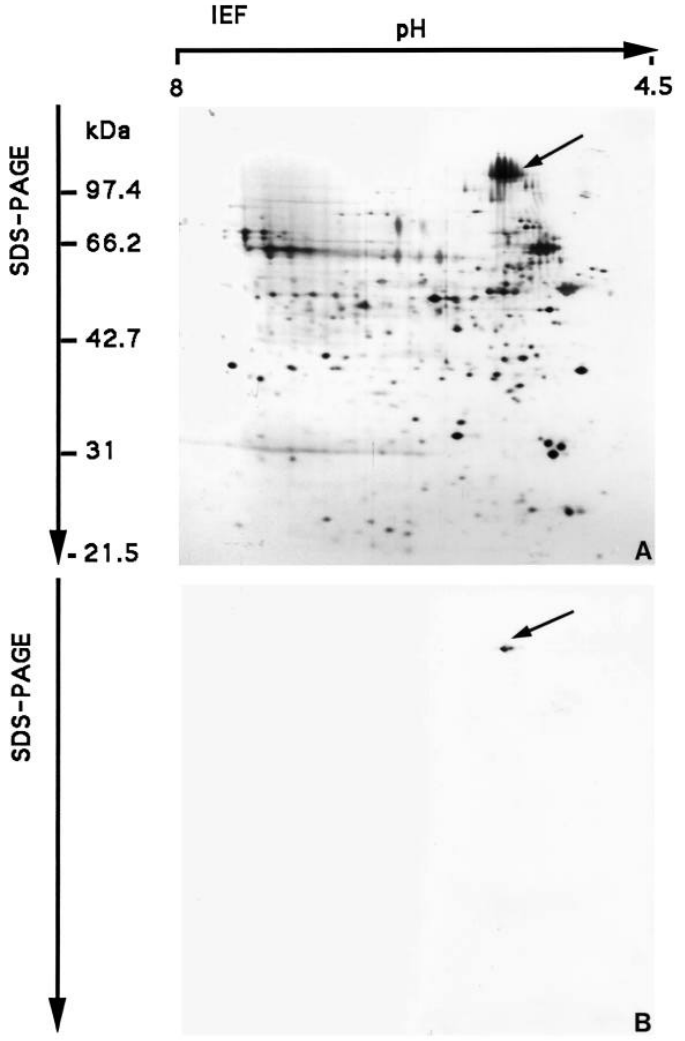

Fig. 5. Two-dimensional electrophoretograms of polypeptides solubilized from tonoplast-enriched fraction from cauliflower. (A) Silver-stained, 2-D polypeptide pattern from the tonoplast. (B) 2-D immunoblot probed with mAb TeM 106 obtained directly from the undiluted supernatant of the clone G6D2. Two-dimensional PAGE and immunoblotting were carried out as described in Materials and Methods. 80 μg proteins were loaded onto the IEF gels.

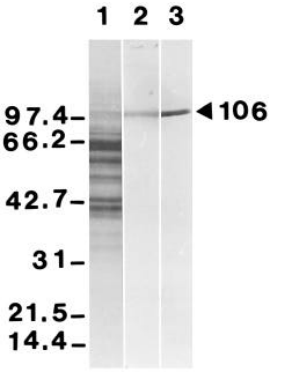

Fig. 6. Affinity blotting of the 106 kDa tonoplast polypeptide with Con A. Lane 1, tonoplast proteins were separated by SDS-PAGE under reducing conditions, and electroblotted onto nitrocellulose membrane. Nonspecific binding sites were blocked with TBSCT for 1 hour at room temperature and Con A-peroxidase conjugate (10 μg ml^{-1} in TBSCT) was added for 1 hour at room temperature. Lane 2, the band containing the 106 kDa polypeptide was first cut out from a preparative polyacrylamide gel and loaded on a 7-15% acrylamide gel for SDS-PAGE, electroblotted onto nitrocellulose membrane, and probed with Con A-peroxidase conjugate as described above. Lane 3, immunoblot analysis of tonoplast proteins separated by SDS-PAGE under reducing conditions, electroblotted onto nitrocellulose membrane, and probed with mAb TeM 106 obtained directly from the undiluted supernatant of the clone G6D2. 15 μg proteins were loaded on lanes 1-3. Molecular mass standards (kDa) are shown at the left.

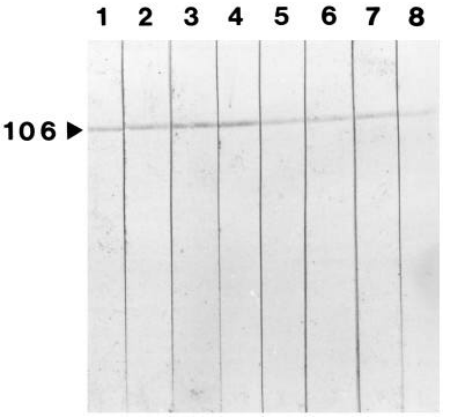

Fig. 7. Immunoblot analysis of tonoplast polypeptides after metaperiodate oxidation. Tonoplast proteins were separated by SDS-PAGE under reducing conditions, electroblotted onto nitrocellulose membrane, and treated with 0 mM (lane 1), 0.1 mM (lane 2), 0.5 mM (lane 3), 1 mM (lane 4), 5 mM (lane 5), 10 mM (lane 6), 20 mM (lane 7) and 30 mM (lane 8) sodium metaperiodate for 1 hour at room temperature. Nonspecific binding sites were blocked with 1% (w/v) glycine in PBS for 30 minutes at room temperature. The blots were washed 3 times with PBS, and probed with mAb TeM 106 obtained directly from the undiluted supernatant of the clone G6D2.

relative molecular mass of 106,000 and a pI of about 5-6 bound the mAb. The location of the labeled spot precisely coincides with a major polypeptide (106 kDa, 5<pI<6) in silver-stained gels, as indicated in Fig. 5.

Glycosylation

Because previous studies have demonstrated that many tonoplast proteins are glycosylated, we wished to know whether the antigen recognized by mAb TeM 106 was a glycoprotein. Con A-peroxidase assays were used to visualize glycopeptides on affinoblots of tonoplast polypeptides that had been subjected to electrophoresis. A number of polypeptides were readily detected (Fig. 6, lane 1). The polypeptide recognized by the mAb was weakly labeled indicating that it is glycosylated to a small extent (Fig. 6, lanes 2 and 3). Because the antigen was labeled by Con A-peroxidase, it can be concluded that it contains glucose and/or mannose residues.

Metaperiodate oxidation

To determine the nature of the epitope in the glycoprotein that recognized mAb TeM 106, polypeptides from the tonoplast-enriched fraction were subjected to electrophoresis using reducing SDS-PAGE, transferred by electroblotting to nitrocellulose membrane, treated by sodium metaperiodate (0-30 mM), and subsequently probed with mAb TeM 106 (Fig. 7). The metaperiodate treatment did not alter the immunoreactivity of the antigen, thus leading us to conclude that the epitope

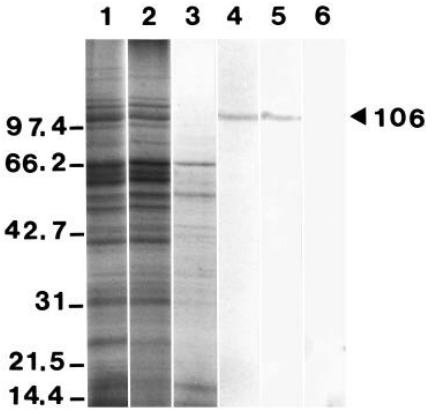

Fig. 8. The release of polypeptides by KCl washing of tonoplast vesicles. Packed membranes (ca. 100 μg proteins) were treated for 30 minutes at 37°C with 10 mM Tris-HCl, pH 6.5 (lanes 1 and 4), or Tris-buffered KCl 1.5 M (lanes 2, 3, 5, and 6), each containing 1 mM EDTA and 0.1 mM PMSF, then centrifuged at 16,000 ***g*** for 40 minutes. Pellet (lanes 2 and 5) and supernatant (lanes 3 and 6) fractions were sampled for SDS-PAGE under reducing conditions. Proteins were either stained with Coomassie Blue in the gel (lanes 1-3) or electroblotted onto Immobilon-P^{TM} membrane, and probed with mAb TeM 106 (lanes 4-6). The same amount of proteins (15 μg) were loaded on each lane. Molecular mass standards (kDa) are shown at the left.

does not result from the oligosaccharide, but is likely contained within the peptide.

Transverse distribution of the polypeptide within the membrane

To obtain preliminary information on the transverse distribution of the polypeptide within the membrane and to determine whether it is a peripheral or intrinsic protein, tonoplast vesicles were sequentially washed by KCl (0 or 1.5 M). A number of polypeptides were differentially eluted from the membrane and they were tentatively identified as peripheral proteins (Fig. 8, lane 3). Other membrane proteins, including the antigen recognized by mAb TeM 106, were not released by salt treatment but were retained in the residual membrane (Fig. 8, lanes 2 and 5). Therefore, it is likely that the polypeptide recognized by the mAb is tightly bound to the membrane core components and can be tentatively identified as a tonoplast intrinsic protein.

DISCUSSION

The large vacuole, together with trafficking vesicles coming from the Golgi apparatus and from the plasma membrane, make up the vacuolar apparatus of differentiated plant cells. This is a highly dynamic, pleiomorphic and multifunctional system which undergoes dramatic morphological as well as physiological changes during cell differentiation. It is reasonable to infer that these changes are induced by the remodeling of molecular assemblies in the membranes. Meristematic tissues, where intense cell vacuolation continuously occurs, are suitable sources for obtaining a panel of components from the vacuolar membranes.

A major limitation is the small size of the meristems which restricts the availability of large quantities of material for isolating tonoplast-enriched fractions. However, the edible part of cauliflower (*Brassica oleracea* L. var. *botrytis*) which results from the proliferation of shoot apical meristems with their associated stems (De Candolle, 1824; Masters, 1869; Sadik, 1962) is a convenient plant material for such studies. This spontaneous mutation and the resulting amplification of meristematic tissues in cauliflower offers an opportunity to study the expression of a series of tonoplast components in vacuolating cells. The uppermost region of the head is truly meristematic. It consists of 10^5 to 10^6 shoot apical meristems, 100-200 μm wide, arrested at an early stage of floral development (Medford et al., 1991). The cells from the two most superficial layers are not vacuolated or contain small vacuoles whereas the thickened short stems from the inner portion consist of enlarged and vacuolated parenchymatous cells. A sharp (acropetal) gradient of cell differentiation is observed; vacuoles at all stages of development are present in the superficial layers of cauliflower head and they are potentially amenable to isolation by cell fractionation.

To reach this goal, we have used a large scale, mechanical apparatus specifically designed to cut open cells from firm tissues like cauliflower (Leigh and Branton, 1976). Vacuoles at different stages of development were directly liberated into a suitable osmoticum, together with other cellular organelles. This method is well-suited for the bulky meristematic tissues of cauliflower. Membranes are not altered by prolonged exposure to cell wall degrading enzymes as they are in procedures requiring the preparation of protoplasts. Finally, the method is rapid and the possible redistribution of membrane components is minimized. Therefore, the vacuole membranes isolated according to this procedure retained their in vivo properties and intact sets of native membrane proteins. The purified fraction likely contained membrane fragments (vesicles) from vacuoles at various stages of formation and, thus, contained a large panel of proteins expressed in the tonoplast at different stages. Despite the low yield of the technique, sufficient amounts of purified membranes can be prepared due to large ready available quantities of cauliflower.

Our use of *Brassica oleracea* L. var. *botrytis* is a way of isolating and purifying a sufficient amount of protein from a particular compartment in differentiating cells so as to use and isolate genes from *Brassica* and/or *Arabidopsis*, the 'botanical *Drosophila*' (Whyte, 1946). *Arabidopsis thaliana* (thale cress) is a weed which has a series of features that make it a convenient plant organism for classical as well as molecular genetics (Meyerowitz, 1987, 1989). For instance, the mature plant has a small size and its generation time is short (5-6 weeks only). Large-scale mutagenesis is facilitated by the small size of the seeds and the small number of cells in the embryo: indeed many mutants have already been described. The cells have a very small haploid nuclear genome (70 megabase pairs) and several genetic maps of marker genes and DNA fragments exist (Chang et al., 1988; Koorneef, 1987; Nam et al., 1989). DNA-mediated transformation can be applied with *Arabidopsis* using *Agrobacterium*, microprojectiles and infiltration technologies (An et al., 1986; Bechtold et al., 1993; Lloyd et al., 1986; Seki et al., 1991; Valvekens et al., 1988). The small size of the adult plant, however, limits the amount of material available for analysis. Moreover, the shoot apical meristem of *Arabidopsis* is among the smallest in higher plants (Vaughan,

1955) and makes it unsuitable for the isolation and purification of components present in differentiating cells.

The *Brassica oleracea* L. var. *botrytis* (cauliflower) is closely related to *Arabidopsis* and its bulky head provides large quantities of tissue for biochemical purifications. Indeed, the haploid nuclear genome size of *Brassica oleracea* L. var. *botrytis* is only 4 times larger than that of *Arabidopsis* but 25 times smaller than that of wheat (Arumuganathan and Earle, 1991). Both species belong to the Brassiceae family, and the similarity between cauliflower and thale cress extends to the DNA sequences. For instance, we found that the NH_2-terminal amino acid sequences of γ-TIPs (Tonoplast Intrinsic Proteins, γ-isoform) from *Brassica oleracea* L. var. *botrytis* and from *Arabidopsis* are identical. In addition, cauliflower is a crop plant of economic importance which means that basic genetic information exists due to a long history of domestication (De Candolle, 1824; Hervé, 1992), and inbred lines are readily available from selection programs. It is also a popular vegetable in many countries and is available all-year round.

When producing monoclonal antibodies, mixtures of immunogens are often used to immunize mice and, therefore, selection of the clones of interest usually requires fastidious steps of screening. Because we injected animals with purified tonoplast fragments, we readily obtained clones secreting antibodies specific to the membrane under study. Thus it appears that the cell fractionation step allows for the production of antibodies that react with relevant membrane antigens. This is of great advantage when antigens are rare or in low abundance on a particular membrane relative to highly immunodominant antigens from other cellular components.

Out of 14 clones secreting antibodies reacting uniquely to the tonoplast, clone G6D2, which secretes antibodies that bind at enhanced levels to the tonoplast polypeptides, was selected for production of antibodies in amounts sufficient for the characterization of the antigen.

The epitope recognized by the mAb TeM 106 is contained in a single tonoplast polypeptide of M_r 106,000 as shown by western blotting. Preliminary investigations have shown that the antigen is a glycoprotein, containing mannose and/or glucose residues in the oligosaccharide side-chain, with a polypeptide backbone which contains the epitope.

In contrast to several other polypeptides from the tonoplast, the immunoreactive antigen was not eluted from the membrane by salt treatment and is therefore tentatively identified as a tonoplast intrinsic protein referenced as TIP 106.

Results obtained from model membranes, such as that of the red blood cell (Bennett, 1985; Elgsaeter et al., 1986), have shown that integral membrane proteins play crucial roles in the organization and function of the membranes in which they are housed. These proteins are used as anchors around which peripheral membrane proteins are organized and specific domains are created. They themselves play important functions across the membranes such as signal transduction or transport. Therefore, characterization of the intrinsic proteins from the membrane of the vacuolar apparatus is of great importance in understanding its topological organization and its specific functions. The existence of distinct domains in the tonoplast of a single vacuole or in the membranes of separated subcompartments of the vacuolar apparatus is implicit in functional compartmentalization. Investigations are under way to characterize and identify the membrane proteins interacting with TIP 106 and to determine how and when TIP 106 functions in the tonoplast of differentiating cells.

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