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Plasma Membrane Intrinsic Proteins of *Beta vulgaris* L.[1]

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The plasma membrane (PM) of higher plants contains numerous proteins; however, due to their low abundance, only a few have been identified and characterized by direct biochemical approaches. The major intrinsic protein (MIP) family is a class of highly hydrophobic integral membrane proteins thought to function as channels that facilitate the passage of water, small solutes, and possibly other moieties through the membrane. A family of PM intrinsic proteins was purified and characterized from PM vesicles derived from storage tissue of *Beta vulgaris* L. using the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate. This PM intrinsic protein-enriched fraction also contains high levels of UDP-glucose:(1,3)-β-glucan (callose) synthase activity. Dithiothreitol is required to visualize the monomeric species of these highly hydrophobic integral membrane proteins. Sequence analysis of tryptic fragments derived from polypeptides of 31 and 27 kD revealed significant homologies to plant MIPs identified from cloned sequences. These MIPs include clone 7a from pea and RD28 from *Arabidopsis*, both of which are water-stress proteins, a tomato ripening-associated membrane protein, and PIP 2b, a PM-bound water channel protein from *Arabidopsis*. MIPs, therefore, represent abundantly occurring components of PMs derived from beet storage tissue.

The PM is one of the most important but least understood membrane systems in higher plants and possesses numerous functions that include containment, transport, recognition, and biosynthesis. In higher plants, the PM serves as the site of synthesis of cellulose microfibrils, which are a virtually ubiquitous component of plant cell walls, and wound-induced callose, which is an essential part of plant defense responses. Despite a long history of efforts by plant biochemists to isolate and characterize PM-bound enzymes, the low abundance of these proteins has complicated purification attempts. To date, the only integral PM protein from plant sources for which sequence information has been obtained via direct purification of a polypeptide from isolated membranes is the 100-kD H^+-ATPase, which is responsible for generation of proton gradients across the membrane (Sussman, 1994).

There has been a great deal of interest recently in membrane proteins that function as channels for the passage of water, ions, and solutes such as glycerol and urea (Ishibashi et al., 1994; Weaver et al., 1994). One such class, collectively known as the MIP or aquaporin family, has attracted a great deal of attention because it is highly conserved and found in species ranging from bacteria to mammals (Pao et al., 1991; Agre et al., 1993; Chrispeels and Maurel, 1994; Knepper, 1994). MIPs generally range in size from 25 to 30 kD, and reconstitution experiments have now shown that the erythrocyte MIP, CHIP28, aggregates as a tetramer to form water-permeable channels within the PM (Agre et al., 1993). Other MIP systems that have been extensively characterized include GlpF, a bacterial glycerol facilitator (Johnson et al., 1990b; Maurel et al., 1994), NOD26, a peribacteroid membrane protein in the nitrogen-fixing root nodules of soybean (Sandal and Marcker, 1988; Weaver and Roberts, 1992; Weaver et al., 1994), and TIP, the tonoplast intrinsic protein, which has been documented in a wide variety of plants (Johnson et al., 1990a, 1990b; Pao et al., 1991; Hofte et al., 1992; Johnson and Chrispeels, 1992; Ludevid et al., 1992). Other MIPs have now been identified in plant tissue. These include RD28 (Yamaguchi-Shinozaka et al., 1992), a water-stress protein from *Arabidopsis*, TRAMP (or pTOM75) (Fray et al., 1994), a group of peroxisomal membrane proteins (Corpas et al., 1994; Jiang et al., 1994), and two families of integral proteins (PIP 1 and 2) derived from the PM of *Arabidopsis* (Kammerloher et al., 1994). These latter proteins were cloned by immunoselection using a mammalian expression system and were shown to be water channels by osmotic water permeability studies in a *Xenopus* oocyte system (Kammerloher et al., 1994).

This paper documents the existence and properties of a family of MIPs derived from storage tissue of *Beta vulgaris* L. We first observed these polypeptides in fractions that were highly purified in callose synthase activity (Wu and Wasserman, 1993). These fractions were obtained using an approach involving direct purification from tonoplast-free PM vesicles in the detergent CHAPS. Here we show that tryptic fragments derived from the abundant 31- and 27-kD integral PM polypeptides from *Beta* are homologous to several recently cloned plant MIPs, including RD28 (Yamaguchi-Shinozaka et al., 1992), TRAMP (Fray et al.,

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CHIP28, channel-forming integral membrane protein of 28 kD; GlpF, glycerol facilitator; MIP, major intrinsic protein; NOD26, soybean nodulin 26; PIP 2b, plasma membrane intrinsic protein 2b of *Arabidopsis*; PM, plasma membrane; PMIP, plasma membrane intrinsic protein; RD28, responsive to desiccation membrane protein of *Arabidopsis*; TIP, tonoplast intrinsic protein; TRAMP, tomato ripening-associated membrane protein.

1994), PIP 1 and 2b (Kammerloher et al., 1994), and pea clone 7a (Guerrero et al., 1990). The tendency of these hydrophobic proteins to form disulfide-linked aggregates is also described. These findings provide direct biochemical evidence for a family of MIPs localized at the PM of *B. vulgaris* L.

MATERIALS AND METHODS

Membrane Preparation and PMIP Purification

PMIPs were isolated using a modified protocol previously developed for purification of callose synthase (Wu and Wasserman, 1993). Microsomal membranes were isolated from red beet (*Beta vulgaris* L.) storage tissue by differential centrifugation (Wasserman et al., 1989). PM vesicles were prepared by aqueous two-phase partitioning (Wu et al., 1991). PMIPs were solubilized in 1 mM EDTA, 1 mM EGTA, 0.6% CHAPS, and 7.5% glycerol in 50 mM Tris-HCl, pH 7.5, by a two-step solubilization procedure (Sloan et al., 1987; Wasserman et al., 1989), and were subsequently purified by glycerol gradient centrifugation followed by product entrapment (Wu and Wasserman, 1993). Alternatively, PMIPs were partially purified by glycerol gradient centrifugation alone, as indicated. Product entrapment was conducted as described (Wu et al., 1991). Glycerol gradient centrifugation was conducted using 4.2-mL linear glycerol (25–40%, v/v) gradients containing 0.5% CHAPS, 3 mM EDTA, and 3 mM EGTA in 50 mM Tris-HCl, pH 7.5. Solubilized proteins (0.75 mL) were applied to each tube, and the gradients were centrifuged at 200,000*g* for 4 h in an SW 50.1 rotor and fractionated into 14 fractions of 0.34 mL each. PMIP-enriched fractions were identified by SDS-PAGE and fractions were also assayed for callose synthase (Wu and Wasserman, 1993). Protein was determined by Coomassie blue dye-binding with BSA as standard (Bradford, 1976).

Electrophoresis and Immunoblotting

SDS-PAGE (Laemmli, 1970) was performed on 9 to 18% polyacrylamide gradient gels containing 5% glycerol (Porzio and Pearson, 1976). Sample loading buffers consisted of 8 M urea, 4% SDS, 20% glycerol, and 100 mM Tris-HCl, pH 8.0, in the presence or absence of DTT or β-mercaptoethanol, as indicated. Polypeptides were visualized by silver staining after enhancement by Coomassie blue (Daiichi double-staining protocol; Integrated Separation Systems, Enprotech, Hyde Park, MA). Band intensities were monitored by densitometry at 630 nm using a scanning densitometer (LKB Ultroscan XL enhanced laser densitometer).

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose membranes in 0.1% SDS, 100 mM Gly, and 10 mM Tris-HCl, pH 8.0 (Towbin et al., 1979). The blots were soaked for 3 h in 1% BSA, 0.15 M NaCl, and 10 mM Tris-HCl, pH 7.4, incubated with antiserum (1:1000 dilution) for 2 h, and washed three times with 0.15 M NaCl and 10 mM Tris-HCl, pH 7.4. Blots were incubated with secondary antibody (horseradish peroxidase-conjugated with goat anti-rabbit IgG) and visualized using the enhanced chemiluminescence kit (Amersham) according to the manufacturer's protocol.

Peptide Sequence Determination

The 31- and 27-kD proteins were electroeluted, concentrated in Centricon 10 microconcentrators (Amicon, Beverly, MA), subjected again to SDS-PAGE, and blotted onto polyvinylidene difluoride membranes (Bio-Rad) using 10 mM (3-[cyclohexylamino]-1-propanesulfonic acid) buffer containing 30% methanol, pH 11. Sample buffer in the second electrophoresis contained 45 mM DTT to prevent aggregation to higher molecular mass species. Blots were stained with 0.5% Ponceau S (Sigma) in 1% acetic acid for 10 s and destained with 1% acetic acid. The bands of interest were excised for internal peptide sequencing. Special care was taken to excise only the center of the 31-kD band to avoid contamination by the 29-kD polypeptide, which overlaps the leading edge of the 31-kD protein. The polyvinylidene difluoride slices were digested in situ by trypsin and products were separated by HPLC. Sequences were determined using a HP G1000A protein sequencer with a 1090 on-line liquid chromatograph or with an Applied Biosystems 477A protein sequencer equipped with a 120A on-line PTH-AA analyzer (Harvard MicroChem, Cambridge, MA). The sequence data base search was done using both MacDNASIS Pro (Hitachi Software Engineering America, Ltd., San Bruno, CA) and the blast network service (National Center for Biotechnology Information).

RESULTS

PMIP Purification and Disulfide-Linked Aggregation

The PM fraction was prepared by aqueous two-phase partitioning and was shown immunologically to be free of the 54-kD subunit of the tonoplast H^+-ATPase (Wu et al., 1991). We report here that subsequent purification steps designed to enrich for callose synthase activity yielded fractions that were also highly enriched in a family of PMIPs. The purification consisted of solubilization with CHAPS, glycerol gradient centrifugation, and product entrapment (Wu and Wasserman, 1993). Upon analysis by SDS-PAGE using a sample buffer containing β-mercaptoethanol, the purified preparations (Fig. 1, lane 3) contained a minor band at 57 kD, a broad band that spanned the molecular mass range of 47 to 42 kD (referred to as the 43-kD band), and polypeptides of 31 and 27 kD. A 29-kD polypeptide, which migrated slightly ahead of the 31-kD polypeptide, was observed in some preparations (Fig. 2). It should be noted that these PMIPs stained poorly with Coomassie blue or silver stain alone; a combined staining procedure utilizing Coomassie blue enhancement followed by silver staining was required for effective visualization.

The relative distribution of these SDS-denatured polypeptides on gels was readily manipulated by addition of disulfide reducing agents such as DTT. In the absence of DTT and β-mercaptoethanol, the broad 43-kD band was the major component present, but addition of DTT resulted in almost complete conversion of this species to the 31-, 29-, and 27-kD proteins (Fig. 2A). Scanning densitometry fur-

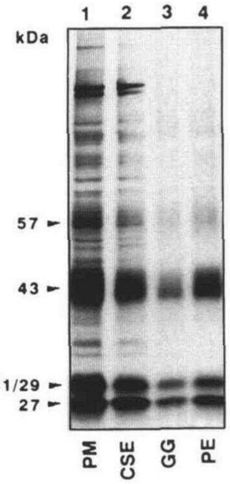

Figure 1. SDS-PAGE of PMs and fractions enriched in PMIPs and callose synthase activity. The PM fraction was prepared by aqueous two-phase partitioning (lane 1) and proteins were solubilized using CHAPS (CSE, lane 2). Further purification consisted of glycerol gradient centrifugation (GG, lane 3) followed by product entrapment (PE, lane 4). The SDS sample buffer contained 50 mM β-mercaptoethanol but no DTT. Polypeptide molecular masses are indicated to the left of the gel.

ther illustrates that loss of the 43-kD band generally corresponded with the combined appearance of the 31-, 29-, and 27-kD proteins (Fig. 2B). Similar results were obtained in a PM fraction isolated from celery (not shown). The 97-kD component was not always present (Fig. 1, lanes 3 and 4), and its position was not affected by DTT (Fig. 2A).

The 27-kD protein cross-reacted with an antibody directed against the carboxy terminus of a TIP from *Arabidopsis* (Fig. 3). In the absence of DTT, this antibody recognized only the 43-kD band, whereas with DTT, this antibody recognized only the 27-kD protein (Fig. 3). This not only confirms the origin of the 27-kD polypeptide, but implicates it as a PM-localized member of the MIP family.

The 31- and 27-kD proteins were highly hydrophobic (approximately 60% uncharged nonpolar amino acids; Table I). Each of these proteins was subject to formation of a ladder of higher molecular mass bands upon electroelution from gels (Fig. 4). The electroeluted 27- and 31-kD proteins polymerized to species of 44 and 48 kD, respectively, when they were electrophoresed in the absence of reducing agent. In addition, higher molecular mass species of 59 and 63 kD were observed. The observation that the calculated molecular masses are not exact multiples of the monomeric molecular mass indicates anomalous migration of one or more of these species (Griffith, 1972; Read and Northcote, 1983). This type of behavior is typical of hydrophobic proteins after their extraction from membranes of plant origin (Maeshima, 1992). Because of the tendency of the 31-, 29-, and 27-kD proteins to form disulfide-linked aggregates, this system bears some similarity to the mitochondrial alternative oxidase, which forms a dimeric complex between two identical subunits (Umbach and Siedow, 1993). However, because the PMIP system from *Beta* contains at least three separate moieties, the nature of possible interactions between these three polypeptides within the PM cannot be readily determined based on the current data.

Sequence Analysis of Peptide Fragments

The 31- and 27-kD polypeptides were each purified by electroelution from SDS gels and subjected to compositional (Table I) and sequence analysis (Fig. 5). Each polypeptide was digested in situ with trypsin and separated by HPLC, and the longest fragments recovered from each were sequenced. Strong homology of both the 31- and 27-kD polypeptides with five recently identified members of the MIP family was found. In the 19-amino acid peptide obtained from the 31-kD protein (Fig. 5A), 11 amino acids were identical to the N-terminal portion of TRAMP from tomato, clone 7a from pea, and the PIP1 family from *Ara-*

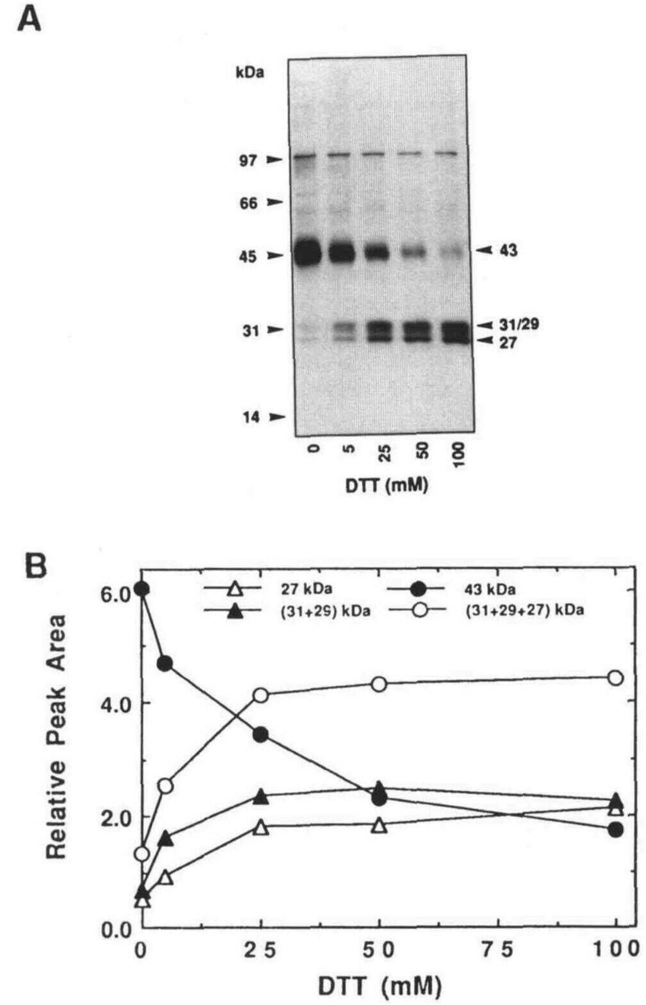

Figure 2. Concentration-dependent conversion of the 43-kD species by DTT. A, The PMIP-enriched fraction was isolated from the CHAPS extract by glycerol gradient centrifugation. Aliquots (5 μg of protein) were combined with various levels of DTT and were incubated at 30°C for 15 min; SDS-PAGE sample buffer prepared without β-mercaptoethanol was added. B, Quantification of polypeptide levels by laser densitometry with relative band intensities determined by integration of peak areas.

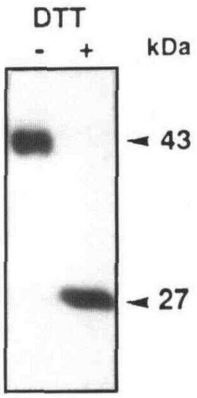

Figure 3. Immunoblot probed with antibodies to TIP of *Arabidopsis*. PM prepared by aqueous two-phase partitioning was electrophoresed (5 μg of protein) in the absence or presence of DTT, transferred to a nitrocellulose membrane, and probed with TIP antibody from *Arabidopsis*. The bands were visualized by enhanced chemiluminescence (see "Materials and Methods").

bidopsis. At least five additional amino acids were nonidentical but related. The segments LGA and QPLG are identical in TRAMP and clone 7a. The RD28 deduced protein sequence lacks this region completely.

The 23-amino acid tryptic fragment derived from the 27-kD protein (Fig. 5B) revealed similar homologies. Fourteen amino acids were identical to an internal region of TRAMP, and 10 aligned with a similarly located region of RD28 and PIP 2b from *Arabidopsis*. Of note, the sequence GGGAN was common to four of the five proteins. A 4-amino acid segment (GYTK) of the 27-kD polypeptide from *Beta* was homologous to TRAMP and clone 7a. These homologies confirm that the 31- and 27-kD polypeptides, which occur abundantly in PMs of beet storage tissue, are members of the MIP family.

Table I. *Experimentally determined amino acid composition of the 31- and 27-kD polypeptides.*

Each polypeptide was obtained by electroelution (see "Materials and Methods"), hydrolyzed, and analyzed for amino acid content.

Amino Acid	31-kD	27-kD	Ratio
		%	
Asx	7.5	5.5	1.36
Glx	6.0	6.5	0.92
Ser	6.5	5.2	1.25
Gly	12.0	13.0	0.92
His	2.4	2.6	0.92
Arg	3.7	3.2	1.16
Thr	6.1	6.4	0.95
Ala	12.2	12.9	0.95
Pro	5.2	5.5	0.92
Tyr	2.8	2.9	1.00
Val	7.3	6.5	1.12
Met	1.5	2.1	0.71
Ile	6.2	6.9	0.90
Leu	9.6	9.1	1.05
Phe	6.2	6.8	0.91
Lys	4.8	4.7	1.02

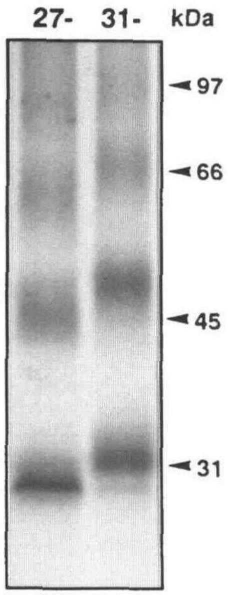

Figure 4. Electroelution-induced aggregation of the 27- and 31-kD polypeptides. Each polypeptide (as indicated) was electroeluted as described in "Materials and Methods" and was electrophoresed in the absence of reducing agent.

DISCUSSION

Sequence analysis of cDNA clones obtained from a range of species and tissue types has shown that members of the MIP or aquaporin family occur widely in plants (Chrispeels and Maurel, 1994). Here, a direct biochemical approach consisting of detergent-based solubilization and subsequent fractionation, was taken to demonstrate that PM vesicles from storage tissue of *Beta* possess at least two members of the MIP family. Based on several independent approaches, i.e. the purification-based strategy employed here, immunoselection from a mammalian expression system used to obtain PIP 1 and 2 from *A. thaliana* (Kammerloher et al., 1994), and immunodetection of RD28-PIP in *A. thaliana* (Daniels et al., 1994), the occurrence of MIPs in the PM of higher plants is now firmly established.

The precise physiological function of MIPs or aquaporins in higher plants is not clear, but suggested functions include the transport of water and an increasing spectrum of solutes (Chrispeels and Maurel, 1994; Ishibashi et al., 1994; Knepper, 1994; Weaver et al., 1994). A mild nondenaturing procedure for isolating PMIPs offers the potential for developing reconstituted systems to further probe physiological functions mediated by members of the MIP family. We note that the purified PMIP fraction contained callose synthase activities in excess of 1500 nmol min^{-1} mg^{-1}. The co-purification of PMIPs with callose synthase raises questions of a speculative nature concerning a possible role in the translocation of nascent biopolymers, such as the β-glucans callose and cellulose, across the PM. All available models of PM callose or cellulose synthases, whether derived by morphological (Mueller and Brown, 1980; Reiss et al., 1984; Herth, 1985; Giddings and Staehelin, 1988) or biochemical (Wu and Wasserman, 1993) means, generally

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A.   31-kD Tryptic Fragment

Beta vulgaris L.         L G A D K Y P D R Q P L G T S V Q T R
                         . . .   .         . . . . .     . .
                         . . . . . .   .   . . . . . . . . .
Tomato TRAMP        11   L G A N K F R E T Q P L G T A A Q T D
                         . . .   .   .   . . . . .       .
                         . . . . . . . . . . . . .   . . . .
Pea Clone 7a        10   L G A N K F P E R Q P L G I A A Q S Q
                           . .   .   .   . . .   . . .   .
                         . . . . . . . . . . . . . . . . . .
Arabidopsis PIP 1   10   V G A N K F P E R Q P I G T S A Q S D

B.   27-kD Tryptic Fragment

Beta vulgaris L.         G F Q P T P - Y M T A G G G A N Y V H H G Y T K
                           . .         .       . . . . .         . .
                         . . . . . .   . .     . . . . . . .   . . .
Arabidopsis RD28    143  A F Q S S H - Y V N Y G G G A N F L A D G Y N T
                         . .       .   .       . . . . .   .     . . . .
                         . .       .   .       . . . . .   . .   . . . .
Tomato TRAMP        153  G F M V G P - Y Q R L G G G A N V V N P G Y T K
                         . .                     . . . .   .     . . . .
                         . .           .         . . . . . .     . . . .
Pea Clone 7a        155  G F E G K Q R F G D L N G G A N F V A P G Y T K
                           . .         .       . . . . .         . .
                           . . . .     .       . . . . .   .   . . .
Arabidopsis PIP 2b  143  S F Q S S Y - Y D R Y G G G A N S L A D G Y N T
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Figure 5. Alignment of PMIP tryptic fragments with sequences of plant MIPs. Sequences obtained from beet tryptic fragments were compared to cDNA clones of TRAMP (Fray et al., 1994), RD28 (Yamaguchi-Shinozaka et al., 1992), clone 7a (Guerrero et al., 1990), and members of the PIP family (Kammerloher et al., 1994). PIP 1 refers to isoforms a, b, and c, which share identical sequences from amino acids 10 to 28. Double circles indicate complete identity with *Beta*; single circles indicate nonidentical but related amino acids.

accept the notion that the UDP-Glc-binding domain of these enzyme complexes are oriented toward the cytoplasmic surface of the PM, and that during catalysis, Glc units are translocated through the PM to elongating microfibrils or amorphous callose deposits. Thus, a PMIP closely associated with callose synthase could channel callose synthesized at the cytoplasmic face of the PM to the apoplastic space, and it could perform a similar function for the (1,4)-β-linked glucan chains that assemble into cellulose microfibrils outside the membrane. The latter possibility is consistent with the hypothesis that synthesis of wound callose or cellulose reflects differential regulation of the same enzyme complex (Jacob and Northcote, 1985; Delmer, 1987). Polypeptides that form pores traversing the PM might be particularly good candidates for conservation between mechanisms for synthesis of callose and cellulose. The establishment of more definitive associations between specific PMIPs and cell wall biopolymer translocation is the subject of ongoing research in our laboratory.

In summary, direct biochemical evidence is presented demonstrating abundant levels of MIPs localized within PM vesicles obtained from callose synthase-rich storage tissue of *B. vulgaris* L. This purification procedure and enhanced understanding of the biochemical properties of plant-derived PMIPs will provide the necessary tools for probing their topology within the PM, genetic regulation, and physiological function.

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