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Characterization of two integral membrane proteins located in the protein bodies of pumpkin seeds

Kaori Inoue[1,2], Yuka Takeuchi[1], Mikio Nishimura[1,2] and Ikuko Hara-Nishimura[1,*]
[1] *Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan, (*author for correspondence);* [2] *Department of Molecular Biomechanics, School of Life Science, Graduate University for Advanced Studies, Okazaki 444, Japan*

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Abstract

Two integral membrane proteins, MP28 and MP23, were found in protein bodies isolated from pumpkin (*Cucurbita* sp.) seeds. Molecular characterization revealed that both MP28 and MP23 belong to the seed TIP (tonoplast intrinsic protein) subfamily. The predicted 29 kDa precursor to MP23 includes six putative membrane-spanning domains, and the loop between the first and second transmembrane domains is larger than that of MP28. The N-terminal sequence of the mature MP23 starts from residue 66 in the first loop, indicating that an N-terminal 7 kDa fragment that contains one transmembrane domain is post-translationally removed. During maturation of pumpkin seeds, mRNAs for MP28 and MP23 became detectable in cotyledons at the early stage, and their levels increased slightly until a rapid decrease occurred at the late stage. This is consistent with the accumulation of the 29 kDa precursor and MP28 in the cotyledons at the early stage. By contrast, MP23 appeared at the late stage simultaneously with the disappearance of the 29 kDa precursor. Thus, it seems possible that the conversion of the 29 kDa precursor to the mature MP23 might occur in the vacuoles after the middle stage of seed maturation. Both proteins were localized immunocytochemically on the membranes of the vacuoles at the middle stage and the protein bodies at the late stage. These results suggest that both MP28 and the precursor to MP23 accumulate on vacuolar membranes before the deposition of storage proteins, and then the precursor is converted to the mature MP23 at the late stage. These two TIPs might have a specific function during the maturation of pumpkin seeds.

Introduction

Protein bodies are protein-storage organelles that are widely distributed in the seeds of higher plants. In pumpkin, protein bodies are formed from vacuoles during seed maturation [7]. During seed germination and subsequent seedling growth, protein bodies undergo dynamic changes. After

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers D45077 (MP23) and D45078 (MP28).

imbibition of seeds, the protein bodies swell, start to fuse with one another and are converted into a large central vacuole [6]. This transformation of protein bodies into vacuoles is accompanied by the breakdown of the protein constituents in protein bodies and by the incorporation of newly synthesized proteins into the vacuoles [23]. The degradation of protein-body components includes the breakdown of the membrane proteins [15, 23, 26].

To elucidate the mechanism responsible for the transformation of protein bodies into vacuoles, we have characterized the five major membrane proteins of the protein bodies in the dry seeds of pumpkin (*Cucurbita* sp.). These proteins are designated MP23, MP27, MP28, MP32 and MP73. We previously reported that two of these membrane proteins, MP27 and MP32, are synthesized as a single precursor that is composed of a hydrophobic signal sequence, MP27 and MP32, in that order. A putative site of cleavage between MP27 and MP32 was located on the C-terminal side of residue 278, asparagine, an indication that the post-translational cleavage might occur as a result of the action of a vacuolar processing enzyme that converts proprotein precursors of seed proteins into the mature forms [8, 10, 12]. Among the five membrane proteins, MP27 and MP32 disappear most rapidly during seedling growth. The degradation of MP27 and MP32 starts just after seed germination, and they are not detected in the developing vacuoles, which are formed by the fusion of the protein bodies [15].

TIP (tonoplast intrinsic protein) is an integral membrane protein that is originally found in plant seeds and belongs to the MIP (major intrinsic protein) family, the members of which are widely distributed in bacteria, animals and plants [29, 30]. TIP of plant seeds is abundant and is conserved among both monocots and dicots. Johnson *et al.* discussed that TIP is involved in the desiccation and rehydration process of the seeds [17]. Molecular cloning of seed TIP from kidney bean (*Phaseolus vulgaris*) revealed that the seed TIP exists as two isoforms, α-TIP and β-TIP [18]. The predicted amino acid sequence of α-TIP includes six putative membrane-spanning domains. In *Arabidopsis*, three genes for TIP were isolated and their products were designated α-TIP, β-TIP and γ-TIP, respectively. α-TIP and β-TIP are seed-specific, whereas γ-TIP is expressed in the vegetative organs of plants and not in seeds [14]. Expression of α-TIP–and γ-TIP in *Xenopus* oocytes revealed that γ-TIP can function as a water-specific channel, but α-TIP failed to exhibit such activity [25]. The physiological role of seed TIP remains to be determined.

In the present study, we showed that two integral membrane proteins, MP28 and MP23, are homologues of TIP and that MP23 is synthesized as a 29 kDa precursor that is converted into the mature form at the late stage of maturation of pumpkin seeds.

Materials and methods

Plant materials

Seeds of pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin), castor bean (*Ricinus communis*), common garden petunia (*Petunia hybrida* Vilm), pea (*Pisum sativum*), rice (*Oryza sativa*), wheat (*Triticum* sp.) and maize (*Zea mays*) were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). Pumpkin seeds were soaked in water for 16 h and then grown at the experimental farm of the National Institute for Basic Biology during the summer season. Cotyledons from maturing seeds on specific days after flowering (DAF) were collected and frozen in liquid nitrogen for extraction of RNA.

Preparation of protein-body membranes

Protein bodies were isolated from dry pumpkin seeds by the previously reported method [9]. Light microscopy and assays of marker enzymes indicated that the prepared protein bodies were barely contaminated by other organelles and cytosolic components. Membranes were prepared from the isolated protein bodies as described previously [15].

Production of antiserum to MP23-specific peptide

Antibodies against MP28 and those against MP23 that we had prepared previously [15] were used for the present experiments. Immunoblot analysis using these antibodies showed that MP28 and MP23 were immunochemically related. To prepare MP23-specific antibodies, we chemically synthesized an MP23-specific peptide as a tetrameric multiple-antigen peptide [35] using a peptide synthesizer (model 430A; Perkin Elmer/Applied Biosystems, Foster City, CA). The peptide was based on a sequence of 15 amino acids, GDGYGRRRTDTGRGA, that is found in the loop between the first and second transmembrane domains of MP23 but is not present in the MP28 polypeptide (Figs. 2B, 3). The peptide dissolved in PBS was emulsified with complete Freund's adjuvant and injected subcutaneously into two rabbits (1 mg each). After three weeks, three booster injections of the peptide with incomplete adjuvant were given at 7-day intervals. After one further week, the blood was drawn and the antiserum was prepared.

Immunoblot analysis

Total proteins from maturing cotyledons of pumpkin and dry seeds of pumpkin, castor bean, petunia, pea, rice, wheat and maize were extracted by the previously described method [17]. The proteins were separated by either SDS-PAGE [21] or Tricine-SDS-PAGE [33], blotted onto a polyvinylidene difluoride (PVDF) membrane (0.22 μm; Nihon Millipore, Tokyo, Japan) and then subjected to immunoblot analysis by the method described previously [15].

Immunocytochemical analysis

Maturing pumpkin seeds were freshly harvested at 22 and 28 DAF. The cotyledons were cut into slices of less than 1 mm in thickness and subjected to fixation, dehydration and embedding by the previously described method [12]. Immunogold-labeling procedures were essentially the same as those described previously [28], except for the use of the antiserum against MP28. The antiserum was diluted either 500-fold or 2000-fold. The sections were examined with a transmission microscope (model 1200EX; JEOL, Japan).

Determination of N-terminal amino acid sequences and peptide mapping

Membranes of protein bodies were treated with 10 mM NaOH as described previously [15] with the single modification that the membranes were suspended with distilled water before extraction with alkali. The membrane fraction remaining after treatment was subjected to SDS-PAGE and the separated proteins were transferred electrophoretically to a PVDF membrane by the method of Matsudaira [24]. Proteins on the blot were stained with Coomassie Blue R-250 and the bands corresponding to MP28 and MP23 were cut out from the blot for application to a gas-phase sequence analyzer (model 470A; Perkin Elmer/Applied Biosystems, Foster City, CA). Since the N-terminal amino acid residue of MP28 was blocked, we cleaved MP28 with cyanogen bromide to determine the internal amino acid sequences, as described previously [12].

Protein-body membranes that had been treated with alkali were subjected to SDS-PAGE and subsequent staining with Coomassie blue. The bands on gels that corresponded to MP28 and MP23, respectively, were excised and digested with 5 μg of either trypsin or V8 protease (Sigma, St. Louis, MO) by the method of Cleveland *et al.* [3]. After Tricine-SDS-PAGE, the separated peptides were stained with Coomassie blue for peptide-mapping analysis. The peptides from MP28 were transferred to a ProBlott membrane (Perkin Elmer/Applied Biosystems, Foster City, CA) and subjected to automatic Edman degradation on a protein sequencer.

Isolation of poly (A)$^+$ RNA

Pumpkin cotyledons were freshly harvested from seeds at various stages of maturation. Total RNA was extracted from the cotyledons by the SDS-phenol method [1] and poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose as described by Aviv and Leder [2].

Screening of cDNAs for MP28 and MP23

Poly(A)$^+$ RNA was prepared from maturing pumpkin cotyledons at 24 DAF. A cDNA library in pBluescript II SK (Stratagene, La Jolla, CA) was constructed with the poly(A)$^+$ RNA and the vector-primer as described previously [27]. *Escherichia coli* cells (DH5α) were transformed with the library.

A degenerate oligonucleotide, 5′-GGITA(T/C)GGI(C/A)GI(C/A)GI(C/A)GIACIGA(T/C)ACIGG-3′, was designed on the basis of the N-terminal amino acid sequence of MP23 and synthesized on a DNA synthesizer (Pharmacia LKB Biotechnology, Uppsala, Sweden). The polymerase chain reaction (PCR) [31] was performed using both the oligonucleotide primer and the KS primer (Toyobo, Osaka, Japan) and DNA from 4×10^4 transformants as a template. An 810 bp fragment was amplified. The 810 bp fragment was labelled with [α-^{32}P]dCTP by the random primer method [4] and used as a probe in 6 × SSPE, 5% (v/v) Irish Cream liqueur (Baileys, UK), 0.1% SDS and 20 μg/ml denatured salmon sperm DNA (Sigma). Colony hybridization was carried out at 65 °C for 18 h [5]. The filters were washed at 65 °C with either solution of high stringency (0.1 × SSC and 0.1% SDS) or low stringency (2 × SSC and 0.1% SDS) and subjected to autoradiography.

Sequence analysis

A series of deletion mutants was constructed using a Deletion Kit (Takara, Otsu, Siga, Japan). Nucleotide sequences were determined by the dideoxy chain-termination method [32] with either T7 DNA polymerase (Pharmacia LKB Biotechnology) or BcaBEST DNA polymerase (Takara), T3 and T7 primers (Toyobo) and [α-^{32}P]dCTP. The nucleotide and the deduced amino acid sequences were analyzed using DNA analytical software (DNASIS, Hitachi Software, Yokohama, Japan; GeneWorks, IntelliGenetics, CA). Hydrophobicity profiles for the amino acid sequences were computed by application of the algorithm of Kyte and Doolittle [20], with a window size of 10 amino acid residues.

Northern blot analysis

A 2 μg portion of poly(A)$^+$ RNA was subjected to electrophoresis on a 1.0% agarose gel containing 0.66 M formaldehyde [1] and transferred onto a Hybond N(+) membrane (Amersham Japan, Tokyo). The isolated cDNAs for MP28 and MP23 were used as probes after labeling by the random primer method, as described above. The cDNA for the peripheral membrane proteins MP27-MP32 [15] and the cDNA for the major seed protein 11S globulin [13] were also used as probes. Hybridizations were performed at 42 °C for 24 h in 6 × SSPE, 50% formamide, 5% (v/v) Irish Cream liqueur, 0.1% SDS and 20 μg/ml denatured salmon sperm DNA. Membranes were washed under the high stringent condition in a solution of 0.1 × SSPE and 0.1% SDS at 65 °C and subjected to autoradiography. MP28 cDNA is 67% identical in nucleotide sequence to MP23 cDNA. At the high stringency, the cDNA probes which were used for MP28 and MP23 were not cross-hybridized. The levels of mRNA were determined by using a densitometer (AE-6920-M, ATTO Corporation, Tokyo, Japan).

Results

Characterization of MP28 and MP23 from pumpkin seeds

Five major proteins, MP23, MP27, MP28, MP32 and MP73, are located in the membranes of the

protein bodies of pumpkin seeds, as shown in Fig. 1A (lane 1). MP28 and MP23 cannot be extracted with alkali, an indication that these proteins are integral membrane proteins [15]. Immunoblot analysis of membranes from protein bodies of pumpkin showed that antibodies raised against MP23 recognized not only MP23 but also MP28 (Fig. 1A, lane 2). Antibodies against MP28 also recognized both MP28 and MP23 (data not shown). The results suggest that MP23 has the same epitopes as MP28. We performed peptide mapping analysis of MP28 and MP23. MP28 and MP23, purified from protein-body membranes, were digested with trypsin and the resultant peptides were separated by SDS-PAGE on a 16.5% polyacrylamide gel. Peptide bands derived from MP28 and MP23 migrated similarly, but a 4 kDa peptide was only detected in the case of MP28 (Fig. 1B, arrowhead). These results suggest that the primary structures of MP28 and MP23 are similar to each other, but that MP28 has an additional peptide as compared to MP23.

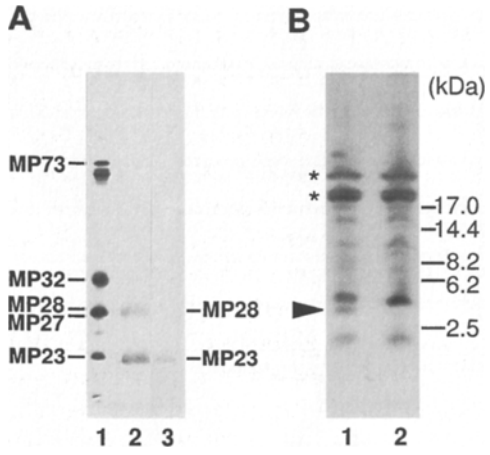

Fig. 1. Two integral membrane proteins, MP28 and MP23, in protein bodies of dry pumpkin seeds. A. The isolated membranes of the protein bodies of dry seeds were subjected to SDS-PAGE and subsequent staining with Coomassie blue (lane 1). Five major proteins, MP23, MP27, MP28, MP32 and MP73, were detected in the protein-body membranes, as indicated on the left. Immunoblot analysis of the membrane proteins was performed using either antibodies against the purified MP23 protein (lane 2) or antibodies against an MP23-specific peptide that had been synthesized chemically (lane 3). The sequence of the MP23-specific peptide can be found in the loop between the first and second transmembrane domains of MP23 but not in the sequence of MP28, as indicated in Fig. 3 by double underlining. B. MP28 (lane 1) and MP23 (lane 2), purified by SDS-PAGE, were digested with trypsin and then subjected to SDS-PAGE on a 16.5% gel with subsequent staining with Coomassie blue. An arrowhead shows a band of a polypeptide of 4 kDa that was detected in the analysis of MP28 (lane 1) but not in that of MP23 (lane 2). The bands of trypsin are indicated by asterisks. The molecular mass of each marker protein is given on the right in kilodaltons.

Johnson *et al.* reported that two bands with molecular masses of 25 kDa and 23 kDa were detected in an analysis of seed TIP of kidney bean, and suggested that a 23 kDa polypeptide might be a product of the degradation of a 25 kDa polypeptide [17]. Molecular characterization of pumpkin MP28 and MP23 was, therefore, required to determine whether or not both membrane proteins are encoded by the same gene. The N-terminal sequence of MP23 was determined to be GYGRRRTDTGXGASDLVVIAIAHAFXLF (X represents an undetermined amino acid) and an oligonucleotide primer for PCR was designed on the basis of this sequence. An 810 bp fragment was amplified using a cDNA library from maturing pumpkin cotyledons as a template and the combination of the primer with a degenerate sequence and a universal primer. We isolated two cDNAs using the amplified fragment as a probe; one gave a strong signal even under highly stringent conditions during hybridization analysis and the another one gave a signal only at lower stringency.

Their nucleotide sequences were determined, as shown in Fig. 2. MP28 cDNA is 67% identical in nucleotide sequence to MP23 cDNA. The above N-terminal sequence of 28 amino acids of MP23 was from Gly-66 through Phe-93 of the sequence predicted from the first cDNA, which had high affinity to the probe, an indication that the cDNA encoded MP23 (Fig. 2B). The second cDNA had low affinity to the probe. To examine whether the cDNA encoded MP28, we determined the amino acid sequence of MP28. Since no N-terminal amino acid of MP28 was detected by automatic Edman degradation, the purified MP28 was digested with either cyanogen bromide or V8 protease to allow us to determine the sequences of two internal peptides. One sequence,

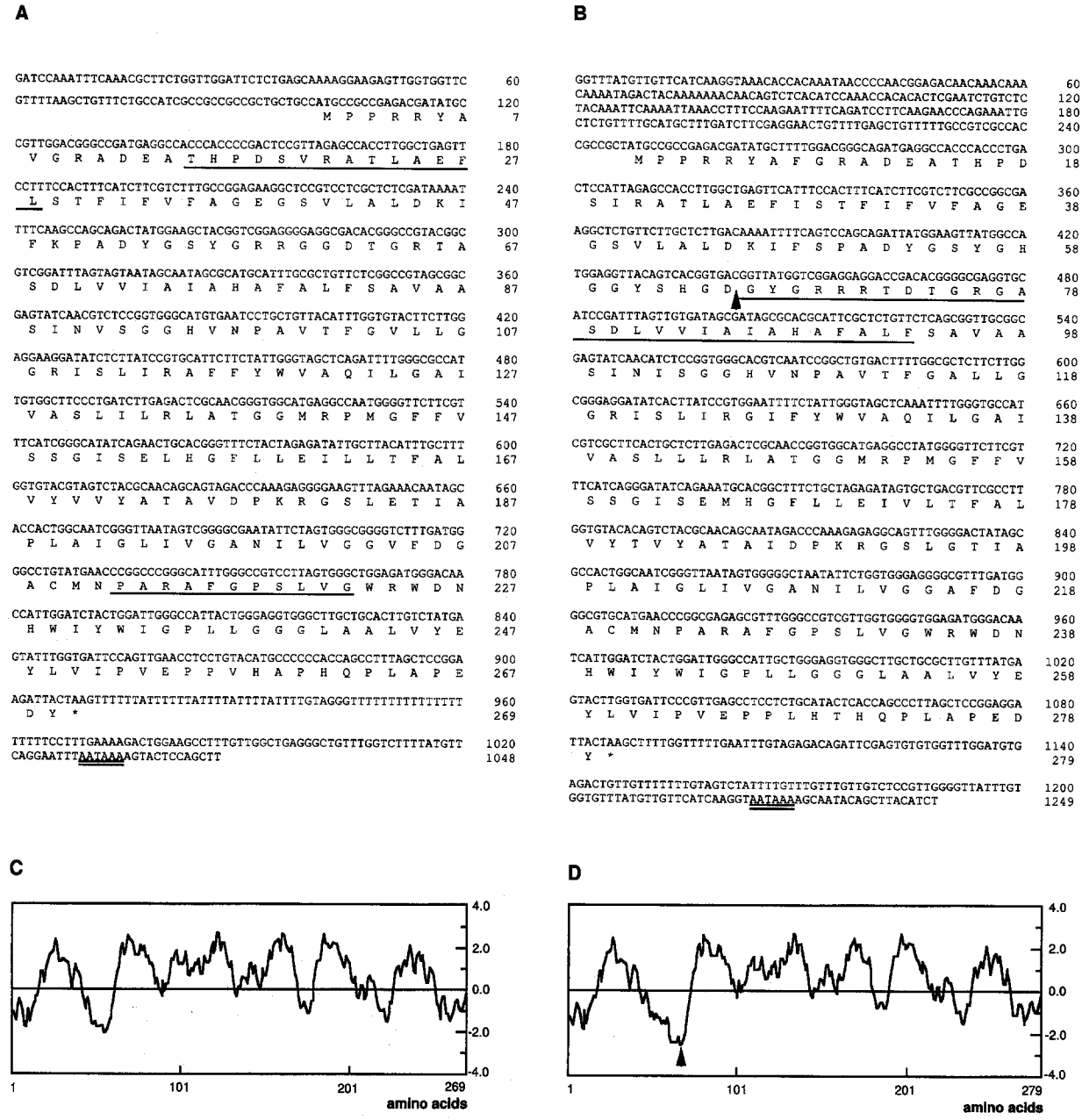

Fig. 2. Molecular structures of MP28 and MP23. A, B. Nucleotide and the deduced amino acid sequences of MP28 (A) and of a precursor to MP23 (B) are shown. N-terminal amino acid sequences that were determined directly are indicated by underlining. A possible site of cleavage for production of MP23 is shown by an arrowhead. Double underlining indicates putative polyadenylation signals. C, D. Hydrophobicity profiles of MP28 (C) and of the precursor to MP23 (D), as constructed by the method of Kyte and Doolittle [20] with a window of 10 residues. The possible site of cleavage for production of MP23 is located in the hydrophilic loop between the first and second transmembrane domains of the precursor, as indicated by an arrowhead.

TXPDXVXATLAEFL, of MP28 corresponded to the sequence from Thr-15 to Leu-28 of the sequence predicted from the second cDNA, an indication that the cDNA encoded MP28 (Fig. 2A). However, another internal sequence, PAXAFGPXLVG, of MP28 was found in both

predicted sequences: from Pro-212 to Gly-222 of the sequence of MP28 and from Pro-223 to Gly-233 of the sequence of MP23. In these regions, the two sequences are highly homologous.

To confirm that MP28 and MP23 are the products from different genes, we raised antibodies against a tetrameric peptide of 15 amino acids [35], whose sequence was found specifically from Gly-64 to Ala-78 of the predicted sequence of MP23 (Fig. 3, double underlining). Immunoblot analysis showed that the antibodies recognized MP23 but not MP28 (Fig. 1A, lane 3). The result clearly indicated that MP28 and MP23 are encoded by different genes.

The amino acid sequences predicted from the cDNAs were found to be 88% identical to each other, and 65% to 67% identical to those of α-TIPs of kidney bean [18] and of *Arabidopsis* [14] (Fig. 3). Thus, pumpkin MP28 and MP23 are homologues of α-TIP. Hydrophobicity profiles of MP28 and MP23 indicate that both include six hydrophobic regions that are sufficient

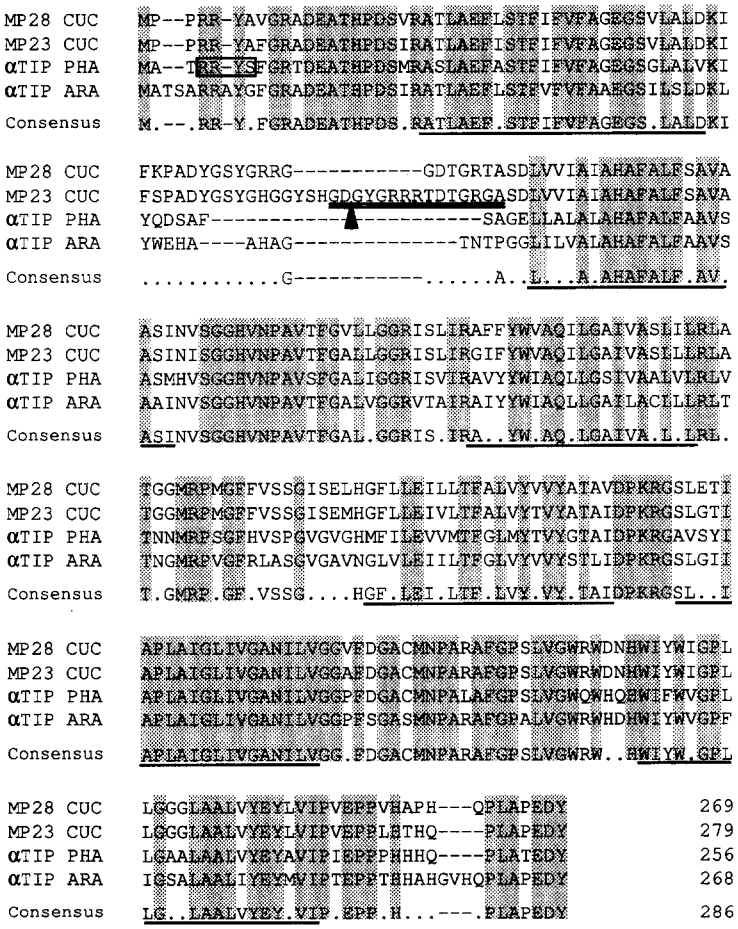

Fig. 3. Alignment of MP28 and MP23 with seed-specific α-TIPs of kidney bean (PHA) and *Arabidopsis* (ARA). Sequence alignment was performed using GeneWorks (DNA analytical software from IntelliGenetics, CA, USA). Shading indicates identical amino acids. Underlining indicates putative transmembrane domains. The precursor to MP23 has an insert in the loop between the first and second transmembrane domains. A peptide with the sequence shown by double underlining was synthesized for production of MP23-specific antibodies. The N-terminus of mature MP23 is located at Gly-66, and the arrowhead indicates a putative cleavage site for generation of mature MP23. A box indicates a phosphorylation signal for a calcium-dependent protein kinase [19].

to span a lipid bilayer, as do other TIPs (Figs. 2C, 2D). The hydrophilic region of the first loop between the first and second membrane-spanning domains is least conserved in terms of amino acid sequence, and the loop of MP23 is the longest (Fig. 3). Two NPA sequences, namely, consensus sequences of the members of MIP family, are conserved in the second and the fifth loops of both MP28 and MP23. Ser-7 in the N-terminal hydrophilic region of α-TIP of kidney bean (Fig. 3, a box) has been reported to be phosphorylated [16]. However, no serine residue was found at an analogous position in pumpkin MP28 or MP23.

The molecular mass of 28 580 Da that was calculated from the predicted sequence of MP28 was consistent with the 28 kDa determined directly for MP28 from protein-body membranes. However, the mass of 29 592 Da, calculated from the predicted sequence of MP23, was much larger than the 23 kDa of MP23 from the membrane. The N-terminal sequence of the purified MP23 protein starts from Gly-66 in the first loop of the predicted sequence, indicating that MP23 is synthesized as a 29 kDa precursor and that an N-terminal 7 kDa fragment that contains one transmembrane domain is cleaved off post-translationally to produce MP23. The site of the post-translational cleavage is the C-terminal side of Asp-65 in the long first loop of the 29 kDa precursor (Figs. 2B, 2D, 3; arrowheads).

Immunoblot analysis with antibodies against MP23 was performed to examine the possibility of such post-translational cleavage in various dry seeds, such as those of pumpkin, castor bean, petunia, pea, wheat and maize, as shown in Fig. 4. One or two bands were detected in the homogenates from the seeds of all plant species, suggesting that TIPs are conserved in plant seeds [17]. However, the differences of the molecular masses between the two bands are less than 2 kDa in the seeds except for pumpkin, and are smaller than a 4 kDa difference between pumpkin MP28 and MP23. A relatively small molecular species of less than 23 kDa, lacking one membrane-spanning domain, was not detected in any seeds except those of pumpkin. Thus, it is likely that a post-translational removal of one membrane spanning domain might not occur in TIPs of the seeds of castor bean, petunia, pea, wheat and maize.

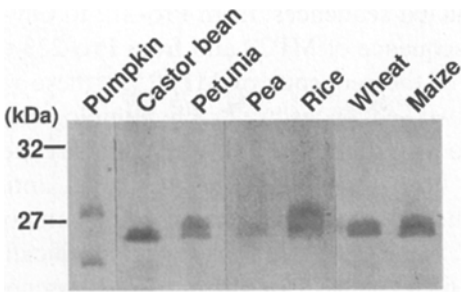

Fig. 4. Presence of multiple homologues of TIP in dry seeds of various plants. Homogenates from seeds of pumpkin, castor bean, petunia, pea, rice, wheat and maize were subjected to Tricine-SDS-PAGE (10% acrylamide) and subsequent immunoblot analysis using antibodies against MP23. The molecular mass of each prestained marker protein is given on the left in kilodaltons.

Developmental changes in levels of pumpkin MP28 and MP23 and their mRNAs during the maturation of seeds

The cotyledons were formed in pumpkin seeds after 18 DAF, and they grow with the accumulation of storage proteins including 11S globulin. To analyze the changes in levels of the transcripts for pumpkin MP28 and MP23 during seed maturation, poly(A)$^+$ RNA was prepared from pumpkin seeds at 19 DAF to 26 DAF and northern blot analysis was performed under the high stringent condition to differentiate between MP23 and MP28 mRNAs, as shown in Fig. 5A and B. The levels of the transcripts for 11S globulin and the peripheral membrane proteins, MP27 and MP32, were very low in the seeds at 19 DAF. Their level dramatically increased from 21 DAF to 24 DAF. By contrast, the transcripts for MP28 and MP23 had accumulated in young seeds by 19 DAF. The levels of both increased gradually from 19 DAF to 24 DAF. The levels of all the transcripts that we analyzed decreased rapidly after desiccation of the seeds began. These results suggest that the changes in the levels of the transcripts for TIP are

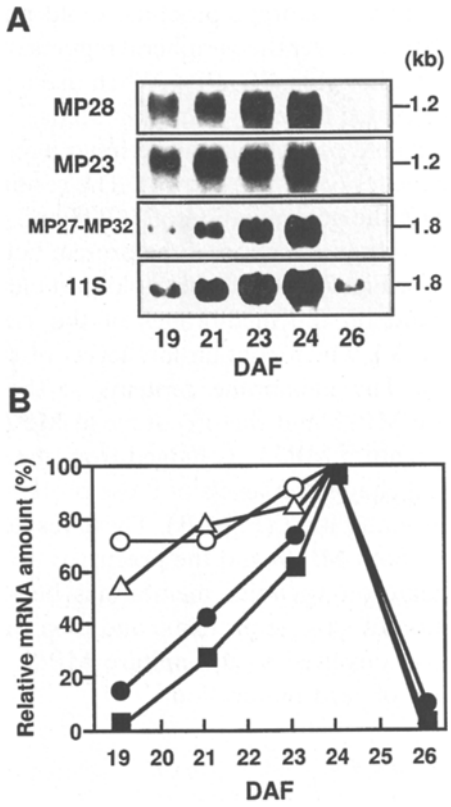

Fig. 5. Developmental changes in levels of the transcripts for protein-body proteins that include MP28, MP23, peripheral membrane proteins and a major storage protein during maturation of pumpkin seeds. A. Pumpkin cotyledons were harvested from the seeds at 19, 21, 23, 24 and 26 DAF for preparation of poly(A)$^+$ RNA. Two micrograms of poly(A)$^+$ RNA were subjected to northern blot analysis. cDNAs for both MP28 and MP23 were ^{32}P-labelled and used as probes. Transcripts for peripheral membrane proteins MP27 and MP32, which are synthesized as a single precursor (MP27-MP32) [15], and that for a major seed protein 11S globulin (11S) [13] were also examined. The size of each transcript is given on the right in kilobases. (B) The levels of mRNAs for MP28 (open circles), MP23 (open triangles), MP27-MP32 (closed squares) and 11S globulin (closed circles) on the blot (A) were determined by using a densitometer (AE-6920-M, ATTO Corporation, Tokyo, Japan). The relative mRNA amount is given as a percentage of the maximum value.

not associated with the accumulation of the storage proteins in maturing pumpkin seeds.

From the results shown in Fig. 5, the development of maturing pumpkin seeds was divided into three stages, early (19 DAF to 21 DAF), middle (22 DAF to 25 DAF) and late (26 DAF to 43 DAF). To analyze the changes in levels of MP28 and MP23 proteins during seed maturation, we prepared homogenates from pumpkin seeds at 19 DAF to 43 DAF and subjected them to immunoblot analysis using antibodies against MP23 (Fig. 6B). The accumulation of the membrane proteins appeared to be independent of the deposition of storage proteins, such as 11S globulin and 7S protein, in the vacuoles (Fig. 6A), as in the case of kidney bean [17]. Two proteins, MP28 and a 29-kDa protein, were detected at the early

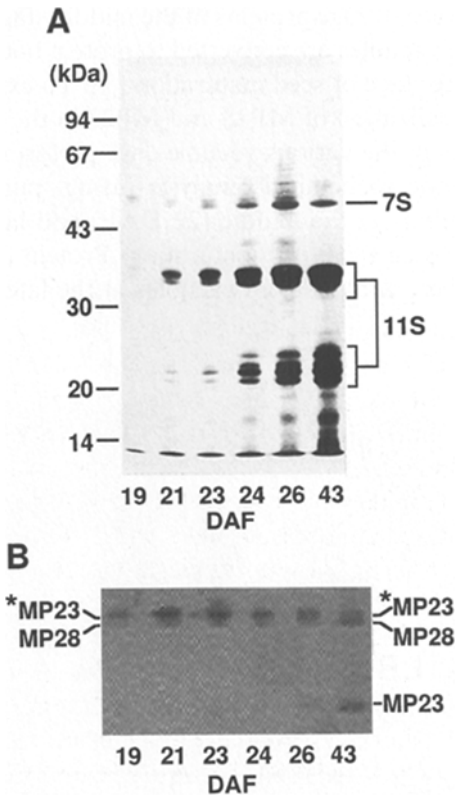

Fig. 6. Accumulation of MP28, MP23 and major storage proteins in pumpkin cotyledons during seed maturation. Pumpkin cotyledons were harvested at 19, 21, 23, 24, 26 and 43 DAF. A crude extract of cotyledons was subjected to SDS-PAGE and subsequent staining with Coomassie blue (A) or immunoblot analysis with antibodies against MP23 (B). A. 7S and 11S indicate the storage proteins 7S globulin and 11S globulin, respectively. The molecular mass of each marker protein is given on the left in kilodaltons. B. The antibodies against MP23 recognized not only both MP28 and MP23 but also a 29 kDa polypeptide (*MP23) that is the putative precursor to MP23.

stage (19 and 21 DAF) of seed maturation. The level of MP28 was unchanged from 21 DAF to 43 DAF. By contrast, the MP23 appeared in the seeds from 26 DAF and the level increased in parallel with the reduction of the 29 kDa precursor to MP23, which had accumulated at 19 to 24 DAF. This result suggests that the conversion of the 29 kDa precursor to MP23 might occur after the late stage of seed maturation.

Morphological changes in vacuoles occur in the cotyledonary cells during seed maturation. Many, apparently empty, small vacuoles that are found at the early stage increase in size and accumulate storage proteins at the middle stage [7]. These vacuoles are converted to protein bodies at the late stage of seed maturation [7]. To examine the localization of MP28 and MP23 in the membranes of the various vacuoles, we performed an immunocytochemical analysis using pumpkin cotyledons at the middle (22 DAF) and late (28 DAF) stages of seed maturation. Protein bodies that were derived from vacuoles at the late stage are stuffed with storage proteins. Gold particles were observed over the peripheral regions of these protein bodies (Fig. 7B, PB). When used protein bodies isolated from dry pumpkin seeds, we also observed gold particles in the peripheral region of the organelles (data not shown). The result coincides with the accumulation of MP28 and MP23 in the membrane fraction of the protein bodies of dry seeds. Fig. 7A shows that gold particles were also found on the membranes of the vacuoles (Fig. 7A, V), which contain low levels of storage proteins. The membrane proteins at the early stage are MP28 and the precursor to MP23, but not the mature MP23, as judged from the developmental changes in levels of these proteins during seed maturation (Fig. 6B). These results suggest that both MP28 and the precursor to MP23 accumulate on vacuolar membranes before the deposition of storage proteins, and then the precursor is converted to the mature MP23 at the late stage of seed maturation.

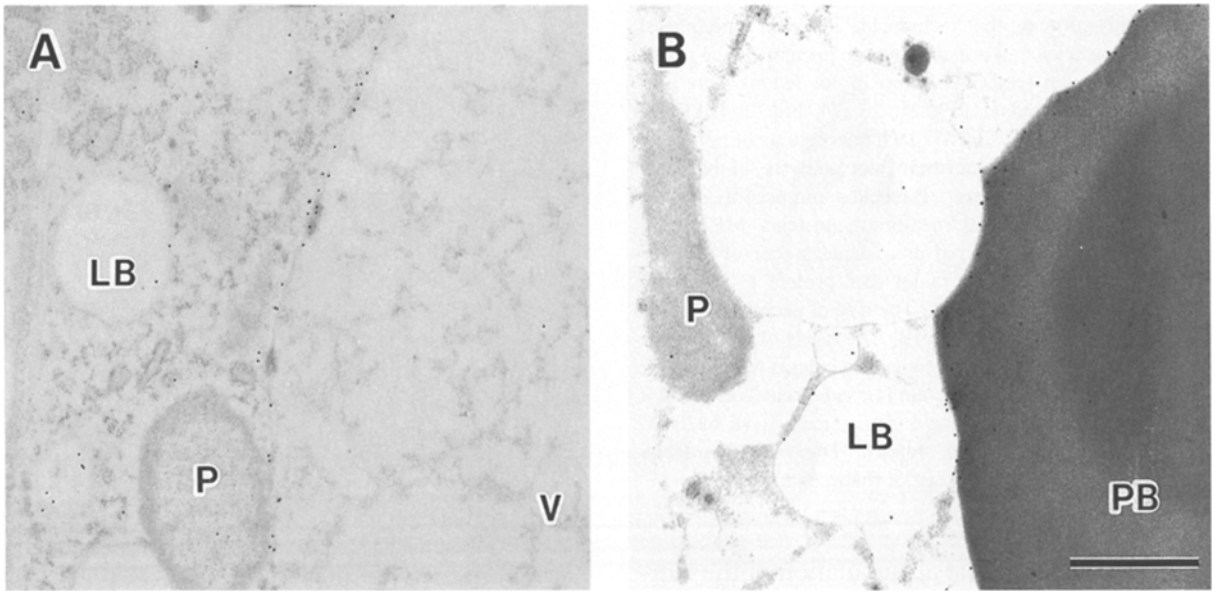

Fig. 7. Immunocytochemical localization of MP28 and MP23 in pumpkin cotyledons at the early and late stages of maturation. Pumpkin cotyledons were harvested at the early stage (22 DAF) and at the late stage (28 DAF) of seed maturation. Immunogold analysis was performed using antiserum against MP28 (1:500 dilution). Gold particles were localized on the membranes of vacuole (V) in the 22 DAF cotyledon (A) and on the membranes of protein body (PB) in the 28 DAF cotyledon (B). P and LB indicate a plastid and a lipid body, respectively. Bar = 1 μm.

Discussion

The presence of two isoforms of TIP, namely, α-TIP and β-TIP, was reported in the seeds of kidney bean [18] and *Arabidopsis* [14]. The molecular structure of β-TIP has not been reported but that of α-TIP has been well characterized. In the present study, we examined the molecular structures of two seed TIPs, MP28 and MP23, that are found in the protein-body membranes of pumpkin seeds (Fig. 3). It is likely that the two seed TIPs of pumpkin correspond to α-TIP and β-TIP of kidney bean and *Arabidopsis*.

Our molecular characterization of MP23 suggested that MP23 is synthesized as a 29 kDa precursor that is converted post-translationally to the mature MP23. Such cleavage has not been reported for the other members of the MIP family. The post-translational cleavage occurs on the C-terminal side of Asp-65 of the precursor molecule (Fig. 2B). The site of the cleavage is located in a hydrophilic region between the first and the second membrane-spanning domains (Fig. 2D). The region can form a loop that faces the interior of the vacuole [18]. The loop of MP23 is the largest yet found in a seed TIP (Fig. 3), and such a large loop might be particularly susceptible to proteolytic cleavage in the vacuole.

The proprotein precursors of many storage proteins are converted post-translationally to their respective mature forms by the action of a vacuolar processing enzyme (VPE) [8, 10, 12]. The VPE cleaves a peptide bond on the C-terminal side of an asparagine residue that is exposed on the surface of the precursor molecule [10, 11]. This substrate specificity of the VPE is not consistent with the nature of the cleavage of the precursor to MP23, which is cleaved on the C-terminal side of Asp-65. We reported recently that VPE itself is also synthesized as a proprotein precursor that is converted to the mature form by the cleavage of propeptides and that one possible site of the post-translational cleavage is on the C-terminal side of an aspartic acid residue [34]. Thus, a protease that recognizes an aspartic acid and cleaves a peptide bond on the C-terminal side of this residue might be responsible for the maturation of both MP23 and VPE.

The 29 kDa precursor to MP23 was detected in pumpkin seeds until the middle stage of seed maturation. Mature MP23 appeared at the late stage, with the concomitant disappearance of the 29 kDa precursor (Fig. 6B). Precursors to storage proteins are processed post-translationally to their mature forms immediately after their entry into the vacuoles and no accumulation of precursor is observed in maturing seeds. However, the precursor to MP23 accumulated in seeds prior to maturation of seeds, suggesting that the protease responsible for the maturation of MP23 might appear at the late stage of seed maturation and that the cleavage might regulate the function of the MP23 protein. It remains to be determined whether or not the N-terminal fragment is bound to MP23 molecule after the post-translational cleavage, and whether or not MP23 can function without the first membrane-spanning region.

The function of plant TIPs apart from γ-TIP are unclear. Plant γ-TIP has been shown to serve as a water channel, but α-TIP does not have such water-channel activity in the plasma membrane of *Xenopus* oocytes [25]. The possibility that MP28 and MP23, homologues of α-TIP, could function as a water channel remains to be established. In the cells of maturing pumpkin seeds, large amounts of storage proteins and phytic acids are dramatically accumulated into the vacuoles and the osmotic condition in the vacuoles is changed. MP28 and MP23 might function as transporters of low molecular compounds or osmolytes across the membranes and might regulate the osmotic condition during the seed maturation.

The accumulation of TIP in the vacuolar membranes occurs after the deposition of the storage proteins into the vacuoles in maturing soybean seeds [26]. By contrast, TIPs appear in pumpkin seeds at the early stage of seed maturation prior to the accumulation of the storage proteins (Figs. 6, 7). At the early to the middle stages of seed maturation, small vacuoles increase in size. Seed TIPs of pumpkin might be responsible for the enlargement of the vacuoles in the young

seeds. We reported recently that the seed TIPs of pumpkin disappear during the germination of seeds and growth of seedling, with the simultaneous appearance of γ-TIP [23]. The expression of γ-TIP is tightly correlated with expansion of plant cells where the large vacuoles are formed [22]. Therefore, it is also possible that seed TIP is rather responsible for the formation of the protein bodies from the vacuoles in maturing seeds, and that γ-TIP is responsible for the formation of a large vacuole from the protein bodies in growing seedlings. Further analyses of the two seed TIPs of pumpkin and the post-translational cleavage of MP23 are required if we are eventually to understand the physiological role of seed TIPs.

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