Biochemical studies on PV72, a vacuolar sorting receptor of higher plants

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Abbreviations

CTPP, C-terminal propeptide; EDTA, ethylenediamine tetraacetic acid; EGF, Epidermal Growth Factor; EGTA, ethylene glycol bis (β-aminoethylether)-tetraacetic acid; ER, endoplasmic reticulum; PAC, precursor-accumulating; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR; polymerase chain reaction; SDS, sodium dodecyl sulfate; SNAP, soluble NSF-attachment protein; NTPP, N-terminal propeptide; SNARE, SNAP receptor

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Vacuolar proteins are synthesized on rough endoplasmic reticulum (rER) and are transported to respective vacuoles. Unlike the transport of lytic proteins, the molecular mechanism of the transport of seed storage proteins is still unclear. Precursor accumulating (PAC) vesicles that had accumulated large amounts of proprotein precursors of storage proteins were purified from maturing pumpkin seeds. PV72, a type I membrane protein, was found to be localized on the membranes of the PAC vesicles. PV72 is composed of lumenal domain, transmembrane domain, and cytoplasmic domain. The lumenal domain contains three epidermal-growth factor (EGF)-like motifs. The third EGF-like motif is a Ca^{2+} -binding type.

To clarify the function of PV72 as a vacuolar sorting receptor for seed storage protein, I performed molecular studies on PV72. I found that the lumenal domain of PV72 (rPV72) expressed in insect cells bound to proprotein precursor of 2S albumin in a Ca^{2+} -dependent manner. I analyzed the domain of PV72 by expressing modified PV72s, rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3 in insect cells. Then I examined these modified PV72s with the internal propeptide (the 2S-I peptide) of pro2S albumin, a seed storage protein precursor, by affinity chromatography and surface plasmon resonance analysis. rPV72 specifically bound to the 2S-I peptide with a K_D value of 0.2 μM, which was low enough for it to function as a receptor.

Lytic type sorting receptors have been reported to bind to ligand in a pH-dependent manner. Thus I examined the possibility of pH as a regulation factor of interaction and clarified that the binding of Ca^{2+} stabilizes the receptor-ligand complex even at pH 4.0. I focused on Ca^{2+} as a regulatory factor for the association and dissociation of PV72 with the ligand and clarified that PV72 bound to the ligand in a Ca^{2+}-dependent manner. Furthermore, I clarified that the EGF-like motifs modulated a

Ca^{2+}-dependent conformational change of PV72 to form a functional pocket for the ligand binding. The association and dissociation of PV72 with the ligand is modulated by the Ca^{2+} concentration (EC_{50} value = 40 μM) rather than the environmental pH. The overall results suggested that PV72 functions as a sorting receptor for pro2S albumin and that Ca^{2+} regulates the sorting mechanism.

As described above, PV72 seemed to be a key molecule for sorting of vacuolar proteins. In Arabidopsis, seven receptor homologues are found. These receptors must have different functions. However the difference among them is unclear. Thus, we further characterized authentic PV72 and PV82 derived from pumpkin seeds and found that PV72 and PV82 showed different characters. Further, we performed biochemical characterization of AtELP that has been reported as sorting receptor for lytic protease in Arabidopsis. We found that rAtELP bound to the AtPAP peptide derived from AtALEU in a Ca^{2+}-dependent. It is possible that Ca^{2+} is a key regulator for transport of vacuolar proteins in higher plants.

In contrast to well characterization *in vitro*, the demonstration for the *in vivo* function of vacuolar sorting receptors has been poorly done. PV72 bound both to the peptide derived from 2S albumin and to the peptide derived from aleurain. It implied that receptor bound to various types of ligands including storage protein and lytic protease. Then, I performed *in vivo* characterization of vacuolar sorting receptor in transgenic Arabidopsis cells. To elucidate a physiological function of such vacuolar sorting receptor in plant cells, we produced transgenic Arabidopsis plants that expressed a fusion protein (PV72-HDEL) composed of the lumen domain of PV72 with a His-tag and an endoplasmic reticulum (ER)-retention signal, HDEL.

The ectopic overexpression of PV72-HDEL induced the accumulation of a precursor of a cysteine proteinase, AtALEU, which contains a vacuolar targeting signal, NPIR, in the N-terminal propeptide. On the other hand, no influence was observed on another cysteine proteinase, RD21, which contains no NPIR sequence. Subcellular

fractionation revealed that the AtALEU precursor was associated with PV72-HDEL in the ER of the transgenic plants. To clarify the interaction between the receptor and the ligand, we analyzed their ability to bind the NPIR-containing propeptide (AtPAP peptide) of the AtALEU precursor. rPV72 bound to the propeptide with a K_D value of 0.1 μM, which was low enough for it to function as a receptor. The association of rPV72 with the AtPAP peptide was stabilized in the presence of 1 mM $CaCl_2$. rPV72 bound to the ligand even at pH 4.0 in the presence of Ca^{2+}. Deletion of three repeats of EGF-like motifs of the lumenal domain reduced the affinity (K_D value of 1.2 μM) between the rPV72Δ1,2,3 and the propeptide. Overall results suggest that PV72 homolog(s) functions as a sorting receptor for the NPIR-containing protease AtALEU to be transported to the lytic vacuoles and that the receptor-mediated transport is regulated by Ca^{2+} concentration rather than the environmental pH.

Introduction

A typical plant cell contains 5,000 to 10,000 of different polypeptide sequences and billions of individual protein molecules. For proper function of the cell, these proteins must be directed to specific metabolic compartments, cytoplasmic structures, and membrane systems. Cells, therefore, require the necessary machinery to sort each protein and direct it to its proper destination (Bar-Peled, et al., 1996).

The first sorting event for all proteins made in the cytosol separates them into two groups. The proteins in the first group are released in the cytosol and may be retained in that compartment or are targeted to various subcellular organelles, such as the mitochondria, chloroplasts, peroxisomes and nuclei. By contrast, proteins in the second group are targeted to the ER by signal peptides located in the N terminus. Translation of a signal peptide causes the ribosome to bind to ER membrane during protein synthesis. ER studded with such ribosomes is referred to as rough ER. Proteins synthesized on the rough ER enter the secretary pathway (Bar-Peled, et al., 1996). This pathway is composed of the ER, Golgi complex, plasma membrane, small vesicles; also part of this system are vacuoles (in plant cells and yeast) and lysosomes (in mammalian cells). The mechanism for ER retention/retrieval is conserved between plant, mammalian and yeast cells. The KDEL-like sequence, when fused to normally secreted reporter proteins, retain in the ER of plant, yeast and mammalian cells (Munro and Pelham, 1987) (Pelhum, 1988) (Vitale, et al., 1993). Proteins that have the KDEL-like signal are recognized and bound by a KDEL receptor in the Golgi complex (Hsu, et al., 1992) (Lewis and Pelhum, 1990) (Lewis and Pelhum, 1992).

The transport of proteins from the ER to the Golgi complex is the first vesicle mediated step in the process of protein secretion. Transport between the membrane bound compartments of the secretary pathway is thought to involve the formation of coated vesicle intermediates that bud from one membrane compartment and are targeted

and subsequently fuse with an acceptor membrane compartment (Pryer, et al., 1992). The transport of proteins along the Golgi complex is mediated by small vesicles budding from one cisternae and fusing with the next. The *trans* Golgi network is thought to be the compartment where proteins are sorted to the vacuole or the plasma membrane for secretion. The proteins with specific signals are transported to the vacuole/lysosome, and the proteins with no signals are secreted out. A unique feature of the secretary pathway is that proteins are transported in small membrane bound vesicles.

Transport of proteins to mammalian lysosomes

The lysosome is considered to be the mammalian equivalent of the yeast vacuole. A transport mechanism of proteins to this organelle has been extensively studied. Most acid hydrolases are transported to lysosome by a secretary pathway to the *trans* Golgi network, where they are sorted from secretary proteins and transported to lysosomes via endosomes (Traub and Kornfeld, 1997).

Most lysosomal proteins contain a carbohydrate targeting signal consisting of phosphorylated mannose residues (Von Figura and Hasilik, 1986). The mammose-6-phosphate receptors bind to this mannose 6-phosphate residues of lysosomal proteins at the *trans* Golgi network, and transport them to endosome (Von Figura and Hasilik, 1986) (Kornfeld, 1992). The low pH (pH 6.0) of the acidified endosome causes the mammose-6-phosphate receptors to release lysosomal hydrolases (Dahms, et al., 1989) and the mammose-6-phosphate receptors travel back to the *trans* Golgi network by sorting determinants in the cytoplasmic domains (Rohn, et al., 2000).

The sorting of lysosomal proteins at the *trans* Golgi network is mediated by clathrin coated vesicles that are ubiquitous in eukaryotes. The main component on clathrin-coated vesicles is clathrin, a trimeric scaffold protein, which organizes itself into cage-like lattices (Kirchhausen, 1993). The major proteins that drive clathrin formation are clathrin adapter protein complexes that couple coated pit assembly to the

entrapment of membrane receptors. This adapter proteins connect the clathrin coat to the vesicle membrane through interaction with the Tyr-based signals (YXXΦ sequence: Y stands for Tyr; X, for any residue; Φ, for a bulky, hydrophobic residue) present in the cytoplasmic tail of receptors (Kirchhausen, et al., 1997).

Transport of proteins to yeast vacuole

In yeast, newly synthesized proteins that reach the *trans* Golgi network are sorted and transported either to the vacuole or to the cell surface (Conibear and Stevens, 1998) (Wendland, et al., 1998). Yeast vacuole is an acidified organelle that is responsible for the degradation of macromolecules. Sorting of the vacuolar proteins occurs at the *trans* Golgi network like mammals. By contrast, transport of proteins to the yeast vacuole does not require a carbohydrate modification; rather, peptide sequences are responsible for targeting (Wendland, et al., 1998). A vacuolar proteins carboxypeptidase Y (CPY) has a QRPL signal as the vacuolar targeting signal in its N-terminal propeptide which is cleaved in the vacuole to yield mature form (Valls, et al., 1990). The transport pathway of CPY from the *tarns* Golgi network to the vacuoles is well analyzed. The transport pathway of CPY carries soluble hydrases to vacuole via a prevacuolar compartment. *VPS10* gene was identified as a gene encoding a vacuolar sorting receptor for CPY (Marcusson, et al., 1994). VPS10p is a type I membrane protein that can be cross-linked to CPY precursor, but not to CPY mature form (Marcusson, et al., 1994). At the *tarns* Golgi network, CPY is captured by VPS10p and is then recruited to a transport vesicle. This vesicle then fuses with the prevacuolar compartment. Based on these facts, a model could be drawn for the sorting of CPY to the yeast vacuole that is analogous to the sorting of lysosomal proteins by mannose-6-phosphate receptors. CPY is recognized by a specific receptor in the *trans* Golgi network and delivered to an prevacuolar compartment that may be similar to the endosome of mammalian cells.

A SNARE (SNAP receptors) hypothesis offers a widely accepted explanation of

the mechanism of specificity in vesicle docking and fusion. A conserved function of many of these components has led to a generalized hypothesis for vesicle fusion. SNAREs are membrane proteins found on both transported vesicles (v-SNARE) and target organelles (t-SNARE). In this model, specific proteins on the vesicle (v-SNARE) interacted with partner proteins on the target membrane (t-SNARE). SNARE proteins are essential for membrane fusion and are conserved from yeast to mammals (Sollner, et al., 1993). The specific interactions between t-SNARE and v-SNARE ensure that vesicles are targeted to the correct compartment and lead to membrane fusion. Pep12p a t-SNARE on the prevacuolar compartment is required for correct sorting of CPY (Becherer, et al., 1996).

Differentiation of plant vacuoles; protein storage vacuoles and lytic vacuoles

Higher plants have two types of vacuoles: protein storage vacuoles that develop mainly in storage organs, such as seeds, and lytic vacuoles that develop in the vegetative organs and contain various lytic enzymes,.

Each Vacuole can be characterized by the presence of specific tonoplast intrinsic proteins (TIPs): α-TIP has been associated with protein storage vacuoles (Johnson, et al., 1989) (Johnson, et al., 1990), whereas γ-TIP has been found in lytic vacuoles (Paris, et al., 1996) and δ-TIP has been found in pigment-containing vacuoles (Jauh, et al., 1998). TIPs have also often found in various combinations in single vacuoles (Jauh, et al., 1998) (Jauh, et al., 1999). Both types of vacuoles are found in the same cells of barley roots (Paris, et al., 1996) and of maturing pea seeds (Hoh, et al., 1995). In these cells, vacuolar proteins synthesized on the rER are sorted and delivered to their respective vacuoles. Thus, each type of vacuole must have different targeting machinery for protein transport in these cells.

Vacuolar targeting signals

Recently, vacuolar targeting signals on the polypeptide sequences of soluble vacuolar proteins have been well characterized. The signals are separated into three classes, sequence specific vacuolar-sorting signal, C-terminal vacuolar-sorting signal and protein structure based vacuolar-sorting signal (Matsuoka and Neuhaus, 1999).

The sequence specific vacuolar-sorting signal was first defined in the propeptides of sweet potato sporamin and barley aleurain. After cleavage of the signal peptide, prosporamin carries a N-terminal propeptide that is removed to form mature form in sweat potato tubers (Matsuoka, et al., 1990). Aleurain is a cysteine protease which is synthesized as the proenzyme and transported to lytic vacuoles where it is processed to mature form (Holwerda, et al., 1990). An NPIR sequence conserved in the N-terminal propeptides (NTPPs) both of aleurain, SSSSFADSNPIRPVTDRAAST and sporamin, RFNPIRLPTTHEPA, has been shown to function as a vacuolar targeting signal *in vivo* (Holwerda, et al., 1992) (Matsuoka and Nakamura, 1991). The consensus composition of the NPIRL sequence of sporamin is <preferably Asn>, <not acidic>, <Ile or Leu>, <any amino acid>, <large and hydrophobic> (Matsuoka and Nakamura, 1999). Although both aleurain and sporamin signals are found at their N terminus, the position is less important than the sequence specificity feature since the sporamin vacuolar propeptide is still functional when put at the C-terminus (Koide, et al., 1997). Transport to the vacuole of proteins with a sequence specific vacuolar-sorting signal was found to be relatively resistant to wortmannin in tobacco BY-2 cells (Matsuoka, et al., 1995), compared with another group of vacuolar proteins described below.

Only a few C-terminal propeptides have been positively identified: in a barley lectin (Dombrowski, et al., 1993), in a chitinase (Melchers, et al., 1993), a glucanase (Neuhaus, et al., 1991) and an osmotin (Sticher, et al., 1992) from tabacco and in 2S albumin storage proteins from Brazil nut (Saalbach, et al., 1991) and pea (Higgins, et al., 1986). By contrast to the sequence specific vacuolar-sorting signal, the C-terminal

propeptides have no significant conserved sequence among C-terminal propeptides (Matsuoka and Neuhaus, 1999). Several different segments of the barley lectin propeptide, VFAEAIAANSTLVAE, were each functional *in vivo*. Instead, the first four or the last four residues each were sufficient. The sequence requirements for vacuolar sorting of chitinase A were characterization by deletion and mutation analysis. Many single mutation had little effect and several random sequences could also function as signal (Neuhaus, et al., 1994). In both cases, the function of this vacuolar-sorting signal could be most efficiently reduced by the addition of one or several Gly residues to the end of the propeptide. This means that C-terminal vacuolar-sorting signal must be accessible from the end. In contrast to the sequence specific vacuolar-sorting signal, vacuolar proteins with C-terminal propeptide were secreted when BY-2 cells were treated with wortmannin (Matsuoka, et al., 1995).

There is a third heterogeneous group of proteins for which neither of the two first classes of vacuolar-sorting signals seems to apply. Some storage proteins are thought to have a sorting signal within the mature polypeptide chain. An internal sequence specific vacuolar-sorting signal is possible, but for several seed storage proteins, another sorting system is likely to be involved. These are the vicilin-like and legumin-like proteins that accumulate into dense vesicles at the *trans* Golgi network and are transported without involvement of clathrin coated vesicles (Robinson and Bäumer, 1998). Aggregation has been proposed as a non-receptor-mediated sorting mechanism and is known to occur in a pH-dependent manner in animal cells (Vitale and Chrispeels, 1992) (Castle, et al., 1997). An extreme form of aggregation is presented by cereal prolamins which aggregation is presented by cereal prolamins which aggregate within the ER to form protein bodies (Okita and Rogers, 1996). Pea prolegumin is more hydrophobic in the ER and Golgi than is legumin in the protein bodies (Hinz, et al., 1997).

Vacuolar sorting receptors

Shimada et al. identified two potential vacuolar sorting receptors (PV72 and PV82) from the PAC vesicle (descrived below) fraction prepared from maturing pumpkin seeds (Shimada, et al., 1997). PV72 is a type I integral membrane protein with a large N-terminal lumenal domain containing three potential N-glycosylation sites and a C-terminal domain of 38 amino acids. Within the N-terminal domain the first 400 amino acids represent a unique region with no homology to yeast or animal sequences. This region does show homology to sequences from maize, rice and Arabidopsis and within pea and Arabidopsis there appears to be gene families for the protein. The remaining intralumenal region of PV72 is occupied by the Epidermal Growth Factor (EGF) repeats. The third EGF motif is a Ca^{2+}-binding type. In the cytoplasmic domain, there are Tyr-based motifs as described above.

PV72 bound both to the 2S-I peptide and to the 2S-C peptide derived from pumpkin 2S albumin (Shimada, et al., 1997). The 2S-I peptide included an internal propeptide while the 2S-C peptide included the C-terminal region of precursor. Binding to the 2S-I peptide (MRGIEN/PWR**RE**G) was traced down to a region containing a processing site, and a Gly scan identified two important residues, the R and E indicated in bold (Shimada, et al., 1997). The 2S-I peptide (KAR**NLPS**MCGIRPQRCDF) contains an NLPS motif conserved in 2S albumins of other species. Replacement of the four residues by GGGG abolished the binding. PV72 also bound to the NPIR-containing propeptide derived from barley aleurain (Shimada, et al., 1997).

PV72 homologue, BP-80, which bound to the NPIR-containing propeptide derived from barley aleurain was identified from the extract of clathrin coated vesicles derived from pea developing cotyledon (Kirsch, et al., 1994) (Paris, et al., 1997). In mammalian systems, the mannose-6-phosphate receptors involved in the recovery of lysosomal precursors from the *trans* Golgi network becomes incorporated into clathrin coated vesicles (Leborgue and Hoflack, 1997). By analogy, it was reasoned that a

receptor for the targeting determinants for vacuolar delivery in plants would also be present in clathrin coated vesicles. Based on the knowledge obtained on the mammalian mannose-6-phosphate receptors it was hypothesized that a plant receptor for acid hydrase would bind its ligand in a pH-dependent manner. *In vitro* analysis revealed that, BP-80 bound to the NPIR-containing propeptide in a pH-dependent manner (Kirsch, et al., 1994). Immunoelectrom microscopy revealed that, in addition to be localized in clathrin coated vesicles, BP-80 was found at the *trans* face of the Golgi and in small compartments adjacent to the large vacuole, prevacuoles (Hinz, et al., 1999). By screening the EST database for EGF receptor repeat sequence, AtELP, was identified from Arabidopsis (Ahmed, et al., 1997). It was associated with two membrane fractions, one enriched in clathrin and its associated adapter containing vesicles and an unidentified compartment (Sanderfoot, et al., 1998).

Golgi-independent and Golgi-dependent transport of seed storage proteins to protein storage vacuoles

The molecular mechanism responsible for the transport of storage proteins that is unique to higher plants is scarcely elucidated. Storage proteins are separated into four types: albumin, globulin, proramin, and gluterin. In maturing seeds, storage protein precursors are synthesized on the rER and then transported to protein storage vacuoles, where the precursor proteins are converted into the respective mature forms by the action of vacuolar processing enzyme (Hara-Nishimura, et al., 1991; Hara-Nishimura, et al., 1998a; Hara-Nishimura and Nishimura, 1987; Hara-Nishimura, et al., 1995; Hara-Nishimura, et al., 1993; Kinoshita, et al., 1999).

Most seed proteins, including globlins and some prolamins, have been shown to be transported to vacuoles in a Golgi-dependent pathway (Shotwell and Larkins, 1988) (Chrispeels, 1991) (Herman and Larkins, 1999). Hohl et al. (Hohl, et al., 1996) and Hinz et al. (Hinz, et al., 1999) demonstrated immunocytochemically that dense vesicles with a

diameter of about 100 nm associated within *trans* Golgi network contain storage proteins in maturing pea cotyledons. Dense vesicles contain the precursor of the major storage proteins legumin and vicilin (Hinz, et al., 1999). Protein aggregation within the Golgi has been suggested to be another way in which proteins destined for the vacuole are segregated away from secreted proteins.

In contrast to Golgi-dependent transport, studies on the transport of seed storage proteins in legumes and cereals have shown that storage proteins can be sorted in a Golgi-independent manner (Marty, 1999). Levanony et al. reported that wheat prolamins are transported to the protein storage vacuoles without any contribution by the Golgi apparatus (Levanony, et al., 1992). A unique pathway has been suggested in cells from maturing seeds of pumpkin and caster bean (Hara-Nishimura, et al., 1998b). In these cells, seed proteins were shown to be transported from the rough ER to the protein storage vacuoles via large vesicles (Hara-Nishimura, et al., 1998b). The vesicles responsible for delivery of seed storage protein precursors to the vacuoles were designated them PAC (precursor-accumulating) vesicles (Hara-Nishimura, et al., 1998b). Isolation of the PAC vesicles revealed that they contained a large amount of proprotein precursors of various seed proteins, including 11S globulin and 2S albumin but not mature products (Hara-Nishimura, et al., 1998b). It was suggested that the core of storage proproteins, contained in these large vesicles might derive directly from protein aggregates that are formed in the ER. In addition to that, the PAC vesicles also accumulate ER resident proteins such as BiP. The increased accumulation of storage proteins parallels the increase in the amount of BiP during maturation of pumpkin (Hatano, et al., 1997). It seems likely that BiP might play some role in the formation of the aggregates in the ER of pumpkin cotyledons. In maturing pumpkin cotyledons, where the majority of storage proteins are not glycosylated, the PAC vesicles the Golgi such that their transport is not inhibited by the carboxylic ionophore monenesin (Hayashi, et al., 1988). Thus, the PAC vesicles mediate a transport for storage proteins

directly to protein storage vacuoles in Golgi-independent manner. PV72 has been identified from the membrane fraction of PAC vesicles (Shimada, et al., 1997). However, the function of PV72 is still clear.

Transport of vacuolar proteins to lytic vacuoles

The transport of proteins to lytic vacuoles is thought to pass through the *trans* Golgi network by analogy to the yeast and mammalian system. The presence of clathrin coated vacuoles at the *trans* Golgi network of plant cells invites the speculation that, in analogy to the situation in animal and yeast cells, they should participate in the delivery of proteins to the plant vacuole. Cell fractionation studies suggested that BP-80 and AtELP were enriched in a membrane fraction of clathrin coated vacuoles (Hinz, et al., 1999; Sanderfoot, et al., 1998). In pea cotyledons, clathrin coated vacuoles that form at the *trans* Golgi network do not contain the abundant storage proteins (Hinz, et al., 1999).

AtALEU which is aleurain homologue in Arabidopsis contains the NPIR sequence in the N-terminal propeptide (Lohman, et al., 1994). On the contrary, RD21 did not contain the NPIR sequence (Koizumi et al. 1993). AtALEU and RD21 which are members of the same papain family of lytic vacuoles are known to be induced by desiccation in *Arabidopsis* (Lohman, et al., 1994) (Koizumi et al. 1993). However, the transport mechanism of these proteins to lytic vacuoles is still unclear.

Biochemical analysis of vacuolar sorting receptors

My interest is the biochemical characterization of a plant vacuolar sorting receptor, PV72. PV72 was identified as a putative sorting receptor from pumpkin cotyledon seeds. However the functional characterization as receptor was not performed. How the association and dissociation of a receptor and the respective ligand is regulated is unclear. Thus we focussed on the functional biochemical characterization of PV72 to answer the question. The well known biochemical characterization of a vacuolar sorting

receptor, there are no report of *in vivo* functional characterization. Thus, we performed further *in vivo* demonstration of PV72 after biochemical characterization.

Materials and Methods

Plant materials

Pumpkin (Cucurbita sp cv Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). The seeds were planted in the field. The cotyledons of maturing seeds, freshly harvested 22-28 days after anthesis, were used for the experiments. The leaf of the cotyledon freshly harvested 6 days after seedling, were used for the experiments.

Ultrastructural analysis

Maturing pumpkin seeds were vacuum-infiltrated for 1 hr with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The tissues were then cut into slices of less than 1 mm in thickness with a razor blade and treated for another 2 hr with freshly prepared fixative.

Isolation of PAC vesicles from maturing pumpkin seeds

PAC vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation as described previously (Dahms, et al., 1989). The cotyledons were homogenized in buffer A (20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA and 0.3 M mannitol) with an ice-chilled mortar and pestle and filtered through three layers of cheesecloth. The filtrate was centrifuged at 3,000 *g* for 15 min and the supernatant was centrifuged again at 8,000 *g* for 20 min at 4 °C. The pellet was suspended in buffer B (10 mM HEPES-KOH, pH 7.2, 1 mM EDTA and 0.3 M mannitol) and layered on 28% Percoll (Amersham Pharmacia Biotech Tokyo, Japan) solution. After centrifugation at 40,000 *g* for 35 min, the vesicle fraction was pooled and washed once in buffer B. PAC vesicles were collected by the centrifugation at 10,000 *g* for 20 min, and resuspended in buffer B. The isolated vesicles were subjected to immunoblot analysis.

Immunoblot analysis

Immunoblot analysis was performed essentially as described previously (Mitsuhashi, et al., 2000). We used specific rabbit polyclonal antibodies against PV72 (diluted 5,000-fold) (Shimada, et al., 1997) and RD21 (diluted 5,000-fold) (Yamada, et al., 2001), and monoclonal antibody against barley aleurain which is a homologe of AtALEU (2F5-5B2; diluted 1,000-fold) that was kindly donated by Dr. J. C. Rogers of Washinton State University (Rogers, et al., 1997). We also used horseradish peroxidase-conjugated donkey antibodies against either rabbit IgG (diluted 5,000-fold; Amersham Pharmacia Biotech, Tokyo, Japan) or mouse IgA (diluted 1,000-fold; Amersham Pharmacia Biotech). Immunodetection was performed with an enhanced chemiluminescence kit (an ECL system, Amersham Pharmacia Biotech).

Plasmid construction

The cDNA insert of pBS pro2S albumin was inserted into pET28a (Novagen, Madison, WI) to yield pET2proSalbumin, which was used for expression of His-tagged pro2Salbumin in *E. coli*. For the chimeric gene encoding His-tagged pro2Salbumin, two annealing complementary oligonucleotides, 5'-TAT GGA TCC TAC CGC ACC ACC ATC -3' as an antisense strand and 5'-TAA CTC GAG TCA GAA GTC GCA TCG -3' as an antisense strand, were synthesized and annealed to produce a double-strand DNA with protruding ends to be ligated with the Eco RV site of pBS-SK. The *Ban* HI-*Xho* I fragment of pBS pro2S albumin was ligated with the *Ban* HI-*Xho* I -digested pET28a to produce the pETpro2Salbumin. The chimeric gene was composed of a His-tag, followed by the pro2S albumin.

Four modified PV72s were expressed in insect cells of *Spodoptera frugiperda* (Sf21) with a baculovirus expression system (Invitrogen, San Diego, CA). The system includes a transfer vector pBlueBac 4.5 and an expression vector Bac-N-Blue DNA composed of engineered baculoviral *Autographa california* multiple polyhedrosis virus.

The *Kpn* I-*Sac* I fragment of pPV72 was produced from the amplified DNA with PV72 cDNA and a unique primer of 5'-ATT TGT TTA ACT GAA GAC GTG CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3', and was ligated with the *Kpn* I-*Sac* I-digested pBlueBac 4.5 to produce the pBlueBac-rPV72. The pBlueBac-rPV72 encoded a fusion protein composed of both the signal sequence and the lumenal domain of PV72 followed by a His-tag and an HDEL sequence. Constructs for three other modified PV72s were produced by the same procedure described above, except for using each primer: 5'-GAT GGA GTC CAC ACG TGT GAA CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3' for PV72Δ3, 5'-YAC ACT CAT TGT GAA GCT CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3' for PV72Δ2,3, and 5'-ATT TGT TTA ACT GAA CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3' for PV72Δ1,2,3.

The cDNA for AtALEU and two oligonucleotides, 5' CCACATGTCTGCGAAAACAAT 3' and 5' GTTTCCATGGTAGCTGCTTCTGTGACCT 3' were used to amplify a DNA fragment encoding an N-terminal region (184 amino acids) of preproAtAleu, which included a signal peptide and a propeptide followed a part of the mature protein. The *Afl*III-*Sac*I digested fragment was inserted into the Ti-plasmid pSPGFP (Mitsuhashi, et al., 2000).

The vector containing the *Afl* III - *Sac* I fragment was designated as pSKNPIRGFP. pSKNPIRGFP encoded a fusion protein composed of both the signal sequence and the proregion of AtALEU followed by a GFP sequence. A fragment containing *Xho* I and *Sac* I was ligated to thep35SLM digested with *Xho* I and *Sac* I. The vector containing the *Xho* I - *Sac* I fragment was designated as p35NPIRGFP.

Overexpression and purification of pro2S albumin in *E. coli*

His-tagged pro2S albumin was expressed in *E. coli* BL21 (DE3) and purified with a Hitrap chelating column (Pharmacia) in the presence of 6M Urea. The cells were

collected by centrifugation at 10,000 *g* for 30 min, and gently suspended in 20 mM HEPES, pH 7.0, 500 mM NaCl, 6M Urea. The cells were lysed by three bursts of sonication for 3 min at 10-min intervals on ice and were centrifuged at 750 000 *g* for 30 min. The supernatant was loaded on a Hi-Trap chelating column (Amersham Pharmacia Biotech, Tokyo, Japan) and was eluted with a gradient of 20 to 1000 mM imidazol in the above buffer. The purified His-tagged pro2S albumin fractions were dialyzed against the HEPES buffer (20 mM HEPES, pH 7.0, 150 mM NaCl). Purified His-tagged pro2S albumin was used for ligands of affinity columns.

Expression and purification of the modified PV72s

We cotransfected Sf21 cells with Bac-N-Blue DNA and each of the produced plasmids to generate recombinant baculoviruses. The viruses were purified from the supernatant of the transfected cells by a plaque assay to generate a high-titer recombinant viral stock (24).

For large-scale expression of these modified PV72s, the optimal expression time was determined by monitoring the cellular extract using SDS-PAGE and an immunoblot with anti-PV72 antibodies. Three days after infection, the cells were collected by centrifugation at 500 *g* for 10 min, washed with PBS and gently suspended in 20 mM HEPES, pH 7.0, 150 mM NaCl, 1% CHAPS and 1 mM $CaCl_2$. The cells were lysed by three bursts of sonication for 1 min at 10-min intervals on ice and were centrifuged at 750 000 *g* for 30 min. The supernatant was loaded on a Hi-Trap chelating column (Amersham Pharmacia Biotech, Tokyo, Japan) and was eluted with a gradient of 20 to 1000 mM imidazol in the above buffer. The modified PV72 fractions were dialyzed against the HEPES buffer (20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.4% CHAPS) plus 1 mM $CaCl_2$, were concentrated using Centricon 30 (Amicon Inc., Beverly, MA) and then were loaded on a Superdex-200 column (Amersham Pharmacia Biotech) equilibrated with the HEPES buffer plus 1 mM $CaCl_2$. The purified modified PV72s

were concentrated by Centricon 30 and subjected to a protein assay (Nippon Bio-Rad Laboratories, Tokyo) and a binding assay as described below.

Ligand-binding assay by affinity column chromatography

His-tagged pro2S albumin was immobilized to NHS-activated Sepharose HP (Amersham Pharmacia Biotech) to prepare affinity columns. Five peptides were chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems Inc., Tokyo, Japan) and were used for ligands of affinity columns; SRDVLQMRGIENPWRREG (2S-I), SRDVLQMRGIENPWGGGG (2S-I/3G), SRDVLQMRGIENGWRREG (2S-I/P75G), SRDVLQMRGIGNPWRREG (2S-I/E73G), SRDVLQMRGIENPWRRGG (2S-I/E79G), ANIGFDESNPIRMVSDGLREV (AtPAP), SRFNPIRLPT (SPO), SRFNPGRLPT (SPO-NPGR). Each peptide (10 mg) was immobilized to NHS-activated Sepharose HP (Amersham Pharmacia Biotech) to prepare affinity columns. The modified PV72s were applied to each column equilibrated with the HEPES buffer plus 1 mM $CaCl_2$ and then eluted with the HEPES buffer plus 2.5 mM EDTA (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 0.4 % CHAPS and 2.5 mM EDTA) on an automated chromatography system (ÄKTA, Amersham Pharmacia Biotech).

The modified PV72s were applied to the AtPAP column equilibrated with the HEPES buffer containing 1 mM $CaCl_2$ and then eluted with the buffer containing 2.5 mM EDTA , 20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl and 0.4 % CHAPS on an automated chromatography system (ÄKTA, Amersham Pharmacia Biotech). Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

The crude extract of pumpkin seeds was applied to each column (the, AtPAP, SPO, SPO-NPGR or 2S-I column) equilibrated with the HEPES buffer plus 1 mM $CaCl_2$ and then eluted with the HEPES buffer plus 2.5 mM EDTA (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 0.4 % CHAPS and 2.5 mM EDTA) on an automated chromatography

system (ÄKTA, Amersham Pharmacia Biotech). Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis with both anti PV72 and PV82 antibodies.

Surface plasmon resonance and kinetic assays

We immobilized either 2S-I peptide, 2S-I/3G, AtPAP peptide on a sensor chip for BIACORE-X (BIACORE, Tokyo, Japan) in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.005 % P-20 (HBS, BIACORE). Carboxymethylated dextran on a sensor chip (CM5) was activated with the mixture (70 µl) of 0.05 M N-hydroxysuccinimide (NHS) and 0.05 M N-ethyl-N-(3-diethylaminopropyl) carbodiimide (EDC) and then coupled with either 2S-I peptide or 2S-I/3G peptide at 25 °C and a flow rate of 5 prevacuolar compartment l/min for the solutions used on BIACORE-X. A control flow cell was prepared with no peptide. The amount of the coupled peptide on the sensor chip was found to be 700-1500 resonance units (RU).

The modified PV72s were injected onto the sensor chip for 300 sec and then the HEPES buffer plus 1 mM $CaCl_2$ was eluted for 200-300 sec at 25 °C and a flow rate of 30 µl/min. The sensor chip surface was regenerated with 30 µl of 20 mM HCl to remove residual PV72 from the immobilized peptides. Equal volumes of each protein dilution were also injected over a control flow cell to serve as blank sensorgrams for subtraction of bulk refractive index background and nonspecific binding of analyte. The sensorgrams shown in this study are made by subtracting the sensorgram made with the control flow cell.

Kinetic analysis was performed according to the manufacturer's protocol. The association, dissociation, and regeneration phases were followed in real time as the changes in the relative diffraction. The association phase (0 to 180 sec) was analyzed by nonlinear least squares curve fitting to yield the association rate constants (k_a) as mean values. The dissociation phase (180 to 300 sec) was also analyzed by nonlinear least

squares curve fitting to yield the dissociation rate constants (k_d). To avoid mass transport, we worked at a low immobilization level, a high flow rate (30 μl/min), and using suitable concentrations of analyte. Kinetic constants {the association rate constant (k_a), the dissociation rate constant (k_d) and the dissociation constant ($K_D = k_d / k_a$)} were calculated from the sensorgrams using BIA evaluation software version 2.1 (BIACORE). These kinetic parameters were determined from three independent experiments.

Ca^{2+}-dependent binding to the 2S-I peptide

Ca^{2+}-dependent binding was analyzed by two methods: surface plasmon resonance analysis and affinity chromatography. Both rPV72 and rPV72Δ1,2,3 were dialyzed against the HEPES buffer plus 2.5 mM EGTA, followed by dialysis against the HEPES buffer to remove Ca^{2+} and EGTA. The dialyzed PV72s were injected onto the 2S-I-immobilized sensor chip equilibrated with the HEPES buffer plus 0, 0.02, 0.05 0.1 and 1 mM $CaCl_2$ or 1 mM $MgCl_2$ to obtain each sensorgram.

Alternatively rPV72 was applied to the 2S-I affinity column with the HEPES buffer plus 1 mM $CaCl_2$ and then washed with the HEPES buffer containing decreasing concentration of $CaCl_2$ (500 μM, 100 μM, 50 μM, 20 μM, 0 μM). Finally, the column was eluted with the HEPES buffer plus 1 mM EGTA.

To compare the Ca^{2+}-sensitivity of rPV72 with that of rPV72Δ1,2,3, both modified proteins were applied to the 2S-I column with either the HEPES buffer plus 1 mM $CaCl_2$ or the MES buffer (20 mM MES-NaOH, pH 5.5, 150 mM NaCl, 0.4 % CHAPS) plus 1 mM $CaCl_2$. I used the HEPES buffer plus 50 μM $CaCl_2$ or the MES buffer plus 50 μM $CaCl_2$ as the washing solution for the columns, and the HEPES buffer plus 2.5 mM EGTA or the MES buffer plus 2.5 mM EGTA as the elution buffers. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

To compare the Ca^{2+}-sensitivity of PV72 with that of PV82, Ca^{2+}-dependent binding was analyzed by affinity chromatography. The crude extract was applied to the

AtPAP affinity column with the HEPES buffer plus 5 mM $CaCl_2$ and then washed with the HEPES buffer containing decreasing concentration of $CaCl_2$ (500 μM, 100 μM, 50 μM, 20 μM, 0 μM). Finally, the column was eluted with the HEPES buffer plus 1 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

pH-dependent binding

To investigate the pH-effect on the interaction of modified PV72s with either 2S-I peptide, 2S-I/E73G peptide, 2S-I/P75G peptide, and 2S-I/E79G peptide, the proteins were subjected to each affinity column equilibrated with the HEPES buffer plus 1 mM $CaCl_2$ or the sodium acetate buffer (20 mM sodium acetate, pH 4.0, 150 mM NaCl, 0.4 % CHAPS) plus 1 mM $CaCl_2$. The column was washed with the respective buffer, and eluted with the respective buffer plus 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

The modified PV72s were applied to an AtPAP affinity column equilibrated with HEPES buffer containing 1 mM $CaCl_2$. The column was washed with the same buffer and followed by washing with the sodium acetate buffer (20 mM sodium acetate, pH 4.0, 150 mM NaCl, 0.4 % CHAPS) containing 1 mM $CaCl_2$ and by the sodium acetate buffer containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot.

Spectroscopic measurements

Fluorescence emission spectra were recorded from 300 to 400 nm by a fluorescence spectrophotometer (Hitachi, F-4500, Tokyo, Japan) with an excitation wavelength at 280 nm in mixtures containing 1 μg/ml modified PV72s, in the HEPES buffer plus 1 mM $CaCl_2$ or the HEPES buffer plus 1 mM EDTA, as described by (25).

Preparation of the extract from the maturing pumpkin seeds

The crude extract of the maturing seeds was prepared from pumpkin cotyledons at the middle stage of seed maturation. The cotyledons were homogenized in a solution of 20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA, and 0.3 M mannitol with an ice-chilledmortar and pestle, and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 3000 g for 20 min and the supernatant was centrifuged at 75,000g for 30 min. The supernatant was dialyzed to the 20 mM HEPES (pH 7.0), 150 mM NaCl. The buffer (20 mM HEPES (pH 7.0), 150 mM NaCl, 2% CHAPS, 5 mM $CaCl_2$) was added to the dialyzed supernatant. The supernatant was used as the crude extract in this study.

Transformation of plants

Arabidopsis thaliana (ecotype Columbia) was used throughout this work. Seeds of Arabidopsis were surface-sterilized and then sown on soil or onto 0.5% Gellan Gum (Wako, Tokyo, Japan) that contained Murasige-Skoog's medium and were grown at 22 °C under continuous light. We harvested young seedlings for the experiment of subcellular fractionation. The 2 to 3-week-old plants were homogenized with a SDS-buffer solution and subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies, anti-AtALEU antibody and anti-RD21 antibodies as described below.

Subcellular fractionation

The whole plants were homogenized in homogenizing buffer (20 mM HEPES, pH 7.0, 150mM NaCl, 1 mM $CaCl_2$, and 10 % sucrose). The supernatant of the extraction was collected by centrifugation at 500 g for 10 min, and gently suspended in the homogenizing buffer. The microsome fractions were collected as Ppt by centrifugation at 750, 000 g for 30 min, and gently suspended in CHAPS buffer (20 mM HEPES, pH

7.0, 150 mM NaCl, 1% CHAPS and 1 mM $CaCl_2$). Each extract was lysed by three bursts of sonication for 1 min at 10-min intervals on ice and was centrifuged at 750, 000 g for 30 min. The supernatant of each plant was used as the microsome fraction for this study. Each extract was subjected to SDS-PAGE and immunoblot analysis was done with each antibody specific to aleurain, RD21, and PV72.

Co-purification of proAtALEU with PV72-HDEL

The transgenic plants and wild plants were homogenized in the extraction buffer (20 mM HEPES-NaOH, pH 7.0, 150mM NaCl, 1 mM $CaCl_2$, and 10% (w/v) sucrose), and were filtered through cheesecloth. The filtrate was centrifuged at 8,000 g for 20 min. The supernatant was centrifuged at 100, 000 g for 1 hr to obtain the precipitate as the microsomal fraction. The fraction was gently suspended in CHAPS buffer (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 1% (w/v) CHAPS and 1 mM $CaCl_2$). Each extract was lysed by three bursts of sonication for 1 min at 10-min intervals on ice and was centrifuged at 100, 000 g for 30 min to obtain the soluble fraction of the microsomes.

We subjected the soluble fraction to the Ni-NTA resin (QIAGEN, Tokyo, Japan) and washed the column with the CHAPS buffer. The bound proteins were eluted with the CHAPS buffer containing 400 mM Imidazol. Each fraction was subjected to SDS-PAGE and then to immunoblot analysis with specific antibodies against either PV72 or aleurain.

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filtrate was centrifuged at 3000 g for 20 min and the supernatant was centrifuged at 75,000g for 30 min. The supernatant was dialyzed to the 20 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $CaCl_2$. The supernatant was used as the crude extract in this study.

Fluorescent Microscopy

The transformed Arabidopsis T-75 were inspected with a fluorescence microscope (Axiophot 2, Carl Zeiss, Jena, Germany) using a filter set (an excitation filter; BP-450-490, a dichroic mirror; FT510, a barrier filter; BP 515-565, Carl Zeiss), a CCD camera (CoolSNAP, RS Photomerics, Chiba, Japan), and a light source (Arc HBO 100W, Atto, Tokyo, Japan).

Results

Functional characterization of PV72 as a vacuolar sorting receptor

PV72 is localized on the membranes of PAC vesicles that accumulate a proprotein precursor of 2S albumin, a seed storage protein

Previously, Hara-Nishimura et al. found unique transport vesicles, precursor-accumulating (PAC) vesicles, in maturing pumpkin seeds (Fig. 1) (13). PV72 was identified from the PAC vesicles (Fig. 2). Electron microscopy of the maturing seeds revealed numerous electron-dense PAC vesicles within the cells, as indicated by arrowheads in Fig. 3. Isolation of the PAC vesicles showed that they accumulated proprotein precursors of storage proteins, pro2S albumin, proglobulin and PV100 (Fig. 4, lane 1). PAC vesicles mediate the transport of the storage protein precursors to protein storage vacuoles. The pure PAC vesicles contained PV72, a type I membrane protein with three EGF-like motifs, as the fourth major protein of the vesicles (Fig. 4, lanes 1 and 2). The result implied that PV72 functions as a sorting receptor of 2S albumin.

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Overexpression and purification of pro2S albumin and modified PV72s

To know the interaction between pro2S albumin and PV72, we expressed the chimeric protein, His-tagged pro2S albumin in *E. coli*. The expressed protein was purified with a chelating column. Each final preparation was highly pure as judged from SDS-PAGE analysis (Fig. 5).

To clarify the ligand-binding mechanism of PV72, we expressed four modified PV72s with a His-tag followed by the HDEL sequence in insect Sf21 cells employing a baculovirus expression system. Fig. 6A shows each construct of the modified proteins; rPV72 corresponds to the lumenal domain of PV72, rPV72Δ3 corresponds to the lumenal domain without the third EGF-like motif, rPV72Δ2,3 corresponds to the lumenal domain without the second and third EGF-like motifs, and rPV72Δ1,2,3 corresponds to the lumenal domain with no EGF-like motifs. These expressed proteins were purified with a chelating column and a gel filtration column. Each final preparation was highly pure as judged from SDS-PAGE analysis (Fig. 6B). All of their N-terminal amino acid sequences were determined to be RFVVEKNSLK that corresponds to the N-terminal sequence of authentic pumpkin PV72 as reported by Shimada et al. (1997). The results indicate that a signal peptide of the expressed proteins is correctly processed on the rER.

***In vitro* interaction between pro2S albumin and rPV72, PV72 lacking the EGF-like motifs still specifically binds to the internal propeptide of 2S albumin, a storage protein**

To investigate the binding ability of rPV72 to the pro2S albumin, we performed a binding assay on the affinity column (pro2S albumin column) that was conjugated with the His-tagged pro2S albumin. As shown in Fig. 7, rPV72 bound to the pro2S albumin column and then eluted with EDTA (discussed below). To investigate the binding ability

of rPV72 to the ligand peptides, we performed a binding assay on the affinity column (2S-I column) that was conjugated with the 2S-I peptide, the internal propeptide of pumpkin 2S albumin. As shown in Fig. 8A (upper), rPV72 bound to the 2S-I column and then eluted with EDTA (discussed below). Previously we reported that the isolated PV72 from the maturing seeds of pumpkin binds to the 2S-I peptide but not to the mutant peptide 2S-I/3G with GGG instead of RRE of the internal propeptide. To clarify the specificity of the binding of rPV72, we used another affinity column (2S-I/3G column) that was conjugated with the 2S-I/3G peptide. As shown in Fig. 8A (lower), rPV72 did not bind to the 2S-I/3G column. The results indicate that the characteristics of rPV72 with respect to ligand binding were the same as those of the authentic PV72 has.

To identify the ligand-binding region of PV72, we performed a surface plasmon resonance analysis for four modified PV72s with either the 2S-I sensor chip or 2S-I/3G sensor chip. Each modified PV72 of the same concentration was injected onto the sensor chips. rPV72 bound to the 2S-I peptide, but not to the 2S-I/3G peptide (Fig. 8B), as expected from the result in Fig. 8A. All of the deleted proteins, rPV72Δ3, rPV72Δ 2,3 and rPV72Δ1,2,3, also bound to the 2S-I peptide, but not to the 2S-I/3G peptide (Fig. 8B). This demonstrates that the deleted proteins also specifically recognize the RRE sequence of the 2S-I peptide as rPV72 does. These results indicated the N-terminal region of PV72 corresponding to rPV72Δ1,2,3 includes a ligand-binding site.

The EGF-like motifs modulate the association and dissociation of PV72 with the ligand

We determined the kinetic parameters for the binding of each modified rPV72 to the 2S-I peptide by surface plasmon resonance. Each modified rPV72 was injected onto the 2S-I sensor chip to start the association reaction. Fig. 9A shows the association and dissociation curves obtained from the respective experiment with four different

concentrations (0.07-2 μM) of each protein. The sensorgrams of rPV72Δ3, rPV72Δ2,3 and rPV72Δ1,2,3 exhibited more rapid association followed by more rapid dissociation after the injection was completed than did the sensorgram of rPV72.

The kinetic constants of association and dissociation were calculated from the slopes of the curves, as shown in Fig. 9B. In contrast to small differences of the k_a values among the modified rPV72, large differences of the k_d values were observed. The k_d value of each of rPV72Δ3, rPV72Δ2,3 and rPV72Δ1,2,3 was 16- to 23-fold higher than the k_d value of rPV72. The apparent equilibrium dissociation constant was determined from the ratio of these two kinetic constants (k_d / k_a). rPV72 has a high enough affinity for the 2S-I peptide (K_D = 0.2 μM) to function as a receptor. The K_D values of each of rPV72Δ3, rPV72Δ2,3 and rPV72Δ1,2,3 were 10-, 19- and 21-fold higher than the K_D value of rPV72, respectively. Therefore, the affinity of rPV72 for the ligand peptide is much higher than the affinities of the rPV72s lacking the EGF-like motifs. It seems likely that the EGF-like motifs play a role in stabilizing the receptor-ligand complex.

The EGF-like motifs modulate a Ca^{2+}-dependent conformational change of PV72

The question is how the EGF-like motifs regulate the stability of the ligand binding of PV72. Previously, we found that the third EGF-like motif has a consensus sequence for Ca^{2+}-binding, while the first and second motifs do not have such a sequence (15). Fig. 8A shows that rPV72 was eluted from the 2S-I affinity column by the addition of EDTA. This implied that Ca^{2+}-binding to the third EGF-like motif might be important for the ligand binding. To clarify the requirement of Ca^{2+}, we performed an analysis of surface plasmon resonance with rPV72 in the presence of either Ca^{2+} or Mg^{2+}. Fig. 10A (left) shows that the interaction between rPV72 and the ligand was observed in the presence of Ca^{2+}, but not in the presence of Mg^{2+} instead of Ca^{2+}. This result indicates that Ca^{2+} is required for PV72 to interact with the 2S-I peptide.

Unexpectedly, however, rPV72Δ1,2,3, which lacks the EGF-like motifs, also showed a Ca^{2+}-dependent interaction with the 2S-I peptide, but not a Mg^{2+}-dependent interaction (Fig. 10A, right), as rPV72 did. This result indicates that the N-terminal region corresponding to rPV72Δ1,2,3 has another Ca^{2+}-binding site (s), although no consensus sequence for Ca^{2+} binding was found in the region. It should be noted that the affinity of PV72 (K_D = 0.2 μM) was 20-fold stronger than that of rPV72Δ1,2,3 (K_D = 4.2 μM). Thus, the Ca^{2+}-binding to the EGF-like motif must be required for the high affinity of PV72 for the ligand. The next question raised is whether the Ca^{2+}-binding causes a conformational change of PV72 that results in the higher affinity. To answer the question, we measured Ca^{2+}-dependent changes in the fluorescence emission spectra of Tyr or Trp residues in both rPV72 and rPV72Δ1,2,3. The rPV72 polypeptide includes 23 Tyr residues and 11 Trp residues. The fluorescence of these residues was monitored in the presence or absence of Ca^{2+}. Fig. 10B show a remarkable Ca^{2+}-dependent change in the fluorescence emission spectra of rPV72, but only a little change in the spectra of rPV72Δ1,2,3. The result suggests that the EGF-like motifs induced a much larger conformational change of PV72 in a Ca^{2+}-dependent manner than the N-terminal region corresponding to rPV72Δ1,2,3 did.

The Ca^{2+} concentration rather than pH regulate the ligand binding of PV72

The next issue to be determined was a critical concentration of Ca^{2+} for association and dissociation of PV72. We performed a binding assay under the conditions of various Ca^{2+} concentrations by surface plasmon resonance. The EC_{50} value of the Ca^{2+}-dependent interaction was determined to be 40 μM (Fig. 11A). We also performed another binding assay by affinity chromatography with the 2S-I column. rPV72 that had bound to the 2S-I column was exposed to decreasing concentrations of $CaCl_2$ from 500, 100, 50, 20 and 0 μM. The rPV72 was eluted from the column under $CaCl_2$ concentrations lower than 50 μM (Fig. 11B). The $CaCl_2$ concentration was consistent

with the EC_{50} value determined by the surface plasmon resonance. The results suggested that the interaction of PV72 to the ligand might be regulated by the Ca^{2+} concentration in the respective compartment of the maturing seed cells.

In general, the binding of receptors to their ligands are known to be modulated by the environmental pH. This raised a question whether the interaction of PV72 with the ligand is also regulated by pH. To answer this question, we performed an assay with the rPV72 and rPV72Δ1,2,3 that had bound to the 2S-I column in the presence of 1 mM $CaCl_2$. Fig. 12A (upper) shows that the rPV72 that bound at pH 7.0 was not eluted by decreasing the $CaCl_2$ concentration to 50 μM nor by decreasing the pH to 5.5, but was eluted with an EGTA solution. In contrast, the rPV72Δ1,2,3 bound to the column was easily eluted with the neutral buffer (pH 7.0) containing 50 μM $CaCl_2$ (Fig. 12A, lower). Alternatively, we performed another assay with the rPV72 bound to the 2S-I column at pH 5.5 in the presence of 1 mM $CaCl_2$. The result was similar as shown in Fig. 12A. The bound rPV72 was not eluted at pH 5.5 in the presence of 50 μM $CaCl_2$ (Fig. 12B, upper), while the bound rPV72Δ1,2,3 was easily eluted with the acidic buffer (pH 5.5) containing 50 μM $CaCl_2$ (Fig. 12B, lower).

The results indicated that the pH change did not affect the interaction between rPV72 and the ligand in the presence of 50 μM $CaCl_2$. When a more acidic buffer (pH 4.0) was used instead of the pH 5.5 buffer, an elution profile similar to that in Fig. 12A was obtained (data not shown). The presence of 50 μM $CaCl_2$ made the complex of rPV72 and the ligand stable under acidic conditions. The overall results suggested that the EGF-like motifs might be involved in the stability of the complex in the presence of 50 μM $CaCl_2$. Thus, it appears that the association and dissociation of PV72 with the ligand was modulated by the Ca^{2+} concentration rather than by the pH.

To clarify the effect of the 2S-I peptide sequence on the binding, we prepared affinity columns conjugated with three mutant peptides; 2S-I/P75G with Gly instead of Pro-75, 2S-I/E73G with Gly instead of Glu-73 and 2S-I/E79G with Gly instead of Glu-

79. At neutral pH (pH 7.0), both rPV72 and rPV72Δ1,2,3 bound to all the columns with the mutant peptides in the presence of 1 mM $CaCl_2$, and then eluted with the EDTA solution (Fig. 13, right). Even at pH 4.0, both rPV72 and rPV72Δ1,2,3 bound to the 2S-I column and the 2S-I/P75G column, but they did not bind to either the 2S-I/E73G column or 2S-I/E79G column (Fig. 13, left). The results indicate that the PV72 has an ability to bind to the 2S-I peptide not only at pH 7.0 but also at pH 4.0, in the presence of Ca^{2+}. When Glu-73 or Glu-79 of the 2S-I peptide was substituted by Gly, the affinity of either rPV72 or rPV72Δ1,2,3 for the mutant peptide was reduced at pH 4.0, suggesting that both Glu-73 and Glu-79 are necessary for the binding of PV72 at acidic pH. PV72 might interact with pro2S albumin through the two Glu residues in Ca^{2+}-dependent manner.

Effects of NaCl and detergents on the interaction between PV72 and the 2S-I peptide

To know the effect of NaCl and detergents on the interaction between PV72 and the 2S-I peptide, we performed a binding assay under the conditions of various NaCl concentrations by surface plasmon resonance. As shown in Fig. 14A, the interaction in the 0 mM was reduced more than that in the NaCl. This results indicated that the ionic interaction is involved in the interaction between PV72 and the 2S- I peptide. Next, we investigated that the detergent affected the interaction. As shown in Fig. 14B, the interaction in the 0 mM was reduced more than that in the detergent. These results indicated that the hydrophobic interaction is involved in the interaction between PV72 and the 2S-I peptide.

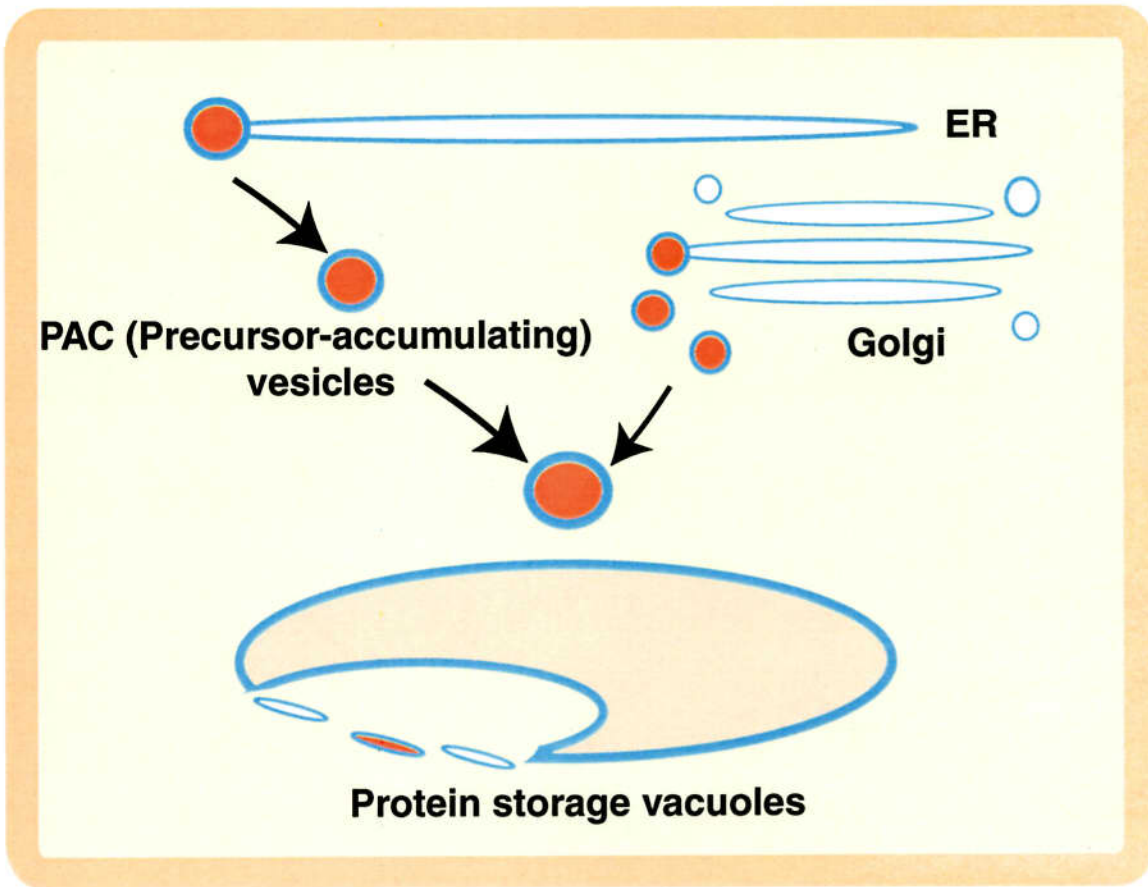


Fig. 1 Intracellular transport of seed storage proteins to protein storage vacuoles.

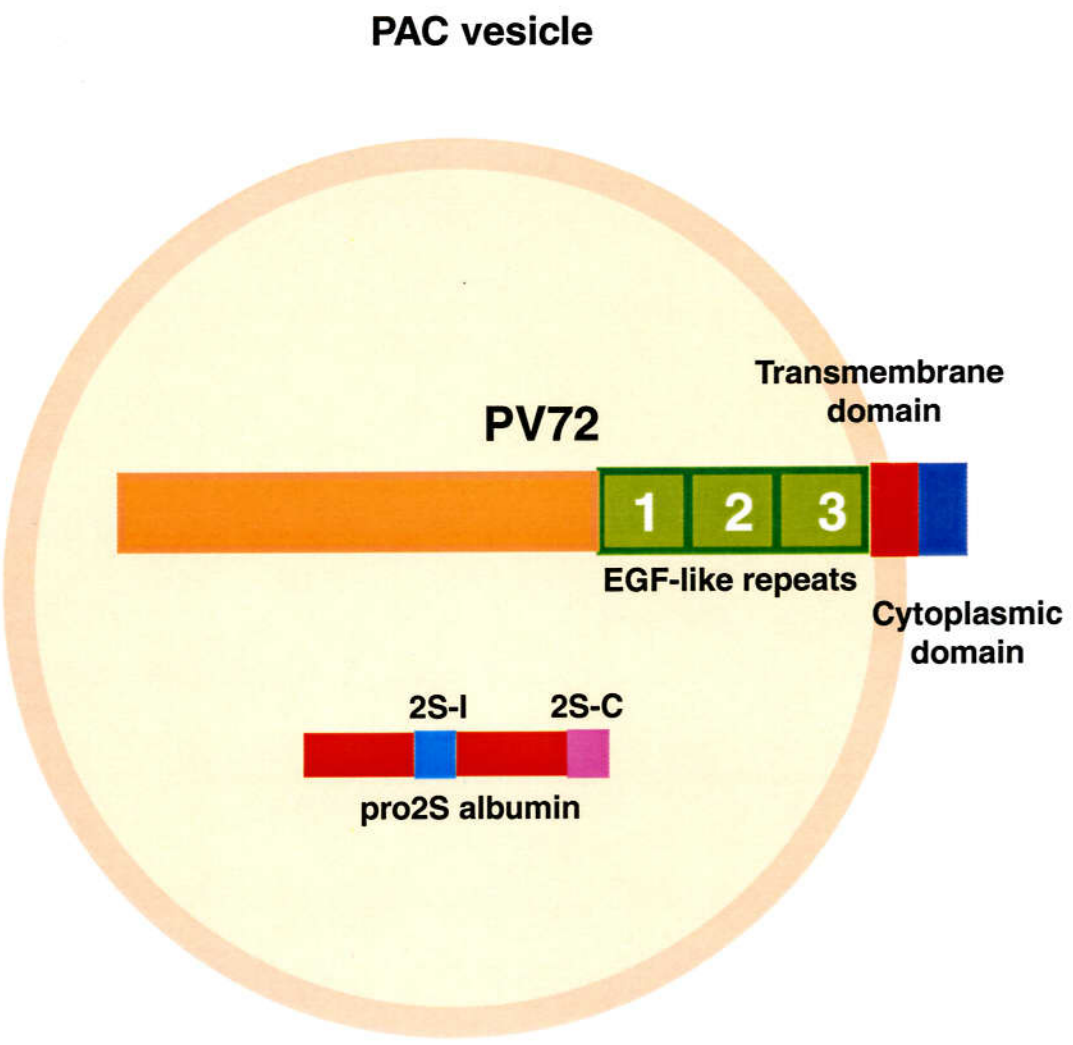


Fig. 2 A domain structure of PV72 on the membrane of the PAC vesicles.

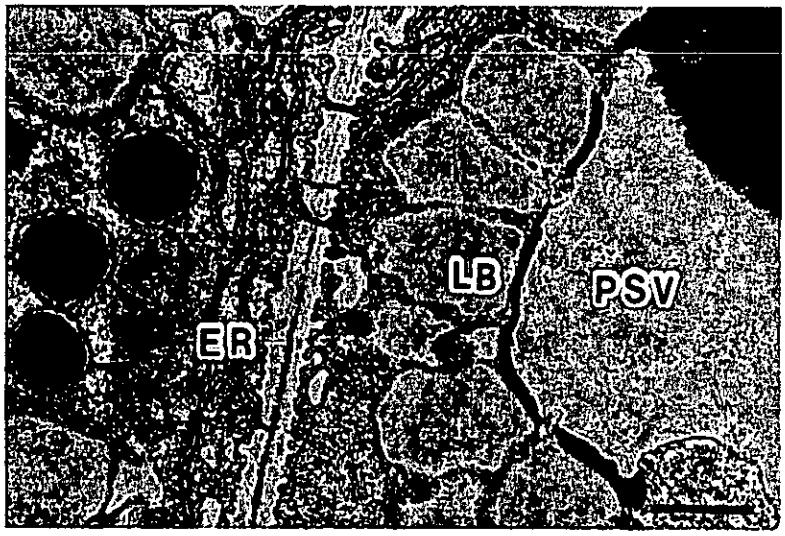


Fig. 3 An electron micrograph of a pumpkin cotyledon at the middle stage of seed maturation. Numerous PAC (precursor-accumulating) vesicles (arrowheads) are visible in the cells. PSV, protein storage vacuole; ER, endoplasmic reticulum; LB, lipid body. Bar = 500 nm.

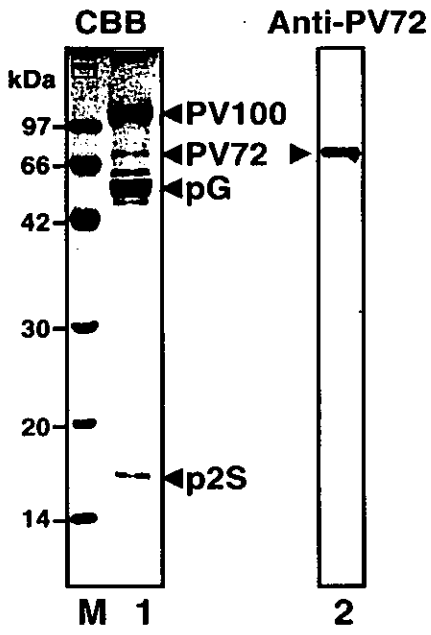


Fig. 4 Localization of PV72 in PAC vesicles that accumulate a proprotein precursor of 2S albumin, a seed storage protein. Isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie blue (lane 1) or immunoblot analysis with anti-PV72 antibodies (lane 2). p2S, pro2S albumin; pG, proglobulin; PV100, a precursor of a protease inhibitor, cytotoxic proteins and 7S globulin. Lane M contains molecular mass markers. The molecular mass of each marker protein is given on the left in kDa.

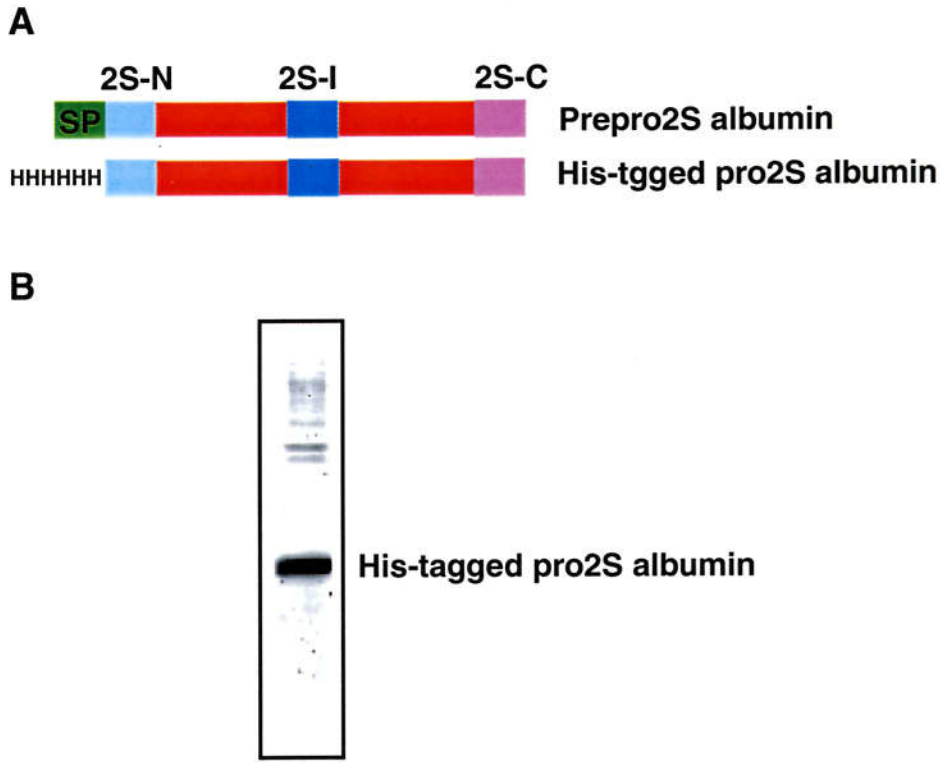


Fig. 5 Expression and purification of a His-tagged pro2S albumin of pumpkin. (A) Schematic representation of prepro2S albumin and His-pro2S albumin of pumpkin. Prepro2S albumin is composed with signal peptide (SP, green box), N-terminal propeptide (2S-N, light blue box), Internal propeptide (2S-C, blue box), the C-terminal region (2S-C, pink box) and mature region (red box). His-pro2S albumin is composed of a His-tag followed by pro2S albumin. (B) The purified His-pro2S albumin that was expressed in *E. coli* were purified with a chelating column. The purified protein was subjected to SDS-PAGE with Coomassie blue staining.

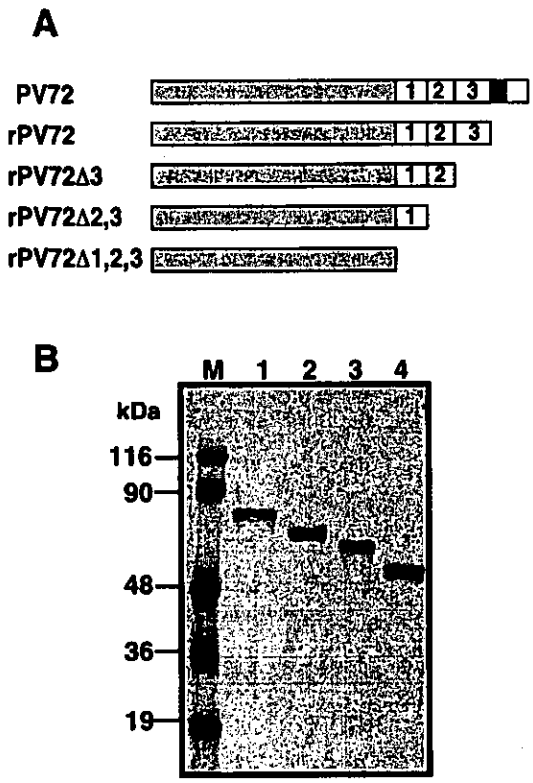


Fig. 6 Constructs and the expressed proteins of four modified PV72s in insect cells. (A) PV72 is a type I integral membrane protein with three EGF-like motifs at the C terminus of the lumenal luminal domain. The N-terminal domain is indicated by a gray box, and each EGF-like motif is indicated by an open box (1, 2 and 3, respectively). The transmembrane domain is indicated by a closed box and the cytoplasmic domain is indicated by an open box. rPV72 was composed of the luminal domain followed by a His-tag and the HDEL sequence. rPV72Δ3, rPV72Δ2,3 and rPV72Δ1,2,3 were composed of rPV72 without the third EGF-like motif, without the second and third EGF-like motifs, and without all three of the EGF-like motifs, respectively. (B) The four modified PV72s that were expressed in insect Sf21 cells were purified with a chelating column and a gel filtration column. Each purified protein was subjected to SDS-PAGE with Coomassie blue staining; rPV72 (lane 1), rPV72Δ3 (lane 2), rPV72Δ2,3 (lane 3) and rPV72Δ1,2,3 (lane 4). The molecular mass of each marker protein (lane M) is given on the left in kDa.

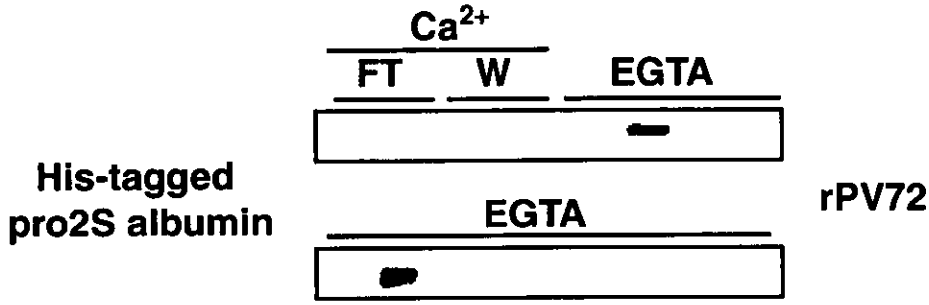


Fig. 7 The luminal domain of PV72 had an ability to bind to pro2S albumin. rPV72 was subjected to an affinity column with His-pro2S albumin in the presence of Ca^{2+} (upper) or EGTA (lower). Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow-through fraction; W, washing fraction with the HEPES buffer (pH 7.0) with 1 mM $CaCl_2$; EGTA, eluted fraction by 2.5 mM EGTA.

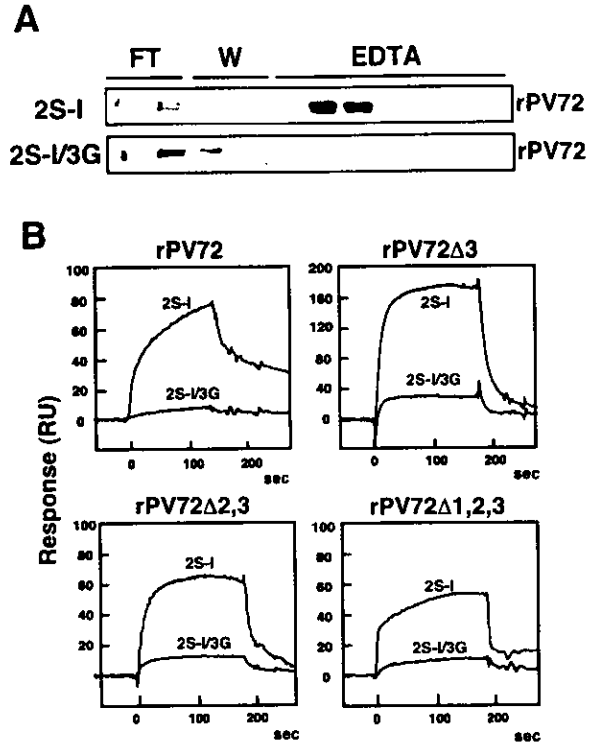
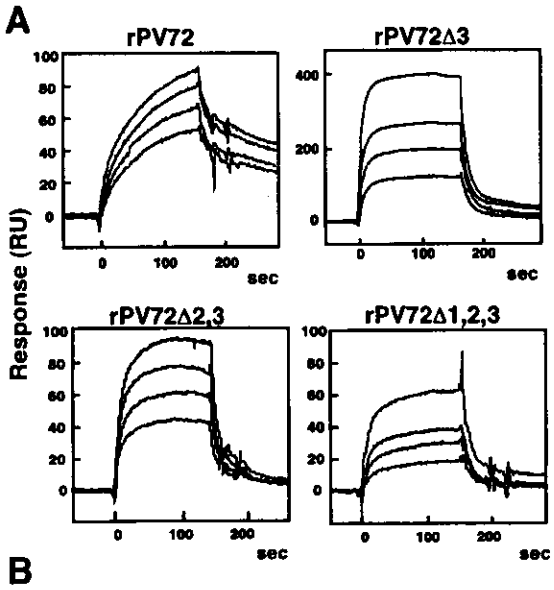


Fig. 8 Modified PV72s showed a specific binding to the 2S-I peptide derived from the internal propeptide of a storage protein, 2S albumin. (A) rPV72 was subjected to an affinity column with either the 2S-I peptide or the mutant peptide with a replacement of RRE by GGG (2S-I/3G). Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow-through fraction; W, washing fraction with the HEPES buffer (pH 7.0) with 1 mM $CaCl_2$; EDTA, eluted fraction by 2.5 mM EDTA. (B) Four modified PV72s were injected onto the sensor chip coupled with either the 2S-I peptide or the 2S-I/3G peptide to obtain the sensorgrams by surface plasmon resonance. The protein concentrations used were 0.7 μM for both rPV72 and rPV72Δ3, 1.6 μM for rPV72Δ2,3 and 1.4 μM for rPV72Δ1,2,3.

A



B

	k_a (per Ms)	k_d (per s)	K_D
rPV72	1.31×10^{4}	2.48×10^{-3}	0.2 μM
rPV72Δ3	2.43×10^{4}	4.95×10^{-2}	2.0 μM
rPV72Δ1,2	1.56×10^{4}	5.89×10^{-2}	3.8 μM
rPV72Δ1,2,3	9.26×10^{3}	3.89×10^{-2}	4.2 μM

Fig. 9 Kinetics for the association and dissociation of the modified PV72s and the 2S-I peptide. (A) The surface plasmon resonance profiles for the association and dissociation curves of the modified PV72s and the 2S-I peptide. The coupling efficiency of the 2S-I peptide to the sensor surface was 1200 RU. Each of the modified PV72s was injected onto the 2S-I sensor chip at different concentrations from 0.07 μM to 1.5 μM. (B) The kinetic constants, an association rate constant (k_a), a dissociation rate constant (k_d) and a dissociation constant ($K_D = k_d / k_a$), were calculated from the above sensorgrams using BIA evaluation software version 2.1. These kinetic parameters were determined from three independent experiments.

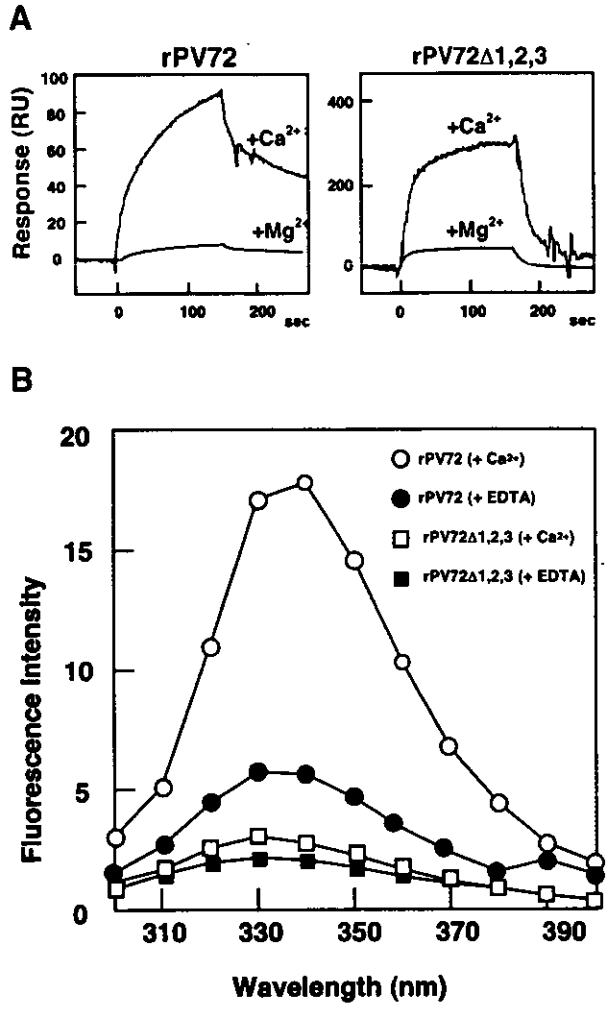


Fig. 10 Ca^{2+}-dependent ligand-binding of PV72 and Ca^{2+}-induced conformational change in PV72. (A) Divalent cation selectivity for the ligand binding. Either rPV72 or rPV72Δ1,2,3 was injected onto the 2S-I sensor chip in the presence of 1 mM $CaCl_2$ or 1 mM $MgCl_2$. (B) Fluorescence emission spectra showing the Ca^{2+}-induced structural change. The fluorescence emission of Trp and Tyr residues in rPV72 and rPV72Δ1,2,3 was measured from 300 to 400 nm after excitation at 280 nm. rPV72 (1 μg/ml) was suspended in the HEPES buffer plus 1 mM $CaCl_2$ (open circles) and the HEPES buffer plus 1 mM EDTA (closed circles). rPV72Δ1,2,3 (1 μg/ml) was in the buffer plus 1 mM $CaCl_2$ (open squares) and the HEPES buffer plus 1 mM EDTA (closed squares).

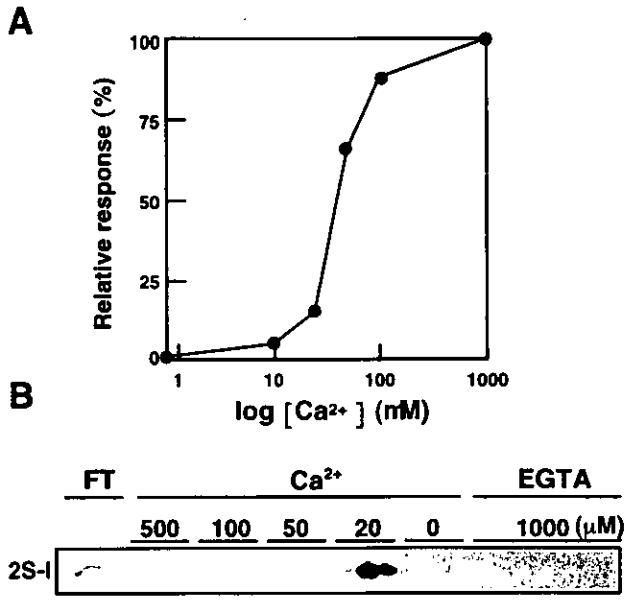


Fig. 11 Regulation of the ligand binding of PV72 by the Ca^{2+} concentration. (A) rPV72 was injected onto the 2S-I sensor chip in the presence of various concentrations of $CaCl_2$. Relative responses were plotted against the concentration of Ca^{2+}. EC_{50} value for Ca^{2+} calculated was 40 μM. (B) rPV72 was subjected to the 2S-I affinity column and eluted by the HEPES buffer containing $CaCl_2$ of various concentrations from 500 μM to 0 μM and finally by 1 mM EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow-through fraction.

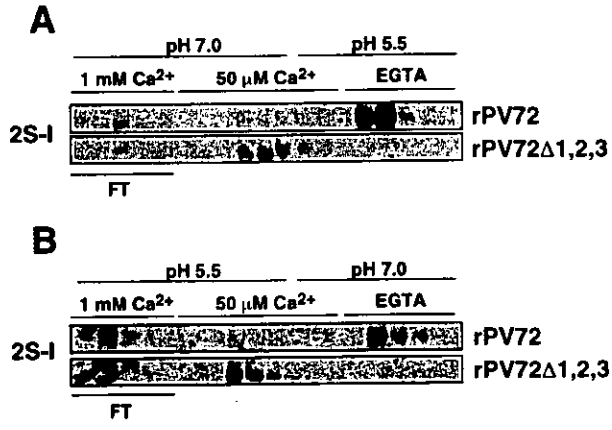


Fig. 12 Modulation of Ca^{2+}-dependent ligand binding of rPV72 by the EGF-like motifs. (A) Either rPV72 or rPV72Δ1,2,3 bound to the 2S-I column in the HEPES buffer (pH 7.0) with 1 mM $CaCl_2$. The elution pattern of each protein was examined with the sequential solutions; the HEPES buffer (pH 7.0) with 50 μM $CaCl_2$, the MES buffer (pH 5.5) with 50 μM $CaCl_2$ and the MES buffer with EGTA. (B) Either rPV72 or rPV72Δ1,2,3 bound to the column in the MES buffer with 1 mM $CaCl_2$. The elution pattern was examined with sequential solutions; the MES buffer with 50 μM $CaCl_2$, the HEPES buffer with 50 μM $CaCl_2$ and the HEPES buffer with EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow-through fraction.

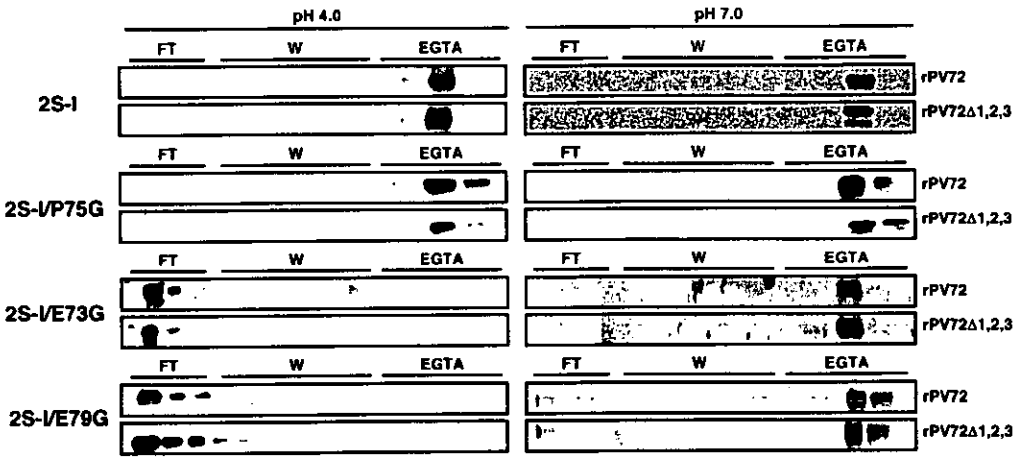


Fig. 13 Effect of pH on the binding of rPV72s to the 2S-I peptide and the mutant peptides. The affinity column conjugated with each of the 2S-I peptide, 2S-I/P75G, 2S-I/E73G or 2S-I/E79G was used, as shown on the left. Either rPV72 or rPV72Δ1,2,3 was applied to the column under neutral conditions (pH 7.0, right panel) or under acidic conditions (pH 4.0, left panel). The column was washed with the respective buffer and finally with the buffer containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow-through fraction; W, washing fraction.

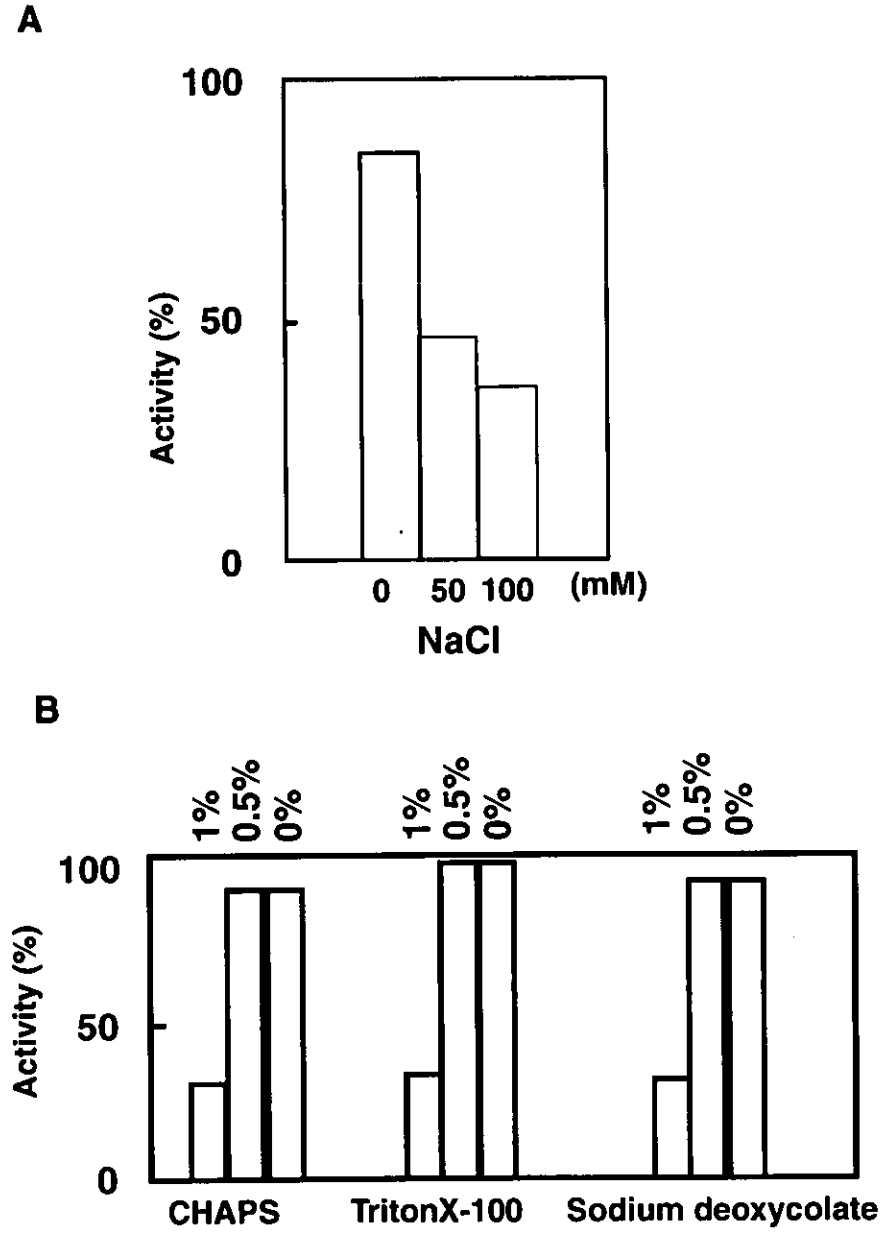


Fig. 14 Effects of NaCl and detergents on the binding of rPV72 to the 2S-I peptide. (A) rPV72 was injected onto the 2S-I sensor chip in the presence of various concentrations of NaCl (0 mM, 50 mM, 100 mM). Relative responses were plotted against the concentration of NaCl. The interaction between rPV72 and the 2S-I peptide was deduced due to increasing concentration of the NaCl. (B) rPV72 was injected onto the 2S-I sensor chip in the presence of various concentrations of CHAPS, TritonX-100, or sodium deoxycolate (1%, 0.5%, 0%). Relative responses were plotted against the concentration of each detergent. The interaction between rPV72 and the 2S-I peptide was reduced by increasing concentration of each detergent.

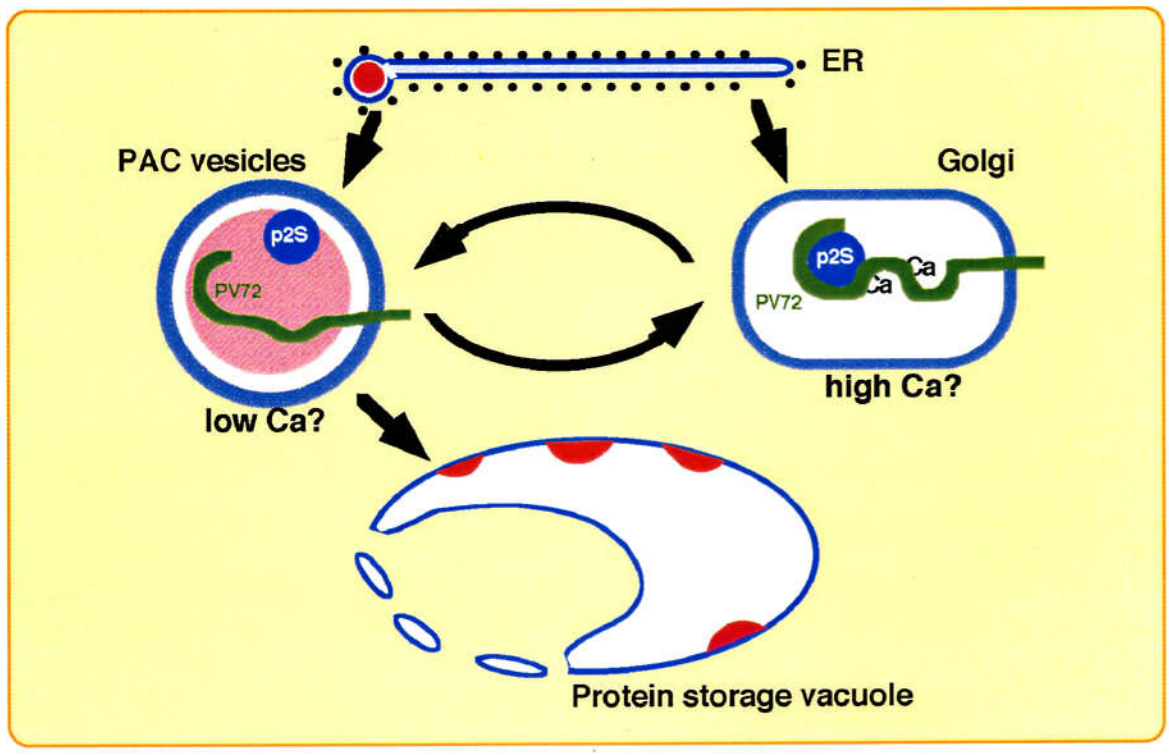


Fig. 15 A schematic model of receptor-mediated transport of a storage protein to protein storage vacuoles.

Organ-specific expression of PV72 and PV82 in pumpkin.

Previously, we identified PV72 and PV82, putative sorting receptors, from maturing pumpkin cotyledons. To elucidate the organ specific expression of receptors, the amounts of these proteins in various organs of pumpkin plants were analyzed. Total proteins were extracted from roots, hypocotyles, leafs, germinating cotyledons and maturing cotyledons. The extracts were subjected to SDS-PAGE followed by immunoblot analysis with anti-PV72 antibodies or anti-PV82 antibodies. Fig. 16 shows that a single major band was detected only on the immunoblot of maturing cotyledons with anti-PV72 antibodies. A very faint band found on the blot of leaves might correspond to PV82, judging from its migration. Fig. 16 shows the result of an immunoblot with anti-PV82 antibodies. A major band in maturing cotyledons, hypocotyls, leaves and germinating cotyledons correspond to PV82. Two bands were detected on the blot of maturing cotyledons. In summary, PV72 was found only in maturing cotyledons, whereas PV82 was present in various organs.

Differential characterization of PV72 and PV82

To compare the biochemical characterization between PV72 and PV82, we did the binding assay with various columns. The pumpkin crude extract was subjected to each column (the AtPAP, SPO and 2S-I column) and finally eluted with the EGTA buffer (Fig. 17). Both of PV72 and PV82 bound to each peptide in a Ca^{2+}-dependent manner. PV72 and PV82 did not bind to the SPO-NPGR peptide (Fig. 17).

To determine the EC_{50} value of PV82, the crude extract was applied to the AtPAP affinity column with the HEPES buffer plus 5 mM $CaCl_2$ and then washed with the HEPES buffer containing decreasing concentration of $CaCl_2$ (5000 μM, 400 μM). Finally, bound proteins were eluted with EGTA. PV82 was eluted with 400 μM $CaCl_2$ (Fig. 18). The EC_{50} value of PV82 was calculated to 400 μM. The value was 10-fold larger than that of rPV72. This result suggests that the Ca^{2+}-sensitivity of PV82 is

different from that of PV72.

Ca^{2+} -dependent binding of AtELP to the AtPAP peptide

rAtELP (Fig. 19A)was expressed in the insect cells. The culture medium was subjected to the Ni-column and eluted with the buffer containing imidazol. The eluted fractions was dialyzed to the HEPES buffer and subjected to the PAP column. The bound fractions were eluted with the sodium acetate buffer. The eluted fractions was dialyzed to the HEPES buffer (0 mM $CaCl_2$) and dried. These results indicated that rAtELP bound to the AtPAP peptide in a pH-dependent manner.

To know the requirement of Ca^{2+} for the binding of rAtELP to the AtPAP peptide, we did the binding assay using the AtPAP peptide. rAtELP was subjected to AtPAP column, washed with same buffer followed by the sodium acetate buffer and finally washed with the buffer containing EGTA. rAtELP was eluted with EGTA (Fig. 19B). These results indicated that rAtELP required to the binding to the AtPAP peptide.

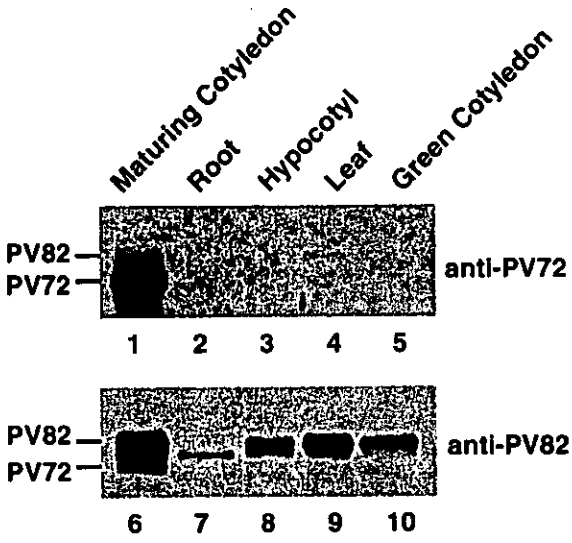


Fig. 16 Organ-specific expression of PV72 and PV82 in pumpkin. Total proteins were extracted from various pumpkin organs, maturing cotyledons (lanes 1 and 6), roots (lanes 2and 7), hypocotyls (lanes 3 and 8), leaves (lanes 4 and 9) and green cotyledons (lanes 5 and 10). Each extract (20 μg protein) was subjected to SDS-PAGE followed by immunoblots with anti-PV72 antibodies (upper) or anti-PV82 antibodies (lower).

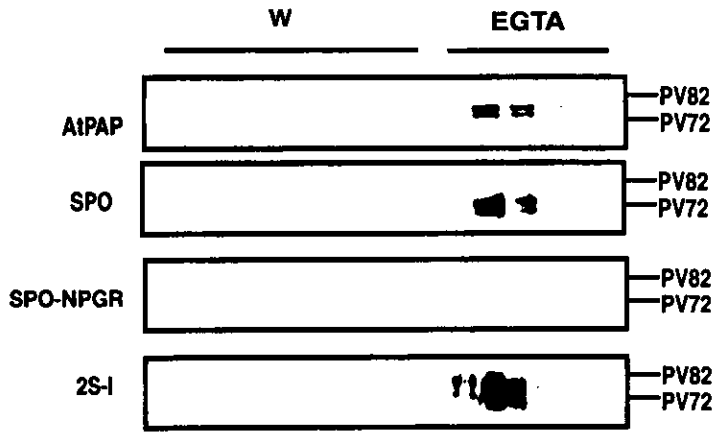


Fig. 17 Ca^{2+}-dependent binding both of PV72 and PV82 to various peptides.
The crude extract from maturing pumpkin seeds were subjected to each affinity column with the AtPAP peptide, the SPO peptide, the SPO-NPGR mutant peptide with a replacement of I by G (SPO-NPGR) and the 2S-I peptide. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 and PV82-antibodies.
W, washing fraction with the HEPES buffer containing 1 mM $CaCl_2$;
EGTA, eluted fraction by 2.5 EGTA.

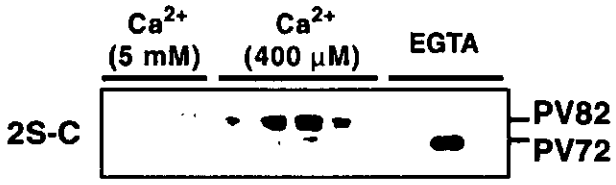
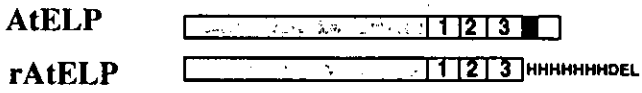


Fig. 18 Differential characterization between PV72 and PV82. Both PV82 and PV72 were bound to the affinity column conjugated with the C-terminal peptide of pro2S albumin (2S-C) in the presence of 5 mM $CaCl_2$. PV82 was eluted from the column with a solution of 400 μM $CaCl_2$, but not PV72. PV72 was eluted with a solution of 1 mM EGTA. Each fraction of the affinity chromatography was subjected to SDS-PAGE followed by an immunoblot with the mixture of anti-PV72 antibodies and anti-PV82 antibodies.

A



B

Fig. 19 Ca^{2+}-mediated interaction between rAtELP and the AtPAP peptide. (A) AtELP is a type I integral membrane protein with three EGF-like motifs at the C terminus of the lumenal domain. The N-terminal domain is indicated by a gray box, and each EGF-like motif is indicated by an open box (1, 2 and 3, respectively). The transmembrane domain is indicated by a closed box and the cytoplasmic domain is indicated by an open box. rAtELP was composed of the lumenal domain followed by a His-tag. (B) rAtELP was applied to an AtPAP affinity column. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. FT, flow through fraction; W, washing fraction with the HEPES buffer (pH 7.0) with 1 mM $CaCl_2$; EGTA, eluted fraction by 2.5 mM EGTA.

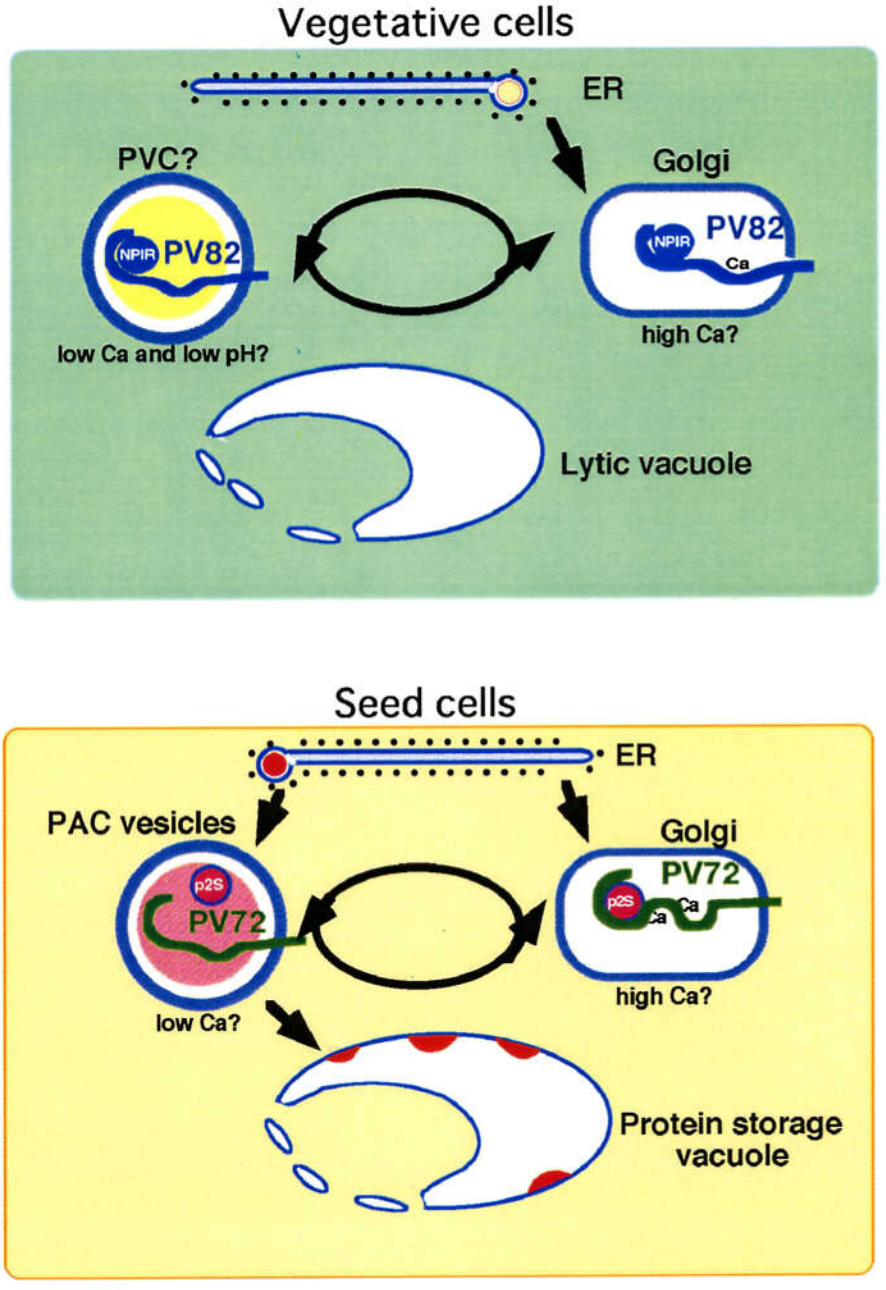


Fig. 20 A schematic model of receptor-mediated transport of a vacuolar protein to each type of vacuole in higher plants.

Demonstration of receptor-dependent transport of AtALEU in Arabidopsis

Overexpression of ER-localized PV72-HDEL results in the Accumulation of a proprotein precursor of AtALEU in the transgenic Arabidopsis plants

BP-80 and AtELP have been reported to be a putative vacuolar sorting receptor responsible for delivery of the NPIR-containing vacuolar proteins to lytic vacuoles. To demonstrate their function *in vivo*, we performed overexpression of the homolog, PV72, in Arabidopsis plants. We introduced a chimeric gene encoding a fusion protein (PV72-HDEL) composed of a signal peptide and the lumenal domain of PV72 followed by a His-tag and an ER retention signal, HDEL, into the Arabidopsis plants under the control of the cauliflower mosaic virus 35S promoter. The transgenic plants accumulated PV72-HDEL in the ER as shown in Fig. 21A (left). If such putative receptors interact with some vacuolar protein synthesized on the ER, the transport to the vacuoles should be affected by the accumulation of the modified receptor molecules in the ER. Arabidopsis has two types of vacuolar cysteine proteases of the papain family in the lytic vacuoles; AtALEU that is a homolog of barley aleurain and RD21 that is a product of responsive-to-desiccation gene 21 (Koizumi et al. 1993); Yamada]. The vacuolar targeting signal, NPIR, is found in the NTPP of AtALEU (Lohman, et al., 1994), but not in that of RD21. This raises the question of whether the transport of these vacuolar proteins is affected by overexpression of the soluble PV72-HDEL molecules in the ER. To answer it, we investigated the transport of the two proteases in the transgenic plants.

We found a specific accumulation of the 41-kD proprotein precursor of AtALEU in the transgenic plants expressing PV72-HDEL (Fig. 21B). On the other hand, wild plants accumulated only the 28-kD mature form of AtALEU, but not the precursor. The molecular masses of 41-kD and 28-kD was larger than the deduced molecular mass from the amino acid sequence of the AtALEU precursor and the mature form,

respectively. N-linked glycosylation at two sites might account for the size discrepancy. On the contrary, a precursor of RD21, which has not the vacuolar-targeting signal NPIR was not detected in both the transgenic and wild plants (Fig. 21C).

Co-purification of PV72-HDEL with the NPIR-containing AtALEU precursor, but not with the mature AtALEU

To clarify the function of the complex of the His-tagged PV72-HDEL and the AtALEU precursor in the microsomal fractions from the transgenic Arabidopsis plants (T/PV72), we performed an affinity chromatography withs the Ni-NTA resin column. The His-tagged PV72-HDEL bound to the Ni-NTA column and was not detect in the flow through (FT) fraction. His-tagged PV72-HDEL was eluted in the CHAPS buffer containing 400 _M imidazol and detected in the eluted (E) fraction (Fig. 22A).

To confirm formation of the complex of PV72-HDEL and the AtALEU precursor, each fraction was subjected to an immunoblot analysis with anti-aleurain monoclonal antibody. The mature form of 28-kD AtALEU (m) and the intermediate form of 38-kD AtALEU (i) were detected in the FT fraction of transgenic plants (Fig. 23B). The 41-kD AtALEU precursor (p) was specifically detected in the E fraction of transgenic plants. On the contrary, mature form of RD21 was not co-purified with PV72-HDEL (data not shown). These results suggest that the AtALEU precursor was recognized by a PV72-like vacuolar sorting receptor *in vivo* to be transported to the vacuoles.

The propeptide of AtALEU at the N terminus as a vacuolar targeting signal

The NTPP of AtALEU contains an NPIR sequence as known a vacuolar targeting signal (Fig. 23A, upper). To confirm the function of the NTPP of AtALEU as a vacuolar targeting signal, we constructed a chimeric gene encoding a signal peptide, the NTPP of AtALEU (T-75). To know the localization of AtALEU-GFP, the transformed cells were inspected with a laser-scanning confocal microscope. AtALEU-GFP was accumulated in

the large compartments as well as lytic vacuoles at 3 day after transformation (Fig. 23B). This result indicated that NTPP of AtALEU actually functions as a vacuolar targeting signal for the lytic enzyme.

Kinetic analysis of the interaction between the modified PV72s and the NTPP of AtALEU

To determine the kinetic parameters for the binding of the receptor and the ligand, we expressed two modified PV72s; rPV72 composed of the lumen domain followed by a His-tag and the HDEL sequence and rPV72Δ1,2,3 composed of rPV72 with no EGF-like motif in insect Sf21 cells employing a baculovirus expression system (Watanabe, et al., 2002). For surface plasmon resonance, each modified PV72s was injected onto the AtALEU sensor chip to start the association reaction. Fig. 24A shows the association and dissociation curves obtained from the respective experiment with four different concentrations (0.15-0.7 μM) of each protein. The sensorgrams of rPV72Δ1,2,3 exhibited more rapid association followed by more rapid dissociation after the injection was completed than did the sensorgram of rPV72. The kinetic constants of association and dissociation were calculated from the slopes of the curves, as shown in Fig. 24B. The apparent equilibrium dissociation constant was determined from the ratio of these two kinetic constants (k_d / k_a). The K_D value of rPV72 for the AtALEU peptide was 0.1 μM and enough high as the vacuolar sorting receptor. The K_D value of rPV72Δ1,2,3 was 10-fold higher than that of rPV72. Previously we demonstrated that the affinity of rPV72 for the ligand peptide is much higher than the affinities of the rPV72s lacking the EGF-like motifs. It seems likely that the EGF-like motifs play a role in stabilizing the vacuolar sorting receptor -ligand complex.

Modified PV72s interact with the NPIR-containing peptide (AtPAP peptide) in a Ca^{2+}-dependent manner

Previously, we found that the the interaction of PV72 with the internal propeptide of 2S albumin was recognized by Ca^{2+} concentration rather than pH (Watanabe, et al., 2002). To clarify a Ca^{2+} -dependent interaction between rPV72 and the AtPAP peptide, we performed binding assay with an affinity column conjugated with the NTPP of AtALEU peptide in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. Modified PV72s bound to the column and was detected in neither the FT nor W fractions (Fig. 25A). Modified PV72s was eluted with the HEPES buffer (pH 7.0) containing 2.5 mM EGTA and was detected in the E fraction (Fig. 25A). These results indicated that modified PV72s interacted with the AtPAP peptide in a Ca^{2+} -dependent manner as in the case of the 2S-I peptide.

The interaction between AtELP and the AtPAP peptide was reported to be pH-dependent (Ahmed, et al., 2000). To investigate the effect of pH on the interaction between modified PV72s and the AtPAP peptide, we performed the binding assay with the affinity column at pH 4.0. rPV72 still bound to the column at pH 4.0 in the presence of 1 mM $CaCl_2$ and eluted by the addition of EGTA. These results indicated that the acidic condition did not affect the interaction between rPV72 and the AtPAP peptide. On the contrary, we found that rPV72Δ1,2,3 without EGF-motifs was detected in the pH 4.0 fractions in the Na-acetate buffer (pH 4.0) containing 1 mM $CaCl_2$ (Fig. 25B). The interaction between rPV72Δ1,2,3 and the peptide was affected by the acidic condition. These results indicated that the binding site for the ligands is located in the N-terminal region of the lumenal domain of PV72 and that the EGF-like motifs function as the stabilizing domain for the complex of interaction and the ligand.

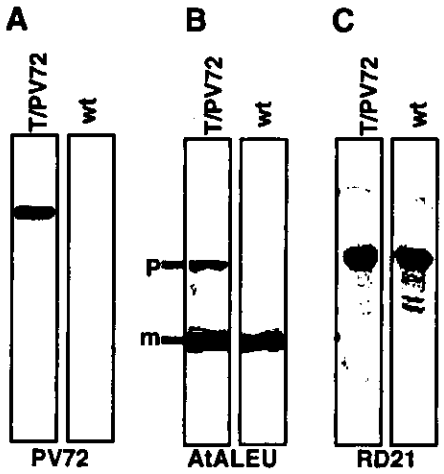


Fig. 21 Overexpression of PV72-HDEL results in the accumulation of a proprotein precursor of AtALEU, which has a vacuolar targeting signal, NPIR, in the transgenic Arabidopsis plants. Arabidopsis plants were transformed with a chimeric gene encoding a PV72 precursor followed by an ER retention signal, HDEL. (A) Crude extracts (10 μg protein) from the transgenic Arabidopsis plants (T/PV72) and wild plants (wt) were subjected to SDS-PAGE followed by an immunoblot analysis with specific antibodies against each of PV72, aleurain and RD21. (B) The 41 kD proprotein precursor (p) of AtALEU was accumulated in the transgenic plants expressing PV72-HDEL, but not in wild plants. (C) On the contrary, a precursor of RD21, which has not a vacuolar targeting signal, NPIR, was not detected in both the transgenic and wild plants. p, the AtALEU precursor; m, the mature form of AtALEU.

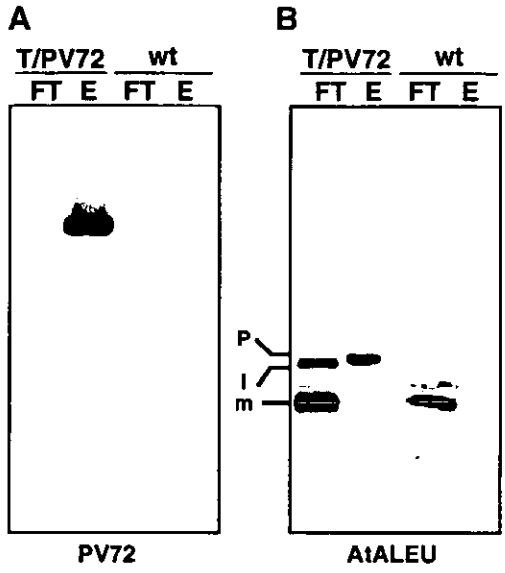


Fig. 22 PV72-HDEL was co-purified with the AtALEU precursor (proAtALEU), but not with the mature form.

Microsome fractions of transgenic Arabidopsis plants (T/PV72) and wild plants (wt) were subjected to Ni-NTA resin and washed with the CHAPS buffer to obtain the flow through fraction. Finally, the bound proteins (E; elution fraction) were eluted with the CHAPS buffer containing 400 mM Imidazol. These fractions were subjected to SDS-PAGE followed by an immunoblot analysis with specific antibodies against each of PV72, aleurain.(A) PV72-HDEL was eluted with the CHAPS buffer containing 400 mM Imidazol in T/PV72. (B) In transgenic Arabidopsis plants (T/PV72) and wild plants (wt), mature AtALEU (28 kDa) and premature AtALEU was detected in the FT fraction. On the contrary, AtALEU precursor (41 kDa) was co-purified with PV72-HDEL. p, the AtALEU precursor; m, the mature form of AtALEU; i, the intermediate form of AtALEU.

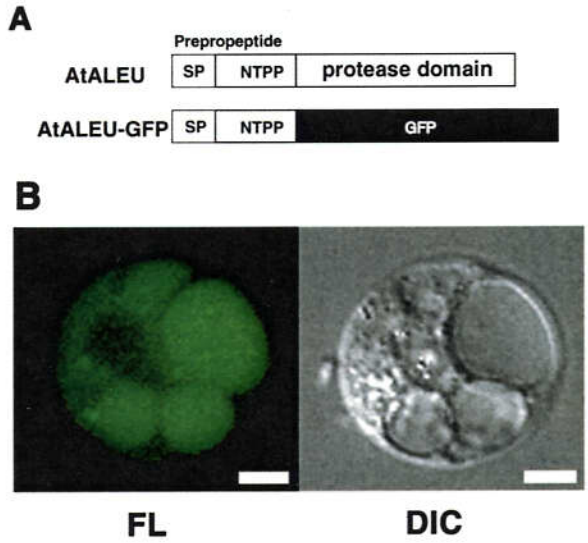
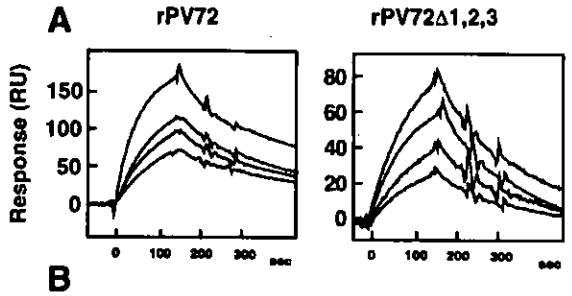


Fig. 23 GFP with the Propeptide of AtALEU at the N terminus (AtALEU-GFP) Was Localized in the Vacuoles of the Transformed Suspension-Cultured Cells of Arabidopsis. (A) Precursors of AtALEU and an AtALEU-GFP are schematically represented. The precursor of AtALEU is composed of three regions; a signal peptide (SP), an N-terminal propeptide (NTPP) that contains a vacuolar targeting signal, NPIR, and a mature protease domain. The protease domain of AtALEU was replaced by GFP to produce AtALEU-GFP. (B) Suspension-cultured cells (T-75) of Arabidopsis were transformed with a chimeric gene encoding AtALEU-GFP. The 3-day-old transformant cells were inspected with a fluorescent (FL) or differential-interference-contrast (DIC) microscope. A fluorescent image shows the localization of the GFP fusion within the cells and a DIC micrograph shows the cellular structures of the respective fields. Bars = 5 μm.



	k_a (per Ms)	k_d (per s)	K_D
rPV72	6.4×10^{4}	6.9×10^{-3}	0.1 μM
rPV72Δ1,2,3	1.5×10^{4}	1.9×10^{-2}	1.2 μM

Fig. 24 Kinetics for the association and Dissociation of rPV72 and the AtPAP Peptide. (A) PV72 has three EGF-like motifs at the C terminus of the lumen Al domain. We expressed two modified PV72s in the insect Sf21cells to use the kinetic analysis; rPV72 composed of the lumen domain followed by a His-tag and the HDEL sequence and rPV72Δ1,2,3 composed of rPV72 with no EGF-like motif. A sensor chip was coupled with the AtPAP peptide on BIACORE-X (a coupling efficiency of 700 resonance units). rPV72 and rPV72Δ1,2,3 were injected onto the sensor chip at different concentrations from 0.17 μM to 0.7 μM to obtain the sensorgrams for the association and dissociation curves by surface plasmon resonance. (B) The kinetic constants, an association rate constant (k_a), a dissociation rate constant (k_d) and a dissociation constant ($K_D = k_d / k_a$), were calculated from the above sensorgrams using BIA evaluation software version 2.1. These kinetic parameters were determined from two independent experiments.

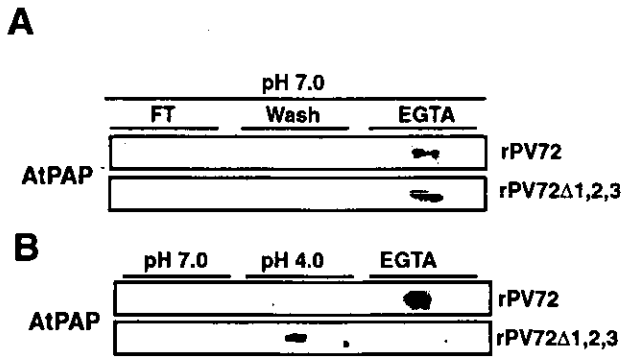


Fig. 25 Ca^{2+}-mediated interaction between modified PV72s and the AtPAP peptide. (A) Either rPV72 (upper) or rPV72Δ1,2,3 (lower) was subjected to an affinity column conjugated with the AtPAP peptide in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. Both proteins were bound to the column and then eluted by addition of the HEPES buffer (pH 7.0) containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies. FT, flow-through fraction, Wash, washing fraction, EGTA, elution fraction. (B) Either rPV72 (upper) or rPV72Δ1,2,3 (lower) were subjected to the AtPAP column in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. The column was washed with the same buffer and followed by the sodium acetate buffer (pH 4.0) containing 1 mM $CaCl_2$, and finally washed with the sodium acetate buffer containing 2.5 mM EGTA. rPV72 was eluted by the addition of the sodium acetate buffer containing 2.5 mM EGTA, while rPV72Δ1,2,3 was eluted by addition of the sodium acetate buffer (pH 4.0) containing 1 mM $CaCl_2$. Each fraction was subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies.

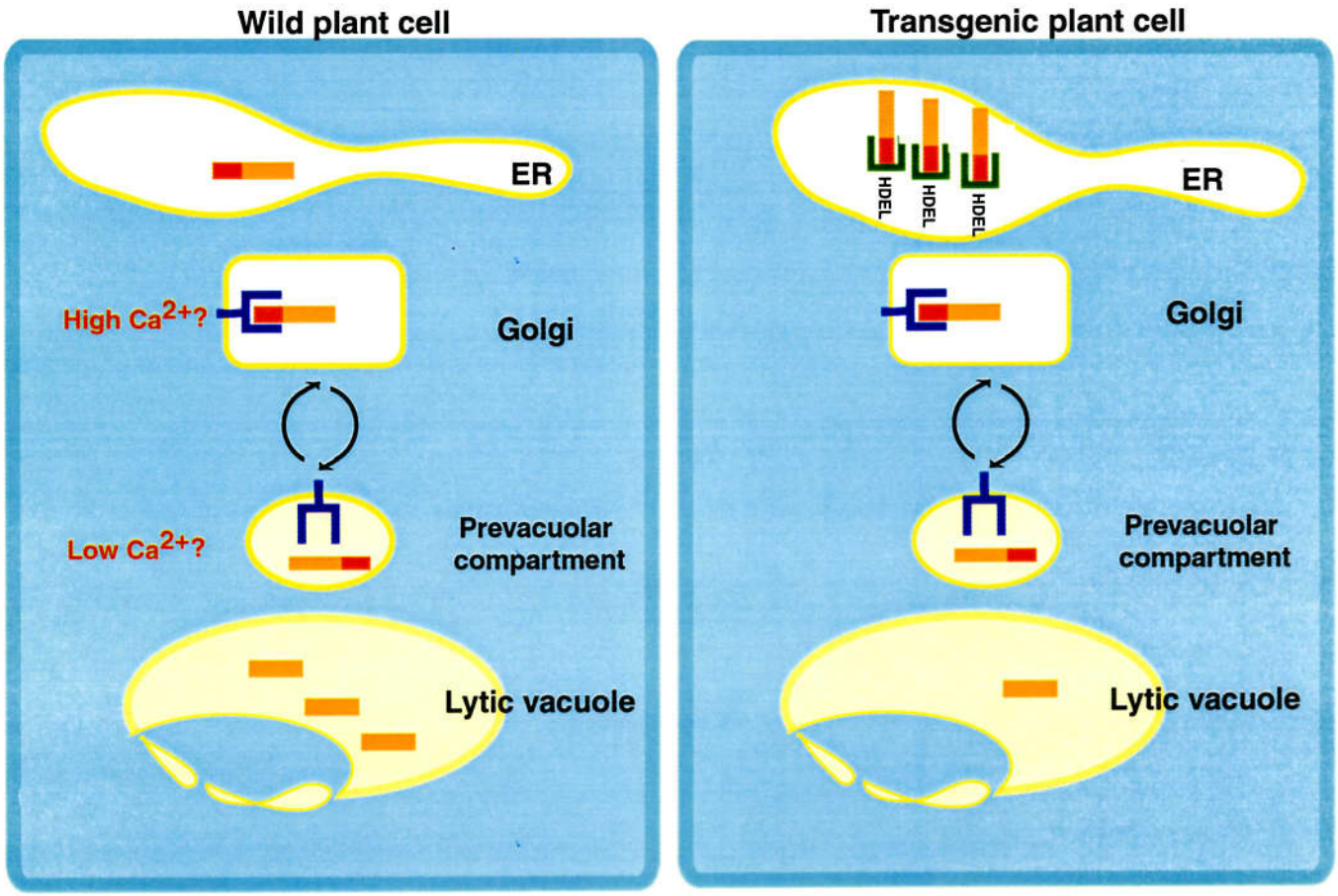


Fig. 26 A schematic model of the transport of NPIR-containig AtALEU.

Discussion

Receptor-mediated transport of seed storage proteins to the protein storage vacuoles

PAC vesicles are involved in the transport of seed storage protein precursors to protein storage vacuoles (13). PV72 was a membrane protein that was found in the PAC vesicles (Fig. 2). PV72 was the most abundant protein of the PAC vesicles after storage protein precursors (Fig. 4). Here we clearly demonstrated that PV72 has an ability to bind pro2S albumin in a Ca^{2+} dependent manner (Fig. 7). These results suggested that PV72 functions as a sorting receptor for a storage protein, 2S albumin. This was supported by the evidence that the expression of PV72 is specific to maturing seeds and the accumulation of PV72 is accompanied by the synthesis of 2S albumin (Shimada et al. submitted).

The amount of PV72 increased in the maturing seeds in parallel with the accumulation of storage protein, and then decreased after seed germination (Shimada et al. submitted). The co-localization of PV72 with pro2S albumin and the developmental change in the level of PV72 support that PV72 is involved in sorting of pro2S albumin to protein storage vacuoles. PV72 has been shown to interact with pro2S albumin, indicating that pro2S albumin is a natural ligand of the receptor. However, there has been only a few report of the interaction of such receptors with natural ligand proteins. A receptor homologue was reported to interact with a protease inhibitor precursor of stigma of *Nicotiana alata* (31). In germinating cotyledons of *Vigna mungo*, a receptor homologue was also reported to interact with a proprotein precursor of a cysteine protease, SH-EP (32).

A homologue, BP-80, has been shown to function as a sorting receptor for a cysteine protease of plants (16,17). Hinz et al. (1999) clearly showed that BP-80 is not localized in the transport vesicles for storage proteins, but in Clathrin-Coated Vesicles,

which contained no storage proteins. Taken together, it appears that PV72 is a sorting receptor for a storage protein to protein storage vacuoles, while BP-80 is a sorting receptor for a lytic enzyme to lytic vacuoles. Arabidopsis has 7 homologues of the sorting receptors, which can be separated into two types; one for storage proteins and another for lytic enzymes.

Localization of receptors

When a fusion protein of green fluorescent protein with a transmembrane domain and a cytoplasmic tail of PV72 was expressed in tobacco BY2 cells, a fluorescent Golgi complex was observed (23). The localization of PV72 in the Golgi complex is supported by the findings that PV72 has a complex glycan (unpublished data) and that anti-complex glycan antibodies stained the peripheral region of the PAC vesicles (13). It is possible that PV72 is recycled between the Golgi complex and the PAC vesicles in maturing seeds.

Previously Hara-Nishimura et al. reported that most of pro2S albumin molecules synthesized on the ER are directly incorporated into the PAC vesicles, in a Golgi-independent manner (12,13). It appears that PV72 might trap the escaped pro2S albumin molecules that leave the rER for the Golgi complex and recruit them from the Golgi complex to the PAC vesicles.

Our results reveled that the association and dissociation of PV72 and pro2S albumin is modulated by the Ca^{2+} concentration. The Ca^{2+} concentration in the Golgi complex was determined to be 300 μM (26), which is high enough for PV72 to bind the ligand. On the other hand, the Ca^{2+} concentration in the ER ranges from 1 μM to 1,500 μM depending on the region of the ER (27). Thus, the ER-derived PAC vesicles possibly have a Ca^{2+} concentration lower than 50 μM, which could dissociate the ligand from the receptor-ligand complex.

Ca^{2+}-mediated association and dissociation of PV72 and the ligand

Receptor-mediated protein sorting involves an association and dissociation of the receptor and the respective ligand by a modulator. In general, the environmental pH is known to regulate it as a modulator. Lysosomal and vacuolar proteins synthesized on the rER are reported to be recognized by a receptor and then delivered to the respective acidic compartment, where the ligands are dissociated from the receptor. The dissociation for both mannose-6-phosphate receptor (1) has been shown to occur in the acidic organelles in mammals.

We found, however, that PV72 binds to the ligand even at pH 4.0 in the presence of Ca^{2+}. It does not appear that the acidic pH is responsible for the dissociation of PV72 from the ligand. Our results suggest that Ca^{2+} functions as a modulator for the association and dissociation of PV72 with the internal propeptide of 2S albumin within the cells of the maturing seeds. The Ca^{2+} concentration in the subcellular compartments was reported to range from 1 μM to 1,500 μM (27). The EC_{50} (Ca^{2+}) value of 40 μM for the ligand binding of rPV72 is reasonable for the regulation of the association and dissociation of the receptor with the ligand within the cells.

PV72 has a consensus sequence for Ca^{2+}-binding in the third EGF-like motif (15). The motif might function as a Ca^{2+}-binding EGF (cbEGF) domain. Binding of Ca^{2+} to the cbEGF domain might cause a conformational change in the PV72 molecule to make the receptor-ligand complex stable. On the other hand, reduction of the environmental Ca^{2+} concentration might cause the dissociation of the ligand from the receptor.

PV72 has another Ca^{2+}-binding site in the N-terminal region corresponding to rPV72Δ1,2,3, which lacks a consensus sequence for Ca^{2+}-binding. However, binding of Ca^{2+} to the region causes only a small conformational change in the PV72. The N-terminal region might play a role in the formation of the ligand-binding pocket with the assistance of Ca^{2+} (discussed below). The binding of Ca^{2+} to both the N-terminal region and the cbEGF domain of PV72 could induce the formation of a functional pocket for

the ligand binding and stabilize the receptor-ligand complex.

The LDL receptor has three EGF motifs, one of which is cbEGF (29). The receptor also has a ligand-binding region with another Ca^{2+}-binding site (30) and the receptor-ligand complex is stabilized by Ca^{2+} in the receptor molecules (Fass, et al., 1997; Kimberly, et al., 1998). In spite of the very low identity of the sequence between the LDL receptor and PV72, the mechanisms of Ca^{2+}-mediated association and dissociation of the receptor and ligand are similar to each other. The Ca^{2+}-mediated regulation through the cbEGF domain is reported to be crucial in mammals. Mutations in a cbEGF domain of the LDL receptor have been shown to cause familial hypercholesterolemia (Fass, et al., 1997) and a mutation in the cbEGF of fibrillin causes Marfan syndrome (Downing, et al., 1996).

The protease-associated domain of PV72 might be involved in the ligand binding

We found that PV72 might form the ligand-binding pocket in the N-terminal region corresponding to rPV72Δ1,2,3. This region contains a protease-associated (PA) domain, which is speculated to be involved in substrate determination for peptidases or to form protein-protein interactions (Luo and Hofmann, 2001; Mahon and Bateman, 2000). It is possible that the PA domain of PV72 mediates the interaction with pro2S albumin. A lytic-enzyme receptor, BP-80, is also reported to have a ligand-binding site in the region corresponding to the PA domain (Cao, et al., 2000). The PA domain is also found in a plant transmembrane protein, ReMembR-H2 whose role is not known (Jiang, et al., 2000).

We found that mutation of either Glu-73 or Glu-79 reduced the affinity of PV72 for the ligand peptide under acidic conditions. Two Glu residues of the 2S-I peptide might be important for stability of the ligand-receptor interaction under acidic conditions. PV72 might interact with pro2S albumin through the two Glu residues, one of that is included in the RRE sequence in the 2S-I peptide. Previously we reported that

the RRE sequence was essential for the interaction with PV72 (Shimada, et al., 1997). The essential sequence is different from the targeting determinant, NPIR, of the lytic enzymes.

Differential characterization between PV72 and PV82

In Arabidopsis, there are seven homologues of PV72. Thus, each receptor must sort respective ligand. From now, the regulators, calcium and pH change, of these interaction was reported. We demonstrated that PV72 bound to the ligand in a Ca^{2+}-dependent manner (Watanabe, et al., 2002). BP-80 and AtELP bound to the ligand by pH-dependent manner (Kirsch, et al., 1994) (Ahmed, et al., 2000). These results suggested that the transport of proteins to lytic vacuoles or protein storage vacuoles is different. However, we showed that the interaction between rAtELP and the NPIR containing AtPAP peptide is in a Ca^{2+}-dependent. The results indicated that these receptors bound to the ligand in a common interaction mechanism. Here, we clarified that the EC_{50} value of PV82 is 10-fold larger than that of PV72. The biochemical characterization of these receptors is different among receptors. That suggested that the function of these receptors is different *in vivo*. This was supported that different localization of PV72/82 supported that each receptor functioned at different organelle in the same cell (submitted, Shimada et al) and the Ca^{2+} concentration in the Golgi complex was determined to be 300 μM (26), which is low for PV82 to bind the ligand. PV82 might bind to the ligand at ER and dissociated at the *trans* Golgi network.

Homologues of the vacuolar sorting receptor including 7 homologues of Arabidopsis can be divided into two or three groups. PV72 and BP-80 belong to different group from each other on the phylogenetic tree of the receptors. The members of the PV72 group might function for protein storage vacuoles, while those of the BP-80 group might function for lytic vacuoles. It is needed for the cDNA cloning of PV82. It is likely that PV82 is analogous to BP-80 that is known to be a sorting receptor for

aleurain to be transported to lytic vacuoles (Humair, et al., 2001). Pea BP-80 expressed at pea developing cotyledon, and functioned as the receptor for the lytic enzymes. These findings implied that sorting receptors should be separated into two types; one for protein storage vacuoles and another for lytic vacuoles.

Receptor-mediated transport of AtALEU in Arabidopsis plants

Recently, Humair et al. (2001) reported that BP-80 interacted with a fusion protein composed of the NTPP of petunia aleurain and GFP in the transformed yeast cells defective for its own vacuolar sorting receptor, VPS10p. This interaction might lead to the transport of the reporter protein through the yeast secretary pathway to the vacuoles (Humair, et al., 2001). However, the physiological function of such putative receptors has not been demonstrated in plant cells. We found that the ectopic expression of PV72-HDEL induced the accumulation of the AtALEU precursor and the formation of the complex of PV72-HDEL and the precursor in the ER. It is noted that PV72-HDEL exhibited an ability to form the complex with the precursor but not with the mature form (Fig. 22). The NPIR-containing propeptide of the AtALEU precursor might be essential for the formation of the complex. The propeptide was shown to function as a vacuolar targeting signal in Arabidopsis cells (Fig. 23). These findings suggest that the receptor binds to the NPIR-containing peptide of the AtALEU precursor within the ER. This is the first demonstration that PV72 homologs function as a vacuolar sorting receptor for a vacuolar targeting signal, NPIR, in Arabidopsis cells.

Here, we clearly demonstrated that PV72 bound to the AtPAP propeptide of AtALEU with K_D value of 0.1 μM, which was larger than 0.037 μM for BP-80 for the NPIR-containing propeptide of barley aleurain (Kirsch, et al., 1994). The difference between the two values might be derived from the difference of assay methods: the value for PV72 was obtained by surface plasmon resonance analysis and that for BP-80 was by an affinity chromatography. , These values are enough low to function as a

vacuolar sorting receptor in plant cells.

Arabidopsis plants has a putative vacuolar targeting receptor, AtELP, that could function as a counter part of PV72. AtELP was reported to be interacted with the NPIR-containing propeptide of AtALEU and localized in the Golgi apparatus (Ahmed, et al., 2000). In wild Arabidopsis plants, the NPIR-containing precursor is transported to Golgi apparatus where they are trapped by the receptors. On the other hand, in the transgenic plants, overexpressed PV72-HDEL prevent the precursor from leaving ER to the Golgi apparatus. It is possible that the interaction between the vacuolar sorting receptor and the NPIR-containing propeptide leads to the transport of the lytic enzyme to the lytic vacuoles in plant cells.

Another transport pathway for vacuolar proteins via ER-derived compartments

PV72 was originally found in the PAC-vesicles that accumulate the precursor proteins of major seed storage proteins including 2S albumin and 11S globulin (Hara-Nishimura, et al., 1998b). Recently, we found that PV72 has an ability to bind with the internal propeptide of 2S albumin of pumpkin (unpublished data). PV72 was specifically and transiently accumulated in maturing seeds in association with the synthesis of seed storage proteins (unpublished data). These results implied that PV72 mediate the transport of 2S albumin in maturing seeds. We reported that the PAC vesicles are derived from ER and mediate a transport of the storage protein precursors directly to protein storage vacuoles (Hara-Nishimura, et al., 1998b). Thus, most of pro2S albumin molecules that are synthesized on ER are directly transported to the PAC vesicles (Hara-Nishimura et al., 1993a). When some pro2S albumin molecules leave the ER for the Golgi complex, the escaped molecules might be recruited from the Golgi complex to the PAC vesicles by PV72. This raises the question of whether pro2S albumin is accumulated in the ER of the transgenic plants as the AtALEU precursor. However, we could not detect the accumulation of pro2S albumin in the microsomal fraction from the

maturing seeds of the transgenic plants from the following two reasons. First, the level of the accumulation of PV72-HDEL was very low in the maturing seeds. This was also supported by the report that the expression of introducing genes in seed is weak than the other tissues under the control of 35S promoter (Zuo, et al., 2001). Second, most of pro2S albumin is transported directly to vacuoles via the PAC vesicles as described above.

In *Vigna mungo* seedlings, a KEDL-tailed cysteine protease, SH-EP, was packed in the KDEL vesicle that was derived from the ER (Mitsuhashi, et al., 1986; Toyooka, et al., 2000). SH-EP was transported to vacuoles via KDEL vesicles in a Golgi-independent manner (Toyooka, et al., 2000). Recently, a receptor homolog that bound to the SH-EP precursor was found in the seedlings (Tsuru-Furuno, et al., 2001). It is possible that the receptor homolog traps the SH-EP precursor leaving ER for Golgi apparatus to deliver it to the KDEL vesicles and/or the vacuoles.

In contrast to vacuolar sorting receptor -mediated transport of the AtALEU precursor, the transport of RD21 to lytic vacuoles is not affected by the accumulation of PV72-HDEL in the ER. The transport of RD21 might be different from that of AtALEU. RD21 is a stress-inducible protease (Kinoshita, et al., 1999; Koizumi, et al., 1993; Yamada, et al., 2001). Recently, The RD21 precursor is accumulated in the ER-body, which is a ER-derived organelle in the epidermal cells of healthy Arabidopsis seedlings (Hayashi, et al., 2001). The ER bodies appear to be a novel protease-sorting system that assists in cell death under the stressed conditions. When seedlings are stressed with a concentrated salt solution, leading to death of the epidermal cells, the ER bodied start to fuse with vacuoles, thereby mediating the delivery of the precursor to the lytic vacuoles.

Unique interaction between PV72 and NTPP in a Ca^{2+}-dependent manner

The pH gradient is known to exist from the ER to the vacuole that is a typical acidic compartment. The abnormal pH of vacuoles induced the miss sorting of the NPIR-

containing protein (Matsuoka, et al., 1997). The binding of BP-80 and AtELP to the ligand was described to be regulated by environmental pH (Ahmed, et al., 2000; Kirsch, et al., 1994). The optimum pH for the ligand binding of the receptors was determined to be pH 6.0 to 6.5 and the dissociation of the receptor and the ligand occurred at pH 4.0 (Kirsch, et al., 1994). It is possible that such receptors bind to the ligand in Golgi apparatus and release the ligand in a late endosomal or prevacuolar compartment whose inside pH might be low (Ahmed, et al., 2000; Paris, et al., 1997).

We demonstrated that rPV72 interacts with the AtPAP propeptide of AtALEU in a Ca^{2+}-dependent manner (Fig. 25). This was consistent with our previous finding that rPV72 bound to the 2S-I peptide in a Ca^{2+} -dependent manner (Watanabe, et al., 2002). These receptors have a consensus sequence for Ca^{2+}-binding in the third EGF-like motif (Shimada, et al., 1997). The motif might function as a Ca^{2+}-binding EGF (cbEGF) domain. Previously, we demonstrated that binding of Ca^{2+} to the cbEGF domain might cause a conformational change in the PV72 molecule to make the receptor-ligand complex stable (Watanabe, et al., 2002). On the other hand, reduction of the environmental Ca^{2+} concentration might cause the dissociation of the ligand from the receptor. The association and dissociation regulated by Ca^{2+} concentration is a unique mechanism for transport of vacuolar proteins.

Under the acidic condition, rPV72Δ1,2,3 bound to the AtPAP propeptide of AtALEU (Fig. 25), but not to the 2S-I peptide (Watanabe, et al., 2002). The different binding ability might be due to the pIs of the ligands. The AtPAP propeptide of AtALEU has pI 4.0 and the 2S-I peptide has pI 10.0. The surface charges of these peptides change in association with the change of environmental pH. The different surface charges might cause the different affinity of rPV72Δ1,2,3 with each peptide.

Vacuolar sorting receptor PV72 and its homologues

PV72 was the most abundant protein of the PAC vesicles after storage protein

precursors (Shimada, et al., 1997). We clearly demonstrated that PV72 has an ability to bind pro2S albumin in a Ca^{2+} dependent manner (unpublished data). PV72 functions as a sorting receptor for 2S albumin. This was supported by the evidence that the expression of PV72 is specific to maturing seeds and the accumulation of PV72 is accompanied by the synthesis of 2S albumin. PV72 is transiently accumulated in the pumpkin cotyledons at the middle stage seed maturation, but not in the vegetative tissues (unpublished data).

On the contrary, PV82, which is a homologue of PV72 (Shimada, et al., 1997), is widely distributed in both maturing seeds and the vegetative tissues of pumpkin (unpublished data). We found that PV82 bound more preferably to the propeptide of barley aleurain than PV72 (unpublished data). It is likely that PV82 is analogous to BP-80 that is known to be a sorting receptor for aleurain to be transported to lytic vacuoles (Humair, et al., 2001). These findings implied that sorting receptors should be separated into two types; one for protein storage vacuoles and another for lytic vacuoles. Homologues of the vacuolar sorting receptor including 7 homologues of Arabidopsis can be divided into two or three groups. PV72 and BP-80 belong to different group from each other on the phylogenetic tree of the receptors.

PV72 has been shown to interact with pro2S albumin, indicating that pro2S albumin is a natural ligand of the receptor. However, there has been only a few report of the interaction of such receptors with natural ligand proteins. A receptor homolog was reported to interact with a protease inhibitor precursor of stigma of *Nicotiana alata* (Miller, et al., 1999). In germinating cotyledons of *Vigna mungo*, a receptor homologue was also reported to interact with a proprotein precursor of a cysteine protease, SH-EP (Tsuru-Furuno, et al., 2001).

The members of the PV72 group might function for protein storage vacuoles, while those of the BP-80 group might function for lytic vacuoles. PV72 is localized in the PAC vesicles, but not in clathrin coated vesicles in maturing pumpkin seeds (unpublished data). The Arabidopsis homologue which is same group of PV72, located

at plasma membrane of leaf, root and silique (Laval, et al., 1999). On the contrary, BP-80 has been shown to be rich in clathrin coated vesicles, but not in dense vesicles responsible for transport seed globulins in maturing pea seeds (Hinz, et al., 1999). BP-80 was localized in clathrin-coated vesicles and *trans*-Golgi network (Hinz, et al., 1999). The Arabidopsis homolog in the same group of BP-80 was expressed in leaves and silique (Laval, et al., 1999). The tissue specific and time specific expression of each receptor is a key to determine the ligand specificity for the receptor.

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Calcium-mediated Association of a Putative Vacuolar Sorting Receptor PV72 with a Propeptide of 2S Albumin*

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PV72, a type I membrane protein with three epidermal-growth factor (EGF)-like motifs, was found to be localized on the membranes of the precursor-accumulating (PAC) vesicles that accumulated precursors of various seed storage proteins. To clarify the function of PV72 as a sorting receptor, we expressed four modified PV72s and analyzed their ability to bind the internal propeptide (the 2S-I peptide) of pro2S albumin by affinity chromatography and surface plasmon resonance. The recombinant PV72 specifically bound to the 2S-I peptide with a K_D value of 0.2 μM, which was low enough for it to function as a receptor. The EGF-like motifs modulated the Ca^{2+}-dependent conformational change of PV72 to form a functional pocket for the ligand binding. The binding of Ca^{2+} stabilizes the receptor-ligand complex even at pH 4.0. The association and dissociation of PV72 with the ligand is modulated by the Ca^{2+} concentration (EC_{50} value = 40 μM) rather than the environmental pH. Overall results suggest that Ca^{2+} regulates the vacuolar sorting mechanism in higher plants.

Most proteins that are synthesized on rough endoplasmic reticulum are delivered to various cellular destinations, including vacuoles and lysosomes. Such sorting involves recognition of targeting signals of proteins by receptors. In mammalian systems, mannose 6-phosphate residues in glycosyl side chains of glycoproteins are known to function as a targeting signal to the lysosomes, and mannose 6-phosphate receptors have been identified as a lysosome sorting receptor (1). In yeast systems, a short stretch sequence of amino acids, QRPL, found in the carboxypeptidase Y, is known to function as a targeting signal to the vacuoles, and Vps10p has been identified as a vacuolar sorting receptor for vacuolar hydrolases (2).

Higher plants have two types of vacuoles: one type, protein storage vacuoles, develop mainly in storage organs, such as seeds, and the other type, lytic vacuoles, which contain various lytic enzymes, develop in the vegetative organs. Both types of vacuoles, however, are found in the same cells of barley roots (3) and of maturing pea seeds (4). In these cells, vacuolar proteins synthesized on the rough endoplasmic reticulum are sorted and delivered to their respective vacuoles. Thus, different targeting machinery for each type of vacuole must be involved in protein transport in these cells.

For the lytic vacuoles, BP-80 was the first putative vacuolar sorting receptor isolated from pea (5). It binds *in vitro* to the vacuolar-targeting determinants (6, 7). Recently, Humair *et al.* (8) demonstrated the *in vivo* binding of BP-80 to the propeptide sequence of barley aleurain in a yeast mutant strain defective for its own vacuolar receptor, Vps10p. An Arabidopsis homolog, AtELP, was also found to interact with the propeptide of an aleurain homolog, AtALEU (9). BP-80 (10) and AtELP (11) are a type I integral membrane protein with epidermal growth factor (EGF)[1]-like motifs. They have been shown to be rich in clathrin-coated vesicles (CCVs) and prevacuolar compartments (12, 13). This implied that the cysteine proteinases might be delivered from the Golgi complex to lytic vacuoles via the CCVs in a receptor-dependent manner (9, 14). In contrast, the CCVs isolated from maturing pea seeds were reported to contain no storage proteins (12). Therefore, the transport machinery for storage proteins should be different from that of the lytic enzymes. However, the molecular mechanism responsible for the transport of storage proteins are scarcely elucidated.

In maturing seeds of plants, seed storage protein precursors are synthesized on the rough endoplasmic reticulum and then transported to protein storage vacuoles, where the precursor proteins are converted into the respective mature forms by the action of vacuolar processing enzyme (15–20). Multiple transport pathways have been shown for storage proteins. Hohl *et al.* (21) and Hinz *et al.* (12) demonstrated immunocytochemically that dense vesicles with a diameter of about 100 nm associated with Golgi complex contain storage proteins in maturing pea cotyledons. We found the other unique vesicles responsible for delivery of precursors of seed storage proteins and a membrane protein into the vacuoles (22–24) and designated them PAC (precursor-accumulating) vesicles (25). The PAC vesicles are derived from the endoplasmic reticulum and mediate a transport for storage proteins directly to protein storage vacuoles. We have found an integral membrane protein, PV72, with EGF-like motifs in the PAC vesicle fraction prepared from maturing pumpkin seeds (26). We have also shown that PV72 exhibits an affinity for peptides derived from pumpkin 2S albumin (26).

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[1] The abbreviations used are: EGF, epidermal growth factor; 2S-I, an internal propeptide of pumpkin 2S albumin; CCVs, clathrin-coated vesicles; PAC vesicles, precursor-accumulating vesicles; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

The question is how the association and dissociation of a receptor and the respective ligand is regulated. In mammalian systems, the affinity of mannose 6-phosphate receptor for their glycoprotein ligands was reported to be reduced by the acidic pH of the lysosomes (1). From the analogy to the system, the binding of BP-80 and AtELP to the ligand was described to be regulated by the pH (5, 9). In this study, however, we found that PV72 still has an ability to bind to the ligand at pH 4.0 in the presence of Ca^{2+} and that the EGF-like motifs modulate a Ca^{2+}-dependent conformational change of PV72. We describe here a unique mechanism by which Ca^{2+} acts as a modulator for the association and dissociation of PV72 with its ligand.

EXPERIMENTAL PROCEDURES

Plant Materials—Pumpkin (*Cucurbita* sp cv Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). The seeds were planted in the field. The cotyledons of maturing seeds, freshly harvested 22–28 days after anthesis, were used for the experiments.

Isolation of PAC Vesicles from Maturing Pumpkin Seeds—PAC vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation as described previously (25). The cotyledons were homogenized in buffer A (20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA and 0.3 M mannitol) with an ice-chilled mortar and pestle and filtered through three layers of cheesecloth. The filtrate was centrifuged at 3,000 *g* for 15 min and the supernatant was centrifuged again at 8,000 *g* for 20 min at 4 °C. The pellet was suspended in buffer B (10 mM HEPES-KOH, pH 7.2, 1 mM EDTA and 0.3 M mannitol) and layered on 28% Percoll (Amersham Biosciences, Tokyo, Japan) solution. After centrifugation at 40,000 × *g* for 35 min, the vesicle fraction was pooled and washed once in buffer B. PAC vesicles were collected by the centrifugation at 10,000 × *g* for 20 min and resuspended in buffer B. The isolated vesicles were subjected to immunoblot analysis.

Ultrastructural Analysis and Immunogold Labeling—Maturing pumpkin seeds were vacuum-infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The tissues were then cut into slices of less than 1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative.

Immunoblot Analysis—Immunoblot analysis was performed essentially as described previously (27), except that in the present study we used specific antibodies against PV72 (diluted 5,000-fold) (26) and horseradish peroxidase-conjugated donkey antibodies against rabbit IgG (diluted 5,000-fold; Amersham Biosciences, Inc.). PV72 was immunologically detected with an enhanced chemiluminescence kit (an ECL system, Amersham Biosciences, Inc.).

Construction of Recombinant Baculoviruses—Four modified PV72s were expressed in insect cells of *Spodoptera frugiperda* (Sf21) with a baculovirus expression system (Invitrogen, San Diego, CA). The system includes a transfer vector pBlueBac 4.5 and an expression vector Bac-N-Blue DNA composed of engineered baculoviral *Autographa california* multiple polyhedrosis virus. The *Kpn*I-*Sac*I fragment of pPV72 was produced from the amplified DNA with PV72 cDNA and a unique primer of 5′-ATT TGT TTA ACT GAA GAC GTG CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3′ and was ligated with the *Kpn*I-*Sac*I-digested pBlueBac 4.5 to produce the pBlueBac-rPV72. The pBlueBac-rPV72 encoded a fusion protein composed of both the signal sequence and the lumen domain of PV72 followed by a polyhistidine tag and an HDEL sequence. Constructs for three other modified PV72s were produced by the same procedure described above, except for using each primer: 5′-GAT GGA GTC CAC ACG TGT GAA CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3′ for PV72Δ3, 5′-YAC ACT CAT TGT GAA GCT CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3′ for PV72Δ2,3, and 5′-ATT TGT TTA ACT GAA CAC CAC CAC CAC CAC CAC GAT GAG CTT TGA GGT ACC GAA TTC-3′ for PV72Δ1,2,3. We cotransfected Sf21 cells with Bac-N-Blue DNA and each of the produced plasmids to generate recombinant baculoviruses. The viruses were purified from the supernatant of the transfected cells by a plaque assay to generate a high titer recombinant viral stock (28).

Expression and Purification of the Modified PV72s—For large scale expression of these modified PV72s, the optimal expression time was determined by monitoring the cellular extract using SDS-PAGE and an immunoblot with anti-PV72 antibodies. Three days after infection, the cells were collected by centrifugation at 500 *g* for 10 min, washed with PBS and gently suspended in 20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 1% CHAPS, and 1 mM $CaCl_2$. The cells were lysed by three bursts of sonication for 1 min at 10-min intervals on ice and were centrifuged at 750,000 × *g* for 30 min. The supernatant was loaded on a Hi-Trap chelating column (Amersham Biosciences, Inc.) and was eluted with a gradient of 20–1,000 mM imidazol in the above buffer. The modified PV72 fractions were dialyzed against the HEPES buffer (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, and 0.4% CHAPS) plus 1 mM $CaCl_2$ were concentrated using Centricon 30 (Amicon Inc., Beverly, MA) and then were loaded on a Superdex-200 column (Amersham Biosciences, Inc.) equilibrated with the HEPES buffer plus 1 mM $CaCl_2$. The purified modified PV72s were concentrated by Centricon 30 and subjected to a protein assay (Nippon Bio-Rad Laboratories, Tokyo) and a binding assay as described below.

Ligand Binding Assay by Affinity Column Chromatography—Five peptides (SRDVLQMRGIENPWRREG (2S-I), SRDVLQMRGIENPWGGGG (2S-I/3G), SRDVLQMRGIENGWRREG (2S-I/P75G), SRDVLQMRGIGNPWRREG (2S-I/E73G), SRDVLQMRGIENPWRRGG (2S-I/E79G)) were chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems Inc., Tokyo, Japan) and were used for ligands of affinity columns. Each peptide (10 mg) was immobilized to *N*-hydroxysuccinimide-activated Sepharose HP (Amersham Biosciences, Inc.) to prepare affinity columns. The modified PV72s were applied to each column equilibrated with the HEPES buffer plus 1 mM $CaCl_2$ and then eluted with the HEPES buffer plus 2.5 mM EDTA (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 0.4% CHAPS, and 2.5 mM EDTA) with an automated chromatography system (ÄKTA, Amersham Biosciences, Inc.). Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis with anti-PV72 antibodies.

Surface Plasmon Resonance and Kinetic Assays—We immobilized either 2S-I peptide or 2S-I/3G peptide on a sensor chip for a BIACORE system (BIACORE, Tokyo, Japan) in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.005% P-20 (HBS, BIACORE). Carboxymethylated dextran on a sensor chip (CM5) was activated with the mixture (70 μl) of 0.05 M *N*-hydroxysuccinimide and 0.05 M *N*-ethyl-*N*-(3-diethylaminopropyl)carbodiimide and then coupled with either 2S-I peptide or 2S-I/3G peptide at 25 °C and a flow rate of 5 μl/min for the solutions used on a BIACORE system. A control flow cell was prepared with no peptide. The amount of the coupled peptide on the sensor chip was found to be 1200–1500 resonance units.

The modified PV72s were injected onto the sensor chip for 300 s, and then the HEPES buffer plus 1 mM $CaCl_2$ was eluted for 200–300 s at 25 °C and a flow rate of 30 μl/min. The sensor chip surface was regenerated with 30 μl of 20 mM HCl to remove residual PV72 from the immobilized peptides. Equal volumes of each protein dilution were also injected over a control flow cell to serve as blank sensorgrams for subtraction of bulk refractive index background and nonspecific binding of analyte. The sensorgrams shown in this study are made by subtracting the sensorgram made with the control flow cell.

Kinetic analysis was performed according to the manufacturer's protocol. The association, dissociation, and regeneration phases were followed in real time as the changes in the relative diffraction. The association phase (0–180 s) was analyzed by nonlinear least squares curve fitting to yield the association rate constants (k_a) as mean values. The dissociation phase (180–300 s) was also analyzed by nonlinear least squares curve fitting to yield the dissociation rate constants (k_d). To avoid mass transport, we worked at a low immobilization level, a high flow rate (30 μl/min), and using suitable concentrations of analyte. Kinetic constants (the association rate constant (k_a), the dissociation rate constant (k_d), and the dissociation constant ($K_D = k_d/k_a$)) were calculated from the sensorgrams using BIA evaluation software version 2.1 (BIACORE). These kinetic parameters were determined from three independent experiments.

Ca^{2+}-dependent Binding to the 2S-I Peptide—Ca^{2+}-dependent binding was analyzed by two methods: surface plasmon resonance analysis and affinity chromatography. Both rPV72 and rPV72Δ1,2,3 were dialyzed against the HEPES buffer plus 2.5 mM EGTA, followed by dialysis against the HEPES buffer to remove Ca^{2+} and EGTA. The dialyzed PV72s were injected onto the 2S-I-immobilized sensor chip equilibrated with the HEPES buffer plus 0, 0.02, 0.05 0.1, and 1 mM $CaCl_2$ or 1 mM $MgCl_2$ to obtain each sensorgram.

Alternatively rPV72 was applied to the 2S-I affinity column with the HEPES buffer plus 1 mM $CaCl_2$ and then washed with the HEPES buffer containing decreasing concentration of $CaCl_2$ (500, 100, 50, 20, 0 μM). Finally, the column was eluted with the HEPES buffer plus 1 mM EGTA.

To compare the Ca^{2+} sensitivity of rPV72 with that of rPV72Δ1,2,3, both modified proteins were applied to the 2S-I column with either the HEPES buffer plus 1 mM $CaCl_2$ or the MES buffer (20 mM MES-NaOH,

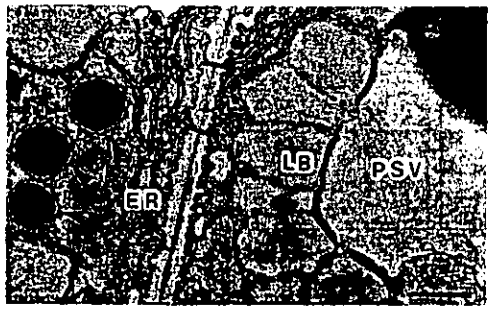


FIG. 1. **Electron microscopy of pumpkin cotyledons at the middle stage of seed maturation.** Numerous PAC vesicles (*arrowheads*) are visible in the cells. *PSV*, protein storage vacuole; *ER*, endoplasmic reticulum; *LB*, lipid body. *Bar* = 500 nm.

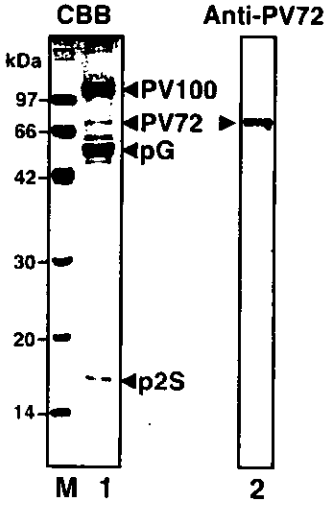


FIG. 2. **Localization of PV72 in PAC vesicles that accumulate a proprotein precursor of 2S albumin, a seed storage protein.** Isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (*lane 1*) or immunoblot analysis with anti-PV72 antibodies (*lane 2*). *p2S*, pro2S albumin; *pG*, proglobulin; *PV100*, a precursor of a proteinase inhibitor, cytotoxic proteins, and 7S globulin (30). *Lane M* contains molecular mass markers. The molecular mass of each marker protein is given on the *left* in kilodaltons.

pH 5.5, 150 mM NaCl, 0.4% CHAPS) plus 1 mM $CaCl_2$. We used the HEPES buffer plus 50 μM $CaCl_2$ or the MES buffer plus 50 μM $CaCl_2$ as the washing solution for the columns and the HEPES buffer plus 2.5 mM EGTA or the MES buffer plus 2.5 mM EGTA as the elution buffers. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

pH-dependent Binding—To investigate the pH effect on the interaction of modified PV72s with either 2S-I peptide, 2S-I/E73G peptide, 2S-I/P75G peptide, and 2S-I/E79G peptide, the proteins were subjected to each affinity column equilibrated with the HEPES buffer plus 1 mM $CaCl_2$ or the sodium acetate buffer (20 mM sodium acetate, pH 4.0, 150 mM NaCl, 0.4% CHAPS) plus 1 mM $CaCl_2$. The column was washed with the respective buffer and eluted with the respective buffer plus 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

Spectroscopic Measurements—Fluorescence emission spectra were recorded from 300 to 400 nm by a fluorescence spectrophotometer (Hitachi, F-4500, Tokyo, Japan) with an excitation wavelength at 280 nm in mixtures containing 1 μg/ml modified PV72s, in the HEPES buffer plus 1 mM $CaCl_2$ or the HEPES buffer plus 1 mM EDTA, as described previously (29).

RESULTS

PV72 Is the Fourth Abundant Protein of PAC Vesicles That Accumulate Storage Protein Precursors—Previously, we found unique vesicles, PAC vesicles, which mediate the transport of the storage protein precursors to protein storage vacuoles in maturing pumpkin seeds (25). Electron microscopy of the maturing seeds revealed numerous electron-dense PAC vesicles within the cells, as indicated by *arrowheads* in Fig. 1. Isolation of the PAC vesicles showed that they accumulated proprotein precursors of seed storage proteins, but not their mature forms at all (Fig. 2, *lane 1*). The precursors included pro2S albumin, proglobulin, and PV100, which is a single precursor of multifunctional proteins including trypsin inhibitors, cytotoxic peptides, and 7S globulin (30). PV72 was detectable as a single band with a molecular mass of 72 kDa on an immunoblot of the PAC vesicles with anti-PV72 antibodies (Fig. 2, *lane 2*). The PV72 content was enough high to be visible on the SDS gel with Coomassie Blue staining (Fig. 2, *lane 1*). The result indicates that the pure PAC vesicles contain PV72 as the fourth abundant protein of the vesicles

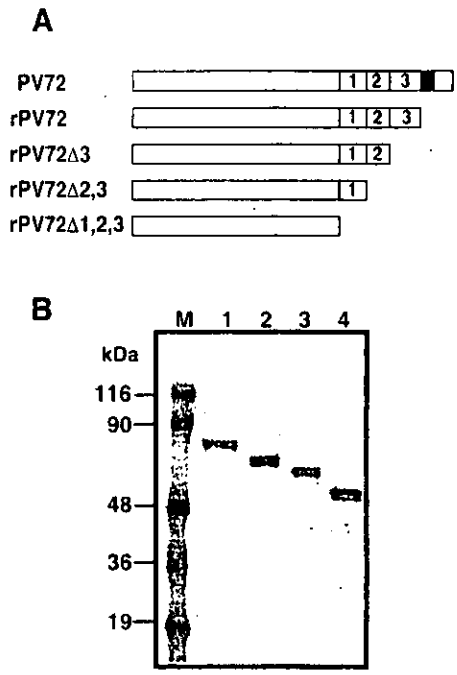


FIG. 3. **Constructs and the expressed proteins of four modified PV72s in insect cells.** *A*, PV72 is a type I integral membrane protein with three EGF-like motifs at the C terminus of the lumenal domain. The N-terminal domain is indicated by a *gray box*, and each EGF-like motif is indicated by an *open box* (*boxes 1, 2,* and *3*, respectively). The transmembrane domain is indicated by a *closed box*, and the cytoplasmic tail is indicated by an *open box*. rPV72 was composed of the lumen domain followed by a His tag and the HDEL sequence. rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3 were composed of rPV72 without the third EGF-like motif, without the second and third EGF-like motifs, and without all three of the EGF-like motifs, respectively. *B*, the four modified PV72s that were expressed in insect Sf21 cells were purified with a chelating column and a gel filtration column. Each purified protein was subjected to SDS-PAGE with Coomassie Blue staining: rPV72 (*lane 1*), rPV72Δ3 (*lane 2*), rPV72Δ2,3 (*lane 3*), and rPV72Δ1,2,3 (*lane 4*). The molecular mass of each marker protein (*lane M*) is given on the *left* in kilodaltons.

PV72 Lacking the EGF-like Motifs Still Specifically Binds to the Internal Propeptide of 2S Albumin—To clarify the ligand-binding mechanism of PV72, we expressed modified rPV72s with a His tag in insect Sf21 cells employing a baculovirus expression system. PV72 is a type I integral membrane protein with EGF-like motifs (26). Fig. 3*A* shows each construct of the modified proteins; rPV72 corresponds to the lumenal domain of PV72, rPV72Δ3 corresponds to the lumenal domain without the third EGF-like motif, rPV72Δ2,3 corresponds to the lumenal domain without the second and third EGF-like motifs, and rPV72Δ1,2,3 corresponds to the lumenal domain with no EGF-like motifs. These expressed proteins were purified with a chelating column and a gel filtration column. Each final preparation was highly pure as judged from SDS-PAGE with Coo-

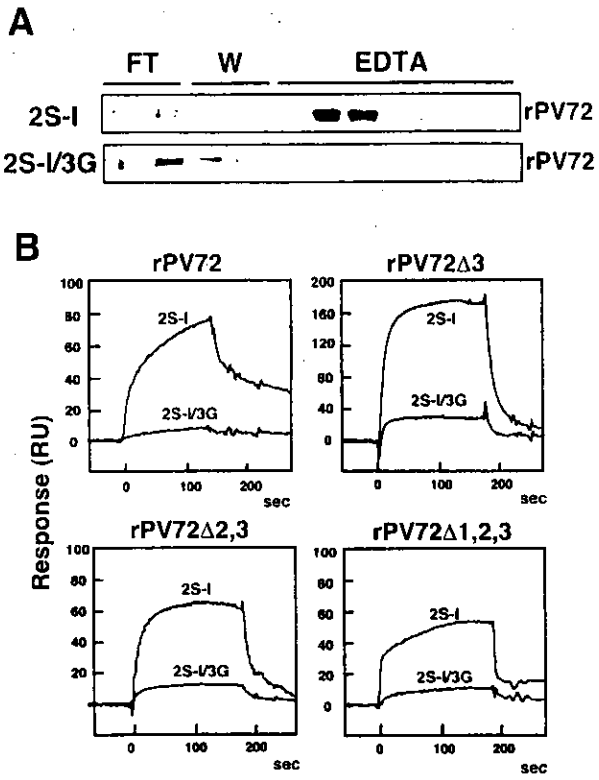
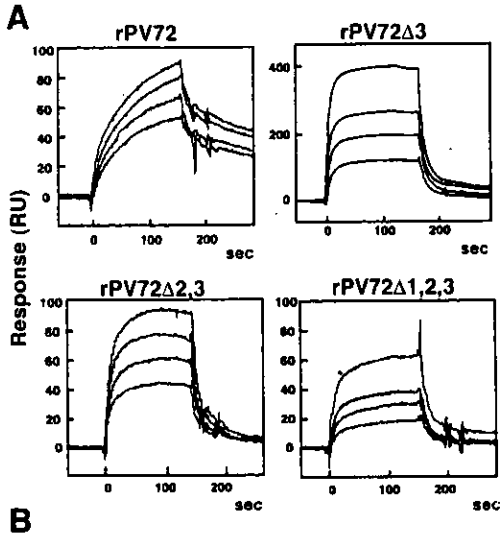


FIG. 4. **Modified PV72s showed a specific binding to the 2S-I peptide derived from the internal propeptide of a storage protein, 2S albumin.** *A*, rPV72 was subjected to an affinity column with either the 2S-I peptide or the mutant peptide with a replacement of RRE by GGG (2S-I/3G). Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. *FT*, flow-through fraction; *W*, washing fraction with the HEPES buffer, pH 7.0, with 1 mM $CaCl_2$; EDTA, eluted fraction by 2.5 mM EDTA. *B*, four modified PV72s were injected onto the sensor chip coupled with either the 2S-I peptide or the 2S-I/3G peptide to obtain the sensorgrams by surface plasmon resonance. The protein concentrations used were 0.7 μM for both rPV72 and rPV72Δ3, 1.6 μM for rPV72Δ2,3, and 1.4 μM for rPV72Δ1,2,3.



B

	k_a (per Ms)	k_d (per s)	K_D
rPV72	1.31×10^{4}	2.48×10^{-3}	0.2 μM
rPV72Δ3	2.43×10^{4}	4.95×10^{-2}	2.0 μM
rPV72Δ1,2	1.56×10^{4}	5.89×10^{-2}	3.8 μM
rPV72Δ1,2,3	9.26×10^{3}	3.89×10^{-2}	4.2 μM

FIG. 5. **Kinetics for the association and dissociation of the modified PV72s and the 2S-I peptide.** *A*, the surface plasmon resonance profiles for the association and dissociation curves of the modified PV72s and the 2S-I peptide. The coupling efficiency of the 2S-I peptide to the sensor surface was 1,200 resonance units. Each of the modified PV72s was injected onto the 2S-I sensor chip at different concentrations from 0.07 μM to 1.5 μM. *B*, the kinetic constants, an association rate constant (k_a), a dissociation rate constant (k_d), and a dissociation constant ($K_D = k_d/k_a$), were calculated from the above sensorgrams using BIA evaluation software version 2.1. These kinetic parameters were determined from three independent experiments.

massie Blue staining (Fig. 3*B*). All of their N-terminal amino acid sequences were determined to be RFVVEKNSLK, which corresponds to the N-terminal sequence of authentic pumpkin PV72 as reported by Shimada *et al.* (26). The results indicate that a signal peptide of the expressed proteins is correctly processed on the rough endoplasmic reticulum.

To investigate the binding ability of rPV72 to the ligand peptides, we performed a binding assay on the affinity column (2S-I column) that was conjugated with the 2S-I peptide, the internal propeptide of pumpkin 2S albumin. As shown in Fig. 4*A* (*upper*), rPV72 bound to the 2S-I column and then eluted with EDTA (discussed below). Previously we reported that the isolated PV72 from the maturing seeds of pumpkin binds to the 2S-I peptide but not to the mutant peptide 2S-I/3G with GGG instead of RRE of the internal propeptide. To clarify the specificity of the binding of rPV72, we used another affinity column (2S-I/3G column) that was conjugated with the 2S-I/3G peptide. As shown in Fig. 4*A* (*lower*), rPV72 did not bind to the 2S-I/3G column. The results indicate that the characteristics of rPV72 with respect to ligand binding were the same as those of the authentic PV72 has.

To identify the ligand-binding region of PV72, we performed a surface plasmon resonance analysis for four modified PV72s with either the 2S-I sensor chip or 2S-I/3G sensor chip. Each modified PV72 of the same concentration was injected onto the sensor chips. rPV72 bound to the 2S-I peptide, but not to the 2S-I/3G peptide (Fig. 4*B*), as expected from the result in Fig. 4*A*. All of the deleted proteins, rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3, also bound to the 2S-I peptide, but not to the 2S-I/3G peptide (Fig. 4*B*). This demonstrates that the deleted proteins also specifically recognize the RRE sequence of the 2S-I peptide as rPV72 does. These results indicated the N-terminal region of PV72 corresponding to rPV72Δ1,2,3 includes a ligand-binding site.

The EGF-like Motifs Modulate the Association and Dissociation of PV72 with the Ligand—We determined the kinetic parameters for the binding of each modified rPV72 to the 2S-I peptide by surface plasmon resonance. Each modified rPV72 was injected onto the 2S-I sensor chip to start the association reaction. Fig. 5*A* shows the association and dissociation curves obtained from the respective experiment with four different concentrations (0.07–2 μM) of each protein. The sensorgrams of rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3 exhibited more rapid association followed by more rapid dissociation after the injection was completed than did the sensorgram of rPV72.

The kinetic constants of association and dissociation were calculated from the slopes of the curves, as shown in Fig. 5*B*. In contrast to small differences of the k_a values among the modified rPV72, large differences of the k_d values were observed. The k_d value of each of rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3 was 16–23-fold higher than the k_d value of rPV72. The apparent equilibrium dissociation constant was determined from the ratio of these two kinetic constants (k_d/k_a). rPV72 has a high enough affinity for the 2S-I peptide (K_D = 0.2 μM) to function as a receptor. The K_D values of each of rPV72Δ3, rPV72Δ2,3, and rPV72Δ1,2,3 were 10-, 19-, and 21-fold higher than the K_d value of rPV72, respectively. Therefore, the affinity of rPV72

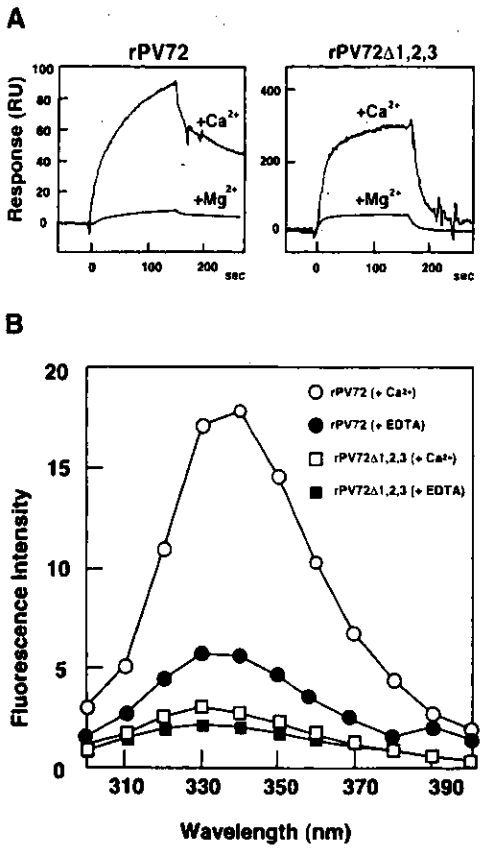


FIG. 6. **Ca^{2+}-dependent ligand-binding of PV72 and Ca^{2+}-induced conformational change in PV72.** *A*, divalent cation selectivity for the ligand binding. Either rPV72 or rPV72Δ1,2,3 was injected onto the 2S-I sensor chip in the presence of 1 mM $CaCl_2$ or 1 mM $MgCl_2$. *B*, fluorescence emission spectra showing the Ca^{2+}-induced structural change. The fluorescence emission of Trp and Tyr residues in rPV72 and rPV72Δ1,2,3 was measured from 300 to 400 nm after excitation at 280 nm. rPV72 (1 μg/ml) was suspended in the HEPES buffer plus 1 mM $CaCl_2$ (*open circles*) and the HEPES buffer plus 1 mM EDTA (*closed circles*). rPV72Δ1,2,3 (1 μg/ml) was in the buffer plus 1 mM $CaCl_2$ (*open squares*) and the HEPES buffer plus 1 mM EDTA (*closed squares*).

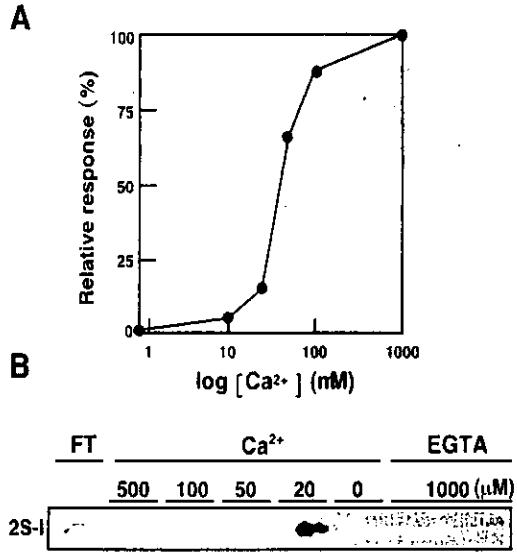


FIG. 7. **Regulation of the ligand binding of PV72 by the Ca^{2+} concentration.** *A*, rPV72 was injected onto the 2S-I sensor chip in the presence of various concentrations of $CaCl_2$. Relative responses were plotted against the concentration of Ca^{2+}. EC_{50} value for Ca^{2+} calculated was 40 μM. *B*, rPV72 was subjected to the 2S-I affinity column and eluted by the HEPES buffer containing $CaCl_2$ of various concentrations from 500 to 0 μM and finally by 1 mM EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. *FT*, flow-through fraction.

for the ligand peptide is much higher than the affinities of the rPV72s lacking the EGF-like motifs. It seems likely that the EGF-like motifs play a role in stabilizing the receptor-ligand complex.

The EGF-like Motifs Modulate a Ca^{2+}-dependent Conformational Change of PV72—The question is how the EGF-like motifs regulate the stability of the ligand binding of PV72. Previously, we found that the third EGF-like motif has a consensus sequence for Ca^{2+} binding, while the first and second motifs do not have such a sequence (26). Fig. 4*A* shows that rPV72 was eluted from the 2S-I column by the addition of chelating agents. This implied that Ca^{2+} binding to the third EGF-like motif might be important for the ligand binding. To clarify the requirement of Ca^{2+}, we performed an analysis of surface plasmon resonance with rPV72 in the presence of either Ca^{2+} or Mg^{2+}. Fig. 6*A* (*left*) shows that the interaction between rPV72 and the ligand was observed in the presence of Ca^{2+}, but not in the presence of Mg^{2+} instead of Ca^{2+}. This result indicates that Ca^{2+} is required for PV72 to interact with the 2S-I peptide.

Unexpectedly, however, rPV72Δ1,2,3, which lacks the EGF-like motifs, also showed a Ca^{2+}-dependent interaction with the 2S-I peptide, but not a Mg^{2+}-dependent interaction (Fig. 6*A*, *right*), as rPV72 did. This result indicates that the N-terminal region corresponding to rPV72Δ1,2,3 has another Ca^{2+}-binding site (s), although no consensus sequence for Ca^{2+} binding was found in the region. It should be noted that the affinity of PV72 (K_D = 0.2 μM) was 20-fold stronger than that of rPV72Δ1,2,3 (K_D = 4.2 μM). Thus, the Ca^{2+}-binding to the EGF-like motif must be required for the high affinity of PV72 for the ligand.

The next question raised is whether the Ca^{2+} binding causes a conformational change of PV72 that results in the higher affinity. To answer the question, we measured Ca^{2+}-dependent changes in the fluorescence emission spectra of Tyr or Trp residues in both rPV72 and rPV72Δ1,2,3. The rPV72 polypeptide includes 23 Tyr residues and 11 Trp residues. The fluorescence of these residues was monitored in the presence or absence of Ca^{2+}. Fig. 6*B* show a remarkable Ca^{2+}-dependent change in the fluorescence emission spectra of rPV72, but only a little change in the spectra of rPV72Δ1,2,3. The result suggests that the EGF-like motifs induced a much larger conformational change of PV72 in a Ca^{2+}-dependent manner than the N-terminal region corresponding to rPV72Δ1,2,3 did.

The Ligand Binding of PV72 Is Regulated by the Ca^{2+} Concentration Rather than pH—The next issue to be determined was a critical concentration of Ca^{2+} for association and dissociation of PV72. We performed a binding assay under the conditions of various Ca^{2+} concentrations by surface plasmon resonance. The EC_{50} value of the Ca^{2+}-dependent interaction was determined to be 40 μM (Fig. 7*A*). We also performed another binding assay by affinity chromatography with the 2S-I column. rPV72 that had bound to the 2S-I column was exposed to decreasing concentrations of $CaCl_2$ from 500, 100, 50, 20, and 0 μM. The rPV72 was eluted from the column under $CaCl_2$ concentrations lower than 50 μM (Fig. 7*B*). The $CaCl_2$ concentration was consistent with the EC_{50} value determined by the surface plasmon resonance. The results suggested that the interaction of PV72 to the ligand might be regulated by the Ca^{2+} concentration in the respective compartment of the maturing seed cells.

In general, binding of receptors to their ligands are known to be modulated by the environmental pH. This raised a question whether the interaction of PV72 with the ligand is also regulated by pH. To answer this question, we performed an assay

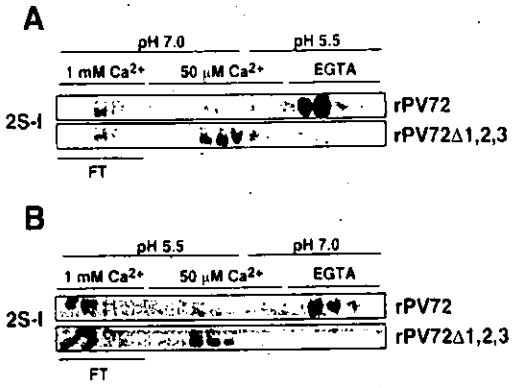


FIG. 8. **Modulation of Ca^{2+}-dependent ligand binding of rPV72 by the EGF-like motifs.** *A*, either rPV72 or rPV72Δ1,2,3 bound to the 2S-I column in the HEPES buffer, pH 7.0, with 1 mM $CaCl_2$. The elution pattern of each protein was examined with the sequential solutions; the HEPES buffer, pH 7.0, with 50 μM $CaCl_2$, the MES buffer, pH 5.5, with 50 μM $CaCl_2$, and the MES buffer with EGTA. *B*, either rPV72 or rPV72Δ1,2,3 bound to the column in the MES buffer with 1 mM $CaCl_2$. The elution pattern was examined with sequential solutions; the MES buffer with 50 μM $CaCl_2$, the HEPES buffer with 50 μM $CaCl_2$, and the HEPES buffer with EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. *FT*, flow-through fraction.

with the rPV72 and rPV72Δ1,2,3 that had bound to the 2S-I column in the presence of 1 mM $CaCl_2$. Fig. 8*A* (*upper*) shows that the rPV72 that bound at pH 7.0 was not eluted by decreasing the $CaCl_2$ concentration to 50 μM nor by decreasing the pH to 5.5, but was eluted with an EGTA solution. In contrast, the rPV72Δ1,2,3 bound to the column was easily eluted with the neutral buffer (pH 7.0) containing 50 μM $CaCl_2$ (Fig. 8*A*, *lower*). Alternatively, we performed another assay with the rPV72 bound to the 2S-I column at pH 5.5 in the presence of 1 mM $CaCl_2$. The result was similar as shown in Fig. 8*A*. The bound rPV72 was not eluted at pH 5.5 in the presence of 50 μM $CaCl_2$ (Fig. 8*B*, *upper*), while the bound rPV72Δ1,2,3 was easily eluted with the acidic buffer (pH 5.5) containing 50 μM $CaCl_2$ (Fig. 8*B*, *lower*).

The results indicated that the pH change did not affect the interaction between rPV72 and the ligand in the presence of 50 μM $CaCl_2$. When a more acidic buffer (pH 4.0) was used instead of the pH 5.5 buffer, an elution profile similar to that in Fig. 8*A* was obtained (data not shown). The presence of 50 μM $CaCl_2$ made the complex of rPV72 and the ligand stable under acidic conditions. The overall results suggested that the EGF-like motifs might be involved in the stability of the complex in the presence of 50 μM $CaCl_2$. Thus, it appears that the association and dissociation of PV72 with the ligand was modulated by the Ca^{2+} concentration rather than by the pH.

To clarify the effect of the 2S-I peptide sequence on the binding, we prepared affinity columns conjugated with three mutant peptides: 2S-I/P75G with Gly instead of Pro-75, 2S-I/E73G with Gly instead of Glu-73, and 2S-I/E79G with Gly instead of Glu-79. At neutral pH (pH 7.0), both rPV72 and rPV72D1,2,3 bound to all the columns with the mutant peptides in the presence of 1 mM $CaCl_2$ and then eluted with the EDTA solution (Fig. 9, *right*). Even at pH 4.0, both rPV72 and rPV72D1,2,3 bound to the 2S-I column and the 2S-I/P75G column, but they did not bind to either the 2S-I/E73G column or 2S-I/E79G column (Fig. 9, *left*). The results indicate that the PV72 has an ability to bind to the 2S-I peptide not only at pH 7.0 but also at pH 4.0, in the presence of Ca^{2+}. When Glu-73 or Glu-79 of the 2S-I peptide was substituted by Gly, the affinity of either rPV72 or rPV72D1,2,3 for the mutant peptide was reduced at pH 4.0, suggesting that both Glu-73 and Glu-79 are necessary for the binding of PV72 at acidic pH. PV72 might interact with pro2S albumin through the two Glu residues in Ca^{2+}-dependent manner.

DISCUSSION

Ca^{2+}-mediated Association and Dissociation of PV72 and the Ligand—Receptor-mediated protein sorting involves an association and dissociation of the receptor and the respective ligand by a modulator. In general, the environmental pH is known to regulate it as a modulator. Lysosomal proteins synthesized on the rough endoplasmic reticulum are reported to be recognized by a receptor and then delivered to the respective acidic compartment, where the ligands are dissociated from the receptor. The dissociation for both mannose 6-phosphate receptor has been shown to occur in the acidic organelles in mammals (1). Similarly in plants, the dissociation for BP-80 was known to occur at pH 4.0 (5).

We found, however, that PV72 binds to the 2S-I peptide even at pH 4.0 in the presence of Ca^{2+}. It does not appear that the acidic pH is responsible for the dissociation of PV72 from the 2S-I peptide. Our results demonstrated that Ca^{2+} functions as a modulator for the association and dissociation of PV72 with the internal propeptide of 2S albumin. The Ca^{2+} concentration in the subcellular compartments was reported to range from 1 to 1,500 μM (31). The EC_{50} (Ca^{2+}) value of 40 μM for the ligand binding of rPV72 is reasonable for the regulation of the association and dissociation of the receptor with the ligand within the cells.

PV72 has a consensus sequence for Ca^{2+}-binding in the third EGF-like motif (26). The motif might function as a Ca^{2+}-binding EGF (cbEGF) domain. Binding of Ca^{2+} to the cbEGF domain might cause a conformational change in the PV72 molecule to make the receptor-ligand complex stable. On the other hand, reduction of the environmental Ca^{2+} concentration might cause the dissociation of the ligand from the receptor.

PV72 has another Ca^{2+}-binding site in the N-terminal region corresponding to rPV72Δ1,2,3, which lacks a consensus sequence for Ca^{2+} binding. However, binding of Ca^{2+} to the region causes only a small conformational change in the PV72. The N-terminal region might play a role in the formation of the ligand-binding pocket with the assistance of Ca^{2+} (discussed below). The binding of Ca^{2+} to both the N-terminal region and the cbEGF domain of PV72 could induce the formation of a functional pocket for the ligand binding and stabilize the receptor-ligand complex.

The low density lipoprotein receptor has three EGF motifs, one of which is cbEGF (32). The receptor also has a ligand-binding region with another Ca^{2+}-binding site (33), and the receptor-ligand complex is stabilized by Ca^{2+} in the receptor molecules (33, 34). Despite the very low identity of the sequence between the low density lipoprotein receptor and PV72, the mechanisms of Ca^{2+}-mediated association and dissociation of the receptor and ligand are similar to each other. The Ca^{2+}-mediated regulation through the cbEGF domain is reported to be crucial in mammals. Mutations in a cbEGF domain of the low density lipoprotein receptor have been shown to cause familial hypercholesterolemia (33) and a mutation in the cbEGF of fibrillin causes Marfan syndrome (35).

The Protease-associated Domain of PV72 Might Be Involved in the Ligand Binding—The lumen domain of BP-80 consists of three domains: an N-terminal domain homologous to RemembR-H2 (RMR) protein, a central domain, and a C-terminal EGF repeat domain (7). It has been shown that the former two domains together determine the NPIR-specific ligand binding site and an EGF repeat domain of BP-80 alters the conformation of the other two domains to enhance ligand binding (7). These results are consistent with our findings that PV72 forms the ligand-binding pocket in the N-terminal region correspond-

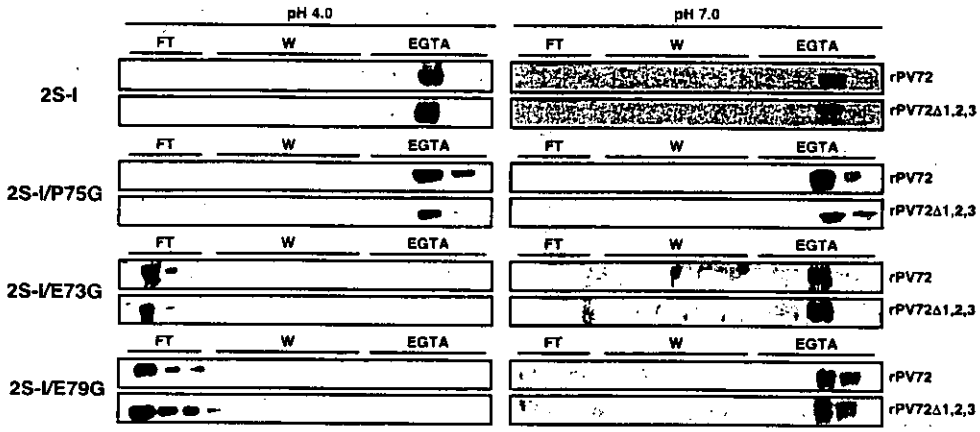


FIG. 9. **Effect of pH on the binding of rPV72s to the 2S-I peptide and the mutant peptides.** The affinity column conjugated with each of the 2S-I peptide, 2S-I/P75G, 2S-I/E73G, or 2S-I/E79G was used, as shown on the *left*. Either rPV72 or rPV72Δ1,2,3 was applied to the column under neutral conditions (pH 7.0, *right panel*) or under acidic conditions (pH 4.0, *left panel*). The column was washed with the respective buffer and finally with the buffer containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and then to an immunoblot with anti-PV72 antibodies. *FT*, flow-through fraction; *W*, washing fraction.

ing to rPV72Δ1,2,3 and that the EGF-like motifs modulate a Ca^{2+}-dependent conformational change of PV72. It seems likely that the EGF-like motifs play a role in stabilizing the receptor-ligand complex. Both the N-terminal region corresponding to rPV72Δ1,2,3 and the RMR homology domain of BP-80 contain a protease-associated domain, which is speculated to be involved in substrate determination for peptidases or to form protein-protein interactions (36, 37). It is possible that each protease-associated domain of PV72 and BP-80 mediates the interaction with the ligand.

We found that mutation of either Glu-73 or Glu-79 reduced the affinity of PV72 for the ligand peptide under acidic conditions. Two Glu residues of the 2S-I peptide might be important for stability of the ligand-receptor interaction under acidic conditions. PV72 might interact with the ligand through the two Glu residues, one of which is included in the RRE sequence in the 2S-I peptide. Previously we reported that the RRE sequence was essential for the interaction with PV72 (26). The essential sequence is different from the targeting determinant, NPIR, of the lytic enzymes.

PV72 Might Be Recycled between the PAC Vesicles and Golgi Complex—When a fusion protein of green fluorescent protein with a transmembrane domain and a cytoplasmic tail of PV72 was expressed in tobacco BY2 cells, a fluorescent Golgi complex was observed. The localization of PV72 in the Golgi complex is supported by the findings that PV72 has a complex glycan.[2] The peripheral region of the PAC vesicles was labeled with gold particles for complex glycans in the immunoelectron micrograph (25). It is possible that PV72 is recycled between the Golgi complex and the PAC vesicles in maturing seeds. It was suggested that the cytosolic tail of BP-80 is responsible for the retrieve from the prevacuolar compartments to the Golgi complex in the protoplasts of the transgenic tobacco (14).

We clearly demonstrated that the interaction of PV72 with the 2S-I peptide is modulated by the Ca^{2+} concentration. The Ca^{2+} concentration in the Golgi complex was determined to be 300 μM (38), which is high enough for PV72 to bind the ligand. On the other hand, the Ca^{2+} concentration in the endoplasmic reticulum ranges from 1 to 1,500 μM depending on the region of the endoplasmic reticulum (31). Thus, the endoplasmic reticulum-derived PAC vesicles possibly have a Ca^{2+} concentration lower than 50 μM, which could dissociate the ligand from the receptor-ligand complex.

[2] E. Watanabe, T. Shimada, M. Nishimura, and I. Hara-Nishimura, unpublished data.

Previously we reported that most of pro2S albumin synthesized on the endoplasmic reticulum are directly incorporated into the PAC vesicles, in a Golgi-independent manner (24, 25). It is possible that PV72 might trap the escaped pro2S albumin that leaves the rough endoplasmic reticulum for the Golgi complex and recruit them from the Golgi complex to the PAC vesicles. On the contrary, BP-80 has been shown to be rich in CCVs, but not in dense vesicles responsible for transport seed globulins in maturing pea seeds (12). BP-80 might transport an NPIR-containing proteinase from Golgi complex to prevacuolar compartments via CCVs. Thus, the possibility cannot be excluded that PV72 mediates the transport of an NPIR-containing proteinase from Golgi complex to the PAC vesicles or from the PAC vesicles to lytic compartments. To clarify the intracellular pathway regulated by the vacuolar sorting receptors, further analysis of subcellular localization of the receptors in Golgi complex or prevacuolar compartments in addition to the PAC vesicles is required. Further investigation of the organ-specific and temporal expression of the receptors will also provide us an insight into the physiological function of them.

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In vivo demonstration of receptor-dependent transport of an NPIR-containing proteinase. （投稿中）

Shimada, T., Watanabe, E., Tamura, K., Hayashi, Y., Nishimura, M., and Hara-Nishimura, I.

A vacuolar-sorting receptor on the membrane of PAC vesicles that accumulate precursors of seed storage proteins. （投稿中）

In Vivo Demonstration of Receptor-Dependent Transport of an NPIR-Containing Proteinase

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Running title: Receptor-dependent transport of an NPIR-containing proteinase

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SUMMARY

PV72 is a putative vacuolar sorting receptor that was originally found in developing pumpkin seeds. To elucidate a physiological function of such putative vacuolar sorting receptor in plant cells, we produced transgenic Arabidopsis plants that expressed a fusion protein (PV72-HDEL) composed of the lumen domain of PV72 with a His-tag and an endoplasmic reticulum (ER)-retention signal, HDEL. The ectopic overexpression of PV72-HDEL induced the accumulation of a precursor of a cysteine proteinase, AtALEU, which contains a vacuolar targeting signal, NPIR, in the N-terminal propeptide, but not a precursor of another cysteine proteinase, RD21, which contains no NPIR sequence. Subcellular fractionation revealed that that the AtALEU precursor was associated with PV72-HDEL in the ER of the transgenic plants. To clarify the interaction between the receptor and the ligand, we expressed the lumenal domain of PV72 (rPV72) in the insect cells and analyzed their ability to bind the NPIR-containing propeptide of the AtALEU precursor by affinity chromatography and surface plasmon resonance. rPV72 bound to the propeptide with a K_D value of 0.1 μM, which was low enough for it to function as a receptor. The association of rPV72 with the NPIR-containing propeptide was stabilized in the presence of 1 mM $CaCl_2$. rPV72 bound to the ligand even at pH 4.0 in the presence of Ca^{2+}. Deletion of three repeats of EGF-like motifs of the lumenal domain reduced the affinity (K_D value of 1.2 μM) between the rPV72Δ1,2,3 and the propeptide. Overall results suggest that PV72 homolog(s) function as a sorting receptor for the NPIR-containing proteinase AtALEU to be transported to the lytic vacuoles and that the receptor-mediated transport is regulated by Ca^{2+} concentration rather than the environmental pH.

INTRODUCTION

Higher plants have two types of vacuoles: one type is lytic vacuoles which contain various lytic enzymes and develop in the vegetative organs, and another type is protein storage

vacuoles which contain storage proteins and develop mainly in storage organs. Both types of vacuoles, however, are found in the same cells of barley roots (Hara-Nishimura, et al., 1993a) and of maturing pea seeds (4). In these cells, vacuolar proteins synthesized on the rough endoplasmic reticulum (ER) are sorted and delivered to their respective vacuoles (Okita and Rogers, 1996) (Neuhaus and Rogers, 1998). The sorting and delivery should be mediated by some machinery. In mammalian system, mannose-6-phosphate in glycosyl side-chains of glycoproteins is known to function as a targeting signal to the lysosomes, and mannose-6-phosphate receptors have been identified as a lysosome sorting receptor for lysosomal proteins (Dahms, et al., 1989). The vacuolar proteins of both plant and yeast cells do not have such mannose 6-phosphate residues. Thus, vacuolar proteins have some sorting information on their polypeptide. In yeast system, a short stretch sequence of amino acids, QRPL, in procarboxypeptidase Y, is known to function as a targeting signal to the vacuoles, and Vps10p has been identified as a vacuolar sorting receptor for vacuolar hydrolases (Marcusson, et al., 1994).

In plants, vacuolar targeting signals have been well characterized for soluble vacuolar proteins (Matsuoka and Neuhaus, 1999) (Beevers and Raikhel, 1998). The signals are separated into three classes (Matsuoka and Neuhaus, 1999). First is sequence-specific vacuolar sorting signals that are located in the N-terminal propeptides (NTPPs) or C-terminal propeptides (CTPPs) as well as within the mature proteins. An NPIR sequence that was conserved in the NTPPs of barley aleurain and sweet potato sporamin has been shown to function as a vacuolar targeting signal in vivo (Holwerda, et al., 1992) (Matsuoka and Nakamura, 1991). A replacement of Ile of the NPIR sequence of sporamin by Gly abolished the vacuolar targeting ability of the protein (Matsuoka and Nakamura, 1991). The consensus composition of the NPIRL sequence of sporamin was determined to be <preferably Asn>, <not acidic>, <Ile or Leu>, <any amino acid>, <large and hydrophobic> (Matsuoka and Nakamura, 1999). Recently, putative vacuolar sorting receptors that interacted with the NPIR-containing propeptide of aleurain were reported; pea BP-80 (Beevers and Raikhel, 1998; Kirsch, et al., 1994) (Paris, et al., 1997), pumpkin PV72 (Shimada, et al., 1997) and

Arabidopsis AtELP (Ahmed, et al., 1997), These proteins are a type-I integral membrane protein with epidermal growth factor (EGF)-like motifs in the lumen domain. These putative vacuolar sorting receptors of BP-80 (Cao, et al., 2000), PV72 (unpublised data, Watanabe et al.) and AtELP (Ahmed, et al., 2000) were reported to bind to the NPIR-containing propeptide of sporamin, but not to the NPGR-containing.

Second class of the vacuolar targeting signals was reported to be located in the CTPPs of barley lectin and tobacco chitinase, which have no significant conserved sequence between them (Bednarek and Raikhel, 1991) (Neuhaus, et al., 1991) (Mitsuhashi, et al., 2000). Since the addition of two glycines at the C terminus of barley lectin propeptide destroyed the vacuolar-targeting signal, the accessibility of the C terminus is crucial for vacuolar targeting. The position at the very end of the polypeptide is essential for such C-terminal signals rather than the amino acid sequences. A putative vacuolar sorting receptor, PV72, was found to bind not only the NPIR-containing propeptide of aleurain but also the CTPP of 2S albumin, one of the major storage proteins (Shimada, et al., 1997). BP-80 was also reported to bind both NPIR-containing propeptide of aleurain and the CTPP of 2S albumin of Brazil nuts (Kirsch, et al., 1996). A BP-80 homolog was co-purified with the protease inhibitor containing a possible vacuolar targeting signal in the CTPP (Miller, et al., 1999).

Third class of the vacuolar targeting signals was reported to be located in the internal propeptide (ITPP) of the ricin precursor (proricin) with a sequence specific determinant for vacuolar sorting (Frigerio, et al., 2001). The sequence, LLIRP, resembles NPIRL of the NTPP of sporamin. The mutation of the Ile residue of the ITPP propeptide affected the sorting of proricin to the vacuoles. PV72 has been shown to bind to the ITPP of 2S albumin (Shimada, et al., 1997).

In contrast to well characterization in vitro, the demonstration for the in vivo function of vacuolar sorting receptors has been poorly done. BP-80 was reported to bind to proprotein precursor of petunia aleurain in yeast cells (Humair, et al., 2001). However, the demonstration of the sorting to vacuoles by such receptor molecules has not been done in plant cells. In this study, we demonstrated that a PV72 homolog functions as a vacuolar

sorting receptor in vivo and mediates the transport of a lytic enzyme to the vacuoles, using the transgenic Arabidopsis plants which accumulated soluble PV72 in the ER. The transgenic plants showed the accumulation of the NPIR-containing cysteine proteinase AtALEU, but not that of non-NPIR-containing proteinase RD21. This is the first demonstration for in vivo function of such putative receptors in plant cells.

RESULTS

Overexpression of ER-localized PV72-HDEL Results in the Accumulation of a Proprotein Precursor of AtALEU in the Transgenic Arabidopsis Plants

BP-80 and AtELP have been reported to be a putative vacuolar sorting receptor responsible for delivery of the NPIR-containing vacuolar proteins to lytic vacuoles. To demonstrate their function in vivo, we performed overexpression of the homolog, PV72, in Arabidopsis plants. We introduced a chimeric gene encoding a fusion protein (PV72-HDEL) composed of a signal peptide and the lumenal domain of PV72 followed by a His-tag and an ER retention signal, HDEL, into the Arabidopsis plants under the control of the cauliflower mosaic virus 35S promoter. The transgenic plants accumulated PV72-HDEL in the ER as shown in Figure 1A (left). If such putative receptors interact with some vacuolar protein synthesized on the ER, the transport to the vacuoles should be affected by the accumulation of the modified receptor molecules in the ER. Arabidopsis has two types of vacuolar cysteine proteinases of the papain family in the lytic vacuoles; AtALEU that is a homolog of barley aleurain and RD21 that is a product of responsive-to-desiccation gene 21 (Koizumi et al. 1993); Yamada]. The vacuolar targeting signal, NPIR, is found in the NTPP of AtALEU (Lohman, et al., 1994), but not in that of RD21. This raises the question of whether the transport of these vacuolar proteins is affected by overexpression of the soluble PV72-HDEL molecules in the ER. To answer it, we investigated the transport of the two proteinases in the transgenic plants.

We found a specific accumulation of the 41-kD proprotein precursor of AtALEU in the

transgenic plants expressing PV72-HDEL (Figure 1B). On the other hand, wild plants accumulated only the 28-kD mature form of AtALEU, but not the precursor. The molecular masses of 41-kD and 28-kD was larger than the deduced molecular mass from the amino acid sequence of the AtALEU precursor and the mature form, respectively. N-linked glycosylation at two sites might account for the size discrepancy. On the contrary, a precursor of RD21, which has not the vacuolar-targeting signal NPIR was not detected in both the transgenic and wild plants (Figure 1C).

The AtALEU Precursor Is Accumulated in the Microsome Fraction of the Transgenic Arabidopsis Plants Expressing PV72-HDEL

The next issue to be resolved is subcellular localization of the accumulation of the AtALEU precursor in the transgenic plants. To answer the question, we performed a subcellular fractionation of both transgenic and wild plants by differential centrifugation. An immunoblot with anti-aleurain monoclonal antibody of each fraction showed that the total homogenates (T) of both the transgenic (T/PV72) and wild (wt) plants contained the 28-kD mature form of AtALEU (m) and 38-kD intermediate form of AtALEU (i), but no 41-kD precursor (Figure 2A and 2B). Overexpression of PV72-HDEL resulted in the accumulation of the AtALEU precursor (p) in the P8 and the P100/microsomal fractions of transgenic plants (Figure 2A). The AtALEU precursor was not detected in the wild plants. The mature form of AtALEU (m) was predominantly found in the S100/vacuolar fraction of both transgenic and wild plants. The results indicated that the overexpression of the vacuolar sorting receptor in the ER affected specifically the transport of AtALEU that has a vacuolar targeting signal, NPIR, sequence in the polypeptide. PV72-HDEL might prevent the AtALEU precursor from leaving the ER by trapping the molecules.

Co-purification of PV72-HDEL with the NPIR-containing AtALEU Precursor, but not with the Mature AtALEU

To clarify the function of the complex of the His-tagged PV72-HDEL and the AtALEU

precursor in the microsomal fractions from the transgenic Arabidopsis plants (T/PV72), we performed an affinity chromatography with the Ni-NTA resin column. The His-tagged PV72-HDEL bound to the Ni-NTA column and was not detect in the flow through (FT) fraction. His-tagged PV72-HDEL was eluted in the CHAPS buffer containing 400 μM imidazol and detected in the eluted (E) fraction (Figure 3A).

To confirm formation of the complex of PV72-HDEL and the AtALEU precursor, each fraction was subjected to an immunoblot analysis with anti-aleurain monoclonal antibody. The mature form of 28-kD AtALEU (m) and the intermediate form of 38-kD AtALEU (i) were detected in the FT fraction of transgenic plants (Figure 3B). The 41-kD AtALEU precursor (p) was specifically detected in the E fraction of transgenic plants. On the contrary, mature form of RD21 was not co-purified with PV72-HDEL (data not shown). These results suggest that the AtALEU precursor was recognized by a PV72-like vacuolar sorting receptor in vivo to be transported to the vacuoles.

The Propeptide of AtALEU at the N terminus as A Vacuolar-Targeting Signal

The NTPP of AtALEU contains an NPIR sequence as known a vacuolar-targeting signal (Figure 4A, upper). To confirm the function of the NTPP of AtALEU as a vacuolar-targeting signal, we constructed a chimeric gene encoding a signal peptide, the NTPP of AtALEU (T-75). To know the localization of AtALEU-GFP, the transformed cells were inspected with a laser-scanning confocal microscope. AtALEU-GFP was accumulated in the large compartments as well as lytic vacuoles at 3 day after transformation (Figure 4B). This result indicated that NTPP of AtALEU actually functions as a vacuolar-targeting signal for the lytic enzyme.

Kinetic Analysis of the Interaction between the Modified PV72s and the NTPP of AtALEU

To determine the kinetic parameters for the binding of the receptor and the ligand, we expressed two modified PV72s; rPV72 composed of the lumen domain followed by a His-tag

and the HDEL sequence and rPV72Δ1,2,3 composed of rPV72 with no EGF-like motif in insect Sf21 cells employing a baculovirus expression system (Watanabe, et al., 2002). For surface plasmon resonance, each modified PV72s was injected onto the AtALEU sensor chip to start the association reaction. Figure 5A shows the association and dissociation curves obtained from the respective experiment with four different concentrations (0.15-0.7 μM) of each protein. The sensorgrams of rPV72Δ1,2,3 exhibited more rapid association followed by more rapid dissociation after the injection was completed than did the sensorgram of rPV72. The kinetic constants of association and dissociation were calculated from the slopes of the curves, as shown in Figure 5B. The apparent equilibrium dissociation constant was determined from the ratio of these two kinetic constants (k_d / k_a). The K_D value of rPV72 for the AtALEU peptide was 0.1 μM and enough high as the vacuolar sorting receptor. The K_D value of rPV72Δ1,2,3 was 10-fold higher than that of rPV72. Previously we demonstrated that the affinity of rPV72 for the ligand peptide is much higher than the affinities of the rPV72s lacking the EGF-like motifs. It seems likely that the EGF-like motifs play a role in stabilizing the vacuolar sorting receptor -ligand complex.

Modified PV72s Interact with the NPIR-Containing Peptide in a Ca^{2+} -Dependent Manner

Previously, we found that the interaction of PV72 with the internal propeptide of 2S albumin was recognized by Ca^{2+} concentration rather than pH (Watanabe, et al., 2002). To clarify a Ca^{2+} -dependent interaction between rPV72 and the NPIR-containing peptide, we performed binding assay with an affinity column conjugated with the NTPP of AtALEU peptide in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. Modified PV72s bound to the column and was detected in neither the FT or W fractions (Figure 6A). Modified PV72s was eluted with the HEPES buffer (pH 7.0) containing 2.5 mM EGTA and was detected in the E fraction (Figure 6A). These results indicated that modified PV72s interacted with the NPIR-containing peptide in a Ca^{2+} -dependent manner as in the case of the 2S-I peptide.

The interaction between AtELP and the NPIR-containing peptide was reported to be pH-

dependent (Ahmed, et al., 2000). To investigate the effect of pH on the interaction between modified PV72s and the NPIR-containing peptide, we performed the binding assay with the affinity column at pH 4.0. rPV72 still bound to the column at pH 4.0 in the presence of 1 mM $CaCl_2$ and eluted by the addition of EGTA. These results indicated that the acidic condition did not affect the interaction between rPV72 and the NPIR-containing peptide. On the contrary, we found that rPV72Δ1,2,3 without EGF-motifs was detected in the pH 4.0 fractions in the Na-acetate buffer (pH 4.0) containing 1 mM $CaCl_2$ (Figure 6B). The interaction between rPV72Δ1,2,3 and the peptide was affected by the acidic condition. These results indicated that the binding site for the ligands is located in the N-terminal region of the luminal domain of PV72 and that the EGF-like motifs function as the stabilizing domain for the complex of interaction and the ligand.

DISCUSSION

Receptor-Mediated Transport of AtALEU in Arabidopsis Plants

Recently, Humair et al. (2001) reported that BP-80 interacted with a fusion protein composed of the NTPP of petunia aleurain and GFP in the transformed yeast cells defective for its own vacuolar sorting receptor, VPS10p. This interaction might lead to the transport of the reporter protein through the yeast secretary pathway to the vacuoles (Humair, et al., 2001). However, the physiological function of such putative receptors has not been demonstrated in plant cells. We found that the ectopic expression of PV72-HDEL induced the accumulation of the AtALEU precursor and the formation of the complex of PV72-HDEL and the precursor in the ER. It is noted that PV72-HDEL exhibited an ability to form the complex with the precursor but not with the mature form (Figure 3). The NPIR-containing propeptide of the AtALEU precursor might be essential for the formation of the complex. The propeptide was shown to function as a vacuolar-targeting signal in Arabidopsis cells (Figure 4). These findings suggest that the receptor binds to the NPIR-containing peptide of the AtALEU precursor within the

ER. This is the first demonstration that PV72 homologs function as a vacuolar sorting receptor for a vacuolar-targeting signal, NPIR, in Arabidopsis cells.

Here, we clearly demonstrated that PV72 bound to the NPIR-containing propeptide of AtALEU with K_D value of 0.1 μM, which was larger than 0.037 μM for BP-80 for the NPIR-containing propeptide of AtALEU (Kirsch, et al., 1994). The difference between the two values might be derived from the difference of assay methods: the value for PV72 was obtained by surface plasmon resonance analysis and that for BP-80 was by an affinity chromatography. , These values are enough low to function as a vacuolar sorting receptor in plant cells.

Arabidopsis plants has a putative vacuolar targeting receptor, AtELP, that could function as a counter part of PV72. AtELP was reported to be interacted with the NPIR-containing propeptide of AtALEU and localized in the Golgi apparatus (Ahmed, et al., 2000). In wild Arabidopsis plants, the NPIR-containing precursor is transported to Golgi apparatus where they are trapped by the receptors. On the other hand, in the transgenic plants, overexpressed PV72-HDEL prevent the precursor from leaving ER to the Golgi apparatus. It is possible that the interaction between the vacuolar sorting receptor and the NPIR-containing propeptide leads to the transport of the lytic enzyme to the lytic vacuoles in plant cells.

Another Transport Pathway for Vacuolar Proteins via ER-Derived Compartments

PV72 was originally found in the PAC-vesicles that accumulate the precursor proteins of major seed storage proteins including 2S albumin and 11S globulin (Hara-Nishimura, et al., 1998). Recently, we found that PV72 has an ability to bind with the internal propeptide of 2S albumin of pumpkin (unpublished data). PV72 was specifically and transiently accumulated in maturing seeds in association with the synthesis of seed storage proteins (unpublished data). These results implied that PV72 mediate the transport of 2S albumin in maturing seeds. We reported that the PAC vesicles are derived from ER and mediate a transport of the storage protein precursors directly to protein storage vacuoles (Hara-Nishimura, et al., 1998). Thus,

most of pro2S albumin molecules that are synthesized on ER are directly transported to the PAC vesicles (Hara-Nishimura et al., 1993a). When some pro2S albumin molecules leave the ER for the Golgi complex, the escaped molecules might be recruited from the Golgi complex to the PAC vesicles by PV72. This raises the question of whether pro2S albumin is accumulated in the ER of the transgenic plants as the AtALEU precursor. However, we could not detect the accumulation of pro2S albumin in the microsomal fraction from the maturing seeds of the transgenic plants from the following two reasons. First, the level of the accumulation of PV72-HDEL was very low in the maturing seeds. This was also supported by the report that the expression of introducing genes in seed is weak than the other tissues under the control of 35S promoter (Zuo, et al., 2001). Second, most of pro2S albumin is transported directly to vacuoles via the PAC vesicles as described above.

In *Vigna mungo* seedlings, a KEDL-tailed cysteine protease, SH-EP, was packed in the KDEL vesicle that was derived from the ER (Mitsuhashi, et al., 1986; Toyooka, et al., 2000). SH-EP was transported to vacuoles via KDEL vesicles in a Golgi-independent manner (Toyooka, et al., 2000). Recently, a receptor homolog that bound to the SH-EP precursor was found in the seedlings (Tsuru-Furuno, et al., 2001). It is possible that the receptor homolog traps the SH-EP precursor leaving ER for Golgi apparatus to deliver it to the KDEL vesicles and/or the vacuoles.

In contrast to vacuolar sorting receptor -mediated transport of the AtALEU precursor, the transport of RD21 to lytic vacuoles is not affected by the accumulation of PV72-HDEL in the ER. The transport of RD21 might be different from that of AtALEU. RD21 is a stress-inducible proteinase (Kinoshita, et al., 1999; Koizumi, et al., 1993; Yamada, et al., 2001). Recently, The RD21 precursor is accumulated in the ER-body, which is a ER-derived organelle in the epidermal cells of healthy Arabidopsis seedlings (Hayashi, et al., 2001). The ER bodies appear to be a novel proteinase-sorting system that assists in cell death under the stressed conditions. When seedlings are stressed with a concentrated salt solution, leading to death of the epidermal cells, the ER bodied start to fuse with vacuoles, thereby mediating the delivery of the precursor to the lytic vacuoles.

Unique Interaction Between PV72 and NTPP in a Ca^{2+}-Dependent Manner

The pH gradient is known to exist from the ER to the vacuole that is a typical acidic compartment. The abnormal pH of vacuoles induced the miss sorting of the NPIR-containing protein (Matsuoka, et al., 1997). The binding of BP-80 and AtELP to the ligand was described to be regulated by environmental pH (Ahmed, et al., 2000; Kirsch, et al., 1994). The optimum pH for the ligand binding of the receptors was determined to be pH 6.0 to 6.5 and the dissociation of the receptor and the ligand occurred at pH 4.0 (Kirsch, et al., 1994). It is possible that such receptors bind to the ligand in Golgi apparatus and release the ligand in a late endosomal or prevacuolar compartment whose inside pH might be low (Ahmed, et al., 2000; Paris, et al., 1997).

We demonstrated that rPV72 interacts with the NPIR-containing propeptide of AtALEU in a Ca^{2+}-dependent manner (Figure 6). This was consistent with our previous finding that rPV72 bound to the 2S-I peptide in a Ca^{2+} -dependent manner (Watanabe, et al., 2002). These receptors have a consensus sequence for Ca^{2+}-binding in the third EGF-like motif (Shimada, et al., 1997). The motif might function as a Ca^{2+}-binding EGF (cbEGF) domain. Previously, we demonstrated that binding of Ca^{2+} to the cbEGF domain might cause a conformational change in the PV72 molecule to make the receptor-ligand complex stable (Watanabe, et al., 2002). On the other hand, reduction of the environmental Ca^{2+} concentration might cause the dissociation of the ligand from the receptor. The association and dissociation regulated by Ca^{2+} concentration is a unique mechanism for transport of vacuolar proteins.

Under the acidic condition, rPV72Δ1,2,3 bound to the NPIR-containing propeptide of AtALEU (Figure 6), but not to the 2S-I peptide (Watanabe, et al., 2002). The different binding ability might be due to the pIs of the ligands. The NPIR-containing propeptide of AtALEU has pI 4.0 and the 2S-I peptide has pI 10.0. The surface charges of these peptides change in association with the change of environmental pH. The different surface charges might cause the different affinity of rPV72Δ1,2,3 with each peptide.

Vacuolar Sorting Receptor PV72 and Its Homologues

PV72 was the most abundant protein of the PAC vesicles after storage protein precursors (Shimada, et al., 1997). We clearly demonstrated that PV72 has an ability to bind pro2S albumin in a Ca^{2+} dependent manner (unpublished data). PV72 functions as a sorting receptor for 2S albumin. This was supported by the evidence that the expression of PV72 is specific to maturing seeds and the accumulation of PV72 is accompanied by the synthesis of 2S albumin. PV72 is transiently accumulated in the pumpkin cotyledons at the middle stage seed maturation, but not in the vegetative tissues (unpublished data).

On the contrary, PV82, which is a homologue of PV72 (Shimada, et al., 1997), is widely distributed in both maturing seeds and the vegetative tissues of pumpkin (unpublished data). We found that PV82 bound more preferably to the propeptide of barley aleurain than PV72 (unpublished data). It is likely that PV82 is analogous to BP-80 that is known to be a sorting receptor for aleurain to be transported to lytic vacuoles (Humair, et al., 2001). These findings implied that sorting receptors should be separated into two types; one for protein storage vacuoles and another for lytic vacuoles. Homologues of the vacuolar sorting receptor including 7 homologues of Arabidopsis can be divided into two or three groups. PV72 and BP-80 belong to different group from each other on the phylogenetic tree of the receptors.

PV72 has been shown to interact with pro2S albumin, indicating that pro2S albumin is a natural ligand of the receptor. However, there has been only a few report of the interaction of such receptors with natural ligand proteins. A receptor homolog was reported to interact with a protease inhibitor precursor of stigma of *Nicotiana alata* (Miller, et al., 1999). In germinating cotyledons of *Vigna mungo*, a receptor homologue was also reported to interact with a proprotein precursor of a cysteine protease, SH-EP (Tsuru-Furuno, et al., 2001).

The members of the PV72 group might function for protein storage vacuoles, while those of the BP-80 group might function for lytic vacuoles. PV72 is localized in the PAC vesicles, but not in clathrin coated vesicles in maturing pumpkin seeds (unpublished data). The Arabidopsis homologue which is same group of PV72, located at plasma membrane of leaf, root and silique (Laval, et al., 1999). On the contrary, BP-80 has been shown to be rich

in clathrin coated vesicles, but not in dense vesicles responsible for transport seed globulins in maturing pea seeds (Hinz, et al., 1999). BP-80 was localized in clathrin-coated vesicles and *trans*-Golgi network (Hinz, et al., 1999). The Arabidopsis homolog in the same group of BP-80 was expressed in leaves and silique (Laval, et al., 1999). The tissue specific and time specific expression of each receptor is a key to determine the ligand specificity for the receptor.

METHODS

Plant Materials

Arabidopsis thaliana (ecotype Columbia) was used throughout this work. Seeds of Arabidopsis were surface-sterilized and then sown on soil or onto 0.5% Gellan Gum (Wako, Tokyo, Japan) that contained Murasige-Skoog's medium and were grown at 22 °C under continuous light. We harvested young seedlings for the experiment of subcellular fractionation. The 2 to 3-week-old plants were homogenized with a SDS-buffer solution and subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies, anti-AtALEU antibody and anti-RD21 antibodies as described below.

Plasmid Construction of PV72-HDEL and Transformation of Arabidopsis Plants

The cDNA for PV72 and two oligonucleotides, 5' GAAACAAATGAGTGTTTGACA 3' and 5'
GGTACCTCAAAGCTCATCGTGGTGGTGGTGGTGGTGTCCGGGCCCGCTTGTCACA GTGCTCCC 3' were used to amplify a DNA fragment encoding a fusion protein (PV72-HDEL) composed of a signal peptide and a lumenal domain of PV72 followed by a poly-histidine tag and an ER-retention signal, HDEL (Chiu, et al., 1996; Mitsuhashi, et al., 2000). The DNA fragment was digested with *Hind*III and *Kpn*I and was inserted into pBS-SK to

produce pBS-PV72-HDEL. The *Xba*I-*Kpn*I-digested pBS-PV72-HDEL was introduced into Ti-plasmid pBI121HmRV (Kinoshita, et al., 1999) to produce the vector, pBI-PV72-HDEL.

The vector pBI-PV72-HDEL was introduced into *Agrobacterium tumefaciens* (strain EHA101; Hood et al. 1986) by electroporation. Arabidopsis plants transformed with the gene via *Agrobacterium tumefaciens* according to the method of Matsuoka and Nakamura (1991)(Bechtold and Pelletier, 1998).

T_1 seeds were surface-sterilized and sown on a Petri dish containing half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), 0.5% (w/v) sucrose, 0.8% (w/v) agar and kanamycinan (75 mg/mL). We selected the transformant (T/PV72) expressing a high amount of PV72-HDEL and used it for the experiments.

Plasmid Construction of AtALEU-GFP and Transformation of Arabidopsis T-75 Cultured Cells

The cDNA for AtALEU and two oligonucleotides, 5' CCACATGTCTGCGAAAACAAT 3' and 5' GTTTCCATGGTAGCTGCTTCTGTGACCT 3' were used to amplify a DNA fragment encoding an N-terminal region (184 amino acids) of preproAtALEU, which included a signal peptide and a propeptide followed a part of the mature protein. The *Afl*III-*Sac*I digested fragment was inserted into the Ti-plasmid pSPGFP (x Mitsuhashi, PCP).

The vector containing the *Afl* III - *Sac* I fragment was designated as pSKNPIRGFP. pSKNPIRGFP encoded a fusion protein composed of both the signal sequence and the proregion of AtALEU followed by an GFP sequence. A fragment containing Xho I and Sac I was ligated to thep35SLM digested with Xho I and Sac I. The vector containing the Xho I - Sac I fragment was designated as p35NPIRGFP.

Immunoblot Analysis

Immunoblot analysis was performed essentially as described previously (Mitsuhashi, et al., 2000). We used specific rabbit polyclonal antibodies against PV72 (diluted 5,000-fold) (Shimada, et al., 1997) and RD21 (diluted 5,000-fold) (Yamada, et al., 2001), and

monoclonal antibody against barley aleurain which is a homologe of AtALEU (2F5-5B2; diluted 1,000-fold) that was kindly donated by Dr. J. C. Rogers of Washinton State University (Rogers, et al., 1997). We also used horseradish peroxidase-conjugated donkey antibodies against either rabbit IgG (diluted 5,000-fold; Amersham Pharmacia Biotech, Tokyo, Japan) or mouse IgA (diluted 1,000-fold; Amersham Pharmacia Biotech). Immunodetection was performed with an enhanced chemiluminescence kit (an ECL system, Amersham Pharmacia Biotech).

Subcellular Fractionation

The seedlings (1.2 to 2.0 g) harvested from the transgenic plants and wild plants were chopped with a razor blade in a Petri dish on ice in 3.6 to 6.0 ml of the chopping buffer that contained 50 mM HEPES-NaOH, pH 7.5, 5 mM EDTA, 0.4 M sucrose and protease inhibitor cocktail (1 tablet per 50 ml, Boehringer mannheim, Tokyo, Japan). The homogenate was filtered through cheesecloth. An aliquot of the filtrate were used as a total (T) fraction. Two ml of the filtrate was centrifuged at 1,000 *g* and 4 °C for 20 min. The pellet (P1) was resuspended in 500 µl of the chopping buffer. The supernatant was centrifuged again at 8,000 *g* and 4°C for 20 min. The pellet (P8) was resuspended in 500 µl of the chopping buffer. The supernatant was ultracentrifuged at 100,000 *g* and 4 °C for 1 h. The pellet (P100) was resuspended in 500 µl of the chopping buffer. The supernatant was designated as S100 fraction and the volume of the fraction was measured. Each fraction (4 µl) of T, P1, P8, P100 and S100 (0.8% v/v) were subjected to SDS-PAGE and subsequently to an immunoblot analysis with anti-aleurain antibodies.

Co-purification of ProAtALEU with PV72-HDEL

The transgenic plants and wild plants were homogenized in the extraction buffer (20 mM HEPES-NaOH, pH 7.0, 150mM NaCl, 1 mM $CaCl_2$, and 10% (w/v) sucrose), and were filtered through cheesecloth. The filtrate was centrifuged at 8,000 *g* for 20 min. The supernatant was centrifuged at 100, 000 *g* for 1 hr to obtain the precipitate as the microsomal

fraction. The fraction was gently suspended in CHAPS buffer (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 1% (w/v) CHAPS and 1 mM $CaCl_2$). Each extract was lysed by three bursts of sonication for 1 min at 10-min intervals on ice and was centrifuged at 100, 000 g for 30 min to obtain the soluble fraction of the microsomes.

We subjected the soluble fraction to the Ni-NTA resin (QIAGEN, Tokyo, Japan) and washed the column with the CHAPS buffer. The bound proteins were eluted with the CHAPS buffer containing 400 mM Imidazol. Each fraction was subjected to SDS-PAGE and then to immunoblot analysis with specific antibodies against either PV72 or aleurain.

Fluorescent Microscopy

The transformed Arabidopsis T-75 were inspected with a fluorescence microscope (Axiophot 2, Carl Zeiss, Jena, Germany) using a filter set (an excitation filter; BP-450-490, a dichroic mirror; FT510, a barrier filter; BP 515-565, Carl Zeiss), a CCD camera (CoolSNAP, RS Photomerics, Chiba, Japan), and a light source (Arc HBO 100W, Atto, Tokyo, Japan).

Expression and Purification of Modified PV72s

Two modified PV72s were expressed in insect cells of Spodoptera frugiperda (Sf21) with a baculovirus expression system (Invitrogen, San Diego, CA) according to the methods as described previously (Watanabe, et al., 2002). The recombinant proteins were purified with a Hi-Trap chelating column (Amersham Pharmacia Biotech) and a Superdex-200 column (Amersham Pharmacia Biotech) as described before (Watanabe, et al., 2002). The purified PV72s were concentrated by Centricon 30 and subjected to a protein assay (Nippon Bio-Rad Laboratories, Tokyo) and binding assays as described below.

Surface Plasmon Resonance and Kinetic Assays

The peptide (AtPAP), ANIGFDESNPIRMVSDGLREV, was chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems Inc., Tokyo, Japan). We immobilized the AtPAP peptide on a sensor chip (carboxymethylated dextran chip; CM5) for BIACORE-X

(BIACORE, Tokyo, Japan) in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.005 % P-20 (HBS, BIACORE) as described previously. A control flow cell was prepared with no peptide. The amount of the coupled peptide on the sensor chip was found to be 700 resonance units (RU).

Modified PV72s were injected onto the sensor chip as described before (Watanabe, et al., 2002). The sensorgrams shown in this study are made by subtracting the sensorgram made with the control flow cell. Kinetic analysis was performed according to the manufacturer's protocol. The association, dissociation, and regeneration phases were followed in real time as the changes in the relative diffraction (RU). The association phase (0 to 180 sec) was analyzed by nonlinear least squares curve fitting to yield the association rate constants (k_a) as mean values. The dissociation phase (180 to 300 sec) was also analyzed by nonlinear least squares curve fitting to yield the dissociation constants (k_d). To avoid mass transport, we worked at a low immobilization level, a high flow rate (30 μl/min), and using suitable concentrations of analyte. Kinetic constants{the association rate constant (k_a), the dissociation rate constant (k_d) and the dissociation constant ($K_D = k_a / k_d$)} were calculated from the sensorgrams using BIA evaluation software version 2.1 (BIACORE). These kinetic parameters were determined from three independent experiments.

Ligand-binding Assay by Affinity Column Chromatography

The AtPAP peptide (10 mg) was immobilized to NHS-activated Sepharose HP (Amersham Pharmacia Biotech) to prepare the AtPAP affinity column. The modified PV72s were applied to the AtPAP column equilibrated with the HEPES buffer containing 1 mM $CaCl_2$ and then eluted with the buffer containing 2.5 mM EDTA, 20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl and 0.4 % CHAPS on an automated chromatography system (ÄKTA, Amersham Pharmacia Biotech). Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot analysis.

The modified PV72s were applied to an AtPAP affinity column equilibrated with HEPES buffer containing 1 mM $CaCl_2$. The column was washed with the same buffer and followed

by washing with the Na-acetate buffer (20 mM Na-acetate, pH 4.0, 150 mM NaCl, 0.4 % CHAPS) containing 1 mM $CaCl_2$ and by the Na-acetate buffer containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to an immunoblot.

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Figure legends

Figure 1. Overexpression of PV72-HDEL Results in the Accumulation of a Proprotein Precursor of AtALEU, Which Has a Vacuolar-Targeting Signal, NPIR, in the Transgenic Arabidopsis Plants.

Arabidopsis plants were transformed with a chimeric gene encoding a PV72 precursor followed by an ER retention signal, HDEL. Crude extracts (10 μg protein) from the transgenic Arabidopsis plants (T/PV72) and wild plants (wt) were subjected to SDS-PAGE followed by an immunoblot analysis with specific antibodies against each of PV72, aleurain and RD21. The 41-kD proprotein precursor (p) of AtALEU was accumulated in the transgenic plants expressing PV72-HDEL, but not in wild plants. On the contrary, a precursor of RD21, which has not a vacuolar-targeting signal, NPIR, was not detected in both the transgenic and wild plants. p, the AtALEU precursor; m, the mature form of AtALEU.

Figure 2. PV72-HDEL Was Co-purified with the AtALEU Precursor (proAtALEU), But Not with the Mature Form.

(A) Microsomal fractions of transgenic Arabidopsis plants (T/PV72) and wild plants (wt) were applied to a Ni-NTA resin column. The column was washed with the CHAPS buffer to obtain the flow through fraction (FT). Finally, the bound protein was eluted with the CHAPS buffer containing 400 mM imidazol to obtain the eluted fraction (E). These fractions were subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies. PV72-HDEL was detected in the eluted fraction of the transgenic plants (T/PV72).

(B) Both eluted (E) and flow through (FT) fractions from the transgenic plants (T/PV72) and wild plants (wt) were subjected to an immunoblot analysis with anti-aleurain monoclonal antibody. The 41-kD AtALEU precursor was co-purified with PV72-HDEL in the eluted fraction from the transgenic plants. The 28-kD mature form and the 38-kD intermediate form of AtALEU were detected in the FT fraction.

Figure 3. The AtALEU Precursor Was accumulated in the Microsomal Fraction of the Transgenic Arabidopsis Plants Expressing PV72-HDEL.

The homogenates from the rosette leaves of 28-day-old transgenic (**A**) and wild (**B**) plants were subjected to subcellular fractionated by differential centrifugation. The differential centrifugation of the total homogenate (T) gave the P1, P8, P100, and S100 fraction. Each fraction was subjected to SDS-PAGE followed by an immunoblot analysis with anti-aleurain monoclonal antibody. The 41-kD AtALEU precursor was detected in the P8 and P100 fraction. The molecular mass of each marker protein is given on the left in kD. p, the AtALEU precursor; m, the mature form of AtALEU; i, the intermediate form of AtALEU.

Figure 4. GFP with the Propeptide of AtALEU at the N terminus (AtALEU-GFP) Was Localized in the Vacuoles of the Transformed Suspension-Cultured Cells of Arabidopsis.

(**A**) Precursors of AtALEU and an AtALEU-GFP are schematically represented. The precursor of AtALEU is composed of three regions; a signal peptide (SP), an N-terminal propeptide (NTPP) that contains a vacuolar targeting signal, NPIR, and a mature proteinase domain. The proteinase domain of AtALEU was replaced by GFP to produce AtALEU-GFP. (**B**) Suspension-cultured cells (T-75) of Arabidopsis were transformed with a chimeric gene encoding AtALEU-GFP. The 3-day-old transformant cells were inspected with a fluorescent (FL) or differential-interference-contrast (DIC) microscope. A fluorescent image shows the localization of the GFP fusion within the cells and a DIC micrograph shows the cellular structures of the respective fields. Bars = 5 μm.

Figure 5. Kinetics for the Association and Dissociation of rPV72 and the AtPAP Peptide.

(**A**) PV72 has three EGF-like motifs at the C terminus of the lumenal domain. We expressed two modified PV72s in the insect Sf21cells to use the kinetic analysis; rPV72 composed of the lumen domain followed by a His-tag and the HDEL sequence and rPV72Δ1,2,3 composed of rPV72 with no EGF-like motif. A sensor chip was coupled with the AtPAP peptide on BIACORE-X (a coupling efficiency of 700 resonance units). rPV72 and

rPV72Δ1,2,3 were injected onto the sensor chip at different concentrations from 0.17 μM to 0,7 μM to obtain the sensorgrams for the association and dissociation curves by surface plasmon resonance.

(**B**) The kinetic constants, an association rate constant (k_a), a dissociation rate constant (k_d) and a dissociation constant ($K_D = k_a / k_d$), were calculated from the above sensorgrams using BIA evaluation software version 2.1. These kinetic parameters were determined from two independent experiments.

Figure 6. Calcium-Mediated Interaction between Modified PV72s and the AtPAP Peptide. (**A**) Either rPV72 (upper) or rPV72Δ1,2,3 (lower) was subjected to an affinity column conjugated with the AtPAP peptide in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. Both proteins were bound to the column and then eluted by addition of the HEPES buffer (pH 7.0) containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies. FT, flow-through fraction, Wash, washing fraction, EGTA, elution fraction. (**B**) Either rPV72 (upper) or rPV72Δ1,2,3 (lower) were subjected to the AtPAP column in the HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. The column was washed with the same buffer and followed by the Na-acetate buffer (pH 4.0) containing 1 mM $CaCl_2$, and finally washed with the Na-acetate buffer containing 2.5 mM EGTA. rPV72 was eluted by the addition of the Na-acetate buffer containing 2.5 mM EGTA, while rPV72Δ1,2,3 was eluted by addition of the Na-acetate buffer (pH 4.0) containing 1 mM $CaCl_2$. Each fraction was subjected to SDS-PAGE followed by an immunoblot analysis with anti-PV72 antibodies.

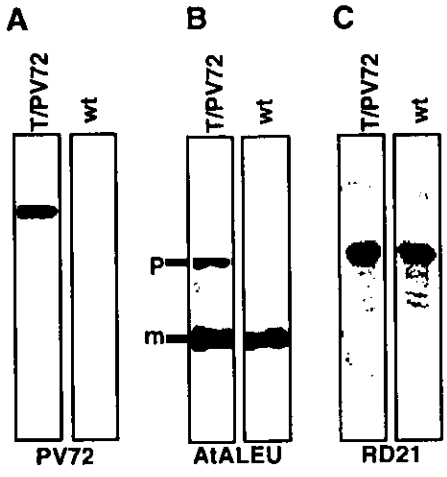


Figure 1. Hara-Nishimura et al.

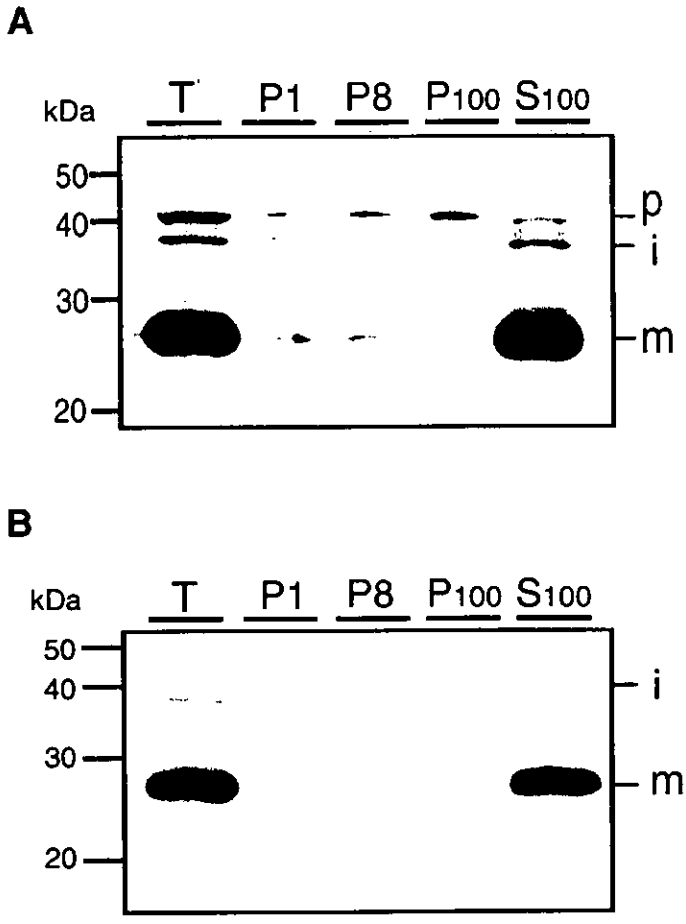


Figure 2. Hara-Nishimura et al.

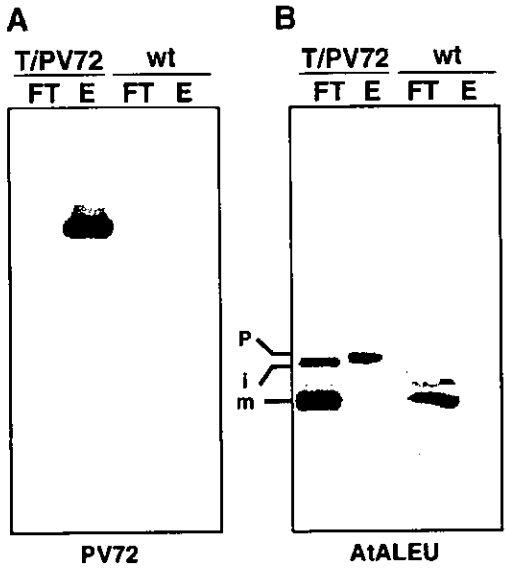


Figure 3. Hara-Nishimura et al.

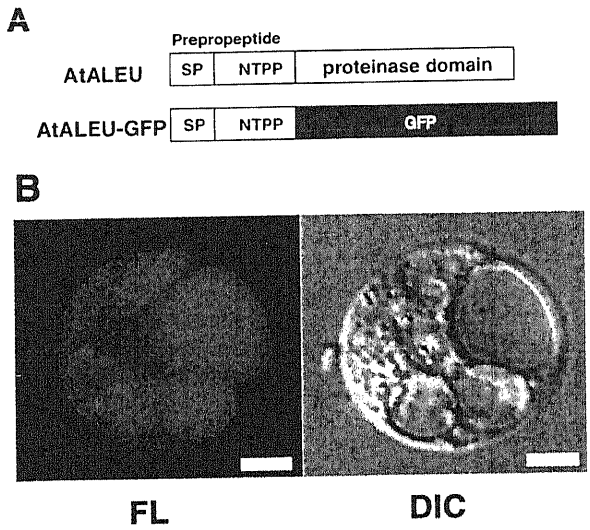
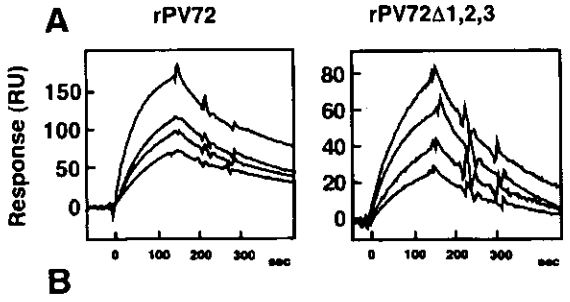


Figure 4. Hara-Nishimura et al.



	k_a (per *Ms*)	k_d (per *s*)	K_D
rPV72	6.4×10^{4}	6.9×10^{-3}	0.1 μM
rPV72Δ1,2,3	1.5×10^{4}	1.9×10^{-2}	1.2 μM

Figure 5. Hara-Nishimura et al.

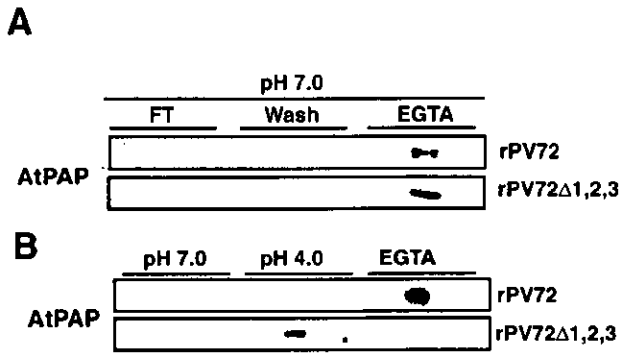


Figure 6. Hara-Nishimura et al.

A Vacuolar-Sorting Receptor on the Membrane of PAC Vesicles that Accumulate Precursors of Seed Storage Proteins

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Running title: A vacuolar sorting receptor on PAC vesicles

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ABSTRACT

A novel vesicle, referred to as a precursor-accumulating (PAC) vesicle, mediates a Golgi-independent transport of storage proteins to protein-storage vacuoles in maturing pumpkin seeds. PV72, a type I integral membrane protein with three repeats of epidermal-growth factor, was found on the membrane of the PAC vesicles that accumulated proprotein precursors of storage proteins, including pro2S albumin. Affinity column chromatography revealed that PV72 had an ability to bind to pro2S albumin in a calcium-dependent manner, via the C-terminal region of pro2S albumin, which was found to function as a vacuolar-targeting signal. It appears that PV72 is a vacuolar-sorting receptor of the storage protein. PV72 was specifically and transiently accumulated at the middle stage of seed maturation in association with the synthesis of storage proteins. Subcellular fractionation of the maturing seeds showed that PV72 was also accumulated in the microsome fraction. The transmembrane domain and the cytosolic tail of PV72 localized the GFP fusion protein on Golgi-like structures. PV72 in the isolated PAC vesicles had a complex type of oligosaccharide, indicating that PV72 passed though the Golgi complex. These results suggest that PV72 is recycled between PAC vesicles and Golgi complex or post-Golgi compartments. It appears that PV72 is responsible for recruitment of the escaped pro2S albumin molecules leaving the endoplasmic reticulum for Golgi complex to the PAC vesicles.

INTRODUCTION

Most seed storage proteins and lectins have been shown to be transported to protein storage vacuoles via the Golgi complex (Chrispeels, 1985; Shotwell and Larkins, 1988). Chrispeels (1983) reported the presence of electron-dense vesicles close to the Golgi stacks in maturing bean cotyledons. Hohl et al. (1996) demonstrated immunocytochemically that electron-dense vesicles with a diameter of about 100 nm associated with cisternae of the Golgi complex

contain storage proteins in maturing pea cotyledons. These results suggest that the storage proteins are transported to protein storage vacuoles via the Golgi-derived dense vesicles. Transport of seed proteins from the Golgi complex to the vacuole requires specific vacuolar targeting signals and their receptors. However, the molecular mechanism responsible for transport of seed proteins has not been characterized or determined.

In contrast to such Golgi-derived vesicles, we found novel vesicles with diameters of 200 to 400 nm that are responsible for a Golgi-independent transport of storage proteins to the vacuoles in maturing pumpkin seeds. The vesicles accumulate large amounts of proprotein precursors of storage proteins and were designated precursor-accumulating (PAC) vesicles. They contain an electron-dense core of storage proteins surrounded by an electron-translucent layer, and some vesicles also contained small vesicle-like structures. The electron-dense cores of the PAC vesicles originate from electron-dense aggregates of storage proteins formed within the endoplasmic reticulum (ER). The PAC vesicles might mediate a transport pathway for insoluble aggregates of storage proteins directly to protein storage vacuoles. A pathway bypassing the Golgi apparatus is presumed to have evolved for the efficient transport of proteins in maturing seeds that synthesize a large amount of storage proteins.

To elucidate the molecular mechanism responsible for a unique transport pathway provided by the PAC vesicles, we have analyzed protein components of the highly purified PAC vesicles. The isolated PAC vesicles have been shown to accumulate proglobulin, pro2S albumin and a unique protein PV100, which is a single precursor of multifunctional proteins such as trypsin inhibitors, cytotoxic proteins and 7S globulin. In addition to such storage proteins, a precursor of a novel membrane protein, MP73, of protein storage vacuoles is also localized on the PAC vesicle membrane. Thus, PAC vesicles mediate delivery of not only soluble storage proteins but also a membrane protein of the vacuoles.

We previously identified two membrane proteins, PV72 and PV82, in the isolated PAC vesicle fraction (Shimada et al., 1997). After the storage protein precursors, these proteins were the next most abundant proteins in PAC vesicles. PV72 and PV82 were not detected in the membrane fraction of the isolated protein storage vacuoles (Shimada et al., unpublished

data). Unlike the proprotein precursors of vacuolar proteins, PV72 and PV82 functional proteins in the PAC vesicle.

PV72 and PV82 are members of vacuolar sorting receptors of higher plants (ref). PV72 has been shown to exhibit a receptor-like structure that is composed of a lumenal domain of 550 amino acids followed by a transmembrane domain of 17 amino acids, and a cytosolic tail of 37 amino acids. Three repeats of epidermal growth factor (EGF)-like motifs of 150 amino acids are found in the C-terminal region of the lumenal domain. The cytosolic tail contains a YMPL sequence, a potential Tyr-based motif. The motif is recognized by an adapter complex of clathrin-coated vesicles (CCVs).

Two proteins homologous to PV72 have been identified: pea BP-80 () and Arabidopsis AtELP (). BP-80 was originally identified in maturing pea cotyledons. Hinz et al. reported that BP-80 was highly enriched in purified CCVs containing no major storage proteins and was markedly reduced in the dense vesicles responsible for the transport of storage proteins. Thus, BP-80 might play a role in the transport to lytic vacuoles rather than in the transport to protein storage vacuoles. Both BP-80 (Kirsch et al., 1994) and AtELP (Ahmed et al., 2000) have been shown to bind to a proaleurain peptide including a vacuolar targeting signal, NPIR. Sanderfoot et al. reported that AtELP was located at the trans Golgi and prevacuolar compartments in Arabidopsis root cells. Based on these observations, BP-80 and AtELP are proposed to function as a sorting receptor for vacuolar proteins in a manner similar to those of yeast and animals.

What is the physiological function of PV72 and PV82, which are homologues of the vacuolar sorting receptor, and which are abundant in the PAC vesicles? In this study, we demonstrated that PV72 has an ability to bind to pro2S albumin via its putative vacuolar targeting signal and is specifically and transiently accumulated in association with the synthesis of storage proteins. We show here a unique recruitment pathway for pro2S albumin between the Golgi complex and the PAC vesicles.

RESULTS

PV72 Is Associated with the Membrane of Precursor-Accumulating Vesicles (PAC Vesicles)

PV72 and PV82 are type-I integral membrane proteins, which we identified previously in the isolated PAC vesicle fraction from maturing pumpkin seeds (Shimada et al., 1997). We wished to determine the subcellular and suborganellar localization of PV72 in maturing seeds. The maturing pumpkin cotyledons were subjected to Percoll self-gradient centrifugation. The PAC vesicles formed a single band in the Percoll gradient (data not shown). The vesicle fraction was further applied on a 30-55% (w/w) linear sucrose gradient. The protein profile of each fraction of the gradient is shown in Figure 1A. Most of the precursors of storage proteins were found in fractions 11-14, indicating that the PAC vesicles sedimented in these fractions. The density of fraction 13 was determined to be 1.24 g/cm^3, in good agreement with the density of the PAC vesicles reported previously (Hara-Nishimura and Nishimura, 1987). The PAC vesicles accumulated pro2S albumin, proglobulin and PV100 which is a single precursor of multifunctional proteins including trypsin inhibitors, cytotoxic peptides and 7S globulin (Yamada et al., 1999), whereas they accumulated no mature form of these proteins.

To clarify the distribution of PV72, we performed an immunoblot analysis of each fraction of the gradient with anti-PV72 antibodies. Figure 1B shows that PV72 was sedimented together with the precursors of storage proteins in the gradient. The peak fraction for PV72 was fraction 13 with a density of 1.24 g/cm^3, which was also the peak fraction for precursors of storage proteins (Fig. 1A). This result indicates that PV72 is associated with the PAC vesicles.

To demonstrate the sub-organellar localization of the membrane protein PV72, we performed immunoelectron microscopy of the isolated PAC vesicles with the anti-PV72 antibodies. Figure 1C shows an electron micrograph of the isolated PAC vesicles, indicating that the PAC vesicles were highly pure. Immunoelectron micrographs show that only the

periphery of the PAC vesicles were labeled with gold particles specific for PV72 (Fig. 1D), in contrast to uniform labeling inside the PAC vesicles with gold particles specific for 2S albumin (Fig. 1E). Statistical analysis of immunoelectron micrographs revealed that 90.8% of the gold particles were found on the periphery of the PAC vesicles and 9.2% were found inside the vesicles. This result indicates that PV72 is associated with the membrane of the PAC vesicles. This was also supported by an immunocytochemical analysis of the maturing cotyledons using a high-pressure-freezing and freeze-substitution method (data not shown).

Calcium-Dependent Binding of PV72 to a Proprotein Precursor of 2S Albumin via its vacuolar targeting signal

To determine the physiological function of PV72 on the PAC vesicle membrane, we examined the interaction between PV72 and a proprotein precursor of 2S albumin, pro2S albumin. The C-terminal region of pro2S albumin is a candidate for the signal of vacuolar targeting. We introduced a chimeric gene encoding a signal peptide and a green-fluorescent protein (GFP) fused with the C-terminal 18 amino-acids of pro2S albumin into the suspension-cultured cells of Arabidopsis. The GFP-fluorescence was observed in the vacuoles of the cells after 24 h of transformation (Fig. 2A). This result indicates that the C-terminal region of pro2S albumin is sufficient for vacuolar targeting.

We next examined the interaction between the C-terminal peptide of pro2S albumin and PV72 and PV82. Protein extracts from maturing pumpkin seeds were applied to an affinity column conjugated with the C-terminal region of pro2S albumin. Both PV72 and PV82 were bound to the column in the presence of 5 mM $CaCl_2$. PV72 was eluted by addition of 1 mM EGTA, while PV82 was eluted by addition of 400 mM of $CaCl_2$ (Fig. 2B). The sensitivities of both proteins to the Ca^{2+} concentration for the ligand binding were different from each other.

To clarify the binding ability of PV72 to the pro2S albumin molecule, we expressed a His-tagged lumenal domain of PV72 (rPV72) in insect Sf21 cells (Watanabe et. al. 2002) and a His-tagged pro2S albumin to prepare an affinity column conjugated with pro2S albumin.

rPV72 was applied to the affinity column. rPV72 bound to the affinity column in the presence of Ca^{2+} and was eluted with a solution of EGTA (Fig. 2C). No rPV72 molecule bound to the affinity column in the presence of EGTA at all (Fig. 2C). This result indicates that PV72 has an ability to bind to pro2S albumin in a Ca-dependent manner.

PV72 Is Associated with PAC Vesicles, but not with CCVs

PV72 has a cytoplasmic tail with a YMPL sequence, a potential Tyr-based signal, YΦ, in which X represents any amino acid and Φ represents a hydrophobic residue with a bulky side chain. Tyr-based signals are known to be recognized by the adapter complex of CCVs. To determine whether the PAC vesicles, like CCVs, are associated with clathrin and β-adapter complex, we performed subcellular fractionation of the maturing seeds with a 30-60% (w/w) linear sucrose gradient followed by an immunoblot analysis with antibodies against either clathrin-heavy chain or β-adaptin.

Both proglobulin and pro2S albumin were sedimented in fractions 9-12 (Fig. 3). The density of fraction 11 was determined to be 1.24 g/cm^3, which was the same as the value of the PAC vesicles reported previously (Hara-Nishimura and Nishimura, 1987). Thus, the PAC vesicles were sedimented around fractions 9-12. An immunoblot of each fraction of the gradient with anti-clathrin-heavy chain antibodies showed that clathrin-heavy chain was localized only in microsomal fractions 2-3 (Fig. 3, CHC). On the other hand, an immunoblot with anti-β-adaptin antibodies detected β-adaptin not only in the microsomal fractions 2-3 but also in fractions 7-9 (Fig. 3, βAd). The density of the peak fraction 8 was 1.21g/cm^3. Fractions 7-9 might contain CCVs lacking clathrin. The sedimentation pattern of β-adaptin did not correspond to that of storage protein precursors. Thus, the vesicles might not be responsible for the transport of storage proteins.

BP-80, a pea homolog of PV72, has been reported to be localized on CCVs in maturing pea cotyledons. To determine whether PV72 and/or PV82 are associated with the CCVs of fractions 7-9 of the gradient, each fraction of the gradient was examined with anti-PV72 antibodies and anti-PV82 antibodies in an immunoblot. PV72 was sedimented together with

the precursor of storage proteins in the PAC vesicle fraction, but not with the β-adaptin in the CCVs (Fig. 3, PV72). PV82 was rarely detectable in the PAC vesicle fraction (Fig. 3, PV82). With a longer exposure, a PV82 signal was detected on the blot of the PAC vesicle fraction, but not on the CCV fraction (data not shown). These results indicate that the PAC vesicles are a different population from the CCVs and are not associated with clathrin coats.

PV72 Might Be Recycled between PAC Vesicles and Golgi Apparatus

Figure 3 also shows that both PV72 and PV82 were predominantly localized in microsomal fractions 2-3, although small amount of PV72 and PV82 must be derived from PAC vesicles broken during the subcellular fractionation procedures. To determine which cellular compartments accumulate PV72 and PV82, we prepared a chimeric gene encoding a fusion protein of GFP and introduced it into suspension-cultured cells of Arabidopsis. The fusion protein SP-GFP-72C was composed of a signal peptide and GFP followed by the transmembrane domain and the cytoplasmic tail of PV72. Figure 4 shows that the GFP-fluorescence was observed on the small particles in the cells 24 h after transformation. The image is consistent with the fluorescent Golgi complex that was reported by Nebenfuhr et al. (1999).

Incubation of the transformant cells for 72 h resulted in the appearance of fluorescent vacuoles instead of the distinct localization of GFP fluorescence on the small particles (data not shown). This suggests that the transmembrane domain and cytoplasmic tail of PV72 was probably cleaved from GFP-PV72C to produce GFP. The latter could have diffused within the vacuoles and caused them to fluoresce. GFP-fluorescence was not observed in the ER network. These results suggest that GFP-72C traveled from the Golgi complex to prevacuolar compartments and then to the vacuoles. Thus, it is possible that most PV72 resides in the Golgi complex or in prevacuolar compartments.

This result raises the question of whether PV72 and PV82 on the PAC-vesicle membrane are derived from the Golgi complex. Previously we reported that the periphery of the PAC vesicles was labeled with gold particles specific for complex glycans (Hara-

Nishimura et al., 1998). This result suggests that Golgi-derived proteins are incorporated into the PAC vesicle membranes. To examine the possibility of the delivery of PV72 and PV82 from the Golgi complex into the PAC vesicles, we characterized the type of oligosaccharides of PV72 and PV82 (Shimada et al., 1997). Both PV72 and PV82 in the isolated PAC vesicles were treated with two glycosidases and then subjected to immunoblot analysis. We used anti-PV82 antibodies to detect PV72 and PV82 at a similar level on the blot of the PAC vesicles, which accumulate much larger amount of PV72 than that of PV82. Treatment with N-glycosidase F caused a 2-3 kDa reduction of the molecular masses of PV72 and PV82 in the PAC vesicles (Fig. 6). On the contrary, both PV72 and PV82 were resistant to endoglycosidase H. These results indicated that they had complex-type oligosaccharides, but not high-mannose-type oligosaccharides. This provided evidence that PV72 and PV82 passed through the Golgi complex and then were transported to the PAC vesicles. It is possible that PV72 and PV82 are recycled between the Golgi complex/post-Golgi compartments and the PAC vesicles.

PV72 Is Specifically and Transiently Accumulated in Maturing Seeds in Association with the Synthesis of Seed Storage Proteins

To clarify the distribution of the two proteins, we analyzed the amounts of the proteins in various organs of pumpkin plants. Total proteins (20 μg each) from roots, hypocotyls, leaves, green cotyledons and maturing cotyledons were subjected to an immunoblot analysis with either anti-PV72 antibodies or anti-PV82 antibodies. A single major band of PV72 was detected only on the immunoblot of maturing cotyledons (Fig.6, upper panel). A very faint band found on the blot of leaves corresponded to PV82. A sequence similarity between PV72 and PV82 resulted in a slight cross-reactivity of each protein with the two antibodies. A major band of PV82 was detected on the blots of maturing cotyledons, hypocotyls, leaves and green cotyledons (Fig. 6, lower panel). A lower and minor band on the blot of maturing cotyledons corresponded to PV72. Another isoform with a molecular mass of 75 kDa was found on the blot of roots with anti-PV82 antibodies, but not with anti-PV72 antibodies. This

result indicates that PV72 is specifically accumulated in maturing cotyledons, whereas PV82 is widely distributed in various organs except for roots.

To demonstrate the different expression between PV72 and PV82 in greater detail, we investigated developmental changes in the levels of PV72 and PV82 in pumpkin cotyledons during seed maturation and after seed germination. Figure 7 (upper) shows the accumulation of major storage proteins during seed maturation followed by their degradation after seed germination. Both 7S globulin and 11S globulin started to be accumulated in maturing cotyledons on day 21, whereas 2S albumin started to be accumulated on day 24. These storage proteins were degraded after seed germination. In association with the active synthesis of the storage proteins, PV72 appeared rapidly during days 21-26 (Fig. 7, lower panel). It should be noted that the level of PV72 decreased during the late stage of seed maturation and that PV72 was no longer detectable in the cotyledons of dry seeds or seedlings. On the other hand, PV82 appeared much earlier than PV72, so that PV82 was detected in very young cotyledons that had not yet accumulated any storage proteins. The level of PV82 also decreased during the late stage of seed maturation and no PV82 was detected in dry seeds. In contrast to the absence of PV72 in the cotyledons of the seedlings, PV82 appeared again in the cotyledons after seed germination. This was also supported by the finding as shown in Figure 6 (lanes 5 and 10). Overall results indicated that PV72 is transiently appeared in the cotyledons at the middle stage of seed maturation. Thus, the physiological function of PV72 in the cells of maturing seeds may be different from that of PV82.

DISCUSSION

Differential Expression of Vacuolar-Sorting Receptors

We have previously identified two homologous protein, PV72 and PV82, in developing pumpkin seeds. In this paper, we addressed the possibility that PV72 and PV82 function as

sorting receptors for seed proteins. The differential expression of these two homologs was found with specific antibodies against either PV72 or PV82. PV72 was transiently expressed in the cotyledons at the middle stage of seed maturation, but not in the vegetative organs at all (Fig. 6 and 7). The appearance of PV72 was exactly accompanied with the active synthesis of storage proteins. In contrast to the limited expression of PV72, PV82 was widely distributed in both vegetative organs and storage organs (Fig. 6). The appearance of PV82 in maturing seeds was much earlier than the accumulation of storage proteins (Fig. 7). In such very young cotyledons of pea, lytic vacuoles were known to develop rather than protein storage vacuoles (Robinson et al., 1995). These results implied that PV72 is a sorting receptor for protein storage vacuoles, while PV82 is a receptor for lytic vacuoles.

A number of homologs of the receptors have been registered in the database. For example, Arabidopsis has 7 homologs. The homologs could be separated into two or three groups. It is possible that one group including BP-80 is involved in the traffic to the lytic and another group including PV72 is involved in the traffic to protein storage vacuoles. Functional differentiation of receptor homologs might be involved in the cells of higher plants.

Ligand Binding Properties of the Receptors

We investigated the interaction between PV72 and pro2S albumin. PV72 could bind the C-terminal 18 amino acid peptide of pro2S albumin (Fig. 2B). This peptide sequence was sufficient for the vacuolar targeting of GFP in Arabidopsis suspension cells (Fig. 2A). These results suggest the possibility that PV72 sorts pro2S albumin to the vacuoles. PV82 also bound to the C-terminal peptide. Interestingly, the Ca2+ sensitivities of these proteins were different to each other. Ca2+ dependent ligand binding properties of PV72were fully investigated.

The interaction of PV72 to the full length of the pro2S albumin was also confirmed (Fig. 2C). So far, there were only two examples that demonstrated the interaction between the sorting receptor homologs and their putative ligand proteins. VmVSR, a receptor homolog

expressed in the cotyledon cells of germinating *Vigna mungo* seeds was shown to bound in vitro with the precursor of SH-EP, a papain-type protease found in the same cells. Cross-linking and affinity precipitation studies has revealed that a BP-80 homolog of *Nicotiana alata* bound to proNa-PI, a proteinase inhibitor in vivo.

It has been shown that the seed proteins expressed in vegetative cells are correctly targeted to LV and easily degraded x. Therefore the sorting machinery for LV is seems to be similar to that for PSV enough to recognize the targeting signal of seed proteins. The vacuolar-targeting signal of Brazil nut 2S albumin was determined using leaf cells of transgenic tobacco plants (Saalbach et al., 1996). The signal is located at the C-terminal end of this protein. BP-80 was shown to recognize this 2S albumin signal in vitro (Kirsch et al., 1996). These results suggest that a protein homologous to BP-80 functions in the sorting of 2S albumin in tobacco leaf cells and in maturing Brazil nut seeds.

Localization of the Sorting Receptors

The possibility that PV72 function as a sorting receptor for storage proteins is also come from its intracellular localization. PV72 was shown to be localized on the PAC vesicle membranes by subcellular fractionation and immunogold labeling (Figs. 1 and 3). This localization is quite interesting because this is the first report showing the co-localization of a vacuolar-sorting receptor and precursors of seed storage proteins. PV82 was less abundant in the PAC vesicles compared with PV72 (Fig. 3). Pea BP-80 was identified in developing pea cotyledons. BP-80 was reported to be enriched in the CCVs in which storage proteins were not found (Hinz et al., 1999). Thus, BP-80 is considered to act as a receptor for LV, in stead of a receptor for storage proteins. It is possible that PV82, like BP-80, might function as a receptor for vacuolar proteins to be transported into LV. We can not ruled out the possibility that a minor portion of the receptors are localized in CCVs.

In this paper, we revealed that both PV72 and PV82 were abundant in microsome fractions (Fig. 3). Using GFP as a reporter, it was revealed that the GFP-fluorescence was observed on the small particles in the cells (fig. 4). Prolonged incubation of the transformant

cells for 72 hr resulted in the fluorescent vacuoles (data not shown). The fluorescence within the vacuoles indicated that the GFP-fusion protein with a transmembrane domain was converted into a soluble form. Delivery of GFP to vacuoles instead of the secretion suggest that the fusion protein was located in a cellular compartment to the vacuoles. Thus, it is possible that most of PV72 resides in Golgi complex or post-Golgi compartments.

In the analysis of the transient expression of phaseolin, a storage protein in common bean (*Phaseolus vulgaris*), it was shown that vacuolar sorting of phaseolin was saturable and saturation led to the Golgi-mediated secretion from tobacco leaf cells (Frigerio et al., 1998). This result suggested that the sorting machinery that is located at the Golgi complex in tobacco leaf cells could recognize the information for vacuolar targeting of phaseolin. The information is not yet found until now. However, C-terminal four amino acids of phaseolin were shown to be necessary for the targeting of phaseolin to the LV in transgenic tobacco leaves (Frigerio et al., 1998).

Transport route from the Golgi Apparatus to the PAC Vesicles

In maturing pumpkin seed cells, electron-dense aggregates of storage proteins are often observed within the rER (ref). It is likely that these aggregates develop into the cores of vesicles and then leave the ER to make PAC vesicles. Therefore, it was suggested that the electron-dense cores bypass the Golgi complex and are directly transported into the PSV (Hara-Nishimura et al., 1998b). The major storage proteins in pumpkin seeds are not glycosylated and need not pass through the Golgi complex. In the peripheral region of PAC vesicles, however, there exist components with complex glycans, suggesting that the Golgi-derived proteins are also incorporated into the PAC vesicles (Hara-Nishimura et al., 1998b). PV72 was shown to have a complex glycan(s) (Fig. 5). Therefore, it is clear that PV72 is sorted to PAC vesicles via the Golgi complex.

It seems that PV72 is recycled between the Golgi complex and the PAC vesicles in maturing pumpkin seeds. PV72 might function as a sorting receptor for seed proteins that are being transported from the Golgi complex to PAC vesicles. Pro2S albumin is a candidate

protein that is sorted by PV72 in pumpkin. Most of pro2S albumin molecules that are synthesized on rER are directly transported to the PAC vesicles (Hara-Nishimura et al., 1993a). When some pro2S albumin molecules leave the rER for the Golgi complex, the escaped molecules might be recruited from the Golgi complex to the PAC vesicles by PV72. Ricin and Ricinus communis agglutinin are candidate proteins in castor bean (*Ricinus communis*). Transport pathway of proricin has been well characterized. Proricin synthesized on rER is transported via the Golgi complex to the periphery of the PAC vesicles and eventually to the PSV. They have complex glycans, indicating that they travel though the Golgi complex. Recently, it was shown that the internal propeptide of the ricin precursor carries a signal for vacuolar targeting (Frigerio et al., 2001). The authors speculated that proricin could be recognized at the trans-Golgi by BP-80-like receptor for sorting to the PAC vesicles.

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FIGURE LEGENDS

Figure 1. PV72 is associated with the membrane of the PAC vesicles. (A) Percoll-purified PAC vesicles were layered on a sucrose density gradient (30-55%, w/w) and centrifuged. Fractions were numbered from the top to the bottom of the gradient. Each fraction was subjected to SDS-PAGE followed by Coomassie blue staining. PAC-vesicle fractions 12-14 are indicated by a horizontal bar at the top. 2S, 2S albumin; p2S, pro2S albumin; G, 11S globulin; pG, proglobulin; 7S, 7S globulin; PV100, a precursor of multiple functional proteins including trypsin inhibitors, cytotoxic peptides and 7S globulin. (B) An immunoblot of each fraction in (A) with anti-PV72 antibodies. (C) An electron micrograph of the isolated PAC vesicles. (D) An immunoelectron micrograph of the isolated PAC vesicles with anti-PV72 antibodies. (E) An immunoelectron micrograph of the isolated PAC vesicles with anti-2S albumin antibodies. Bar = 500 nm.

Figure 2. Calcium-dependent binding of PV72 to pro2S albumin via its vacuolar targeting signal. (A) Suspension-cultured cells of Arabidopsis were transformed with a chimeric gene encoding SP-GFP-2SC that was composed of a signal peptide and GFP followed by the C-terminal 18-amino-acid sequence of pro2S albumin. GFP fluorescence was observed within the vacuoles of the transformant cells (left). A differential-interference-contrast image of the same field as shown in the left (right). Bars = 20 μm. (B) Both PV82 and PV72 were bound to the affinity column conjugated with the C-terminal peptide of pro2S albumin (2SC) in the presence of 5 mM $CaCl_2$. PV82 was eluted from the column with a solution of 400 μM $CaCl_2$, but not PV72. PV72 was eluted with a solution of 1 mM EGTA. Each fraction of the affinity chromatography was subjected to SDS-PAGE followed by an immunoblot with the mixture of anti-PV72 antibodies and anti-PV82 antibodies. (C) rPV72, the lumenal domain of PV72, was bound to the affinity column conjugated with pro2S albumin (p2S) in the presence of 1 mM $CaCl_2$, and was eluted with a solution of 1 mM EGTA (upper). rPV72 was not bound to the column that had been equilibrated with 1 mM EGTA (lower). Each

fraction of the affinity chromatography was subjected to SDS-PAGE followed by an immunoblot with anti-PV72 antibodies.

Figure 3. Subcellular fractionation of maturing pumpkin cotyledons. Whole homogenate of maturing pumpkin cotyledons was layered on a sucrose density gradient (30-60%, w/w) and centrifuged. Fractions were numbered from the top to the bottom of the gradient. Each fraction was subjected to SDS-PAGE followed by immunoblots with antibodies against each of 11S globulin (pG), 2S albumin (p2S), clathrin heavy chain (CHC), β–adaptin (βAd), PV72 and PV82. Microsomal fractions 2-3 and PAC-vesicle fractions 9-12 are indicated by horizontal bars at top.

Figure 4. Subcellular localization of the fusion protein of GFP with a C-terminal domain of PV72 in Arabidopsis suspension cells. (A) Suspension-cultured cells of Arabidopsis were transformed with a chimeric gene encoding SP-GFP-PV72C that was composed of a signal peptide and GFP followed by the C-terminal 68-amino-acid sequence of PV72. The C-terminal sequence includes a transmembrane domain and a cytosolic tail with a tyrosine-based signal, YMPL. GFP fluorescence was observed on small particles throughout cytoplasm of the transformant cells. (B) A differential-interference-contrast image of the same field as shown in (A). Bars = 20 μm.

Figure 5. Both PV72 and PV82 have complex-type oligosaccharide(s). PV72 and PV82 in the isolated PAC vesicles were incubated in the absence (-) or presence (+) of either N-glycosidase F or endoglycosidase H at 37 °C for 3 h and then subjected to SDS-PAGE followed by an immunoblot with anti-PV82 antibodies. PV72ΔΨ and PV82ΔΨ indicate deglycosylated PV72 and PV82, respectively.

Figure 6. Organ-specific expression of PV72 and PV82 in pumpkin. Total proteins were extracted from various pumpkin organs, maturing cotyledons (lanes 1 and 6), roots (lanes

2and 7), hypocotyls (lanes 3 and 8), leaves (lanes 4 and 9) and green cotyledons (lanes 5 and 10). Each extract (20 μg protein) was subjected to SDS-PAGE followed by immunoblots with anti-PV72 antibodies (upper) or anti-PV82 antibodies (lower).

Figure 7. Developmental changes in the level of PV72 and PV82 in pumpkin cotyledons during seed maturation and after seed germination. Pumpkin cotyledons were harvested at the indicated days after pollination (18-38) or after seed germination (0-6). Whole homogenate from the cotyledons (75 mg fresh weight) was subjected to SDS-PAGE followed by either staining with Coomassie blue (upper) or immunoblots with anti-PV72 antibodies and anti-PV82 antibodies (lower). 7S, 7S globulin; G, 11S globulin; 2S, 2S albumin.

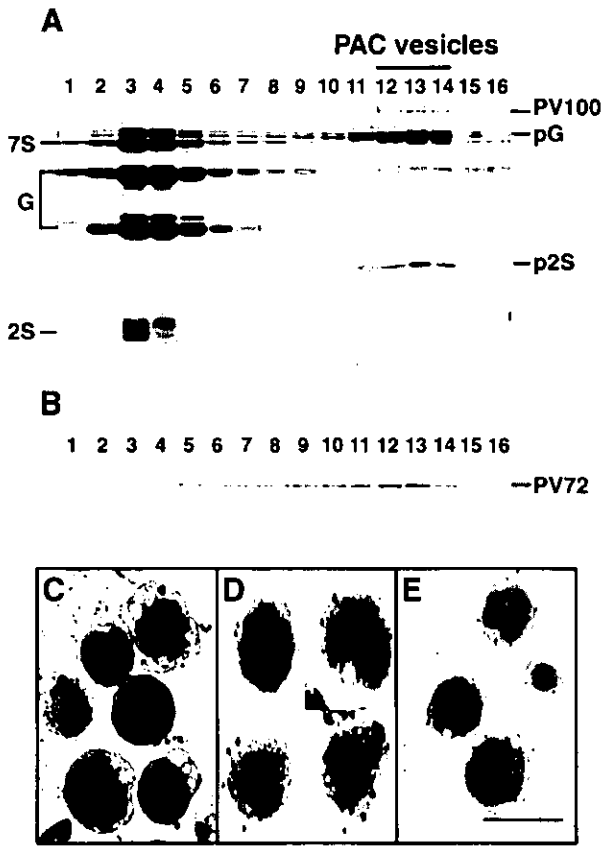
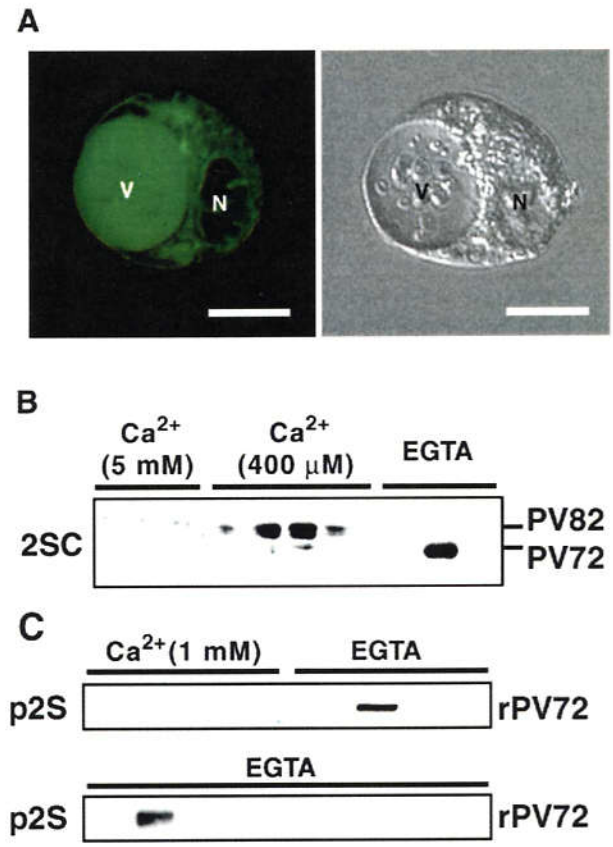


Figure 1 Shimada et al.



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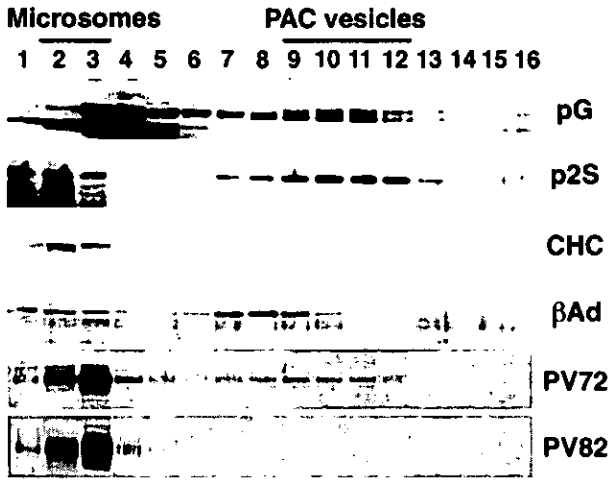

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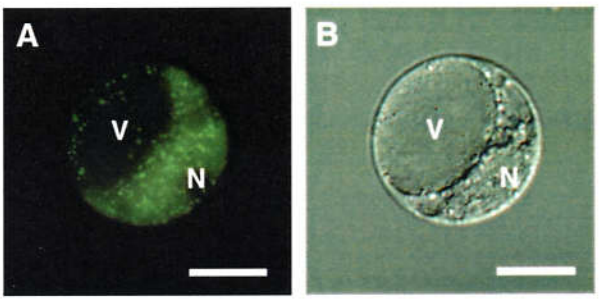

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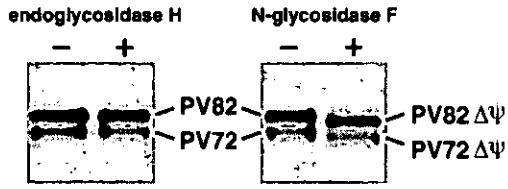

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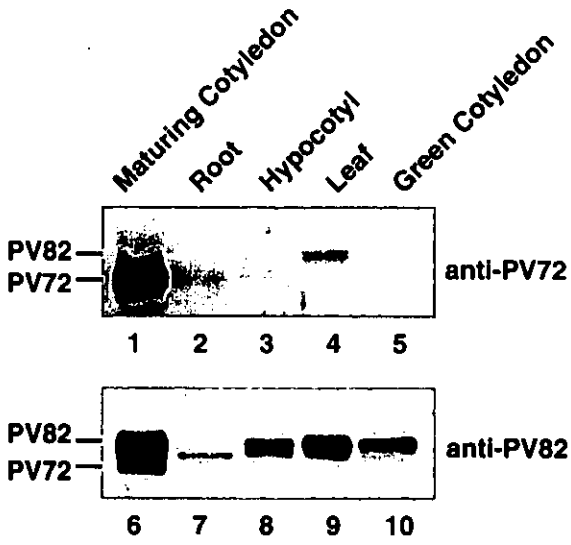

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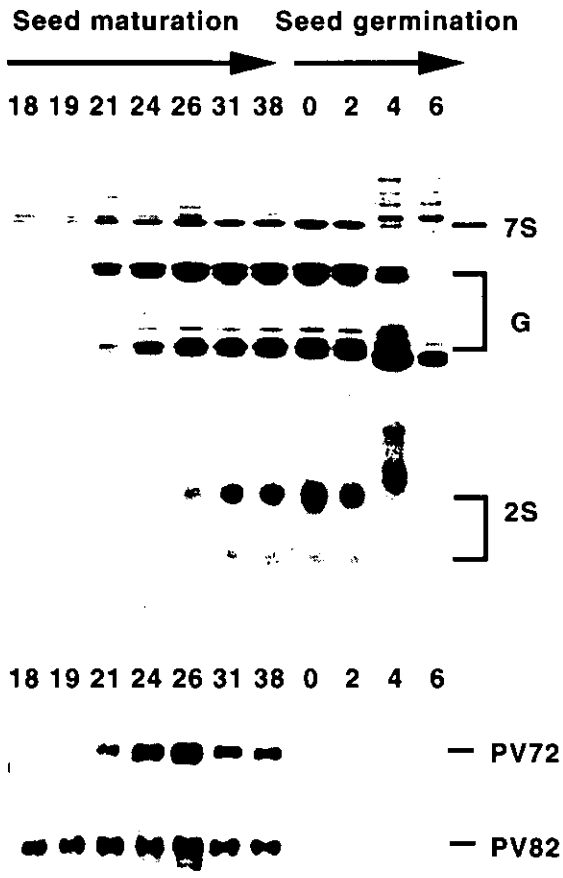

Figure 7 Shimada et al.