An ER-Localized Form of PV72, a Seed-Specific Vacuolar Sorting Receptor, Interferes the Transport of an NPIR-Containing Proteinase in Arabidopsis Leaves

Etsuko Watanabe 1, Tomoo Shimada 1, Kentaro Tamura 1, Ryo Matsushima 1, Yasuko Koumoto 1, Mikio Nishimura 2,3 and Ikuko Hara-Nishimura 1,4

1 Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan
2 Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444-8585 Japan
3 Department of Molecular Biomechanics, School of Life Science, Graduate University for Advanced Studies, Okazaki, 444-8585 Japan

Putative vacuolar sorting receptors that bind to the vacuolar targeting signals have been found in various plants; pumpkin PV72, pea BP-80 and Arabidopsis AtELP. PV72 is a seed-specific receptor that is predicted to sort seed storage proteins to protein storage vacuoles. Analysis by surface plasmon resonance showed that the lumenal domain of PV72 bound to an NPIR (a typical vacuolar targeting signal)-containing peptide of the precursor of a cysteine proteinase, AtALEU, in the presence of Ca\(^{2+}\) (\(K_D = 0.1\mu M\)). To elucidate the receptor-dependent transport of vacuolar proteins in plant cells, we produced transgenic Arabidopsis plants that expressed a fusion protein (PV72-HDEL) composed of the lumenal domain of PV72 and an endoplasmic reticulum (ER)-retention signal, HDEL. The expression of PV72-HDEL induced the accumulation of the AtALEU precursor. The accumulation level of the AtALEU precursor was dependent on that of PV72-HDEL. In contrast, it did not induce the accumulation of a precursor of another cysteine proteinase, RD21, which contains no NPIR. Detailed subcellular localization revealed that both the AtALEU precursor and PV72-HDEL accumulated in the ER fraction. We found that most of the AtALEU precursor molecules formed a complex with PV72-HDEL. The AtALEU precursor might be trapped by PV72-HDEL in the ER and not transported to the vacuoles. This in-planta analysis supports the hypothesis that an Arabidopsis homolog of PV72 functions as a sorting receptor for the NPIR-containing proteinase. The overall results suggest that vacuolar sorting receptors for the protein storage vacuoles and the lytic vacuoles share the similar recognition mechanism for a vacuolar targeting signal.

Keywords: Arabidopsis thaliana — Ligand–receptor interaction — PV72 — Vacuolar proteins — Vacuolar sorting receptor — Vacuolar targeting signal.

Abbreviations: CCV, clathrin-coated vesicle; CTPP, C-terminal propeptide; EGF, epidermal growth factor; ER, endoplasmic reticulum; NTPP, N-terminal propeptide; PA domain, protease-associated domain.

Introduction

Most vacuolar proteins are synthesized on the rough endoplasmic reticulum (ER) and are then delivered to the vacuoles via vesicle-mediated transport systems (Okita and Rogers 1996). Such sorting and targeting to vacuoles requires the presence of a specific signal and a receptor. In plants, two types of the targeting signals of vacuolar proteins have been described. One is an N-terminal propeptide (NTPP) and the other is a C-terminal propeptide (CTPP). The NTPP occurs in a barley cysteine proteinase, barley aleurain (Holwerda et al. 1992), and a sweet potato storage protein, sporamin (Matsuoka and Nakamura 1991). Both barley aleurain and sweet potato sporamin have an NPIR sequence conserved in the NTPP. The AtALEU precursor was dependent on that of PV72-HDEL. In contrast, it did not induce the accumulation of a precursor of another cysteine proteinase, RD21, which contains no NPIR. Detailed subcellular localization revealed that both the AtALEU precursor and PV72-HDEL accumulated in the ER fraction. We found that most of the AtALEU precursor molecules formed a complex with PV72-HDEL. The AtALEU precursor might be trapped by PV72-HDEL in the ER and not transported to the vacuoles. This in-planta analysis supports the hypothesis that an Arabidopsis homolog of PV72 functions as a sorting receptor for the NPIR-containing proteinase. The overall results suggest that vacuolar sorting receptors for the protein storage vacuoles and the lytic vacuoles share the similar recognition mechanism for a vacuolar targeting signal.

Keywords: Arabidopsis thaliana — Ligand–receptor interaction — PV72 — Vacuolar proteins — Vacuolar sorting receptor — Vacuolar targeting signal.

4 Corresponding author: E-mail, ihnishi@gr.bot.kyoto-u.ac.jp; Fax, +81-75-753-4142.
β-conglycinin α’ subunit was reported to act as a vacuolar sorting determinant (Nishizawa et al. 2003).

Putative vacuolar sorting receptors that bind to the vacuolar targeting signals have been identified in proteins from various plants: pumpkin PV72 (Shimada et al. 1997), pea BP-80 (Paris et al. 1997), Arabidopsis AtELP (Ahmed et al. 1997) and Black gram VmVSR (Tsuru-Furuno et al. 2001). They are type I integral membrane proteins with three repeats of an epidermal growth factor (EGF)-like motif in the C-terminal region of the luminal domain. The cytosolic tail contains a YMPL sequence, a potential Tyr-based motif, which is known to be recognized by an adapter complex of clathrin-coated vesicles (CCVs). BP-80 has been shown to be localized in CCVs and the Golgi complex of maturing pea cotyledons (Hillmer et al. 2001, Hinz et al. 1999). AtELP was localized in the Golgi complex and prevacuolar compartments (Ahmed et al. 2000, Sanderfoot et al. 1998). A number of homologs of the putative receptors have been registered in the database. For example, Arabidopsis has seven homologs. According to the phylogenetic tree of the homologs, they can be separated into two or three groups. The binding site of these receptors was located in their lumen domain (Cao et al. 2000, Watanabe et al. 2002). BP-80 was the first putative receptor to be identified that binds in vitro to the NPIR-containing peptide of the propeptide of aleurain (Kirsch et al. 1994). BP-80 has been found to bind not only to the NPIR signal but also to the C-terminal sequence that contained a vacuolar targeting signal of Brazil nut 2S albumin (Kirsch et al. 1996). Similarly, PV72 has been shown to bind not only to the NPIR signal of aleurain but also to the C-terminal peptide of pumpkin 2S albumin (Shimada et al. 1997). AtELP bound to the NPIR sequence of the NTPP of AtALEU and sporamin in vitro (Ahmed et al. 2000). These results indicate that these putative receptors have a broad specificity to ligands. PV72 was the next most abundant protein after the storage protein precursors in the precursor-accumulating (PAC) vesicles that are responsible for the mass transport of storage proteins to protein storage vacuoles in maturing pumpkin seeds (Hara-Nishimura et al. 1998, Shimada et al. 1997). Recently we found that PV72 is transiently accumulated at the middle stage of seed maturation in association with the synthesis of storage proteins, but is not expressed in vegetative tissues (Shimada et al. 2002). We also found that the seed-specific PV72 can bind to the 2S albumin precursor (Shimada et al. 2002). Therefore, we suggested that PV72 functions as a sorting receptor that sorts seed storage proteins to protein storage vacuoles.

In transgenic Arabidopsis plants which expressed sporamin, AtELP is colocalized with sporamin at the Golgi complex (Ahmed et al. 2000). In tobacco cells, BP-80 homolog interacts with the precursor of Na-Pi, Nicotiana alata proteinase inhibitor, containing a C-terminal vacuolar targeting signal in vivo (Miller et al. 1999). Humair et al. reported that BP-80 targeted the GFP fusion protein with petunia aleurain NTTP to vacuoles of yeast mutant (Humair et al. 2001). These observations suggested the functional role of the vacuolar sorting receptors. However, no in-planta demonstration of the vacuolar sorting receptors has been given. A dominant-negative strategy in planta should provide a valuable insight into the receptor-dependent vacuolar sorting system. We created transgenic Arabidopsis plants that express the putative receptor PV72 in the ER and characterized the effect of the expression on the transport of a vacuolar protein. We found that the ectopic expression of ER-localized PV72 disturbed vacuolar sorting of an NPIR-containing proteinase, AtALEU, and resulted in the accumulation of the precursor in the leaf cells. Our results provide the evidence for the function of a putative receptor in planta. The results also indicate that the seed-specific PV72 has an ability to recognize a vacuolar targeting signal of a lytic enzyme in the vegetative tissues of the transgenic plants. The transgenic plants accumulate vacuolar protein precursors that are sorted by the receptors. Therefore, the natural ligands for such receptors can now be identified by proteome analysis of the transgenic plants.

**Results**

Expression of an ER-localized soluble PV72 results in the accumulation of a precursor of an NPIR-containing proteinase, AtALEU

To demonstrate the physiological function of the putative vacuolar sorting receptors in plant cells, we created transgenic Arabidopsis plants that expressed an ER-localized soluble form of PV72. The transgene encoded a fusion protein composed of the signal peptide and the luminal domain of PV72 followed by a His-tag and an ER-retention signal, HDEL, under the control of the cauliflower mosaic virus 35S promoter. An immunoblot analysis with anti-PV72 antibodies showed the accumulation of the fusion protein (PV72-HDEL) in the rosette leaves of each line of four independent transgenic plants. Line 4 accumulated a high level of PV72-HDEL, whereas line 3 accumulated a low level of it (Fig. 1a, upper panel).

To determine whether the transport of an NPIR-containing protein is affected by expression of the soluble PV72-HDEL molecules in the ER, we focused on a typical vacuolar cysteine proteinase, Arabidopsis aleurain (AtALEU), which is a homolog of barley aleurain. An immunoblot with anti-aleurain monoclonal antibody showed that a 41-kDa AtALEU precursor accumulates specifically in the rosette leaves of the transgenic plants expressing PV72-HDEL, together with the 28-kDa mature form of AtALEU (Fig. 1a, lower panel). In wild-type plants, only the mature form of AtALEU was detectable and the precursor form was never detected. In three lines (1, 2, 4), which expressed PV72-HDEL at high levels, the accumulation of the AtALEU precursor was very high. On the other hand, in line 3, which expressed a low level of PV72-HDEL, the accumulation of the AtALEU precursor was very low. This implies that the expression level of AtALEU precursor correlates strongly with the accumulation of PV72-HDEL (Fig. 1a).
Fig. 1 Expression of an ER-localized soluble PV72 results in the accumulation of an NPIR-containing precursor of AtALEU in the rosette leaves of transgenic Arabidopsis Plants. (a) Transgenic Arabidopsis plants that expressed an ER-localized form of PV72 (PV72-HDEL) were generated. Proteins were extracted from one rosette leaf of four independent transgenic plants (T/PV72–1 to 4) and wild-type plants (WT) and were subjected to SDS-PAGE followed by immunoblot analysis with anti-PV72 antibody and anti-aleurain monoclonal antibody (2F5). The 41-kDa protein precursor (p) of AtALEU, which has a vacuolar targeting signal, NPIR, was found in the rosette leaves of transgenic plants, in addition to the mature form (m) of AtALEU that were accumulated in both the wild-type and transgenic plants. (b) Crude extracts from the rosette leaves of the transgenic plants (T/PV72–2) and wild-type plants (WT) were subjected to SDS-PAGE followed by immunoblot analysis with anti-PV72 antibodies (upper), anti-aleurain antibody (middle) and anti-RD21 antibodies (lower). In contrast to the accumulation of the AtALEU precursor (p), the precursor of RD21, which does not have an NPIR sequence, was not detected in either the transgenic plants (T/PV72–2) or wild-type plants (WT). m, the respective mature form of AtALEU or RD21; p*, the predicted position of the RD21 precursor.

We used the line 2 of the transgenic plants in the following experiments.

We also examined another vacuolar protease of papain family, RD-21, which is the product of the responsive-to-desiccation gene 21 (Koizumi et al. 1993, Yamada et al. 2001). A typical vacuolar targeting signal, the NPIR sequence, is not found in the propeptide of RD21. An immunoblot with anti-RD21 antibodies showed that the mature form of RD21 accumulated in the leaves of both the wild-type plants and the transgenic plants, but no precursor was detected (Fig. 1b, lower panel). This result was in contrast to that of AtALEU. These results indicate that the expression of seed-specific putative receptor in the ER caused the accumulation of the precursor of an NPIR-containing proteinase in the rosette leaves, but not the precursor of non-NPIR-containing proteinase.

The AtALEU precursor forms a complex with PV72-HDEL in the ER and is prevented from leaving the ER

To determine the subcellular localization of the accumulation of the AtALEU precursor, the rosette leaves of both the transgenic and wild-type plants were subjected to differential centrifugation to obtain four subcellular fractions: a 1,000×g pellet (P1), an 8,000×g pellet (P8), a 100,000×g pellet (P100) and a 100,000×g supernatant (S100). Fig. 2 shows the immunoblot of each fraction with anti-aleurain monoclonal antibody. The mature form of AtALEU and the intermediate were predominantly detected in the S100/vacuolar fraction of both the transgenic and wild-type plants. On the other hand, the AtALEU precursor was detected specifically in a total homogenate of the transgenic plants, but not in a homogenate of the wild-type plants. The AtALEU precursor was found to be in the P8 and the P100/microsomal fractions of the transgenic plants, but not in the S100/vacuolar fraction (Fig. 2). The distinct accumulation of the precursor in the ER was caused by expression of PV72-HDEL.

To investigate the subcellular localization of both of PV72-HDEL and the AtALEU precursor in more detail, we performed sucrose density gradient analysis with or without magnesium. A magnesium-dependent density shift is characteristic of ER and it occurs when magnesium is chelated and polyribosomes are dissociated from the ER. Total homogenates from the rosette leaves were subjected to the analysis to characterize not only ER fraction but also vacuolar fraction. In the absence of magnesium, PV72-HDEL was localized in 6–10 fractions which also included BiP, an ER marker (Fig. 3a). In addition, the AtALEU precursor was also colocalized in these fractions. In the presence of magnesium, the band pattern of PV72-HDEL shifted to the 11–15 fractions that also contained BiP. As expected, we also found that the AtALEU precursor shifted to the same fractions (Fig. 3b). These results confirmed colocalization of PV72-HDEL and the AtALEU precursor in ER. Our observations suggest that the expression of PV72-HDEL in the ER prevents the AtALEU precursor from leaving the ER by trapping the precursor molecules. In this fractionation, vacuolar contents including the mature AtALEU were retained in the top of the gradient. A small part of the mature AtALEU was sedimented in the 11–15 fractions in the presence of magnesium.
and absence of magnesium. This might be caused by the cosedimentation with chloroplasts (data not shown).

To detect the interaction between PV72-HDEL and the AtALEU precursor, affinity chromatography was performed. We applied the solubilized proteins of the microsomal fraction of the transgenic plants to a Ni-chelating column. Two fractions were obtained: a flow-through fraction and a fraction eluted with CHAPS buffer containing 400 mM imidazole. Both fractions were subjected to immunoblot analysis with anti-PV72 antibodies. The His-tagged PV72-HDEL fusion protein bound to the Ni-chelating column. As expected, all of the PV72-HDEL molecules were detected in the eluted fraction (Fig. 4, upper panel). Fig. 4 (lower panel) shows that the 41-kDa AtALEU precursor was co-purified with the His-tagged PV72-HDEL in the eluted fraction. This indicates that the AtALEU precursor forms a complex with the PV72-HDEL molecule. Neither the intermediate nor the mature forms of AtALEU bound to the column. RD21, which has no NPIR sequence, was not co-purified with PV72-HDEL (data not shown). These results suggest that the NPIR-containing propeptide of the AtALEU precursor is recognized by PV72-HDEL to form a complex with PV72-HDEL within the ER of the transgenic plants.

The NPIR-containing propeptide, a vacuolar targeting signal, of the AtALEU precursor is recognized by PV72 in the presence of calcium

The amino acid sequence of the NPIR peptide of AtALEU (ANIGFDESNPIRMVSDGLREV) exhibits only minimal homology with that of barley aleurain (SSSFADSNPIRPVT-DRAAST), which is known to be a vacuolar targeting signal. Nonetheless, the NPIR-containing propeptide of AtALEU was sufficient to target GFP to the vacuoles of Arabidopsis (Tamura et al. 2003). It is possible that the NPIR-containing propeptide of AtALEU functions as a targeting signal for the lytic enzymes to the vacuoles as does that of barley.

To determine the affinity of the putative sorting receptor PV72 for the NPIR-containing vacuolar targeting signal of AtALEU, we expressed the lumenal domain of PV72 (rPV72) in insect cells employing a baculovirus expression system (Watanabe et al. 2002). For surface plasmon resonance, rPV72 was injected onto a sensor chip conjugated with the NPIR-containing peptide to start the association reaction. Fig. 5a shows the association and dissociation curves for four different concentrations (0.15–0.7 μM) of rPV72. The kinetic constants of association ($k_a$) and dissociation ($k_d$) were calculated from the slopes of the curves, as shown in Fig. 5b. From the ratio of these two kinetic constants ($k_d/k_a$), the apparent equilibrium dissociation constant ($K_D$) of rPV72 for the peptide was determined to be 0.1 μM. rPV72 included three repeats of an EGF-like motif in the C-terminal region. We also analyzed the surface plasmon resonance with rPV72Δ, a form of rPV72 with
no EGF-like motif. The association and dissociation were more rapid for rPV72Δ than for rPV72. The $K_D$ value of rPV72Δ was 10-fold higher than that of rPV72. It seems likely that the EGF-like motifs play a role in stabilizing the receptor–ligand complex. The kinetic analysis showed that PV72 exhibits a sufficiently high affinity to act as a vacuolar sorting receptor.

Although the receptor–ligand complex should be finally dissociated to release the ligand molecules at their final destinations, there is no insight into how this occurs. Recently, we found that the Ca$^{2+}$ concentration, rather than pH, regulates the interaction of PV72 with the NPIR-containing peptide. A sensor chip was coupled with the NPIR-containing propeptide of AtALEU on BIACORE-X (a coupling efficiency of 700 resonance units). rPV72 and rPV72Δ were injected onto the sensor chip at different concentrations from 0.17 μM to 0.7 μM to obtain the sensograms for the association and dissociation curves by surface plasmon resonance. (b) Three 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 sensograms using BIA evaluation software version 2.1. These kinetic parameters were determined from two independent experiments.

The putative vacuolar sorting receptors including PV72 (Shimada et al. 1997), BP-80 (Kirsch et al. 1994) and AtELP (Ahmed et al. 1997) possess high homology and an affinity for a vacuolar targeting signal, the NPIR-containing motif. But it is still unclear whether these putative receptors function as sorting receptors for vacuolar proteins in the plant cells. AtELP has been shown to be localized in the Golgi complex and the prevacuolar compartments and has been predicted to be involved in transport of AtALEU (Ahmed et al. 2000, Sanderfoot et al. 1998). An efficient sorting of AtALEU should be disturbed in cells that express the ligand-binding domain of a receptor protein in the ER where AtALEU is synthesized. To demonstrate

![Image](https://academic.oup.com/pcp/article-abstract/45/1/9/1889368)

**Fig. 5** Kinetics for the association and dissociation of PV72 and the NPIR-containing propeptide of AtALEU. (a) PV72 has three EGF-like motifs at the C terminus of the lumenal domain. We expressed two modified PV72s in insect cells (Sf21) for the kinetic analysis. rPV72 was composed of the lumen domain followed by a His-tag and the HDEL sequence and rPV72Δ was composed of PV72 with no EGF-like motif. A sensor chip was coupled with the NPIR-containing propeptide of AtALEU on BIACORE-X (a coupling efficiency of 700 resonance units). rPV72 and rPV72Δ were injected onto the sensor chip at different concentrations from 0.17 μM to 0.7 μM to obtain the sensograms for the association and dissociation curves by surface plasmon resonance. (b) Three 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 sensograms using BIA evaluation software version 2.1. These kinetic parameters were determined from two independent experiments.

![Image](https://academic.oup.com/pcp/article-abstract/45/1/9/1889368)

**Fig. 6** Calcium-mediated interaction between PV72 and the NPIR-containing propeptide of AtALEU. (a) rPV72 (upper) and rPV72Δ (lower) were separately subjected to an affinity column conjugated with the NPIR-containing propeptide of AtALEU in HEPES buffer (pH 7.0) containing 1 mM CaCl$_2$. Both proteins bound to the column and were then eluted by addition of HEPES buffer (pH 7.0) containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE followed by immunoblot analysis with anti-PV72 antibodies. FT, flow-through fraction; Wash, washing fraction; EGTA, eluted fraction. (b) Either rPV72 (upper) or rPV72Δ (lower) were applied to the affinity column in HEPES buffer (pH 7.0) containing 1 mM CaCl$_2$. The column was washed with Na-acetate buffer (pH 4.0) containing 1 mM CaCl$_2$, and finally washed with Na-acetate buffer containing 2.5 mM EGTA. rPV72 still bound to the column at pH 4.0 in the presence of 1 mM CaCl$_2$.
A vacuolar sorting receptor in plants

Vacuolar sorting receptors for the lytic vacuoles and the protein storage vacuoles share the similar recognition mechanism for a vacuolar targeting signal

Seed proteins that are expressed in vegetative cells are correctly targeted to the lytic vacuoles. Using this system, various vacuolar targeting signals have been determined for seed proteins; common bean phasatein (Holkeri and Vitale 2001), Brazil nut 2S albumin (Saalbach et al. 1996), barley lectin (Bednarek and Raikhel 1991) and castor bean ricin (Frigerio et al. 2001). Furthermore, the vacuolar sorting of phasatein has been shown to be saturated in over-expression experiments (Frigerio et al. 1998). These results suggested a receptor-mediated transport of the storage protein in the vegetative cells. What kind of receptor functions in the transport of these proteins? BP-80 has been shown to bind to the C-terminal peptide of Brazil nut 2S albumin in vitro (Kirsch et al. 1996). The vacuolar targeting signal of Brazil nut 2S albumin exists in this C-terminal peptide (Saalbach et al. 1996). This result suggests that a BP-80 homolog plays a role in the sorting of 2S albumin in tobacco leaves. The sorting machinery for lytic vacuoles seems to be sufficiently similar to that for protein storage vacuoles to recognize the targeting signal of seed proteins. The reverse might be also true. Our results show that seed-specific PV72 recognizes a vacuolar targeting signal of a lytic enzyme in the vegetative tissues of transgenic Arabidopsis plants. Determining the tissue-specific and time-specific expression of each receptor is key to understanding the natural ligands of the receptor.

PV72 was found on the membrane of PAC vesicles responsible for the transport of seed protein precursors to the protein storage vacuoles (Shimada et al. 1997). Recently we reported that PV72 was specifically and transiently accumulated in maturing pumpkin seeds in association with the synthesis of seed storage proteins (Shimada et al. 2002). PV72 has been shown to bind to the proteptides derived from pumpkin 2S albumin with an affinity high enough to function as a sorting receptor (Shimada et al. 1997, Watanabe et al. 2002). These observations imply that PV72 act as a receptor for 2S albumin. Unexpectedly, no significant difference in the accumulation of pro2S albumin in dry seeds and maturing seeds was found between the transgenic plants and the wild-type plants (data not shown). There are two reasons why the accumulation of pro2S albumin was not affected by the expression of PV72-HDEL. One is that the level of PV72-HDEL was very low in maturing seeds. This was also supported by a report that the expression of introduced genes under the control of the 35S promoter is much weaker in seeds than in other tissues (Zuo et al. 2001). Another reason is the multiple transport pathways for 2S albumin. The PAC vesicle, an ER-derived compartment, mediates the direct transport of pro2S albumin from the ER into protein storage vacuoles with no intervention of PV72 (Harano-Nishimura et al. 1998). It is possible that pro2S albumin which is trapped in the ER is transported to protein storage vacuoles via the direct pathway.
Unique interaction between PV72 and the NPIR-containing peptide in a Ca\textsuperscript{2+}-dependent manner

The ligand-binding abilities of BP-80 (Kirsch et al. 1994) and AtELP (Ahmed et al. 2000) have been reported to be regulated by environmental pH. The optimum pH for the ligand binding of the receptors was determined to be pH 6.0–6.5 and the dissociation of the receptor and the ligand occurred at pH 4.0 (Kirsch et al. 1994). However, we demonstrated that rPV72 still had an ability to bind to the NPIR-containing peptide of AtALEU even at pH 4.0 in the presence of Ca\textsuperscript{2+} (Fig. 6). This indicates that rPV72 interacts with the vacuolar targeting signal in a Ca\textsuperscript{2+}-dependent manner. This was consistent with our previous finding that the Ca\textsuperscript{2+} concentration modulates the interaction of PV72 to either pro2S albumin (Shimada et al. 2002) or the internal propeptide of 2S albumin (Watanabe et al. 2002). PV72 and all of its homologs have a consensus sequence for Ca\textsuperscript{2+} binding in the third EGF-like motif. Binding of Ca\textsuperscript{2+} to the EGF-like motif might cause a conformational change in the PV72 molecule that stabilizes the receptor–ligand complex (Watanabe et al. 2002). The association and dissociation regulated by Ca\textsuperscript{2+} concentration might be a unique mechanism for transport of vacuolar proteins.

In this report, we demonstrated that the N-terminal domain of PV72 also interacted with the AtALEU propeptide (Fig. 5, 6). Previously, we showed that the binding site of PV72 for the internal propeptide of 2S albumin was localized at the N-terminal region and that EGF-motifs functioned as the stabilizing the complex of the ligand and receptor. N-terminal domain contained a PA (protease associated) domain which was conserved among plant vacuolar sorting receptors (Mahon and Bateman 2000). BP-80 has two binding sites which recognized two separate ligand determinants (Cao et al. 2000, Paris and Neuhaus 2002). One is an NPIR-specific binding site contributed by the interaction of the N-terminal domain and central domain. The N-terminal domain of BP-80 also has a PA domain (Mahon and Bateman 2000). The PA domain might function as a ligand-binding site for NTTP. The other binding site is a non-NPIR-specific site defined by the central domain. The PA domain might cause a conformational change in the PV72 molecule that stabilizes the receptor–ligand complex (Watanabe et al. 2002). The association and dissociation regulated by Ca\textsuperscript{2+} concentration might be a unique mechanism for transport of vacuolar proteins.

In this report, we demonstrated that the N-terminal domain of PV72 also interacted with the AtALEU propeptide. The 2- to 4-week-old plants were used for immunoblot analysis. Arabidopsis plants were transformed A. tumefaciens (strain EHA101) (Hood et al. 1986) by electroporation. Arabidopsis plants were transformed with anti-aleurain monoclonal antibody. Immunoblot analysis was performed essentially as described previously (Bechtold and Pelletier 1998). T\textsubscript{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, and 1 mM EDTA with or without 5 mM MgCl\textsubscript{2}. The gradient was centrifuged at 8,000 g for 20 min. The pellet (P1) was digested with Kpn\textsubscript{I} and was inserted into pBS-KS to produce pBS-PV72-HDEL. The XbaI- and KpnI-digested pBS-PV72 was introduced into Ti-plasmid pBH121HmRV (Kinoshita et al. 1999) to produce the vector, pBI-PV72-HDEL.

The vector pBI-PV72-HDEL was introduced into Agrobacterium tumefaciens (strain EHA101) by electroporation. Arabidopsis plants were transformed with anti-aleurain monoclonal antibody. Immunoblot analysis was performed essentially as described previously (Bechtold and Pelletier 1998). T\textsubscript{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 kanamycin (75 mg liter\textsuperscript{-1}). The expression level of PV72 was checked in four independent T\textsubscript{1} plants. Finally, we selected the transformant expressing a high level of PV72-HDEL and used it for the experiments.

Immunoblot analysis

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

Materials and Methods

Plant materials

Arabidopsis thaliana (ecotype Columbia-0) 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 medium. Plants were grown at 22°C under continuous light. The 2- to 4-week-old plants were used for immunoblot analysis, affinity column chromatography and subcellular fractionation, as described below.

Plasmid construction of PV72-HDEL and transformation of Arabidopsis plants

The cDNA of PV72 and two oligonucleotides, 5'-GAAACAAA-TGAGTGTGTGACA-3' and 5'-GGACCTCAGCTACGGG-TTTGTCAGCTGTTGTTGTGTTGC-3' were used to amplify a DNA fragment encoding a fusion protein (PV72-HDEL) composed of a signal peptide and a luminal 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 HindIII and KpnI and was inserted into pBS-KS to produce pBS-PV72-HDEL. The XbaI- and KpnI-digested pBS-PV72 was introduced into Ti-plasmid pBH121HmRV (Kinoshita et al. 1999) to produce the vector, pBI-PV72-HDEL.

The vector pBI-PV72-HDEL was introduced into Arabidopsis thaliana (strain EHA101) by electroporation. Arabidopsis plants were transformed with T-DNA (ecotype Columbia-0) 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 medium. Plants were grown at 22°C under continuous light. The 2- to 4-week-old plants were used for immunoblot analysis, affinity column chromatography and subcellular fractionation, as described below.
trifuged at 85,500 g and 4°C for 2.5 h with a swing rotor. Fractions were collected from the bottom of gradient and the sucrose concentration of each fraction was determined using hand refractometer. Each 20 μl fraction was subjected to SDS-PAGE and subsequently to immunoblot analysis with anti-aleurain monoclonal antibody, anti-PV72 antibodies and anti-BiP antibodies.

Co-purification of proαALEU with PV72-HDEL

The transgenic plants and wild-type plants were homogenized in the extraction buffer (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 1 mM CaCl₂, and 10% (w/v) sucrose), and were filtered through cheesecloth. The filtrate was centrifuged at 8,000 rpm for 30 min. The supernatant was centrifuged at 100,000 g for 1 h to obtain the pellet 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 CaCl₂). 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 microsomal fraction. We subjected the soluble fraction to Ni-NTA agarose (Qiagen, Tokyo, Japan) and washed the column with the CHAPS buffer. The bound proteins were eluted with the CHAPS buffer containing 400 mM imidazole. Each fraction was subjected to SDS-PAGE and then to immunoblot analysis with specific antibodies against either PV72 or aleurain.

Expression and purification of modified PV72s

Two modified PV72s were expressed in insect cells of Spodoptera frugiperda (Sf21) using a baculovirus expression system (Invitrogen, San Diego, CA, U.S.A.) as described previously (Watanabe et al. 2002). The recombinant proteins were purified using 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 Centriiicon 30 and subjected to a protein assay (Nippon Bio-Rad Laboratories, Tokyo, Japan) and binding assays as described below.

Surface plasmon resonance and kinetic assays

The peptide, ANIGFDESNPRMVDGLREV, which was derived from the propeptide of αtAULEU was chemically synthesized with a peptide synthesizer (model 431A; Applied Biosystems Inc., Tokyo, Japan). We immobilized the peptide on a sensor chip (carboxymethylated dextran chip; CM5) for BIACORE-X (BIACORE, Tokyo, Japan) and binding assays as described below.

Ligand-binding assay by affinity column chromatography

The synthesized peptide (10 mg, see above) was immobilized on NHS-activated Sepharose HP (Amersham Pharmacia Biotech) to prepare an affinity column. The modified PV72s were applied to the column equilibrated with the HEPS buffer containing 1 mM CaCl₂ 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 immunoblot analysis.

The modified PV72s were applied to the affinity column equilibrated with HEPS buffer containing 1 mM CaCl₂. 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 CaCl₂ and by the Na-acetate buffer containing 2.5 mM EGTA. Each fraction was subjected to SDS-PAGE and subsequently to immunoblot analysis.

Note added in proof

We have recently published an important paper demonstrating that an Arabidopsis homolog (AtVS1) of PV72 functions as a vacuolar-sorting receptor for seed storage proteins (Shimaba et al. Proc. Natl. Acad. Sci. USA 100: 16095–16100).

Acknowledgments

We thank Dr. J. C. Rogers of Washington State University for providing mouse monoclonal antibodies against aleurain (2F5). We are grateful to Ms. S. Osawa (NIBB, Japan) for helpful support on peptide synthesis. This work has been supported by Core Research of Science and Technology (CREST) of Japan Science and Technology Corporation (JST), Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 12138205), and Grants-in-Aid for the ‘Human Frontier Science Program’ (RG0018/2000-M 103).

References

A vacuolar sorting receptor in plants


(Received September 24, 2003; Accepted November 9, 2003)