Protein Mobilization in Germinating Mung Bean Seeds Involves Vacuolar Sorting Receptors and Multivesicular Bodies

Junqi Wang, Yubing Li, Sze Wan Lo, Stefan Hillmer, Samuel S.M. Sun, David G. Robinson, and Liwen Jiang*

Department of Biology and Molecular Biotechnology Program (J.W., Y.L., S.W.L., S.S.M.S., L.J.) and Institute of Plant Molecular Biology and Agricultural Biotechnology (J.W., S.S.M.S., L.J.), Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; and Department of Cell Biology, Heidelberg Institute for Plant Sciences, University of Heidelberg, D-69120 Heidelberg, Germany (S.H., D.G.R.)

Plants accumulate and store proteins in protein storage vacuoles (PSVs) during seed development and maturation. Upon seed germination, these storage proteins are mobilized to provide nutrients for seedling growth. However, little is known about the molecular mechanisms of protein degradation during seed germination. Here we test the hypothesis that vacuolar sorting receptor (VSR) proteins play a role in mediating protein degradation in germinating seeds. We demonstrate that both VSR proteins and hydrolytic enzymes are synthesized de novo during mung bean (Vigna radiata) seed germination. Immunogold electron microscopy with VSR antibodies demonstrate that VSRs mainly locate to the peripheral membrane of multivesicular bodies (MVBs), presumably as recycling receptors in day 1 germinating seeds, but become internalized to the MVB lumen, presumably for degradation at day 3 germination. Chemical cross-linking and immunoprecipitation with VSR antibodies have identified the cysteine protease aleurain as a specific VSR-interacting protein in germinating seeds. Further confocal immunofluorescence and immunogold electron microscopy studies demonstrate that VSR and aleurain colocalize to MVBs as well as PSVs in germinating seeds. Thus, MVBs in germinating seeds exercise dual functions: as a storage compartment for proteases that are physically separated from PSVs in the mature seed and as an intermediate compartment for VSR-mediated delivery of proteases from the Golgi apparatus to the PSV for protein degradation during seed germination.

The seeds of high plants accumulate large amounts of storage proteins during seed development and seed maturation, which are mobilized to provide building blocks and energy for seed germination and early seedling growth upon seed germination (Bewley and Black, 1994). During seed development and maturation, newly synthesized storage proteins are usually transported and stored in a specialized compartment, termed protein storage vacuole (PSV), which is defined by the presence of α- and δ-tonoplast intrinsic protein (TIP) in their tonoplasts (Jiang et al., 2000).

Upon seed germination, storage proteins, oil body, and starch are degraded and used for seedling growth (Bewley and Black, 1994; Poxleitner et al., 2006). However, relatively little is known about the mechanisms of protein mobilization in germinating seeds. Several mechanisms have been proposed. First, both storage proteins and proteases are transported to the same PSVs during seed development, but proteases remain inactive due to the presence of inhibition factors, which are removed upon seed germination and thus lead to activation of stored proteases and subsequent protein degradation within PSVs (Muntz et al., 2001). Second, the PSV globoid may represent a lytic compartment with proteases that are physically separated from storage proteins in the PSV (Jiang et al., 2001). Proteases inside the globoid will be released into the matrix upon seed germination, leading to protein degradation. Third, some proteases are newly synthesized in response to germination and transported to PSVs for protein degradation in germinating seeds. For example, a Cys protease, termed sulfhydryl-endopeptidase (SH-EP), is synthesized de novo in the cotyledons of germinating Vigna mungo seeds, where the proenzyme proSH-EP is packaged into KDEL-tailed vesicles (KV) and transported to the PSV for protein breakdown (Toyooka et al., 2000). Physiologically, as seed germination proceeds, PSVs become a degradative compartment with an acidic environment, filled with active proteases from the Golgi apparatus to the PSV for protein degradation during seed germination.
proteases, and essentially are converted into lytic vacuoles (Bewley and Black, 1994).

Transport of soluble proteins to lytic vacuoles in plant cells is a receptor-mediated process involving a protein family termed vacuolar sorting receptors (VSRs; Neuhaus and Rogers, 1998; Neuhaus and Paris, 2005). BP-80, a type I integral membrane protein isolated from pea (Pisum sativum), was the first VSR identified because of its interaction with the vacuolar sorting determinants of a Cys protease from barley (Hordeum vulgare) called aleurain (Kirsch et al., 1994). Later studies demonstrated that BP-80 specifically targeted aleurain to the lytic vacuole via clathrin-coated vesicles (CCVs) and a Golgi-dependent pathway in plant cells (Paris et al., 1997; Ahmed et al., 2000; Cao et al., 2001; Humair et al., 2001; Jiang and Rogers, 2003; Lam et al., 2005, 2007). In addition, the transmembrane domain and cytoplasmic tail (CT) of BP-80 were specific and sufficient for targeting BP-80 via the Golgi apparatus to its final destination (Jiang and Rogers, 1998; Tse et al., 2004; daSilva et al., 2005, 2006). More recently, BP-80 and its VSR homologs have been found to be concentrated in prevacuolar compartments (PVCs) that were identified as multivesicular bodies (MVBs) in tobacco (Nicotiana tabacum) Bright-Yellow (BY)-2 cells (Li et al., 2002; Tse et al., 2004, 2006; Mo et al., 2006). It has also been suggested that BP-80 and VSRs are recycled from PVCs to Golgi for further binding/sorting of cargo proteins, a process involving the recently identified retromer complex (Lam et al., 2005; Oliviusson et al., 2006). In contrast, nonrecycled receptors are believed to be delivered to the lytic vacuole for degradation. Like the RMR receptor (Jiang et al., 2000; Park et al., 2005), VSRs may also mediate the transport of storage proteins to the PSV (Shimada et al., 1997, 2003). It thus seems that VSR proteins might have dual roles in protein sorting in plant cells. Research to date indicates that most VSRs are concentrated on PVCs (Li et al., 2002; daSilva et al., 2005; Miao et al., 2006), even though a proteomic analysis points to the possibility of VSR isoforms located to the endoplasmic reticulum (ER) and/or Golgi apparatus in Arabidopsis (Arabidopsis thaliana) cultured cells (Dunkley et al., 2006).

Relatively little is known about the possible roles of VSR proteins during seed germination. VSRs have been found, via whole-genomic analysis and reverse transcription-PCR analysis, universally and are differentially expressed in various cell and tissue types during plant development and seed development (Laval et al., 2003; Neuhaus and Paris, 2005). Furthermore, antisense knockout VSR transgenic Arabidopsis seeds with undetectable VSRs failed to germinate, indicating an important role of VSR proteins during seed germination (Laval et al., 2003). However, VSR-interacting proteins have not been identified in germinating seeds so far.

In this study, we have tested the hypothesis that VSR proteins play a role in mediating protein degradation during seed germination. Toward this goal, we demonstrate that hydrolytic enzymes and, most likely, VSR proteins as well, are synthesized de novo during mung bean (Vigna radiata) seed germination. Whereas VSRs seem to be internalized into the lumen of MVBs for degradation, VSRs specifically interact with a newly synthesized Cys protease aleurain and colocalize together to MVBs in germinating mung bean seeds. Finally, aleurain reached PSVs in germinating seeds. Thus, VSRs transport newly synthesized proteases via PVCs/MVBs to PSVs for protein degradation during seed germination.

RESULTS

VSR Proteins Are Present in Germinating Seeds

Seven VSR isoforms with conserved amino acid sequences and structures have been identified in the Arabidopsis genome (Hadlington and Denecke, 2000). Most of the studies on the roles of VSRs in plants have been focused on vegetative cells and developing seeds, but little is known about the possible roles of VSRs during seed germination. As a first step to address this question, we wanted to find out whether VSRs are present in germinating seeds. Total protein was thus extracted from day 3 germinating seeds of various plant species and separated by SDS-PAGE for western-blot analysis with VSR antibodies. As shown in Figure 1, VSRat-1 antibodies, directed against a recombinant truncated protein representing the N-terminal region of VSRat-1 (Tse et al., 2004), detected a single band at 80 kDa from total proteins isolated from a variety of day 3 germinating seeds (lanes 2–10), including Arabidopsis, tomato (Lycopersicon esculentum), tobacco, red bean (Vigna angularis), pea, mung bean, red kidney bean (Phaseolus vulgaris), pumpkin (Cucurbita maxima), sweet bean (Lathyrus odoratus), and rice (Oryza sativa; data not shown) where tobacco-cultured BY-2 cells were also used as a positive control (lane 1), indicating the universal presence of VSRs in germinating seeds and the specificity of VSRat-1 antibodies in detecting VSRs in plants.

To study the possible roles of VSR proteins in germinating seeds, we then studied protein profiles during seed germination of mung bean seeds. Mung bean was chosen because the seed germinates uniformly almost 100% and rapidly with radicle appearance at day 1 after imbibition. Thus, seeds at different stages of germination were used for protein analyses. Proteins from day 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 after imbibition were separated by SDS-PAGE and analyzed (Figure 1). The content of VSRat-1 proteins present in germinating seeds of mung bean (Vigna radiata) is shown in Figure 1. Western-blot analysis of VSR proteins with VSRat-1 antibodies in total protein extracts of various germinating seeds. Asterisk indicates the position of VSRs at 80 kD.
stages of imbibition were collected for protein extraction and western-blot analysis using various antibodies. As shown in Figure 2A, VSR proteins are present in all stages of germinating seeds with gradually decreased amounts from day 0 to day 4 after imbibition, where a new band with lower Mr was detected from day 2 germinating seeds. When the BP-80 CT antibodies, raised against a synthetic peptide corresponding to the CT of BP-80 (Tse et al., 2004), were used, the detected VSR proteins showed similar decreasing profiles in germinating seeds. Similarly, both α- and δ-TIP, markers for PSVs, are present during the early stages of germination from day 0 to day 3 with decreased amounts, indicating the degradation of storage contents in germinating seeds. In contrast, the lytic vacuole marker γ-TIP (Paris et al., 1996) and the two vacuolar Cys proteases, the Golgi-derived aleurain (Holwerda et al., 1990), and the ER-derived SH-EP (Toyooka et al., 2000; Okamoto, 2006), were not detected until 2 d after seed imbibition and increased thereafter from day 2 to day 4, indicating the transition of PSVs into a lytic vacuole for protein mobilization during seed germination. Because aleurain and SH-EP were newly synthesized during seed germination, they must play a role in mediating protein degradation in germinating seeds at a later stage.

To further ascertain whether new VSR proteins are synthesized de novo during seed germination, we performed a two-dimensional (2-D) gel/western-blot analysis with both VSR and BP-80 CT antibodies on proteins isolated from mature (day 0) and day 3 germinating seeds. As shown in Figure 2B, at least two VSR isoforms (indicated by an arrow) were detected by VSR antibodies in day 0 dry seeds, whereas two additional VSR isoforms (indicated by arrowheads) were found in day 3 germinating seeds, indicating that these VSRs are probably synthesized de novo during seed germination, even though the overall amounts of VSR proteins were decreased during seed germination as shown in Figure 2A. To further confirm that the two protein spots detected by VSR antibodies in day 3 germinating seeds were real VSR isoforms and not modifications of the day 0 VSRs, we performed similar western-blot analyses on both day 0 and day 3 protein samples using BP-80 CT antibodies. As shown in Figure 2B, BP-80 CT antibodies detected the same VSR proteins in both day 0 and day 3 samples (as indicated by an arrow) as those detected by VSR antibodies in day 0 samples, but did not recognize the two additional spots (arrowheads) in day 3 samples. Therefore, the two new spots detected by VSR antibodies in day 3 samples most likely represent newly synthesized VSR isoforms distinct from those detected by both VSR and BP-80 antibodies in day 0 samples. Thus, both preexisting and newly synthesized VSRs are detected in germinating seeds in which the old VSRs might be important in triggering the onset of seed germination, whereas the new VSRs function at later stages of seed germination. These results are consistent with a previous study in which mRNAs of newly synthesized VSR proteins in germinating cotyledons of Arabidopsis were compared to dry seeds (Laval et al., 2003). Thus, the presence of VSRs in germinating seeds might indicate their possible roles in transporting proteases via the Golgi-dependent pathway to a place where protein degradation occurs, a hypothesis being tested in this study.

**VSR Proteins Are Located in MVBs in Germinating Seeds**

As a first step to study the functional roles of VSRs during seed germination, we study the subcellular localization of VSR proteins via both confocal immunofluorescence and immunogold electron microscopy (EM) with VSRat-1 antibodies in germinating mung bean seeds. As shown in Figure 3, confocal immunofluorescence with anti-VSRat-1 and anti-BP-80 CT antibodies in mature dry day 0 (D0) and day 3 (D3) germinating mung bean seeds.
with their PVC/MVB localization in tobacco BY-2 cells (Tse et al., 2004). Immunogold EM with anti-VSRat-1 specifically labeled MVBs with little background labeling in ultrathin sections prepared from high-pressure frozen/freeze-substituted cotyledon samples of both day 1 and day 3 germinating mung bean seeds (Fig. 3B, images 2–5; Table I). When numerous VSR-labeled sections from four independent labeling experiments were counted and analyzed for the distribution of gold particles, the majority of gold particles were found in MVBs with an average of 8.5 gold particles per MVB and 1.2 particles per Golgi stack (Table I). In contrast, very small numbers of gold particles were found over other organelles, including PSVs and mitochondria (Table I). All these differences between two organelles are statistically significant (Tables I and II). Interestingly, when distribution of the gold particles between peripheral membranes and inside the lumen of MVBs was analyzed in day 1 and day 3 germinating samples, more than 80% of the gold particles were found in the peripheral membranes of the labeled MVBs in the day 1 sample, whereas only 45% of the gold particles located in the MVB peripheral membranes and more than 54% were found inside the lumen of MVBs in day 3 germinating samples (Fig. 3B; Table II). Similar results of VSR distributions were obtained when BP-80 CT antibodies were used in immunogold EM labeling in ultrathin sections prepared from high-pressure frozen/freeze-substituted cotyledon samples of both day 1 and day 3 germinating mung bean seeds, even though the MVB labeling intensity was much lower than that of VSRat-1 antibodies (Supplemental Fig. S1). The MVB membrane-located VSRs may represent the recycling receptors during early stages of seed germination, whereas the internalized VSR inside MVBs are targeted for degradation upon delivery to vacuoles. Because VSRs are known to be recycled from PVC/MVB to Golgi for a further round of cargo binding and selection whereas proteins delivered to lytic vacuoles from PVC are degraded (Bethke and Jones, 2000; Jiang and Rogers, 2003; Lam et al., 2005; Oliviussen et al., 2006), it is thus possible that the detection of major VSRs inside the lumen of MVBs at day 3 germination might represent the population of internalized VSR proteins in the MVBs targeted for degradation in vacuoles. In contrast, the majority of VSRs detected in the MVB membrane might represent the population of recycling receptors in PVC/MVB.

To find out whether MVBs are present in developing, mature, and germinating seeds and study their morphology, we performed structural studies on MVBs using chemically fixed mung bean seed samples. As shown in Figure 4, typical MVBs with typical internal vesicles were found in developing seeds (5 and 10 d after flowering), mature dry seeds, and germinating seeds (day 1 to day 3 after germination). These

### Table I. Distribution of GP for VSR antibodies of immunogold EM images in germinating (day 1 and day 3) mung bean seeds

| Organelle        | GP No. | Organelle No. | GP per Organelle |
|------------------|--------|---------------|------------------|
| Golgi            | 50     | 40            | 1.25**           |
| MVB              | 343    | 40            | 8.58**           |
| PSV              | 22     | 40            | 0.55**           |
| Mitochondrion    | 7      | 40            | 0.18**           |

Significant difference between two organelles was analyzed using one-sided paired t test ($P < 0.01$ [**]). Data were collected and analyzed from four independent labeling experiments. GP, Gold particle.
results indicated that MVBs are universally present during the life cycle of seeds from development, maturation, and germination. Because we could not prepare high-pressure frozen/freeze-substituted samples of dry seeds for immunogold EM study, labeling of VSRs in dry seeds was not possible.

To further confirm the presence of VSR proteins in MVBs, we isolated VSR-enriched fractions via continuous Suc density gradients (Tse et al., 2004) for use in immunogold negative staining with VSR antibodies. As shown in Figure 5, the identified VSR-enriched fractions (17 and 19; Fig. 5A) contained MVB-like vesicles that were specifically labeled by VSRat-1 and BP-80 CT antibodies (Fig. 5B). Thus, isolated MVBs also contain VSR proteins and VSR proteins were concentrated on the MVB-like structures in germinating mung bean seeds.

Isolation and Identification of VSR-Interacting Proteins in Germinating Seeds

To study the possible VSR cargos during seed germination, we next carried out biochemical cross-linking studies to identify VSR-interacting proteins in germinating seeds. We first prepared microsome fractions from day 3 germinating mung bean cotyledons, followed by chemically cross-linking and subsequent protein purification using an affinity column conjugated with VSRat-1 antibodies (Fig. 6A). VSR-interacting proteins were then eluted from the column (termed S3 fraction) and subjected to matrix-assisted laser-desorption ionization (MALDI)-time-of-flight (TOF) analysis for protein identification (Fig. 6A). As controls, we used a column without VSR antibodies (C1) and microsome proteins without chemical cross-linking treatment eluted from columns conjugated with VSR antibodies (C2). As shown in Figure 6B, eluted proteins of cross-linked samples from anti-VSR columns in S3 contained the VSR band (asterisk) and eight other visible bands (numbered 1–8) in a Coomassie Blue-stained gel in which the VSR band, band 1, and band 4 were identified as a VSR homolog, a PV72 homolog (a pumpkin VSR), and aleurain, respectively, via MALDI-TOF analysis (see also Table III for detailed information on amino acids and matching). As expected, the bands corresponding to VSR and PV72 were also identified from non-cross-linked control samples (C2) due to binding of VSR antibodies. In addition, the identified aleurain was a specific VSR-interacting protein because no such band was detected in C2 (Fig. 6B). The identification of other protein bands via MALDI-TOF analysis was unsuccessful due to the poor quality of the generated fingerprints and lack of a database for mung bean (data not shown).

To further confirm that the eluted aleurain was due to specific interaction between VSR and aleurain, we performed western-blot analysis on various fractions of proteins using antibodies against known markers (Fig. 6C). As expected, VSR antibodies detected VSR proteins predominantly in S3 and C2 fractions (eluted from an anti-VSR column) with much less in S1 and S2 (Fig. 6C, image 1). Similar results were obtained when BP-80 CT antibodies were used (Supplemental Fig. S2). Similarly, the 42-kD proaleurain (indicated by a single

| Samples     | No. | Percentage | No. | Percentage |
|-------------|-----|------------|-----|------------|
| Day 1       | 37  | 19.3%**    | 155 | 80.7%**    |
| Day 3       | 82  | 54.3%**    | 69  | 45.7%**    |

Table II. Distribution of immunogold VSR in MVBs

Significant difference between the day 1 and day 3 samples was analyzed using one-sided paired t test (P < 0.01 [**]). Data were collected and analyzed from four independent labeling experiments.

GP, Gold particle.
asterisk) was mainly found in S3, probably in the presence of the VSR-interacting motif NPIR at its N terminus, whereas the 32-kD mature aleurain (indicated by a double asterisk) was found in S1 and S2, but absent in S3 because aleurain lacks NPIR (Fig. 6C, image 2). In contrast, the other Cys protease, SH-EP, and the two storage proteins, 2S albumin and phaseolin, as well as Rha1, a small GTP-binding protein of the Ras family (Sohn et al., 2003), were almost missing in S3, but present in both S1 and S2 (Fig. 6C, images 3–6). These results indicated that the interaction between VSR proteins and aleurain in germinating seeds is specific and consistent with the demonstrated interaction between BP-80 and the aleurain NPIR vacuolar sorting motif by which BP-80 was first shown to function as a sorting receptor for transporting hydrolases via the Golgi apparatus to the lytic vacuole (Kirsch et al., 1994; Paris et al., 1997; Ahmed et al., 2000; Cao et al., 2000).

VSR Proteins Colocalized with Aleurain to MVBs in Germinating Seeds

To further demonstrate that the interaction between VSR and aleurain in germinating mung bean seeds is due to in vivo protein interaction, we next performed subcellular localization to compare VSR with aleurain. Paraffin sections prepared from day 3 germinating mung bean seeds were double labeled with two antibodies and observed under confocal microscopy for their localization and relationship to one another. As shown in Figure 7, the punctate signals generated by VSR and aleurain antibodies were largely (more than 85%) overlapped (Fig. 7A; Table IV), indicating colocalization of VSR and aleurain to the same organelles during seed germination. In contrast, PVC/MVB organelles detected by anti-VSR and anti-BP-80 were largely separated from the KV organelles labeled by SH-EP antibodies in the same cells of germinating seeds (Fig. 7, B and C). Similarly, when the two Cys proteases were compared directly in the same cell, they remained largely separated (Fig. 7D; Table IV). To further confirm the MVB localization of aleurain in germinating seeds, we performed an immunogold EM study with ultrathin sections prepared from high-pressure frozen/freeze-substituted cotyledon samples of day 3 germinating mung bean seeds. As shown in Figure 8, anti-aleurain specifically labeled MVBs (Fig. 8A), whereas SH-EP antibodies labeled KV vesicles (Fig. 8C), but not MVB (Fig. 8B) in germinating seeds. These results demonstrated that VSR proteins colocalized with aleurain in PVCs/MVBs that are distinct from the KV organelles in germinating mung bean seeds.

SH-EP is a Cys protease that was newly synthesized during seed germination and transported to PSV via ER-derived KV vesicles for protein degradation in germinating mung bean seeds (Toyooka et al., 2000). In contrast, aleurain is transported by BP-80 via Golgi and PVC to the vacuole (Holwerda et al., 1990; Paris et al., 1997). These results are consistent with previous studies on SH-EP and aleurain for their distinct transport pathways leading to vacuoles.

To further ascertain whether aleurain and SH-EP reached PSV in germinating seeds, we next performed subcellular localization to compare the PSV marker α-TIP (Jauh et al., 1999) with aleurain and SH-EP. Paraffin sections prepared from day 3 germinating mung bean seeds were double labeled with two antibodies and observed under confocal microscopy for their localization and relationship to one another. As shown in Figure 9, some punctate signals generated by aleurain and SH-EP antibodies, representing MVB and KV
vesicles, respectively, were found located inside the PSVs labeled by α-TIP antibodies (green). These results indicated that both aleurain and SH-EP reached PSVs in germinating seeds, likely for protein degradation.

DISCUSSION

Roles of VSR Proteins in Germinating Seeds

VSR proteins may perform dual functions in plant cells. BP-80 was the first VSR protein to be identified based on its interaction with the vacuolar sorting determinant NPIR (Kirsch et al., 1994). Subsequently, several in vitro and in vivo studies demonstrated that BP-80 and its Arabidopsis homolog AtVSR1 appear to function as a sorting receptor for transporting the Cys protease aleurain from the Golgi apparatus to lytic vacuoles (Jiang and Rogers, 1998; Humair et al., 2001; Hernandez et al., 2005). In contrast, the VSR homolog PV72 from pumpkin, as well as Arabidopsis AtVSR1, may function as sorting receptors for transporting storage proteins to the PSV in developing seeds (Shimada et al., 1997, 2003; Paris and Neuhaus, 2002; Hernandez et al., 2005; Park et al., 2005). However, little is known about the functional roles of VSR proteins during seed germination.

Seven VSR homologs have been found in Arabidopsis that are present in different temporal and spatial expression profiles (Laval et al., 2003). In mature Arabidopsis seeds, some VSR mRNAs have been detected, but upon seed germination, some new isoforms of VSR are synthesized, which are not present in mature seeds (Laval et al., 2003). These results indicated that VSR proteins might have distinct functional roles in developing and germinating seeds. More recently, green fluorescent protein fusions with the seven Arabidopsis

Figure 6. Isolation and identification of VSR-interacting proteins in germinating mung bean seeds. A, Flow chart of cross-linking experiments for the isolation and identification of VSR-interacting proteins. S1, Total proteins prior to binding with column conjugated with VSR antibodies; S2, proteins passing through the column; S3, VSR-interacting proteins eluted from the column. DSP, Dithiobis(succinimidylpropionate). B, Identification of VSR-interacting proteins in Coomassie Blue-stained SDS-PAGE gels. S3, Eluted proteins from a VSR antibody column after binding with cross-linked protein samples; C1, proteins eluted from a control column without VSR antibodies; C2, proteins eluted from a VSR antibody column after binding with non-cross-linking protein samples. The identity of individual proteins from MALDI-TOF analysis is indicated (see also Table III). M, Molecular marker in kilodaltons. C, Western-blot analysis of eluted proteins with various known antibodies. Rha1, A small GTPase. Asterisk indicates the position of the detected proteins. Asterisk and double asterisk in image 2 indicate the detected proaleurain and mature aleurain, respectively. Detection of proaleurain in C3 only, probably because it contains VSR-interacting NPIR motif, which is missing from mature aleurain.

Wang et al.

Paris and Neuhaus, 2002; Hernandez et al., 2005). In contrast, the VSR homolog PV72 from pumpkin, as well as Arabidopsis AtVSR1, may function as sorting receptors for transporting storage proteins to the PSV in developing seeds (Shimada et al., 1997, 2003; Paris and Neuhaus, 2002; Hernandez et al., 2005; Park et al., 2005). However, little is known about the functional roles of VSR proteins during seed germination.

Seven VSR homologs have been found in Arabidopsis that are present in different temporal and spatial expression profiles (Laval et al., 2003). In mature Arabidopsis seeds, some VSR mRNAs have been detected, but upon seed germination, some new isoforms of VSR are synthesized, which are not present in mature seeds (Laval et al., 2003). These results indicated that VSR proteins might have distinct functional roles in developing and germinating seeds. More recently, green fluorescent protein fusions with the seven Arabidopsis

Plant Physiol. Vol. 143, 2007

Downloaded from on August 30, 2017 - Published by www.plantphysiol.org
Copyright © 2007 American Society of Plant Biologists. All rights reserved.
VSRS proteins were found to localize to PVCs/MVBs in transgenic tobacco BY-2 cells (Miao et al., 2006). However, the functions of individual VSRS populations remain to be illustrated.

In this study, we have shown that two Cys proteases, aleurain and SH-EP, and most likely a VSRS as well, were synthesized de novo during seed germination. However, we cannot rule out the possibility that BP-80 CT antibodies might fail to discriminate various VSRS isoforms due to VSRS turnover and thus lost their epitopes for anti-BP-80 CT. Confocal immunofluorescence and immunogold EM studies further demonstrated that VSRS and aleurain colocalized to MVBs in germinating mung bean seeds, indicating that VSRS proteins transport aleurain to PSVs via MVBs for protein degradation during seed germination. MVBs were found in developing, mature, and germinating mung bean seeds. Nevertheless, it is reasonable to assume that VSRSs also located in MVBs in mature dry seeds because VSRSs were specifically present in MVBs in day 1 and day 3 germinating seeds. So what are the functional implications of MVB localization of VSRS proteins in mature dry seeds? Because VSRSs seem to transport proteases to MVBs during seed development, the MVB compartmentation of VSRS proteins in mature seeds suggests that the proteases might also locate to MVBs in mature seeds, thus providing a physical separation from PSV-localized storage proteins. Early in seed germination, these stored proteases would then be released into the PSV to degrade storage proteins. In this way, protein degradation in the PSV can be initiated before the new synthesis of VSRSs and proteases can take effect. Therefore, our results support a model that proteases are newly synthesized and transported to PSVs via MVBs by VSRS proteins for protein degradation in germinating seeds.

### Table III. Identification of isolated proteins via MALDI-TOF and mass database searching

| Band No. | Molecular Mass | Identity from Searching | pI | Theoretical Molecular Mass | Matched Peptides |
|----------|----------------|-------------------------|----|---------------------------|-----------------|
| 1        | Approximately 70,000 | PV72—Cucurbita cv Kurokawa Amakuri | 5.5 | 69,028.3 | 106 AWNAQNGGAAAlVAv DDR 123 |
|          |                | Vacular targeting receptor | 5.41 | 69,255.4 | 122 DEPLITMDNPEDTGTVK 117 160 |
|          |                | BP-80—wheat (Triticum aestivum) | 5.41 | 69,255.4 | 122 DEPLITMDNPEDTGTVK 117 160 |
|          |                | SR homolog (fragment)—maize (Zea mays) | - | - | - |
| 4        | Approximately 43,000 | Putative Cys protease | 6.26 | 38,982 | 55 HVLSFAR 61 102 LGVNQFADLTWQEFR |

**Mechanisms of VSRS Turnover in Germinating Seeds**

It is believed that VSRS proteins sort cargo proteins at the trans-Golgi network and pack them into CCVs for delivery to a lytic PVC, from where the receptors are recycled back to the Golgi via the newly characterized retromer complex (Oliiviousson et al., 2006) for a new round of sorting and binding (Jiang and Rogers, 2003). The CT of VSRSs is essential for interaction with adaptor proteins and subsequent packaging into CCVs (Happel et al., 2004; Murphy et al., 2005) and for PVC trafficking (Jiang and Rogers, 2003). When a truncated BP-80 missing its CT was expressed in transgenic tobacco cells, its half-life was about 40 min, a dramatic reduction when compared to that of the full-length BP-80 with a half-life of more than 2 h in the same transgenic tobacco cells (Jiang and Rogers, 1998). This result indicates that the CT of BP-80 is important for the stability of the receptor, which is most likely due to the inability of the truncated receptor to recycle back to the Golgi from PVC and results in its delivery to the vacuole for degradation (Jiang and Rogers, 2003). This conclusion was further confirmed by a recent study demonstrating that the CT of BP-80 was important for PVC targeting of the receptor in tobacco leaf cells (daSilva et al., 2006).

In this study, we demonstrated that, whereas VSRSs are being newly synthesized, the total amounts of VSRS proteins in germinating seeds, as detected by western-blot analysis with VSRSat-1 antibodies, gradually decreased as germination proceeded from day 1 to day 3. However, the differential subcellular distribution of VSRS proteins within the PVCs/MVBs as shown by immunogold EM has to be taken into account: Whereas the majority (more than 80%) of the VSRS proteins were found on the peripheral membrane of MVBs in day 1 germinating seeds, VSRS proteins were mainly found inside the lumen of MVBs in day 3 germinating seeds. This novel observation indicates that the membrane-localized VSRSs at steady state may represent the population of recycling receptors, whereas those VSRSs inside the lumen of MVBs are destined for degradation, presumably after fusion of the PVCs/MVBs with the vacuole.
vacuole (Jiang et al., 2002; Jiang and Rogers, 2003). It would thus be interesting to ascertain what triggers the internalization of VSR proteins in MVBs and whether these internalized VSRs begin to be degraded within the lumen of MVBs or first inside the PSVs.

MATERIALS AND METHODS

Plant Materials

Mung bean (Vigna radiata) seeds were germinated on wet filter paper at 26°C in the dark. The cotyledons of germinating mung bean were used for protein extraction, organelle isolation via fractionation, and sample preparation for transmission EM.

Antibodies

Characterization of the following antibodies used in this study has been described as follows: polyclonal VSRat-1 and BP-80 CT antibodies (Tse et al., 2004), various TIP antibodies (Jash et al., 1999), monoclonal and polyclonal antibodies against aleurain (Jiang and Rogers, 1998; Jiang et al., 2000), monoclonal and polyclonal antibodies against SH-EP (Toyooka et al., 2000) kindly provided by Dr. K. Toyooka and Dr. T. Okamoto (Tokyo Metropolitan University), and Rha1 antibody (Sohn et al., 2003) was a gift from I. Hwang (Pohang University of Science and Technology).

Protein Extraction, Protein Gel, and Immunoblotting

Cotyledons of germinating mung bean at different stages were collected and ground to a fine powder in liquid nitrogen. Protein extraction buffer (Tris-HCl 50 mM, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 0.1 mM

| Antibodies Compared         | Colocalization Percentage (Mean ± SD) | n  |
|-----------------------------|---------------------------------------|----|
| VSR versus aleurain         | 85 ± 6                                 | 20 |
| VSR versus SH-EP            | 9 ± 5                                  | 20 |
| BP-80 CT versus SH-EP       | 12 ± 7                                 | 20 |
| Aleurain versus SH-EP       | 10 ± 7                                 | 20 |

Table IV. Quantitation of antibody colocalization in confocal immunofluorescence images from two independent labeling experiments
phenylmethylsulfonyl fluoride, 1% SDS, and 5 mg/mL leupeptin) was then added to the powder for extraction of proteins. Protein extracts were boiled for 10 min and centrifuged at 14,000 rpm for 5 min. Supernatants were collected for determination of protein concentrations (Bradford, 1976) using a Bio-Rad protein assay kit. Equal amounts of proteins were separated via 10% SDS-PAGE, followed by western-blot analysis with various antibodies at 4 mg/mL as previously described (Tse et al., 2004).

For 2-D gel electrophoresis, protein samples were prepared as recommended by the manufacturer (Amersham Biosciences). Briefly, cotyledon powder was suspended in 10% TCA, 0.3% dithiothreitol in acetone, and stored at −20°C overnight. After centrifugation, the pellet was washed with acetone and air dried, followed by addition of the rehydration buffer (8 M urea, 4% CHAPS, 60 mM dithiothreitol, 2% Pharmalyte 3–10, 0.002% bromphenol blue) to solubilize the pellet before protein samples were directly loaded onto the Immobiline DryStrip (pH 3–10, 7 cm, nonlinear; Amersham Biosciences) and rehydrated overnight. One-dimensional isoelectric focusing (IEF) was performed with the Amersham Biosciences IEFphor II IEF system. Upon completion of IEF electrophoresis, the gel strip was equilibrated according to the manufacturer’s instructions. Proteins in the gel strip were separated via SDS-PAGE using a Bio-Rad Protean II mini electrophoresis unit, followed by protein transfer to the membrane and immunoblot analysis with VSR antibodies as previously described (Tse et al., 2004).

Cross-Linking Studies

The cotyledons of day 3 germinating mung bean were collected and ground in grinding buffer (HEPES, pH 7.2 containing 0.4 M Suc), followed by centrifugation at 800g for 10 min. The resulting supernatant was further subjected to ultra centrifugation at 100,000g for 1 h before the pellet was resuspended in grinding buffer and used as a microscope fraction. For the cross-linking study, dithiobis(succinimidylpropionate) (20 mM, dissolved in dimethyl sulfoxide; Pierce) was added to the microscope fraction at 2 mM (final concentration) and incubated at room temperature for 30 min. To quench the reaction, Tris-HCl (pH 8.0) was added at 20 mM (final concentration), followed by incubation at room temperature for 30 min. Triton X-100 was then added at 1% (final concentration) to lyse the microsomes, followed by incubation at room temperature for 4 h before the insoluble materials were removed by centrifugation at 20,000g for 10 min. For isolation of VSR-interacting proteins, 4 volumes of 1× Tris-buffered saline containing 0.25% NP-40 were added to the supernatant of the above cross-linked samples and mixed with the resin conjugated with VSR antibodies, followed by rotation at room temperature overnight before the resin was transferred to a column and washed with phosphate-buffered saline. The bound proteins were eluted from the column with 0.2 M Gly-HCl (pH 2.0) and collected in equal volumes of 1 M Tris-HCl (pH 8.0) to each fraction. The eluted proteins, along with other controls, including un-cross-linked samples, total protein, and passing-through proteins, were separated by SDS-PAGE analysis, followed by Coomassie Blue staining and protein identification via MALDI-TOF or western-blot analysis with various antibodies.

Confocal Immunofluorescence Studies

Fixation and preparation of tissues from cotyledons of germinating mung bean for paraffin-embedded sections and their labeling and analysis by confocal immunofluorescence have been described previously (Jiang and Rogers, 1998; Jiang et al., 2000; Li et al., 2002). Briefly, cotyledons were cut into small cubes, followed by incubation in fixation solution containing 10% glacial acetic acid for 1 h. After fixation, tissues were washed with 0.2 M glycine-HCl (pH 2.0) and dehydrated in a series of ethanol solutions (30%, 50%, 70%, 90%, 100%) before being transitioned to paraffin. Paraffin sections were cut at 8 μm and mounted on glass slides. For double labeling, sections were incubated with primary antibodies against aleurain (α-TIP, 1:500) and SH-EP (1:100) for 1 h at room temperature, followed by incubation with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594. Immunostaining was detected with a Leica TCS SP2 AOBS confocal microscope equipped with a 40× objective. Images were captured using Leica Confocal Software (Leica Microsystems) and merged using Adobe Photoshop (Adobe Systems).
formaldehyde, 50% ethanol, and 3% acetic acid for 24 h. Dehydration and infiltration of samples were performed with the Enclosed Tissue Processor (Leica TP-1050; Leica Microsystems), followed by samples embedded in paraffin blocks. Thin paraffin-embedded sections were deparaffinized and used for single or double labeling with various antibodies at the following concentrations: 4 μg/mL for anti-VSR, anti-aleurain, and anti-BP-80 CT, and 1:500 dilution for anti-SH-EP. All confocal fluorescence images were collected using a Bio-Rad Radiance 2100 system (Heidelberg). Images were processed using Adobe Photoshop software and the extent of colocalization of two polyclonal antibodies in confocal immunofluorescence images from paraffin-embedded mung bean sections was quantitated as previously described (Jiang and Rogers, 1998).

Isolation of PVCs

PVC isolation was performed according to Tse et al. (2004) with some modifications. The cotyledons of day 3 germinating mung bean (15 g) were ground to a fine paste in 20 mL basic buffer (40 mM HEPES-KOH, pH 7.2, 10 mM KCl, 0.1 mM EDTA) with 15% Suc in mortar with pestle, followed by filtration through three layers of cheesecloth. Another 20 mL of grinding buffer was added into mortar for further grinding before a second filtration. The clear filtrates were combined into a 50-mL Falcon tube and centrifuged at 1,000g for 10 min. The supernatant was layered onto 7 mL of a 60% Suc cushion in basic buffer at the bottom of an ultracentrifuge tube. After centrifugation at 100,000g for 2 h (swing rotor SW28; Beckman), the vesicle layer floating at the 15% to 60% Suc interface was collected and diluted three times with basic buffer with 3 mM MgCl₂ and further layered onto a 20% to 60% linear Suc gradient in basic buffer with 3 mM MgCl₂ and spun at 100,000g for 2 h (swing rotor SW28; Beckman), the vesicle fraction were separated by SDS-PAGE and western-blot analysis with VSR antibodies to identify VSR-enriched fractions. Immunogold EM staining of isolated PVCs with VSR antibodies was performed as previously described (Tse et al., 2004).

EM

The general procedures for conventional thin sectioning of chemically fixed samples of mung bean were performed essentially as described previously (Ritzenthaler et al., 2002; Tse et al., 2004). For ultrastructural analysis, the cotyledons were cut into small cubes (<1 mm) and fixed in primary fixation solution (2% glutaraldehyde, 10% picric acid in 25 mM CaCo buffer, pH 7.2) at 4°C overnight. After washing in CaCo buffer, samples were incubated in secondary fixation solution (2% osmium tetroxide, 0.5% potassium ferrocyanide in 25 mM CaCo buffer, pH 7.2), followed by steps of dehydration and infiltration before embedding in Spurr’s resin. Ultrathin sections were post-stained with uranyl acetate/lead citrate before EM observation.

Immunogold EM on ultrathin sections prepared from high-pressure frozen/ freeze substitution of germinating mung bean cotyledons was performed essentially as previously described (Tse et al., 2004). Small blocks of cotyledons were frozen in a high-pressure freezing apparatus (HPF010; Bal-Tec). Substitution was performed in an APS freeze substitution unit (Leica). Samples were stepwise infiltrated with HM20 at 20°C, embedded, and UV polymerized. Immunolabeling on HM20 sections was done using standard procedures as described (Tse et al., 2004), with VSR and aleurain antibodies at 40 μg/mL, SH-EP antibodies at 1:50 dilution, and gold-coupled secondary antibodies at 1:50. Aqueous uranyl acetate/lead citrate poststained sections were examined in a JOEL JEM-1200 EXII transmission electron microscope (JOEL) operating at 100 kV or a Philips CM10 transmission electron microscope operating at 80 kV as previously described (Tse et al., 2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Immunogold EM localization of VSR proteins by BP-80 CT antibodies to MVVs in day 1 (image 1) and day 3 (image 2) germinating mung bean seeds.

Supplemental Figure S2. Western-blot analysis of eluted proteins with BP-80 CT antibodies. Asterisk indicates the position of the detected proteins.

ACKNOWLEDGMENTS

We are grateful to Dr. T. Okamoto and Dr. K. Toyooka (Tokyo Metropolitan University), Dr. K. Matsuoka (RIKEN) for sharing various SH-EP antibodies, and Prof. I. Hwang (Pohang University of Science and Technology) for the Rha1 antibody.

Received January 22, 2007; accepted February 12, 2007; published February 23, 2007.

LITERATURE CITED

Ahmed SU, Rojo E, Kovaleva V, Venkataraman S, Dombrowski JE, Matsuoka K, Raikhel NV (2000) The plant vacuolar sorting receptor AtELP is involved in transport of NH₂-terminal propeptide-containing vacuolar proteins in Arabidopsis italiana. J Cell Biol 149: 1335–1344

Betheke PC, Jones RL (2000) Vacuoles and prevacuolar compartments. Curr Opin Plant Biol 3: 469–475

Bewley JD, Black M (1994) Seeds: Physiology of Development and Germination, Ed 2. Plenum Press, New York

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Cao X, Rogers SW, Butler J, Beeverus L, Rogers JC (2000) Structural requirements for ligand binding by a probable plant vacuolar sorting receptor. Plant Cell 12: 493–506

daSilva LL, Foresti O, Denecke J (2006) Targeting of the plant vacuolar sorting receptor BP80 is dependent on multiple sorting signals in the cytosolic tail. Plant Cell 18: 1477–1497

daSilva LL, Taylor JP, Hadlington JL, Hanton SL, Snowden CJ, Fox SJ, Foresti O, Brandizzi F, Denecke J (2005) Receptor salvage from the prevacuolar compartment is essential for efficient vacuolar protein targeting. Plant Cell 17: 132–148

Dunkley TP, Hester S, Shadforth IP, Runions J, Weimar T, Hanton SL, Griffith J, Bessant C, Brandizzi F, Hawes C, et al. (2006) Mapping the Arabidopsis organelle proteome. Proc Natl Acad Sci USA 103: 6518–6523

Hadlington JL, Denecke J (2000) Sorting of soluble proteins in the secretory pathway of plants. Curr Opin Plant Biol 3: 461–468

Happei N, Honing S, Neuhaus JM, Paris N, Robinson DG, Holstein SE (2004) Arabidopsis mu A-adaptin interacts with the tyrosine motif of the vacuolar sorting receptor VSR-PS1. Plant J 37: 678–693

Hernandez DH, Paris N, Neuhaus JM, Deloche O (2005) The yeast Saccharomyces cerevisiae is not an efficient tool for in vivo studies of plant vacuolar sorting receptors. Plant Cell 17: 1339–1342

Holwerda BC, Galvin NJ, Baranski TJ, Rogers JC (1990) In vitro processing of auleain, a barley vacuolar thiol protease. Plant Cell 2: 1091–1106

Humair D, Hernandez Felipe D, Neuhaus JM, Paris N (2001) Demonstration in yeast of the function of BP-80, a putative plant vacuolar sorting receptor. Plant Cell 13: 781–792

Jauh GY, Phillips TE, Rogers JC (1999) Tonoplast intrinsic protein isoforms as markers for vacuolar functions. Plant Cell 11: 1867–1882

Jiang L, Erickson A, Rogers J (2002) Multivesicular bodies: a mechanism to package lytic and storage functions in one organelle? Trends Cell Biol 12: 362–367

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, Rea PA, Maeshima M, Rogers SW, Rogers JC (2001) The protein storage vacuole: a unique compound organelle. J Cell Biol 155: 991–1002

Jiang L, Phillips TE, Rogers SW, Rogers JC (2000) Biogenesis of the plant storage vacuole crystalloid. J Cell Biol 150: 755–770

Jiang L, Rogers JC (1998) Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways. J Cell Biol 143: 1183–1199

Jiang L, Rogers JC (2003) Sorting of lytic enzymes in the plant Golgi apparatus. Annu Plant Rev 9: 114–140

Kirch T, Paris N, Butler JM, Beeverus L, Rogers JC (1994) Purification and initial characterization of a potential plant vacuolar targeting receptor. Proc Natl Acad Sci USA 91: 3403–3407

Lam SK, Sui CL, Hillmer S, Jiang S, An G, Robinson DG, Jiang L (2007) Rice SCAMP1 defines clathrin-coated, trans Golgi–located tubular vesicular structures as an early endosome in tobacco BY2 cells. Plant Cell 19: 296–319

Lam SK, Tse YC, Jiang L, Oliviusson P, Heinzerling O, Robinson DG (2005) Plant prevacuolar compartment and endocytosis. Plant Cell Monogr 1: 37–61

1638 Plant Physiol. Vol. 143, 2007

Downloaded from on August 30, 2017 - Published by www.plantphysiol.org
Copyright © 2007 American Society of Plant Biologists. All rights reserved.
Laval V, Masclaux F, Serin A, Carriere M, Roldan C, Devic M, Pont-Lezica RF, Galaud JP (2003) Seed germination is blocked in Arabidopsis putative vacuolar sorting receptor (atbp80) antisense transformants. J Exp Bot 54: 213–221

Li YB, Rogers SW, Tse YC, Lo SW, Sun SS, Jauh GY, Jiang L (2002) BP-80 and homologs are concentrated on post-Golgi, probable lytic prevacuolar compartments. Plant Cell Physiol 43: 726–742

Miao Y, Yan P, Kim H, Hwang I, Jiang L (2006) Localization of GFP fusions with the seven Arabidopsis vacuolar sorting receptors to prevacuolar compartments in tobacco BY-2 cells. Plant Physiol 142: 945–962

Mo BX, Tse YC, Jiang L (2006) Plant prevacuolar/endosomal compartments. Int Rev Cytol 253: 96–129

Muntz K, Belozerksky MA, Dunaevsky YE, Schlereth A, Tiedemann J (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. J Exp Bot 52: 1741–1752

Murphy AS, Bandyopadhyay A, Holstein SE, Peer WA (2005) Endocytotic cycling of PM proteins. Annu Rev Plant Biol 56: 221–251

Neuhaus JM, Paris N (2005) Plant vacuoles: from biogenesis to function. Plant Cell Monogr 1: 63–82

Neuhaus JM, Rogers JC (1998) Sorting of proteins to vacuoles in plant cells. Plant Mol Biol 38: 127–144

Okamoto T (2006) Transport of proteases to the vacuole: ER export bypassing Golgi? Plant Cell Monogr 4: 125–139

Oliviusson P, Heinzerling O, Hillmer S, Tse YC, Jiang L, Robinson DG (2006) Plant retromer, localized to the prevacuolar compartment and multivesicular bodies in Arabidopsis, may interact with prevacuolar sorting receptors. Plant Cell 18: 1239–1252

Paris N, Neuhaus JM (2002) BP-80 as a vacuolar sorting receptor. Plant Mol Biol 50: 903–914

Paris N, Rogers SW, Jiang L, Kirsch T, Beveres L, Phillips TE, Rogers JC (1997) Molecular cloning and further characterization of a probable plant vacuolar sorting receptor. Plant Physiol 115: 29–39

Paris N, Stanley CM, Jones RL, Rogers JC (1996) Plant cells contain two functionally distinct vacuolar compartments. Cell 85: 563–572

Park M, Lee D, Lee GJ, Hwang I (2005) AtRMR1 functions as a cargo receptor for protein trafficking to the protein storage vacuole. J Cell Biol 170: 757–767

Poxleitner M, Rogers SW, Samuels AL, Browne J, Rogers J (2006) A role for calnexin in degradation of oil-body storage lipid during seed germination. Plant J 47: 917–933

Rüttenhaller C, Nebenfuhr A, Movafeghi A, Stussi-Garaud C, Behnia L, Pimpi P, Staehelin LA, Robinson DG (2002) Reevaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. Plant Cell 14: 237–261

Shimada T, Fuji K, Tamura K, Kondo M, Nishimura M, Hara-Nishimura I (2003) Vacular sorting receptor for seed storage proteins in Arabidopsis thaliana. Proc Natl Acad Sci USA 100: 16095–16100

Shimada T, Kuroyanagi M, Nishimura M, Hara-Nishimura I (1997) A pumpkin 72-kDa membrane protein of precursor-accumulating vesicles has characteristics of a vacuolar sorting receptor. Plant Cell Physiol 38: 1414–1420

Sohn EJ, Kim ES, Zhao M, Kim SJ, Kim H, Kim YW, Lee YJ, Sohn U, Jiang L, et al (2003) Rha1, an Arabidopsis Rab5 homolog, plays a critical role in the vacuolar trafficking of soluble cargo proteins. Plant Cell 15: 1057–1070

Toyooka K, Okamoto T, Minamikawa T (2000) Mass transport of proform of a KDEL-tailed cysteine proteinase (SH-EP) to protein storage vacuoles by endoplasmic reticulum-derived vesicle is involved in protein mobilization in germinating seeds. J Cell Biol 148: 453–464

Tse YC, Lo SW, Hillmer S, Dupree P, Jiang L (2006) Dynamic response of prevacuolar compartments to brefeldin A in plant cells. Plant Physiol 142: 1442–1459

Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG, Jiang L (2004) Identification of multivesicular bodies as prevacuolar compartments in Nicotiana tabacum BY-2 cells. Plant Cell 16: 672–693