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Molecular Characterization of Plant Vacuolar Sorting Receptor (VSR) Proteins

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ABSTRACT

Vacuolar sorting receptor (VSR) proteins transport cargoes from Golgi to vacuoles via prevacuolar compartments (PVCs), where VSRs define lytic PVCs as multivesicular bodies (MVBs) in plant cells. The *Arabidopsis* genome contains seven VSR homologs (AtVSR1-7) but little is known about their subcellular localization and function in plants. Here we summarize studies taken to understand the biology of VSR proteins over the past years and present possible strategies to be used in studying the functional roles of individual AtVSRs in future research, which will serve as a first step for exploring VSR function in plants.

Keywords: Secretory pathway, prevacuolar compartment, vacuolar sorting determinant, BP-80, wortmannin
Abbreviations: CCVs, clathrin coated vesicles; **CT**, cytoplasmic tail; **GFP**, green fluorescent protein; **immunoEM**, immunocytochemical electron microscope; **LV**, lytic vacuole; **MVBs**, multivesicular bodies; **PSV**, protein storage vacuole; **PVC**, prevacuolar compartment; **TIP**, tonoplast intrinsic protein; **TMD**, transmembrane domain; **VSDs**, vacuolar sorting determinants; **VSR**, vacuolar sorting receptor

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INTRODUCTION

Secretory pathway in plants

Plant cells contain two functionally and structurally distinct types of vacuole compartments: protein storage vacuole (PSV) and lytic vacuole (LV), which are defined by the presence of aquaporin α-TIP (tonoplast intrinsic protein) and γ-TIP on their tonoplasts, respectively (Jauh *et al*. 1999; Fung *et al*. 2005). PSV, a unique compartment in plant cells, deposits proteins intracellularly to provide a nutrient source for plant seed development and maturation (Paris *et al*. 1996; Jiang *et al*. 2001). LV, equivalent of yeast vacuole and mammalian lysosome, contains hydrolytic enzymes that function in an acidic environment for protein degradation (Paris *et al*. 1996). Vacuolar proteins with signal peptide enter the secretory pathway from the endoplasmic reticulum (ER) where ER chaperones (e.g. calnexin, calreticulin and BIP) facilitate protein folding and their subsequent correct targeting to final destinations (Boyce *et al*. 1994; Pimpl *et al*. 2006). Upon correct folding, proteins are retained in or exported from ER via transport vesicles to *cis*-Golgi depending upon the targeting information inside their polypeptide chain (Vitale and Raikhel 1999). Proteins are then transported to multiple intracellular destinations by passing through the Golgi apparatus from *cis*-Golgi to *trans*-Golgi site that are marked by a glycan processing enzyme mannosidase I and a nucleotide sugar transporter GONST1 protein, respectively (Nebenfuhr *et al*. 1999; Baldwin *et al*. 2001). Prevacuolar compartments (PVCs) receive cargoes via receptor-mediated sorting from Golgi derived transport vesicles and subsequently deliver them to vacuoles by fusion

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Invited Mini-Review

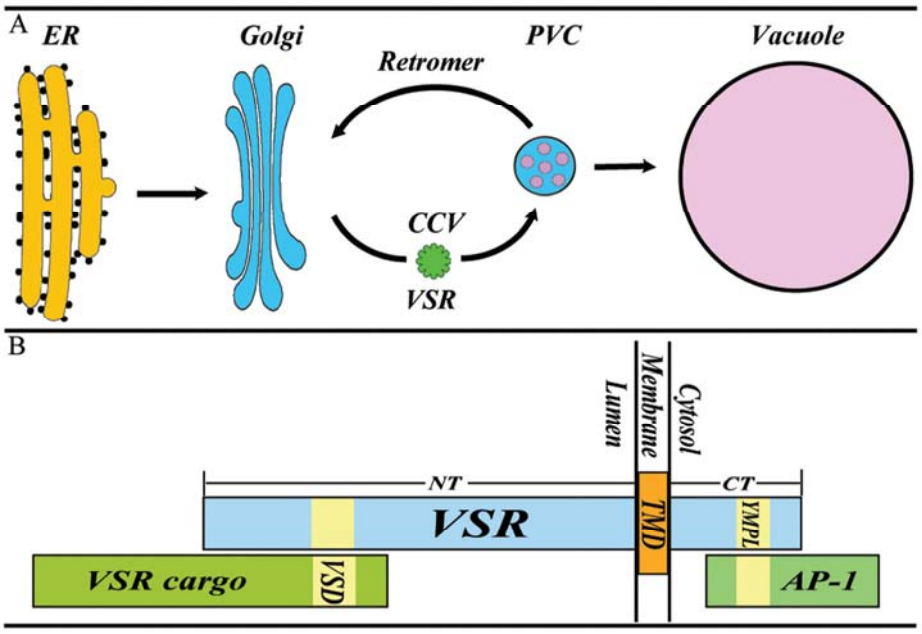

Fig. 1 Working model of receptor-mediated protein trafficking in the plant secretory pathway. (**A**) Vacuolar proteins reach vacuoles from endoplasmic reticulum (ER) and Golgi via a pre-vacuolar compartment (PVC) are mediated by vacuolar sorting receptor (VSR) BP-80 via clathrin coated vesicles (CCVs) (Jiang and Rogers 2003). (**B**) Possible interaction between VSR and its cargo protein from Golgi to PVC. The N-terminal (NT) region of VSR interacts with vacuolar sorting determinant (VSD) of cargo protein, while its C-terminal (CT) interacts with adaptor protein complex AP-1 via a conserved tyrosine YMPL motif.

with the tonoplast. PVCs have been identified by the presence of vacuolar sorting receptor (VSR) proteins as multivesicular bodies (MVBs) in tobacco BY-2 cells (Tse *et al*. 2004) and more recently in *Arabidopsis* seeds (Otegui *et al*. 2006). VSRs function in sorting cargoes from Golgi to vacuole via clathrin coated vesicles (CCVs), where the recycle of VSR from PVC/MVB to Golgi is mediated by retromer complex (Lam *et al*. 2005; Oliviusson *et al*. 2006). **Fig. 1A** summarizes the working model of protein trafficking in the plant secretory pathway.

Vacuolar sorting receptor (VSR) and BP-80

Soluble proteins reach vacuoles because they contain vacuolar sorting determinants (VSDs) that are recognized by a family of proteins termed vacuolar sorting receptors (VSRs) (Vitale and Raikhel 1999; Jiang and Rogers 2003). BP-80 was the first VSR protein isolated from pea (*Pisum sativum*) involved in recognizing the N-terminal VSD of proaleurain, a cysteine protease (Kirsch *et al*. 1994), for its transport from Golgi to the lytic vacuole (Humair *et al*. 2001; Paris and Neuhaus 2002). BP-80 is a type I integral membrane protein containing a single transmembrane domain (TMD) and a cytoplasmic tail (CT). The N-terminus of BP-80 contains three major functional regions: a region homologous to the luminal domain of ReMembR-H2 (RMR) protein, a unique central region and three C-terminal epidermal growth factor (EGF) repeats. It was proposed that the N-terminal/RMR homology domain is responsible for the major direct interaction with Asn-Pro-Ile-Arg (NPIR) motif of proaleurain at *trans-Golgi network* (TGN) for cargo-receptor interaction (Cao *et al*. 2000). The short CT of BP-80 contains a tyrosine-based motif, YMPL, recognized by the AP-1 clathrin adaptor protein complex of clathrin coated vesicles budded from the Golgi (Ahmed *et al*. 1997; Paris *et al*. 1997; Shimada *et al*. 1997; Sanderfoot *et al*. 1998). Eventually, the receptor-cargo complex assembled in CCVs is delivered to PVCs and then vacuoles. Recently, an *Arabidopsis* TGN-localized μ-adaptin was also shown to bind with the C-terminal YMPL motif of BP-80 (Happel *et al*. 2004). **Fig. 1B** shows putative interaction between BP-80 to its ligand in a CCV.

BP-80 and BP-80 reporter

BP-80 was first reported to localize to both Golgi apparatus and a putative PVC in immunocytochemical electron microscope (immunoEM) and confocal immunofluorescence studies in root-tip cells and tobacco (*Nicotiana tabacum*) cells (Paris *et al*. 1997; Li *et al*. 2002). A reporter system was also developed to study the trafficking of BP-80, in which the reporter containing the TMD and CT sequences of BP-80 was colocalized with endogenous VSR proteins after transiently expressed in tobacco suspension cultured cells (Jiang and Rogers 1998). Similarly, a yellow fluorescent protein (YFP)-BP-80 reporter containing the YFP protein and TMD/CT sequences of BP-80 was also colocalized with VSRs to PVCs but separated from the Golgi apparatus in transgenic tobacco BY-2 cells (Tse *et al*. 2004). These results suggest that the TMD and CT regions are specific and sufficient for targeting BP-80 to its final destination. Moreover, immunoEM study in tobacco BY-2 cells demonstrated that VSRs were restricted to the limiting membrane of PVCs/MVBs (Tse *et al*. 2004; Miao *et al*. 2006; Lam *et al*. 2007a). Taken together, VSR proteins and YFP-BP-80 reporter are reliable markers for PVC/MVBs in plant cells.

Using BP-80 reporter to study VSR function and PVC dynamics

BP-80 reporter containing GFP and TMD/CT of BP-80 (GFP-BP-80) (Tse *et al*. 2004) had also been utilized to study the role of BP-80 protein in transporting cargoes to vacuole, where GFP-BP-80 fusion competed with the endogenous VSR proteins for cargo sorting at the retrograde transport route from PVC back to Golgi in tobacco leave protoplast (da Silva *et al*. 2005). When GFP-BP-80 was transiently co-expressed with a BP-80 ligand sporamin, the hypersecretion of sporamin was detected in culture medium (da Silva *et al*. 2005). Similar to endogenous VSR proteins, over-expressed GFP-BP-80 reporter is dominantly localized to PVCs in tobacco cells, which would saturate PVC membrane and thus limit the recycle pathway of functional endogenous VSR from PVC to Golgi and therefore cause missorting of cargoes (da Silva *et al*. 2005). Such positional competition between the truncated BP-80 and endogenous BP-80 may also be true for other VSR family proteins because of their highly conserved sequences and secretory pathway in plant kingdom (Miao *et al*. 2006). Such strategy can also be used to exploit the functional roles of VSR proteins in various cells or tissue types as well as during different developmental stages.

Response of PVC to Wortmannin and Brefeldin A

Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits traffic of internalized proteins from endosome to lysosome in mammalian cells (Arcaro and Wymann 1993;

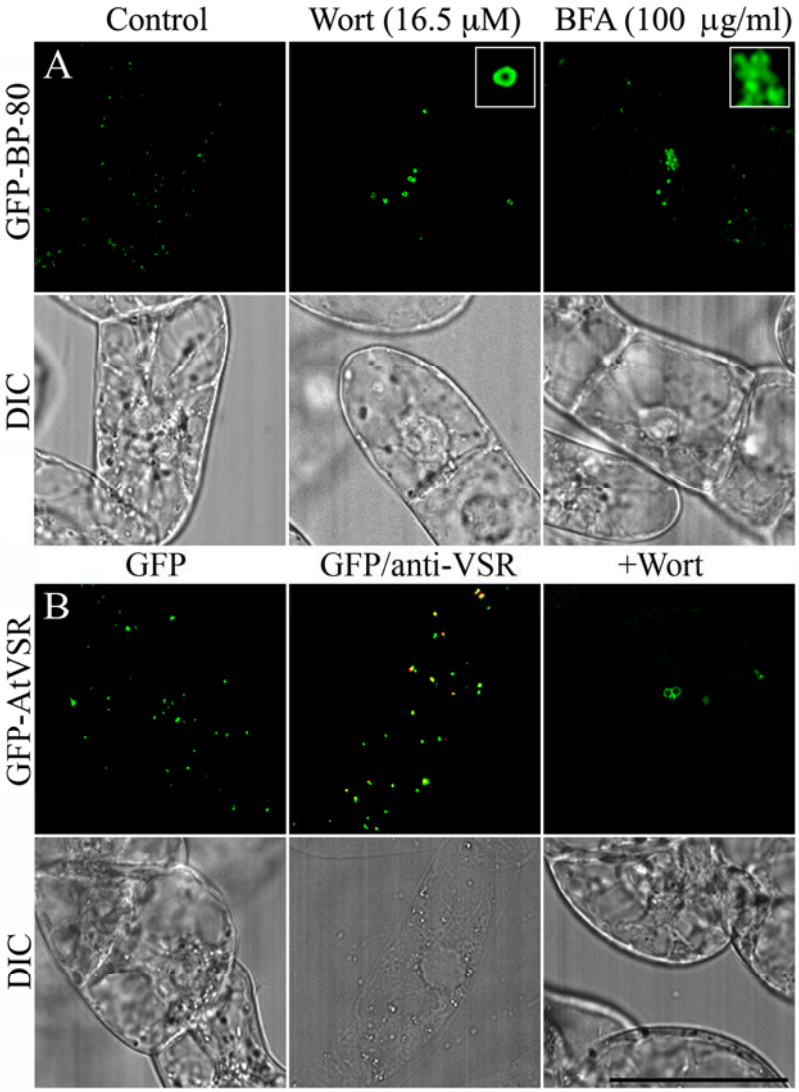

Fig. 2 Dynamics of GFP-marked PVCs in transgenic tobacco BY-2 cells. (**A**) Green fluorescent protein (GFP)-BP-80-tagged PVCs in transgenic tobacco BY-2 cells showed typical punctate patterns that were induced to form small vacuoles by wortmannin treatment at 16.5 μM and form aggregates by brefeldin A (BFA) treatment at 100 μg/mL. (**B**) Transgenic tobacco BY-2 cells expressing GFP-AtVSR1-7 fusions were fixed prior to immunostaining with VSR antibodies to compare the localization between the GFP fusion (green) and VSR (red; GFP/VSR). Cells were also treated with wortmannin (Wort) at 16.5 μM for 2 h before image collection for GFP-marked organelles in living cells. Colocalization of two signals is indicated by a yellow color. DIC, differential interference contrast. Scale bar = 50 μm.

Kjeken *et al*. 2001). Wortmannin affects MVB biogenesis and induces MVB vacuolation by inhibiting the invagination or pinching off of intraluminal vesicles in mammalian cells (Malide and Cushman 1997; Fernandez-Borja *et al*. 1999; Houle and Marceau 2003). Similarly, wortmannin also affect protein transport to vacuoles by inhibiting the retrograde transport of VSR between the PVC and the Golgi apparatus but not the ER-Golgi route in plant cells (Pimpl *et al*. 2003; da Silva *et al*. 2005). In tobacco BY-2 cells and other plant cell types, wortmannin caused the swelling of VSR-marked MVB/PVC (Tse *et al*. 2004; Miao *et al*. 2006; Lam *et al*. 2007a).

Brefeldin A (BFA) has also been widely used to study protein trafficking in eukaryotic cells (Satiat-Jeunemaitre *et al*. 1996; Ritzenthaler *et al*. 2002; Vetterlein *et al*. 2003; Tse *et al*. 2006; Lam *et al*. 2007b). BFA dramatically influence the COP I-coated vesicles that are responsible for retrograde protein transport from Golgi to ER by targeting to guanine nucleotide exchange factors (GEFs) (Jackson and Casanova 2000). BFA seems to trigger cellular response starting from Golgi apparatus, BFA at low concentrations (2 to 10 μg/mL) caused extensive Golgi tubulation and its fusion with the ER (Klausner *et al*. 1992; Tse *et al*. 2006). In plant cells, BFA induced the Golgi apparatus to form ER-Golgi hybrid or BFA compartments (Satiat-Jeunemaitre *et al*. 1996; Wee *et al*. 1998; Baldwin *et al*. 2001; Ritzenthaler *et al*. 2002). Interestingly, BFA at high concentration also caused morphological changes of PVCs in various plant cell types (Tse *et al*. 2006). In transgenic tobacco BY-2 cells expressing GFP-BP-80 reporter, the GFP-marked PVC formed aggregates in response to BFA treatment at higher concentration (50 or 100 μg/mL) and these BFA-induced PVC-derived aggregates still contained VSRs (Tse *et al*. 2006).

Fig. 2A showed the morphological response of VSR-marked PVC to wortmannin and BFA in transgenic tobacco BY-2 cells expressing PVC marker GFP-BP-80. However, mechanisms of PVC response to wortmannin and BFA remain to be illustrated. Several questions can be addressed in future research. How does wortmannin cause the dilation of PVC? Is it derived from the fusion of the outer membrane to intra-luminal vesicles or cytosolic vesicles? What are the molecular targets of PVCs in response to wortmannin and BFA in plant cells? Will the transporting of soluble proteins in plant secretory pathway be impaired due to wortmannin or BFA treatment?

Seven vacuolar sorting receptors in *Arabidopsis*

The *Arabidopsis* genome contains seven BP-80 homologs termed AtVSR1-7, with highly conserved amino acid sequences especially at their N-terminal functional domains (Hadlington and Denecke 2000; Miao *et al*. 2006). **Fig. 3** showed the amino acid sequences alignment of BP-80 and the seven AtVSRs. Due to the availability of well-annotated *Arabidopsis* genomic information, AtVSR1-7 had been investigated recently (Shimada *et al*. 2003; Miao *et al*. 2006; Otegui *et al*. 2006). The mRNA expression levels of individual AtVSRs expressed spatially in different *Arabidopsis* plant tissues (Laval *et al*. 2003). AtELP/AtVSR1 might function in transporting cysteine protease AALP, storage proteins 12S globulin and 2S albumin of *Arabidopsis* (Ahmed *et al*. 2000; Shimada *et al*. 2003; Otegui *et al*. 2006). Antisense of AtVSR1 prevented germination of *Arabidopsis* seed (Laval *et al*. 2003). Study using AtVSR1 specific T-

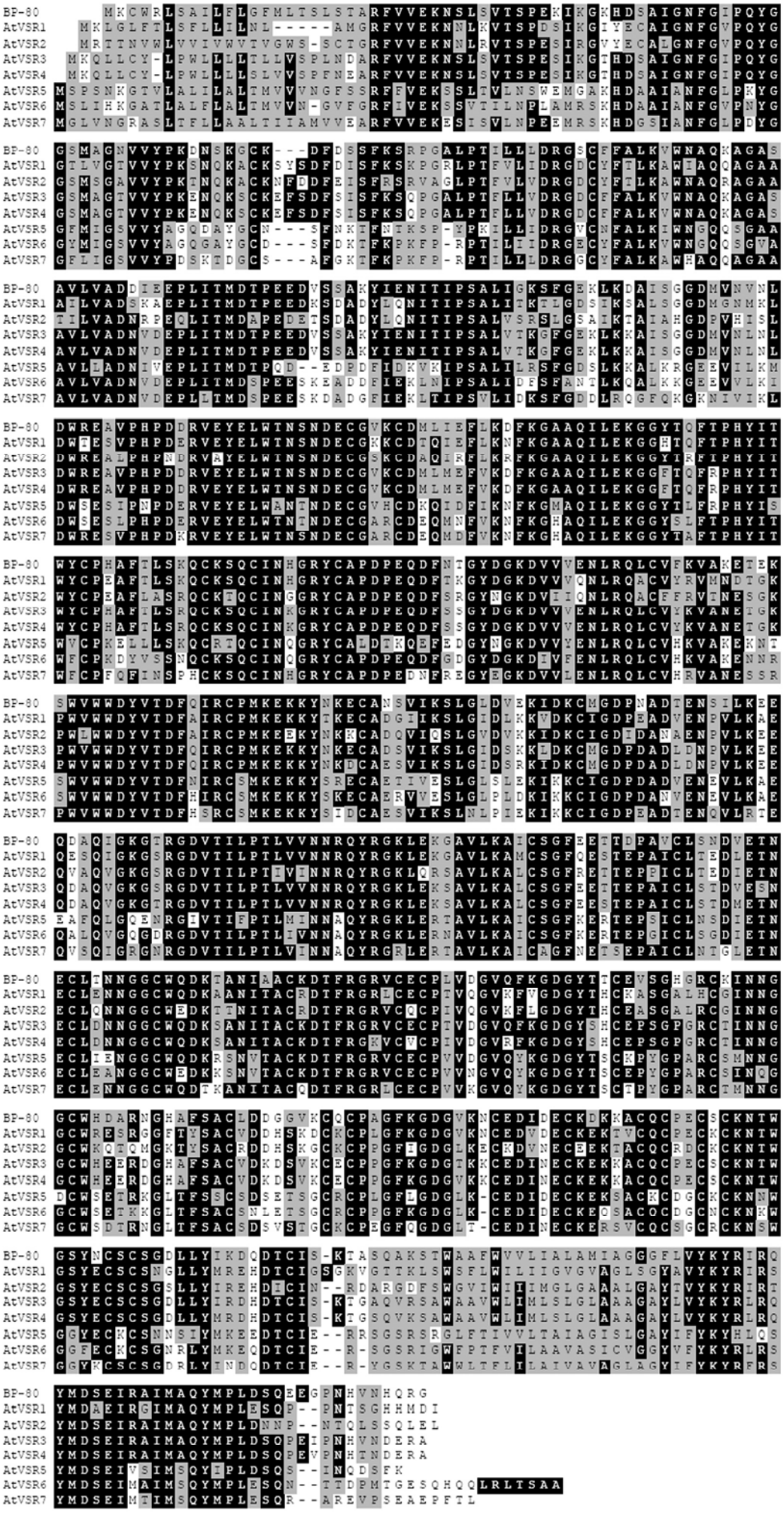

Fig. 3 Alignment of amino acid sequences of *Arabidopsis* vacuolar sorting receptor (AtVSR) proteins. Multiple protein sequence alignment of vacuolar sorting receptors from pea (BP-80) and *Arabidopsis* was performed using ClustalW. The species and the corresponding NCBI access number of each VSR protein are indicated as follows: *Pisum sativum* (BP-80, AAB72110); *Arabidopsis thaliana* (AtVSR1, P93026; AtVSR2, O22925; AtVSR3, O80977; AtVSR4, Q56ZQ3; AtVSR5, O64758; AtVSR6, Q9FYH7; AtVSR7, Q8L7E3). Black represents conserved residues.

DNA insertion mutant demonstrated that the PSV transport of two storage proteins 12S globulin and 2S albumin was impaired and resulted in secretion to extracellular space, indicating the *in vivo* function of AtVSR1 in transporting storage proteins (Shimada *et al*. 2003). Recently, immuno EM studies in *Arabidopsis* embryo cells provided more evidences on the interaction between AtVSR and storage cargoes because AtVSR1 colocalized with 2S albumin in storage protein–containing vesicles. By fusion with enzyme–containing vesicles the storage protein–containing vesicles would start the proteolytic processing of 2S albumin in the MVBs (Otegui *et al*. 2006).

As a first step to understand the functional roles and trafficking of individual AtVSR proteins, we have recently used GFP fusion approach to study the subcellular localization of the seven AtVSR proteins and demonstrated PVC localization of the seven GFP-AtVSR1-7 fusions containing TMD/CT domains of individual AtVSR in transgenic tobacco BY-2 cells (Miao *et al*. 2006). Moreover, PVC organelles marked by GFP-AtVSR1-7 became vacuolated in response to wortmannin treatment but remained unchanged in response to low concentration of BFA at 5-10 μg/mL (Miao *et al*. 2006). **Fig. 2B** showed examples of PVC localization of seven GFP-AtVSR fusions in transgenic tobacco BY-2 cells. Thus, the seven AtVSR1-7 proteins may transport cargoes via PVC-mediated transporting pathway from Golgi to vacuole in tobacco BY-2 cells. However, the subcellular localization of seven AtVSR proteins in *Arabidopsis* plants and their individual functions for cargo trafficking are still unclear, we next discuss several approaches to be used for further study the biology of seven *Arabidopsis* AtVSR proteins.

HOW TO STUDY THE FUNCTIONS OF SEVEN *ARABIDOPSIS* VSR PROTEINS?

Localization study of seven AtVSR proteins

We recently studied the subcellular localization of individual AtVSR proteins in transgenic tobacco BY-2 cells using chimeric fusions containing GFP and TMD/CT sequences of individual AtVSR proteins (GFP-AtVSR) (Miao *et al*. 2006). In order to study the localization of seven AtVSR proteins in *Arabidopsis* plants, the same sets of GFP-AtVSR fusions can be transformed into *Arabidopsis*. Transgenic *Arabidopsis* plants expressing various GFP-AtVSR fusions can then be used for localization identification of GFP fusions in root tip cells by comparing with various known organelle markers via confocal immunofluoresence and immunoEM (Tse *et al*. 2004; Miao *et al*. 2006; Tse *et al*. 2006). In addition, the identity of PVC-, Golgi- or endosome-localization of GFP fusions can also be confirmed by drug treatments with BFA and wortmannin in transgenic plants and transgenic cells as previously described (Tse *et al*. 2004; Miao *et al*. 2006; Tse *et al*. 2006; Lam *et al*. 2007a).

The seven *Arabidopsis* VSRs have a highly conserved N-terminal domain but their CT varied greatly (see **Fig. 3**). $VSR_{at\text{-}1}$ antibody, raised against a recombinant protein corresponding to the N-terminal region of AtVSR1, shall recognize multiple members of the VSR family (Tse *et al*. 2004; Miao *et al*. 2006; Tse *et al*. 2006). It is thus possible to generate antibodies specific for individual AtVSR CT using synthetic peptides corresponding to the unique CT amino acid sequences of individual AtVSR. These antibodies shall be useful tools to study individual AtVSR for their cell- or tissue-type specific expression, localization and function in plants via western blot, confocal and immunoEM analysis.

Functional study of seven AtVSR proteins using two plant model systems

AtVSR1 functioned in transporting both protease and storage proteins in *Arabidopsis* (Ahmed *et al*. 2000; Shimada *et al*. 2003; Otegui *et al*. 2006). However, relatively little is known about the possible cargo proteins for the other six AtVSR proteins in *Arabidopsis*. Thus, identification of cargoes for individual AtVSRs will contribute greatly to our understanding about the functions of individual AtVSR proteins in the post-genomic era. Both transgenic tobacco BY-2 cells and *Arabidopsis* plants are two model systems for such goal.

In vivo identification of VSR cargo proteins in tobacco BY-2 cells

Tobacco BY-2 cells is a quick tool for *in vivo* experiments. Vacuolar sorting signals of cargo proteins can be recognized by the N-terminal domain of AtVSR (**Fig. 1B**). If a truncated VSR lacking its TMD/CT is expressed in BY-2 cells, it will be secreted into the cultured media, along with its interacted cargoes via the default pathway. Therefore, cargo proteins specific for individual AtVSR proteins could be identified via expressing truncated VSR proteins, followed by identification of proteins in cultured media. Thus, cultured media can be collected for protein precipitation, followed by 2-D protein gel electrophoresis and subsequent LC-MS/MS analysis for protein identification. Due to the easy manipulation and fast culture properties of BY-2 cells, large amount of truncated AtVSR proteins and VSR-interacting cargo proteins are likely to be accumulated in the cultured media for protein purification and identification. Such *in vitro* protein-protein interaction study is possibly to generate novel information on VSR cargo proteins in tobacco BY-2 cells.

In vitro identification of VSR cargo proteins in Arabidopsis plants

VSR-interacting cargo proteins can also be identified using affinity columns conjugated with various truncated recombinant AtVSR proteins lacking their TMD/CT regions. These truncated AtVSR proteins can be generated and purified from various expression systems including *E. coli*, yeast and tobacco BY-2 cells. Purified truncated VSR proteins can be used as baits in affinity columns to isolate and identify cargo proteins corresponding to individual AtVSR via *in vitro* pull down binding assay using protein extracts from *Arabidopsis* plants (**Fig. 4A**). Further proofs of cargo-receptor interaction can then be tested as discussed below.

Confirmation of the receptor-ligand interaction

Several strategies can be employed to further prove the interaction between AtVSR proteins and their cargoes. First, antibodies specific for cargo protein and individual AtVSR can be generated and used in immunoprecipitation assay to study their interaction *in vivo*. Second, antibodies or GFP fusions approaches can be used to study the colocalization nature between AtVSR and its cargoes expressed together in either transgenic plants or protoplasts, which would support cargo-receptor interaction *in vivo*. Third, functional competition method (da Silva *et al*. 2005) is also a good choice for studying the interaction between receptors and cargoes via expressing cargoes and GFP-VSR reporters together in plant cells, where cargoes destined to vacuoles will be mis-sorted and detected in extracellular space due to the functional competition between GFP-VSR and endogenous VSR proteins (da Silva *et al*. 2005). Thus, this elegant strategy can be applied here to confirm ligand-AtVSR interaction *in vivo* because the expression level of AtVSR cargoes destined to vacuole decreased in transgenic plants over-expressing GFP-AtVSR fusions comparing to the wild type plants (**Fig. 4B**). The subcellular localization and distribution of affected cargo proteins can also be confirmed via immunoEM with specific antibody.

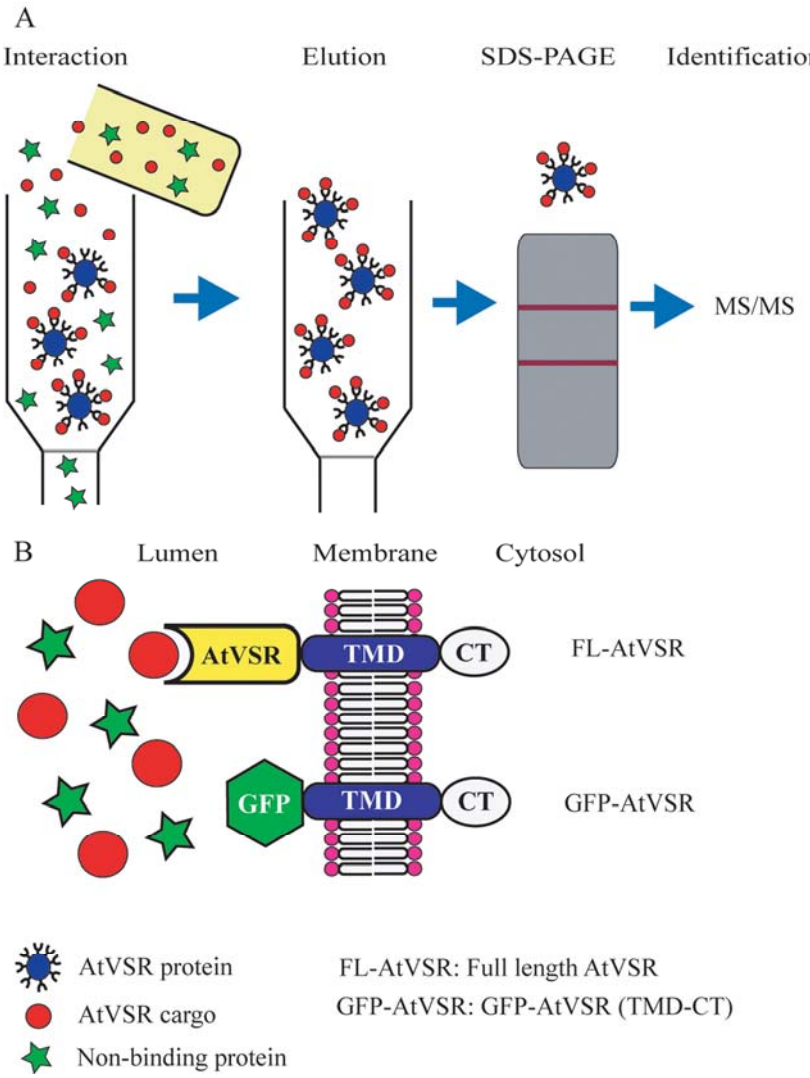

Fig. 4 Approaches to study AtVSR function. (**A**) Pull-down assay to identify new AtVSR cargo proteins. Total *Arabidopsis* proteins are applied onto agarose coated with truncated recombinant AtVSR proteins, cargoes can then isolated and identified via 2-D gel electrophoresis and subsequent LC-MS/MS analysis. (**B**) Functional competition between full length AtVSR proteins (FL-AtVSR) and truncated AtVSR proteins containing GFP and TMD/CT of AtVSR proteins (GFP-AtVSR) (modified from da Silva *et al.* 2005).

Functional studies using loss-of-function approach

Using a number of individual AtVSR T-DNA knockout mutants as platforms, the functional roles of individual AtVSR proteins can also be investigated at both morphological and physiological levels (Shimada *et al*. 2003). In individual AtVSR mutants, the expression and localization of identified particular ligands of individual AtVSR proteins can be studied, which will help to clarify the function of individual AtVSR proteins.

CONCLUDING REMARKS

BP-80 protein is the first VSR protein identified in plant cells that transports cargo proteins to vacuole via a prevacuolar compartment enriched of VSRs. It is not known why plants contain so many VSR proteins expressing in various cells and tissues types including seeds. They might have the same functional roles in transporting the same ligands but at different developmental stages or at different tissue/cell types. They might transport distinct cargoes to the same destination via distinct transport pathways for specific functions. Over the past years, great progress has been made towards our understanding of VSR biology in plants. Using multiple approaches in future research in studying VSR proteins shall be an exciting period for plant cell biologists.

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