Functional Analysis of Arabidopsis and Rice Vacuolar Sorting Receptor (VSR) Proteins

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Statement

All experiment works reported in this thesis were performed by the author, unless specially stated otherwise in the text.

SUEN Pui Kit

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Abstract

Abstract of thesis entitled:

Functional Study of Arabidopsis and Rice Vacuolar Sorting Receptors

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Vacuolar sorting receptors (VSRs) are type I integral membrane family proteins that mediate protein transport from late Golgi or trans-Golgi network (TGN) to vacuole via prevacuolar compartment (PVC) in plant cells. The N-terminus of a VSR is believed to be important for cargo binding while its transmembrane domain (TMD) and cytoplasmic tail (CT) are essential for its correct subcellular localization. In this study, I first developed and tested an expression system using transgenic tobacco BY-2 cells to produce truncated VSR proteins (VSRNT) lacking the TMD/CT into the cultured media. The secreted VSRs bind specifically to the vacuolar sorting determinants (VSDs) of known vacuolar proteins and such binding is calcium dependent *in vitro*. Thus, VSR cargo

proteins are likely secreted into the cultured media along with the truncated VSRs, which enable the identification of various VSR cargo proteins from the cultured media of transgenic cells. I then identified these putative VSR cargo proteins through liquid-chromatography with tandem mass spectrometry (LC-MS/MS) and Fourier transform mass spectrometry (FT-MS) using transgenic *Arabidopsis* cell suspension cultures PSB-D expressing these truncated VSRs. Among the 17 unique proteins found in the cultured media of transgenic *Arabidopsis* PSB-D cell line expressing VSRNT, an *Arabidopsis* glycosyl hydrolase family 3 protein At5g10560 (GH3) was chosen for further study on VSR-cargo protein interaction. GFP-tagged GH3 fusion protein was found to co-localize with VSR-RFP marker protein in PVC, whereas GH3 was also shown to interact with a VSR protein BP-80. Loss-of-function analysis demonstrated that the GH3 contained a vacuolar sorting determinant (VSD) for PVC targeting.

摘要

液泡篩選受體（VSR）蛋白是負責從高爾基網路（TGN）運送液泡蛋白至植物液泡。VSR 的 N 端與液泡蛋白相互作用，而其 TMD 及 CT 則負責 VSR 在植物細胞中的定位。這項研究中，我首先利用轉基因煙草懸浮細胞 BY-2 表達缺乏 TMD/CT 的截斷 VSR 蛋白（VSRNT），並使 VSRNT 分泌於培養介質。液泡蛋白的氨基酸序列上與 VSR 相互作用的區域稱為 VSD。透過 VSRNT 與 VSD 相互作用的分析顯示，它們之間的相互作用是依賴鈣離子，並受鈣離子的濃度影響而有強弱。我從而推斷跟 VSRNT 相互作用的液泡蛋白亦會分泌於轉基因懸浮細胞的培養介質中。故此，我利用轉基因擬南芥懸浮細胞 PSB-D 來表達 VSRNT，並通過液相色譜串聯質譜法（LC-MS/MS）和傅裡葉變換質譜法（FT-MS）去分析培養介質中的分泌蛋白。當中，我共鑒定十七個只有在 VSRNT 轉基因擬南芥 PSB-D 細胞中找到的獨有蛋白。我選擇了一個擬南芥糖苷水解酶家族 3 的液泡蛋白 At5g10560（GH3）作為研究 VSRNT 與液泡蛋白相互作用的模型。透過共聚焦顯微鏡技術，綠色螢光蛋白（GFP）-GH3 融合蛋白被發現定位於液泡前體（PVC），並且與其中一個 VSR（BP-80）蛋白發生相互作用。我並在 GH3 上發現負責其於 PVC 定位的氨基酸序列。總結起來，我已建立一個用於 VSR 蛋白功能研究上快速及高效的研究平台，並且提供了在植物細胞研究領域中有關 VSR 與其相互作用的液泡蛋白的重要資訊。

Chapter 1 Introduction

1.1 The plant secretory pathway

All eukaryotic cells contain a secretory pathway, an endomembrane system that is comprised of several distinct organelles that are defined by specific proteins on or within their membranes (Jiang and Rogers, 1998; Bassham et al., 2000; Lemmon and Traub, 2000; Maxfield and McGraw, 2004; Lam et al., 2005). Although the secretory pathway is responsible for secretion of soluble proteins to the outside of the cell, it also includes membrane-bound organelles that are the final destination of soluble proteins that do not leave the cell; these are the endosome or prevacuolar compartment (PVC) (Bethke and Jones, 2000; Lam et al., 2007a) and the lysosome or vacuole (Okita and Rogers, 1996). Soluble proteins that are destined for the lysosome or vacuole contain positive information that causes them to be sorted away from the flow of proteins to be secreted from the cell (Jiang and Rogers, 1998; Lam et al., 2007a).

In plant cells, receptor-mediated sorting is one of the major mechanisms responsible for sorting soluble proteins to vacuoles in plant cells (Neuhaus and Rogers, 1998; Jiang and Roger, 2003; Surpin and Raikhel, 2004). Soluble vacuolar proteins such as storage proteins and hydrolytic enzymes reach vacuoles because they contain vacuolar sorting determinants (VSDs) that can be

recognized by specific vacuolar sorting receptor (VSR) proteins (Jiang and Roger, 2003) or receptor-like proteins termed RMR (Jiang et al., 2000). BP-80 was the first VSR protein identified in pea (*Pisum sativum*) that recognized the VSD Asn-Pro-Ile-Arg (NPIR) of the barley cysteine protease aleurain (Kirsch et al., 1994; Paris et al., 1997). Similarly, the major seed storage protein 2S albumin and 12S globulin were transported to protein storage vacuole (PSV) by AtVSR1 in *Arabidopsis* seed (Shimada et al., 2003a), whereas the transport of phaseolin to a PSV-like compartment was achieved by the AtRMR1 receptor protein (Jiang et al., 2000) in *Arabidopsis* cells (Park et al., 2005) and tobacco cells (Park et al., 2007). However, the native cargo proteins for most of the studied VSRs in plants remained unknown because either reporters or heterologous vacuolar proteins with known VSDs were used in most of these studies (Lam et al., 2007a).

1.2 Vacuolar sorting receptors

VSRs are type I integral membrane protein containing a single transmembrane domain (TMD) and a cytoplasmic tail (CT) (Paris et al., 1997; Neuhaus and Rogers, 1998; Jiang and Roger, 2003). The N-terminal region of VSR contains cargo-binding domain and epidermal growth factor (EGF)-like repeats (Cao et al., 2000; Paris and Neuhaus, 2002; Watanabe et al., 2002). The N-terminal region of

a VSR is believed to be responsible for sorting cargo proteins at late Golgi or *trans-Golgi network* (TGN) via specific cargo-receptor interaction (Cao et al., 2000), even though a recent study using transient expression system with tobacco protoplasts suggested that such cargo-receptor interaction and protein sorting could occur at the endoplasmic reticulum (ER) (Niemes et al., 2010). Eventually, the receptor-cargo complex assembled in clathrin coated vesicles (CCVs) is delivered to an intermediate prevacuolar compartment (PVC) prior to reaching the vacuoles (Bethke and Jones, 2000; Jiang and Roger, 2003; Lam et al., 2005). In addition, the TMD and CT of a VSR have been shown to be both essential and sufficient for PVC targeting in plant cells (Jiang and Rogers, 1998; Tse et al., 2004). Using VSR antibodies (Li et al., 2002) and the YFP-BP-80 reporter (Tse et al., 2004) as PVC markers, multivesicular bodies (MVBs) were identified as being PVCs in plant cells (Tse et al., 2004; Miao et al., 2006; Wang et al., 2007; Lam et al., 2009).

Calcium also plays an important role in protein-protein interaction, especially in regulating the protein structure (Head and Perry, 1974). The EGF-like repeats presence in VSR has been shown to be important for VSR-cargo interaction (Cao et al., 2000; Paris and Neuhaus, 2002; Watanabe et al., 2002), likely due to its function as calcium binding domain that might

induces conformational changes in the VSR structure upon its interaction with cargo proteins (Watanabe et al., 2002). Such scenario is supported by the resolved 3D structure of the calcium-bound form of EGF motif in animal cells (Selander-Sunnerhagen et al., 1992).

VSR isoforms have been found in many plant species including pea, pumpkin, mung bean, *Arabidopsis* and rice (Kirsch et al., 1994; Paris et al., 1997; Shimada et al., 1997; Ahmed et al., 2000; Paris and Neuhaus, 2002; Shimada et al., 2002; Laval et al., 2003; Wang et al., 2007). The genome of *Arabidopsis thaliana* contains seven VSR isoforms (AtVSR1-7) with highly conserved amino acid sequences especially at their N-terminal domains (Hadlington and Denecke, 2000; Paris and Neuhaus, 2002; Miao et al., 2006) and in rice, an important model crop plant, there are six VSRs. When the seven green fluorescent protein (GFP) fusions with the transmembrane domain (TMD) and cytoplasmic tail (CT) of individual AtVSR1-7 were expressed in transgenic tobacco BY-2 cells, the seven GFP-AtVSR fusions were found to localize to the same PVCs (Miao et al., 2006), where these PVCs mediated the transport of vacuolar reporters for both hydrolytic enzymes and storage protein in suspension cultured *Arabidopsis* and BY-2 cells (Miao and Jiang, 2007; Miao et al., 2008). However, the function of most individual AtVSRs remains elusive. Furthermore, the seven AtVSRs have

different spatial and temporal expression profiles (Paris and Neuhaus, 2002; Laval et al., 2003; Shimada et al., 2003a), thus suggesting their possible functional diversity and redundancy throughout various stages in the plant development. Phylogentic analysis of VSRs of *Arabidopsis*, rice, pea BP-80 and pumpkin PV72 shows that plant VSRs can be divided into 3 subgroups (Figure 1), which suggested different groups of VSRs may serve different functions (Masclaux et al., 2005).

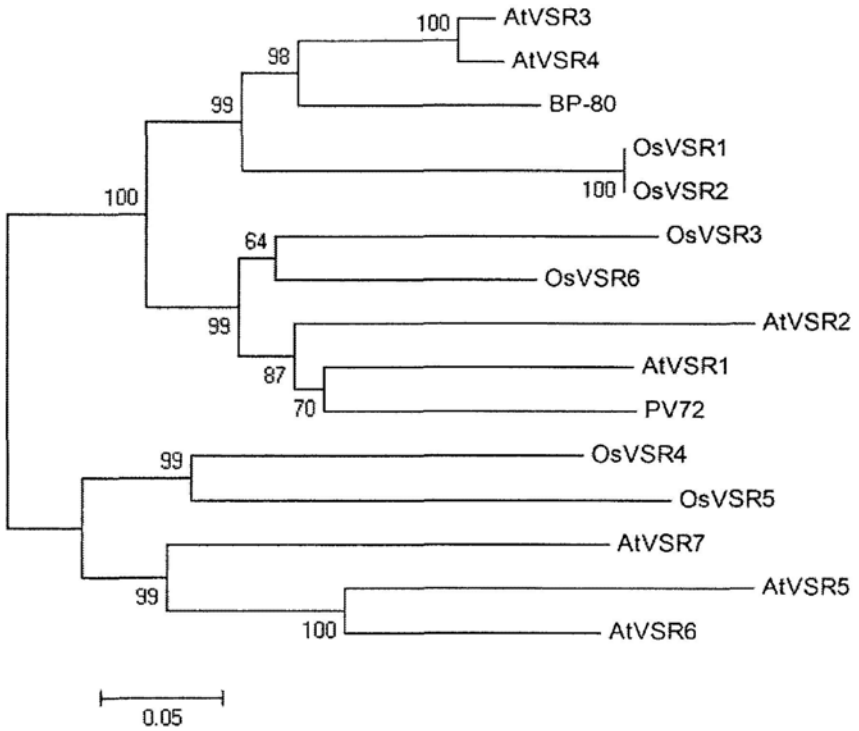

Figure 1 Phylogentic analysis of plant VSR proteins.

Amino acid sequence of pea BP-80, pumpkin PV72, *Arabidopsis* AtVSRs and rice OsVSRs are retrieved from NCBI. Multiple alignment was performed using the ClustalW2 tool with default parameters. Phylogenetic tree was constructed by using MEGA4 software, using Neighbor-Joining with 1000 bootstaps. Numbers represent the bootstap values and the scale bar representing the estimated amino acid substitution per site.

1.3 Functional studies of VSRs

Several approaches have been used to study the possible functions of individual VSR proteins in plants. BP-80 may function in transporting hydrolytic enzymes to lytic vacuole (LV) because it recognized the NPIR motif of barley aleurain (Kirsch et al., 1994; Paris et al., 1997). AtVSR1/AtELP also bound to the NPIR motif for vacuolar transport of proteases in *Arabidopsis* cells (Ahmed et al., 2000). In T-DNA insertion *Arabidopsis* atvsr1 mutant, the PSV transport of two storage proteins 12S globulin and 2S albumin was impaired and resulting in the secretion of these two storage proteins to extracellular space, demonstrating the *in vivo* function of AtVSR1 as a sorting receptor in transporting storage protein to the protein storage vacuoles (PSVs) in *Arabidopsis* seeds (Shimada et al., 2003b). It thus seems that AtVSR1 may also function as a sorting receptor in transporting both cysteine protease and storage proteins in *Arabidopsis* (Ahmed et al., 2000; Shimada et al., 2003b; Otegui et al., 2006). Furthermore, the antisense knock out mutant for *Arabidopsis* VSR proteins is defective in germination (Laval et al., 2003), showing the importance of VSRs in *Arabidopsis* seed germination. Another study using RNAi technology demonstrated that AtVSR3 were important for proper physiological function of the guard cell, because the guard cells of the *atvsr3* RNAi knock-down mutant *Arabidopsis*

plants, in contrast to the wild type plant, did not close properly in response to abscisic acid treatment (Avila et al., 2008). More recently, VSRs and SCAMPs (secretory carrier membrane proteins) were shown to be essential for pollen tube growth (Wang et al., 2009). However, except for AtVSR1 and AtVSR3, we know little about the function of most *Arabidopsis* AtVSRs and VSR proteins in other plants such as rice. It is possible that different VSRs bind different cargo proteins for vacuolar transport in plants.

Previous efforts on functional characterization of VSR protein focus on heterologous expression and purification of VSR proteins in animal culture cells. For example, expression of recombinant truncated BP-80 in Drosophila S2 cells for large scale purification was not feasible because chromatographic purification resulted in large losses and low yield (Cao et al., 2000). Although a further study using the same expression system for a His-tagged *Arabidopsis* VSR AtBP80b (AtVSR1) allowed purification in relatively large amount for crystallization and initial diffraction studies (Rogers et al., 2004), it is technically challenging and economically expensive for functional studies of VSR proteins in the animal cell culture system.

1.4 Functional characterization of VSR proteins using tobacco BY-2 culture cells as an expression system

Protein expression in tobacco BY-2 culture cells may provide a better alternative for VSR protein expression compare with the heterologous expression system in animal culture cells. It has been showed that transgenic tobacco BY-2 cells can highly accumulated protein of interest in the culture medium (Fu et al., 2009). Therefore, expression of VSR proteins in tobacco BY-2 cells should provide a better platform for functional studies of VSR proteins. The native full-length VSR is concentrated on PVCs (Li et al., 2002) whereas its TMD/CT is important for PVC targeting (Jiang and Rogers, 1998; Tse et al., 2004) in plant cells. Thus, the truncated VSR (VSRNT), lacking its TMD and CT, will be secreted into cultured media when expressed in transgenic tobacco BY-2 cells (Figure 2).

As a first step to study the cargo binding of individual VSR proteins in plants, and as proof of principle, I have expressed the soluble truncated forms or N-terminus of five VSR proteins, lacking their TMD and CT, in transgenic tobacco BY-2 cells, including the pea BP-80, the first studied VSR proteins, its close related VSR proteins in *Arabidopsis* AtVSR4, another well studied *Arabidopsis* VSR AtVSR1, and two closely related rice VSR proteins (OsVSR3 and OsVSR6). These expressed N-terminus VSR (BP80NT, AtVSR1NT, AtVSR4NT, OsVSR3NT and OsVSR6NT) are properly secreted into the

cultured media of transgenic BY-2 cells. I have used ligand binding studies with known VSDs to study the specific ligand binding ability of the truncated VSRs. These truncated VSRs had a ligand binding specificity that was indistinguishable from the previously described for full-length BP-80 (Kirsch et al., 1994) and the CHO cells-derived truncated BP-80 (Cao et al., 2000). In addition, such receptor-ligand is calcium-dependent. Therefore, BY-2 cells-derived truncated VSR proteins may represent a useful and reliable tool for studying specific receptor-ligand binding for other members of VSR proteins of *Arabidopsis* and rice. This study will also service as the first essential step for the identification and characterization of new cargo proteins with vacuolar sorting determinants (VSDs) in plant cells.

1.5 Functional study VSR proteins through proteomics approaches

The tobacco BY-2 culture cell system is not a suitable platform for protein identification in the culture media because it lacks a MS/MS database for protein identification. In addition, since tobacco BY-2 cells also produce 3 cationic preoxidase isozymes (34kDa, 38kDa and 40kDa) as the major secreted proteins that contribute around half of the amount of secreted proteins in the cultured media (Narita et al., 1995), thus the presence of these proteins will hinder the

identification of other secreted proteins, especially the cargo proteins in the transgenic cell lines in the culture media of tobacco BY-2 cells. However, these problems do not exist in *Arabidopsis* PSB-D cells, and the fully sequenced *Arabidopsis* genome provides a useful platform for bioinfomatics studies, in recent years, the development of proetomics studies on *Arabidopsis* has grown significantly. Several approaches have been used to dissect the proetomics of the *Arabidopsis*, from the different organs at different development stages (Baerenfaller et al., 2008), specific cell type such as guard cells (Zhao et al., 2008), and organelle proteomics (Mo et al., 2003; Taylor et al., 2003; Carter et al., 2004; Dunkley et al., 2006; Jaquinod et al., 2007). Among them, organelle proteomics is the most changeling one, due to the fact that it is difficult to achieve highly purified organelles, especially those organelles that make up the secretory pathway (Mo et al., 2003; Dunkley et al., 2006). Furthermore, membrane proteins contribute a major part of the protein discovered in these proteome (Carter et al., 2004; Jaquinod et al., 2007). Thus, to search for soluble proteins in the secretory pathway, a highly purified protein fraction which is free of membrane proteins, such as the extracellular secreted fraction of plant culture cells, may provide a more convenient platform for the identification of soluble proteins.

1.6 Hypothesis

Using plant cell as an expression system can provide a promising platform for protein production, as such expression system allows the stable accumulation of protein of interest to be used for functional testing. Thus, I have proposed to use the expression platform to express truncated VSR proteins in tobacco BY-2 and *Arabidopsis* PSB-D culture cells for ligand-receptor characterization, that is based on the following hypothesis and predicted results:

1). The truncated VSR (VSRNT) proteins (the pea BP80NT, *Arabidopsis* AtVSR1NT and AtVSR4NT, and rice OsVSR3NT and OsVSR6NT) are expressed and highly accumulated in the cultured media of transgenic tobacco BY-2 cell culture, which allow subsequent analysis on the functions of VSRNT proteins in terms of calcium dependent interaction with the vacuolar sorting determinants (VSDs).

2). These over-expressed VSRNTs are functionally indistinguishable as native VSRs in terms of calcium dependent interaction with the VSDs. Thus, VSRNTs should interact with various kinds of VSDs and these VSRNT-VSD interactions are calcium dependent.

3). Cargo proteins should be co-secreted with the VSRNT into the culture media of transgenic culture cells. When these VSRNTs are expressed in the

Arabidopsis PSB-D culture cells, VSR interacting cargo proteins that are co-secreted in the culture media can be identified by LC-MS/MS and FT-MS analysis.

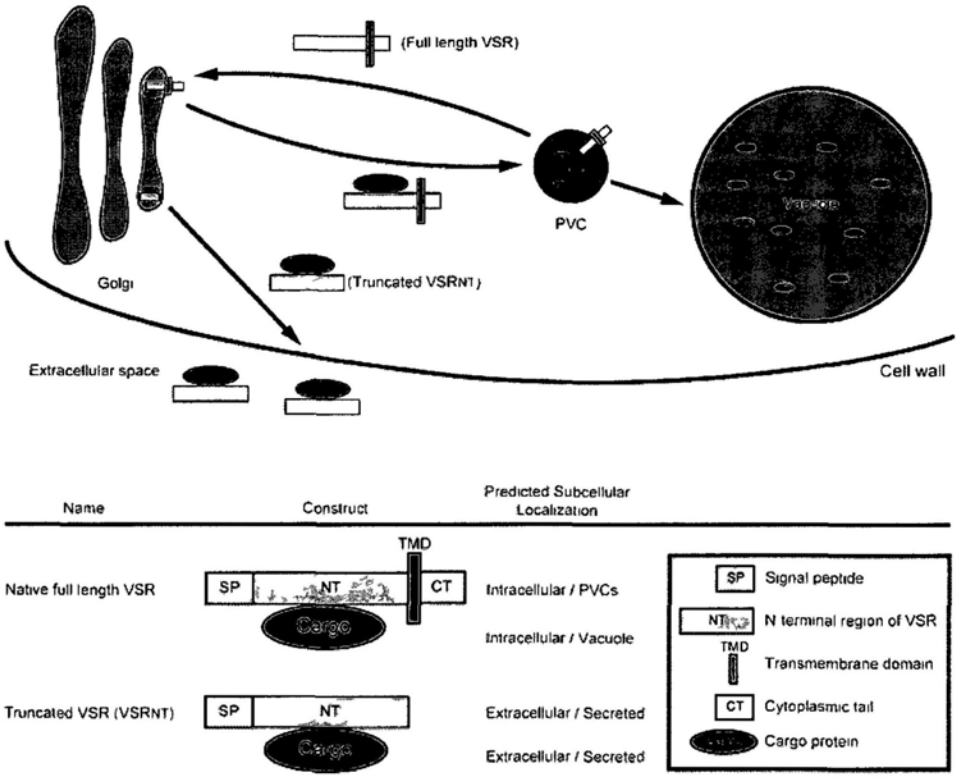

Figure 2 Model of protein trafficking, structure and predicted subcellular localization of full-length VSR, truncated VSR and cargo proteins in cultured cells.

Upper panel, Model of protein trafficking of the full-length, truncated VSR and cargo proteins in the plant secretroy pathway. The full-length VSR is traffic between late Golgi or trans-Golgi network (TGN) to prevacuolar compartment (PVC) and mediated the accumulation of cargo proteins in the vacuole, while the truncated VSR (VSRNT) will be secreted into the cultured media of transgenic culture cells, together with their corresponding cargo proteins.

Lower panel, Structures and predicted subcellular localization of full-length VSR, truncated VSR and cargo proteins. The native VSR contains an N-terminal signal peptide (SP), an N-terminal domain (NT) responsible for binding of cargo protein and deliver the cargo protein into the vacuole, and a single transmembrane domain (TMD) and cytoplasmic tail (CT) responsible for VSRs trafficking to PVCs. The truncated VSR, lacking its TMD and CT, is expected to secrete outside of the cells, together with the corresponding cargo protein when expressed in the *Arabidopsis* PSB-D cells.

Chapter 2 Materials and methods

2.1 Maintenance of cell suspension culture

General methods for the construction and characterization of recombinant plasmids, the maintenance of suspension cultured tobacco (*Nicotiana tabacum*) BY-2 cells and *Arabidopsis thaliana* PSB-D suspension culture cells (ecotype Landsberg erecta), and the preparation and characterization of antibodies have been described previously (Jiang and Rogers, 1998; Jiang et al., 2000; Jiang et al., 2001; Tse et al., 2004; Lam et al., 2007b; Miao and Jiang, 2007).

2.2 Plasmid construction

Nucleotide sequence of primers for amplifying truncated VSR constructs are listed in Table 1. For the construction of truncated BP-80, full length BP-80 from plasmid NP472 (Paris et al., 1997) was used as template for PCR amplification with primers BP80-NTL1 and BP80-NTR1. Full length cDNA of AtVSR1 and AtVSR4 from Riken was used as template, truncated AtVSR1 was PCR amplified by primers AtVSR1-NTL1 and AtVSR1-NTR1 and truncated AtVSR4 was amplified by ATVSR4-NTL1 and AtVSR4-NTR1. Full length cDNA of OsVSR3 and OsVSR6 were from Rice Genome Resources Center (RGRC), truncated OsVSR3 was PCR amplified by primers OsVSR3-NTL1 and OsVSR3-NTR1 and truncated OsVSR6 was amplified by OsVSR6-NTL1 and

OsVSR6-NTR1. For all constructs, the T7 epitode tag is added at the C-terminus. The PCR-amplified fragments were digested with XbaI/SacI for BP80NT, BamHI/SacI for AtVSR1NT, BamHI/SacI for AtVSR4NT, BamHI/SacI for OsVSR3NT and BamHI/SacI for OsVSR6NT and cloned into the plant expression vector pBI121 via replacing the GUS reporter gene. The constructs were checked and verified by restriction mapping and DNA sequencing.

2.3 *Agrobacterium*-mediated transformation of tobacco BY-2 culture cells

Plasmid containing truncated VSR constructs were first introduced into the *Agrobacterium* strain LBA4404 by electroporation prior to transfecting wild-type tobacco BY-2 culture cells as described previously (Tse et al., 2004; Lam et al., 2007b). Kanamycin-resistance calli were transferred into MS liquid medium containing kanamycin to initiate suspension culture. Culture media of transgenic BY-2 cell lines were collected and concentrated by methanol-chloroform for SDS PAGE and western blot analysis using VSR antibodies as described previously (Tse et al., 2004; Wang et al., 2007).

Table 1: Primers used for the construction of truncated VSRNT constructs

PCR Primer	Nucleotide sequence	Restriction enzyme cutting sites
BP80-NTL1	CGGTCTAGAATGAAGTGTTGGAGATTGTCGGCG	XbaI
BP80-NTR1	GCCGAGCTCTCAACCCATCTGCTGACCACCAGTCATCGAAGCCATGGATTTTGCCTGACTGGCAGTTTTAC	SacI
AtVSR1-NTL1	CGGGGATCCATGAAGCTTGGGCTTTTCACTCTC	BamHI
AtVSR1-NTR1	GCCGAGCTCTCAACCCATCTGCTGACCACCAGTCATCGAAGCCATTTTTGTGGTTCCAACTTTGCCTGAAC	SacI
AtVSR4-NTL1	CGGGGATCCATGAAGCAGCTTCTATGTTATCTTCC	BamHI
AtVSR4-NTR1	GCCGAGCTCTCAACCCATCTGCTGACCACCAGTCATCGAAGCCATTGATTTCACTTGTGAACCCGTCTTG	SacI
OsVSR3-NTL1	CGGGGATCCATGACGATGATGGGGTGTGCCG	BamHI
OsVSR3-NTR1	GCCGAGCTCTCAACCCATCTGCTGACCACCAGTCATCGAAGCCATGCCCACTGACGAAGAAGCAACTTTG	SacI
OsVSR6-NTL1	CGGGGATCCATGGGGCTCCGATCCCCCAAC	BamHI
OsVSR6-NTR1	GCCGAGCTCTCAACCCATCTGCTGACCACCAGTCATCGAAGCCATTCCTACCTCTGTTGCTGCATTTTTGC	SacI

Primers for making the truncated VSR constructs for transgenic expression in tobacco BY-2 culture cells. Underlined, restriction enzyme cutting sites for cloning into plant expression vector pBI121. Restriction enzyme cutting sites for BP80NT are XbaI/SacI; AtVSR1NT are BamHI/SacI; AtVSR4NT are BamHI/SacI; OsVSR3NT are BamHI/SacI; and OsVSR6NT are BamHI/SacI.

2.4 Protein precipitation by methanol-chloroform

Culture media were collected by filtering the cultured cells through filter paper. The filtrates with secreted proteins were collected. Proteins were precipitated by methanol-chloroform as described previously (Wessel and Flugge, 1984), by first adding 4 volume of methanol and 1 volume of chloroform, mixed thoroughly and then added 3 volume of double-distilled water. After centrifuge for 5min at 14000rpm, the upper aqueous phase was discarded and 2 volume of methanol were added and mixed with the organic phase. The protein pellet was collected by centrifuge for 5min at 14000rpm. SDS-PAGE sample loading buffer (1X) was used to dissolve the pellet and the protein samples were boiled for 10mins before performing SDS-PAGE.

2.5 Construction of peptide sepharose for pull down assay

Synthetic peptides containing VSDs or their mutated form of vacuolar proteins were synthesized by Genescript. Amino acid sequences and other related information about the synthetic peptides used in this study were listed in Table 2. The synthetic peptides were conjugated into CNBr-activated sepharose (Sigma) according to manufacturer's protocol. Different synthetic peptides are designed to test the specific interaction of truncated VSRs versus different types of known or putative VSDs of vacuolar proteins, including the BP-80-binding NPIR VSD

of aleurain and sporamin, the AFVY VSD of phaseolin. VSDs from other storage proteins such as pro2S albumin, BN2S, beta-conglycinin, vicilin and proConA were also used. Besides, mutation analysis was also performed on VSD of vicilin-like storage protein (VLSP) containing one AFVY-like motif (AVFV) and one WSIV motif, found to be important for correct targeting of phaseolin to protein storage vacuole. VSDs from two tobacco chitinases, the vacuolar basic chitinase and secreted acidic chitinase, together with putative VSDs from 5 *Arabidopsis* chitinases were also used to test the VSR-VSD pull down assay in identifying novel VSDs. The trafficking of tobacco basic chitinase depends on the presence of VSD, with amino acid sequence LLVDTM, on the C-terminal of chitinase. Similar putative VSDs were also found in the C-terminal of *Arabidopsis* chitinases. A "blank column" without peptides was constructed as the same way and used as control.

2.6 Pull down assay for VSRNT-peptide interaction assay and calcium dependent assay

Day-7 culture media were collected by filter the cell culture through a filter paper. The filtrates containing secreted proteins were collected and dialyzed against double distilled water at 4°C overnight, followed by lyophilization. The lyophilized proteins were resuspended in binding buffer (25mM HEPES, pH 7.1,

150mM NaCl, 1mM $MgCl_2$) with 1000μM $CaCl_2$ in the VSRNT-peptide interaction assay. One milliliter of the resuspended proteins was mixed with 10μL of sepharose conjugated with peptides at 4°C overnight. After washing with binding buffer for three times, the sepharose containing the VSRNT peptide complex was boiled in 20μL SDS-PAGE sample loading buffer for 10 min and subject to western blot by VSR antibodies (Figure 3). Calcium-dependent interaction of VSRNT-aleurain under different Ca^{2+} concentration was done by using 0, 10, 25, 50, 100, 1000μM $CaCl_2$ in the binding buffer (Kirsch et al., 1994; Watanabe et al., 2002). Sepharose conjugated with synthetic aleurain peptide was mixed with buffer containing VSRNT proteins. Procedures of calcium-dependent assay are the same as VSRNT-peptide interaction assay.

2.7 Calculation of percentage of binding of VSRNT to synthetic aleurain peptide

The percentage of binding of VSRNT to VSD of aleurain is calculated from comparing the image intensity of the correspond protein bands in western blot analysis, as determined by ImageJ (available in http://rsbweb.nih.gov/ij/index.html), using the signal obtained at 1000μM concentration of calcium as 100%.

Table 2: Amino acid sequence of synthetic peptides containing known VSDs

Peptide	Name	Sequence
a	NPIR	SSSFADS**NPIR**PVTDRAASTYC
b	Mutated NIPR	SSSFADSAAAAPVTDRAASTYC
c	Sporamin	CAHSRF**NPIRL**PTTHEPAS
d	Phaseolin	CKGSHQQEQQKGRKG**AFVY**
e	Mutated Phasoelin	CKGSHQQEQQKGRKGAAAA
f	pro2S Albumin	CSRDVLQMRGIENPW**RREG**
g	2S Albumin	CRCNLSPMRCPMGGS**IAGF**
h	beta-conglycinin	CEGNKGRKG**PLSSILRAFY**
i	vicillin	CDAQPQQRERGSRETRDRLSSV
j	ProConcanavalinA (proConA)	CSFTSKLKSN**EIPDITAVV**
k	VLSP	CSQDKAVFVDGPRGSRSLWSIV
L	mVLSP1	CSQDKAVFVDGPRGSRSLAAAA
m	mVLSP2	CSQDKAAAADGPRGSRSLWSIV
n	Tobacco Acidic Chitinase	CDLCGNQRSFGNG
o	Tobacco Basic Chitinase	CDLCGNQRSFGNG**LLVDTM**
p	At3g12500	CDLCYNQRSFVNGLLEAAI
q	At1g05850	CDLCENQRPFS
r	At1g02360	CDLNENQRPYA
s	At3g16920	CAEQKPFNPSTVPSSSSS
t	At4g01700	CDQEPFSSSSAPPSSGSSS

Synthetic peptides containing vacuolar sorting determinants (VSDs) that are used on the VSR-VSD pull down assay. Bold and underlined, known VSD motif for VSR interaction. Doubleunderlined, mutated motif.

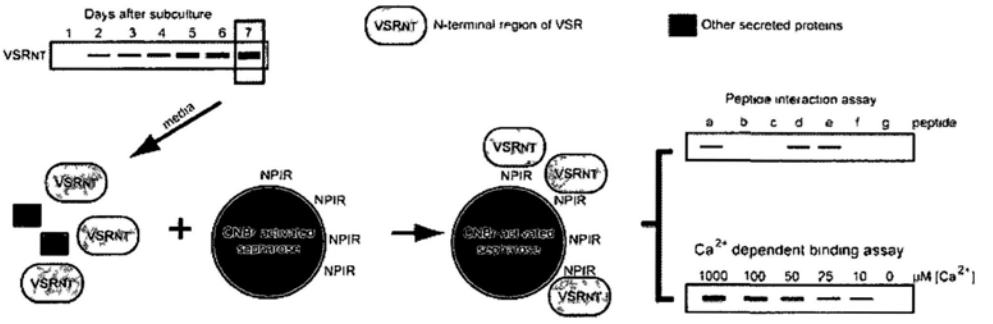

Figure 3 Procedures of pull down assay and Calcium-dependent binding assay.

Day-7 culture media of transgenic BY-2 cells expressing truncated VSR (VSRNT) were collected. After dialysis, the media were lyophilized and resuspended in binding buffer (25mM HEPES, pH 7.1, 150mM NaCl, 1mM $MgCl_2$) with 1000μM $CaCl_2$ in the VSRNT-peptide interaction assay or with 0, 10, 25, 50, 100, 1000μM $CaCl_2$ in the calcium dependent assay. The resuspended proteins were mixed with sepharose conjugated with various synthetic peptides of VSDs or mutated VSDs and washed with binding buffer prior to SDS-PAGE and VSR antibodies were used in western blot analysis.

2.8 Western blot analysis of marker proteins of various cellular compartments

Intracellular and secreted proteins were collected, followed by SDS-PAGE and western blot analysis using antibodies against different marker proteins including ER, Golgi, vacuolar membrane, plasma membrane, endosome and cytoskeleton marker. BiP and calreticulin are the two ER chaperones that assist protein folding (Jia et al., 2009). MannosidaseI (ManI) is a Golgi resident protein and is responsible for protein glycosylation along the secretory pathway (Tse et al., 2004; Saint-Jore-Dupas et al., 2006). Tonoplast marker alpha-tonoplast intrinsic protein (alpha-TIP), is an aquaporin present in the tonoplast membrane of plant cell (Jauh et al., 1999). Plasma membrane and early endosome marker SCAMP (secretory carrier membrane protein) is a protein cycling between endosome and plasma membrane (Lam et al., 2007b), and cytoskeleton marker tubulin is a cytosolic proteins and is the building blocks of microtubules (Yoneda et al., 2007).

2.9 Bioinformatics analysis

Amino acid sequences of VSRs are retrieved from NCBI database under accession numbers, AtVSR1 (At3g52850), AtVSR2 (At2g30290), AtVSR3 (At2g14740), AtVSR4 (At2g14720), AtVSR5 (At2g34940), AtVSR6

(At1g30900), AtVSR7 (At4g20110), OsVSR1 (Os10g0346600), OsVSR2 (AAN05373), OsVSR3 (Os07g0680000), OsVSR4 (Os06g0664300), OsVSR5 (Os04g0611400), OsVSR6 (Os12g0116000), PV72 (BAA25079) and BP-80 (P93484). Multiple alignment was performed using the ClustalW2 tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html) with default parameters (Larkin et al., 2007). Phylogenetic tree was constructed by using MEGA4 software (Tamura et al., 2007), using Neighbor-Joining with 1000 bootstaps.

2.10 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* PSB-D culture cells

Plasmids containing the truncated VSRs from pea BP80NT, *Arabidopsis* AtVSR1NT and AtVSR4NT, and rice OsVSR3NT and OsVSR6NT were first introduced into the *Agrobacterium* strain GV3101 (pMP90) by electroporation and screened by antibiotics (kanamycin, gentamycin and rifampicin) on YEP agar plates. *Agrobacterium* harbouring the constructs was first grown to stationary phase in YEP liquid culture prior to transformation of the *Arabidopsis* culture cells. *Arabidopsis* suspension cells, two days after subcultured, were transformed by co-cultivating with Agrobacterium for 3 days at 130 rpm in an orbital shaker at 25°C with 187μM acetosyringone. Transgenic *Arabidopsis* calli were selected on MS plates containing kanamycin and cefotaxime. The

kanamycin-resistance calli were transferred into MS liquid medium containing kanamycin to initiate suspension culture. Culture media of transgenic PSB-D cell lines were collected and concentrated by methanol-chloroform for SDS-PAGE and western blot analysis of VSRNT using VSR antibodies as described in Section 2.4.

2.11 Secreted protein preparation for mass spectrometry analysis

To prepare secreted proteins presenting in the culture media, Day-4 culture media from wild-type PSB-D and transgenic PSB-D expressing BP80NT, AtVSR1NT and AtVSR4NT were collected by filtering the cultured cells through filter papers. The secreted proteins were precipitate by methanol/chloroform, the protein pellet was then air-dried, rehydrated in 27.5μL of 25mM ammonium bicarbonate containing 300ng of sequencing grade modified trypsin (Promega, Madison, WI). Tryptic digestion was performed overnight at 28°C. Peptides were extracted by repeating twice with 20μL of acetonitrile/0.05% trifluoroacetic acid (1:1), sonicated for 10min, spun and collected the supernatant, and finally extracted with 10μL of acetonitrile, sonicate for 10min spin and collect the supernatant. The extracted peptides were vacuum-dried and re-dissolved in 2% acetonitrile/0.05% trifluoroacetic acid for nano-LC-MS/MS or 0.1% formaic acid

for FT-MS

2.12 Nano liquid chromatography tandem mass spectrometry (Nano-LC-MS/MS)

Digested peptide mixture was loaded to PepMap100 C18 nano column (LC Packings) of UltiMate 3000 Intelligent LC system (Dionex, Sunnyvale) in order to perform nano-LC. A 30-min gradient from 20% to 70% solvent B (Solvent A: 2% acetonitrile/0.05% trifluoroacetic acid; Solvent B: 80% acetonitrile/0.05% trifluoroacetic acid) was set to run at a flow rate of 3μL/min. Samples collected were further analyzed using MALDI TOF/TOF Analyzer (ABI4700 Proteomics Analyzer, Applied Biosystems, Foster City). The search software MASCOT (Matrix Science) was used to manually search against the National Center for Biotechnology Information nonredundant primary sequence database (NCBInr 20071004) with tryptic constraints and initial mass tolerances ±0.13 Da. Search parameters were set as follows: taxonomy, *Arabidopsis thaliana*; enzyme, trypsin; variable modifications, acetyl (N-term), oxidation (Methionines), mass values, monoisotopic; protein mass, unrestricted peptide; precursor tolerance, ±50 ppm; MS/MS fragment tolerance, ± 0.5 Da; max. missed cleavages, 1. Only peptides achieving a significant MASCOT ion score above 30 and containing a sequence tag of at least three consecutive amino acids were accepted. All proteins

identified by MASCOT were further verified by BLAST searches against the TAIR database (http://www.Arabidopsis.org/Blast/index.jsp). Three independent repeats were performed, and for each of the repeats, the digested peptides were injected twice.

2.13 Fourier-transform ion cyclotron resonance mass spectrometry (FT-MS)

Analyses were performed using an electrospray ionization source (Apex ultra Hybrid Qq-FTMS, Bruker Daltonics) coupled to a liquid chromatography (Ultimate 3000; Dionex). The system was controlled with the software package HyStar (version 3.4 build 8, Bruker Daltonics). The analyses were performed by injecting 5μL aliquots of a standard solution or sample extract onto PepMap100 C18 nano column (LC Packings). The sample was eluted at a flow rate of 300nL/min with an elution gradient composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The following gradient profile was used: a linear gradient from 2 to 20% B (0 to 10 min), from 20 to 60% B (10 to 40 min), 70% B (40 to 45 min), and a linear gradient from 70 to 2% B (45 to 45.1 min). Samples were detected in positive ion mode with electrospray ionization parameter values of 1600V for the capillary entrance, 1.7 L/min of dry gas flow, and 180°C for the drying gas temperature. A fourier

transformed full scan was acquired. The spectra were acquired in the m/z range of 250 to 2200. The four most intense peaks were sequentially isolated for the MS/MS experiments using collisionally induced dissociation. To prevent duplication of MS/MS data for the same peptide, dynamic exclusion was set to 1, and selected ions were placed on the exclusion list for 30 s. The MS/MS raw spectra data were converted to mgf files using DataAnalysis 4.0 SP1 (Bruker Daltonics) and analyzed by means of BioTools 3.1 (Bruker Daltonics). The search software MASCOT (Matrix Science) was used to manually search against the National Center for Biotechnology Information nonredundant primary sequence database with tryptic constraints and initial mass tolerances ±0.13 Da. Search parameters were set as follows: taxonomy, *Arabidopsis thaliana*; enzyme, trypsin; variable modifications, oxidation (Methionines); mass values, monoisotopic; charge state, 1+, 2+ and 3+; protein mass, unrestricted peptide; precursor tolerance, ± 0.05 Da; MS/MS fragment tolerance, ± 0.05 Da; Instrument, ESI-FTICR. Only peptides achieving a significant MASCOT ion score above 30 and containing a sequence tag of at least three consecutive amino acids were accepted. All proteins identified by MASCOT were further verified by BLAST searches against the TAIR database (http://www.arabidopsis.org/Blast/index.jsp). Three independent repeats were

performed, and for each of the repeats, the digested peptides were injected twice.

2.14 Analysis of the secreted proteins from the *Arabidopsis* PSB-D culture cells

Secreted proteins identified by MASCOT in the wild type PSB-D were pooled together, termed as the wild-type database (WTDB). Only those proteins identified by MASCOT in the transgenic PSB-D that are found in at least two of the independent repeated experiments of mass spectrometry are accepted as secreted proteins of the transgenic PSB-D cells, termed as the truncated VSR database (VSRNTDB). By comparing VSRNTDB with the WTDB, unique proteins found only in the transgenic *Arabidopsis* PSB-D cells but not in wild type PSB-D cell were identified. The nucleotide acid and amino acid sequences were retrieved from TAIR database. Signal peptide and the signal peptide cleavage site were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane domain was predicted by TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Only those unique proteins that have MASCOT ion score above 50, with at least 2 peptides, and contain a signal peptide but no TMD were accepted as potential cargo proteins.

2.15 Plasmid construction for subcellular localization of *Arabidopsis* glycosyl hydrolase family 3 protein At3g10560 (GH3) and *in vivo* VSR-cargo protein interaction

Green fluorescent protein (GFP) fusion protein was used as reporter for the study of subcellular localization of the *Arabidopsis* glycosyl hydrolase family 3 protein (At3g10560, termed as GH3) through transient expression in *Arabidopsis* PSB-D culture cell protoplast. Aleurain-GFP and 2S albumin-GFP were used as positive control for cargo protein traffic to PVC marked by mRFP-AtVSR2 (Miao et al., 2008). Primers for constructing the GH3-GFP fusion construct were listed in Table 3. Full length GH3 cDNA from Riken was used as template for PCR amplification with primers GH3-L1 and GH3-R1. GFP was amplified from the GFP with the signal peptide from barley (*Hordeum vulgare*) proaleurain (Tse et al., 2004; Miao et al., 2006) using primers GFP-L1 and GFP-L2 without amplifying the signal peptide. The PCR-amplified fragments were digested with XbaI/KpnI for GH3 and KpnI/SacI for GFP and cloned into transient expression vector pBI221 via replacing the GUS reporter gene.

Primers for constructing the BP80-CNX-RFP fusion construct were listed in Table 3. For the construction of BP80-CNX-RFP, monomeric RFP was first PCR amplified from mRFP-AtVSR2 (Miao et al., 2008) using primers RFP-L1 and RFP-R1, followed by digestion with BamHI/SacI and cloning into the transient

expression vector pBI221 via replacing the GUS reporter gene, forming pBI221-mRFP. The TMD and CT of calnexin from GFP-CNX (daSilva et al., 2005; daSilva et al., 2006; Pimpl et al., 2006) was amplified using primers CNX-L1 and CNX-R1 and inserted into the SpeI/KpnI site of pBI221-mRFP, forming pBI221-CNX-RFP. The BP80NT was amplified with primers BP80-NTL1 and BP80-NTR2 for subsequent cloning into pBI221-CNX-RFP via the XbaI-XhoI site, resulting BP80-CNX-RFP. The constructs were checked and verified by restriction mapping and DNA sequencing.

2.16 Deletion mapping of the At3g10560 (GH3)

Green fluorescent protein (GFP) fusion protein was used as reporter for the study of subcellular localization of the GH3 and its deletions through transient expression in *Arabidopsis* PSB-D culture cell protoplast. Primers for cloning the GH3 deletion constructs were listed in Table 3. The full length GH3-GFP fusion consists of 792 amino acids fused with GFP, and the two deletion constructs, GH3-277-GFP consists of the first 277 amino acids of GH3 fused with GFP, and the GH3-534-GFP consists of the first 534 amino acids of GH3 and fused with GFP (Figure 3A). Full length GH3-GFP fusion construct has been described in section 3.2.6. For the construction of GH3-277-GFP, the GH3-277 fragment was

amplified by primers GH3-L1 and GH3-R3 using full length GH3 as template. The PCR-amplified fragments were digested with XbaI/KpnI for GH3-277 and KpnI/SacI for GFP and cloned into transient expression vector pBI221 via replacing the GUS reporter gene. For the construction of GH3-534-GFP, the GH3-534 fragment was amplified by primers GH3-L1 and GH3-R2 from full length GH3. The PCR-amplified fragments were digested with XbaI/KpnI for GH3-534 and KpnI/SacI for GFP and cloned into transient expression vector pBI221 via replacing the GUS reporter gene. The constructs were checked and verified by restriction mapping and DNA sequencing.

2.17 Confocal Microscopy

Transient expression on the wild-type *Arabidopsis* PSB-D culture was performed as described previously (Miao and Jiang, 2007). Confocal images were collected with Fluoview FV1000 (Olympus, Japan) with a 60x objective water lens. The settings for collecting confocal images within the linear range were as described previously (Jiang and Rogers, 1998; Tse et al., 2004). Images were processed using Adobe Photoshop software (Jiang and Rogers, 1998).

Table 3: Primers used for the construction of the *Arabidopsis* At3g12500 GFP (GH3-GFP) fusion protein and its truncated versions.

PCR Primer	Nucleotide sequence	Restriction enzyme cutting sites
GH3-L1	GCCTCTAGAATGAATCTTCAGTTGACTCTAATC TCCTTACTCTTC	XbaI
GH3-R1	GCCGGTACCGAATTCAACAGAGAGAGAATGTT GTAAATCTCCCAAGAAC	KpnI
GH3-R2	GCCGGTACCAGACACAAGATCTTTCTGTTTAC CAGGC	KpnI
GH3-R3	GCCGGTACCACACATCAAGCAGCTAGCTTTAC CAT	KpnI
GFP-L1	GCCGGTACCAGCAAGGGCGAGGAGCTG	KpnI
GFP-R1	GCCGAGCTCTTACTTGTACAGCTCGTCCATGCC GTGAGTG	SacI
BP80-NTL1	CGGTCTAGAATGAAGTGTTGGAGATTGTCGGC G	XbaI
BP80-NTR2	CGGCTCGAGGGATTTTGCCTGACTGGCAGTTT TAC	XhoI
CNX-L1	GGGACTAGTCTCGAGATGGAACTGATTGAGAA AGCCG	5'-SpeI-XhoI
CNX-R1	GGGGGTACCATTATCACGTCTCGGTTGCC	KpnI
RFP-L1	GGGGGATCCGTCGACACTAGTGGTACCATGGC CTCCTCCGAGGACGTCATCAAG	5'-BamHI-SalI -SpeI-KpnI
RFP-R1	GGGGAGCTCTTAGGCGCCGGTGGAGTGGCGG C	SacI

Primers for making the GH3-GFP fusion and GH3 deletion proteins for subcellular localization studies in *Arabidopsis* PSB-D protoplasts. The constructs were cloned into pBI221 for transient expression studies. Underlined, restriction enzyme cutting sites.

Chapter 3 Results

3.1 Generation and characterization of transgenic tobacco BY-2 cells expressing truncated VSRs (VSRNT)

The pea VSR BP-80 consists of a signal peptide, N-terminal cargo binding domain (NT), a single transmembrane domain (TMD) and cytoplasmic tail (CT). The BP-80 NT is believed to be responsible for cargo binding while its TMD/CT is essential for its correct targeting to prevacuolar compartments (PVCs) in plant cells (Jiang and Rogers, 1998). Similarly, the seven *Arabidopsis* VSRs are also localized to the same PVC populations in plant cells (Li et al., 2002; Miao et al., 2006). It has been suggested that individual VSRs might bind to specific cargo(es) for vacuolar targeting in plants.

As a first step to study the cargo binding of individual VSR proteins in plants, I tested a secretion expression system using transgenic tobacco BY-2 cells (Fu et al., 2009). Since the native full-length VSR is concentrated on PVCs (Li et al., 2002) whereas its TMD/CT is important for PVC targeting (Jiang and Rogers, 1998; Tse et al., 2004) in plant cells, I have thus hypothesized that the truncated VSR (VSRNT), lacking its TMD and CT, will be secreted into the cultured media when expressed in transgenic tobacco BY-2 cells (Figure 2). The truncated VSRs (BP80NT, AtVSR1NT, AtVSR4NT, OsVSR3NT and OsVSR6NT) constructs were transformed into tobacco BY-2 cells via *Agrobacterium*-mediated

transformation. Culture media of transgenic BY-2 cells were collected and western blot analysis was performed to confirm the expression of VSRNT proteins in each constructs (Figure 4A). In fact, in wild type (WT) tobacco BY-2 cells, anti-VSR only detected the endogenous VSR protein in the intracellular (I) fraction (Figure 4B, lane 1, double asterisks) but missing from the secreted (S) media (Figure 4B, lane 2). In contrast, in transgenic tobacco BY-2 cells expressing the truncated VSRs, in addition to the presence of the endogenous VSR in the intracellular fraction (Figure 4B, lane 3, 5, 7, 9 and 11, double asterisk), a protein band corresponding to the truncated BP-80 was detected in both the intracellular fraction (I) and the secretion fraction (S) (Figure 4B, lane 3 and 4, asterisk), similar results were obtained from other truncated VSRs, even though AtVSR1NT, AtVSR4NT and OsVSR6NT in the intracellular fraction (I) were below detection level (Figure 4B, lane 5, 7, 11, asterisk). In addition, the accumulation of secreted VSRNTs in the secretion fraction (S) (Figure 4B, lane 4, 6, 8, 10 and 12) are much higher than that of intracellular fraction (I) (Figure 4B, lane 3, 5, 7, 9 and 11), indicating the stable accumulation of secreted VSRNT in the culture media. Furthermore, when culture media of transgenic BY-2 cells expressing VSRNTs were collected from Day 1 to Day 7 after subculture for western blot analysis using VSR antibodies, the detectable amounts of the

truncated VSRNTs in the media were increased gradually from Day 1 to Day 7 (BP80NT, Figure 5A; AtVSR1NT, Figure 5B; AtVSR4NT, Figure 5C; OsVSR3NT, Figure 5D; OsVSR6NT, Figure 5E; lanes 1 to 7, asterisk), indicating the steady accumulation of the secreted VSRNT over the 7-day culture period. Taken together, these results demonstrate that transgenic tobacco BY-2 cells expressing transgenic VSRNTs have resulted in proper secretion of the truncated VSRs in the culture media.

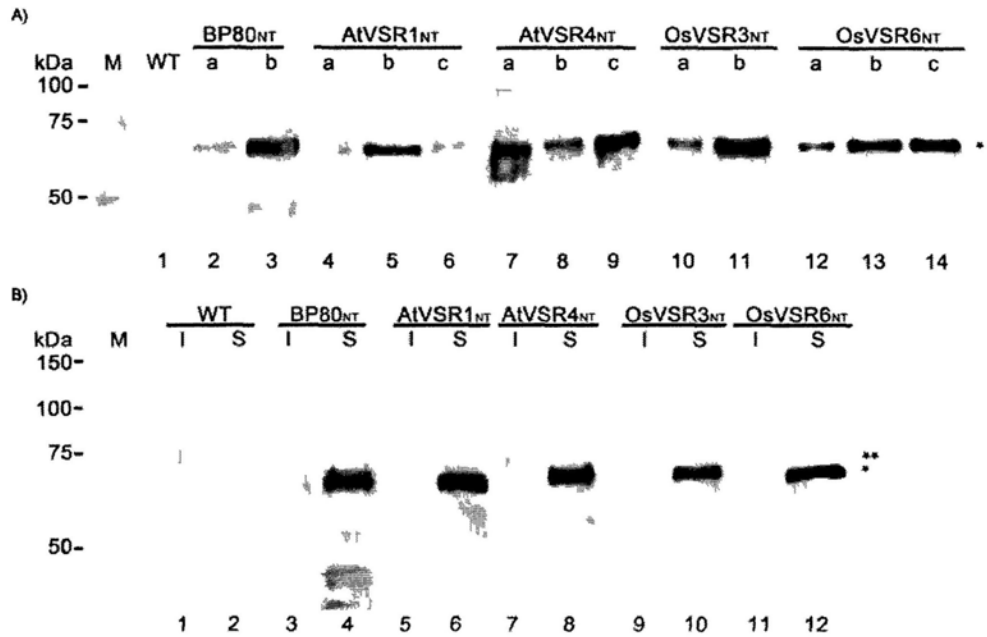

Figure 4 Expression and characterization of transgenic tobacco BY-2 cell lines expressing the truncated VSRs.

(A). Culture media containing secreted protein were isolated from wild type (WT, lane 1) or various cell lines transgenic BY-2 cells expressing BP80NT (Cell lines a and b, lane 2 and 3), AtVSR1NT (Cell lines a, b and c, lane 4 to 6), AtVSR4NT (Cell lines a, b and c, lane 7 to 9), OsVSR3NT (Cell lines a and b, lane 10 and 11) and OsVSR6NT (Cell lines a, b and c, lane 12 to 14), followed by protein separation via SDS-PAGE and western blot analysis using VSR antibodies. (B). The intracellular (I) and secreted (S) proteins were isolated from either wild type (WT, lane 1 and 2) or transgenic tobacco BY-2 cells expressing BP80NT (lane 3 and 4), AtVSR1NT (lane 5 and 6), AtVSR4NT (lane 7 and 8), OsVSR3NT (lane 9 and 10) and OsVSR6NT (lane 11 and 12). M, molecular mass in kilodalton. Double-asterisks and asterisk indicate the full-length endogenous VSR and the expressed truncated VSRNT proteins respectively.

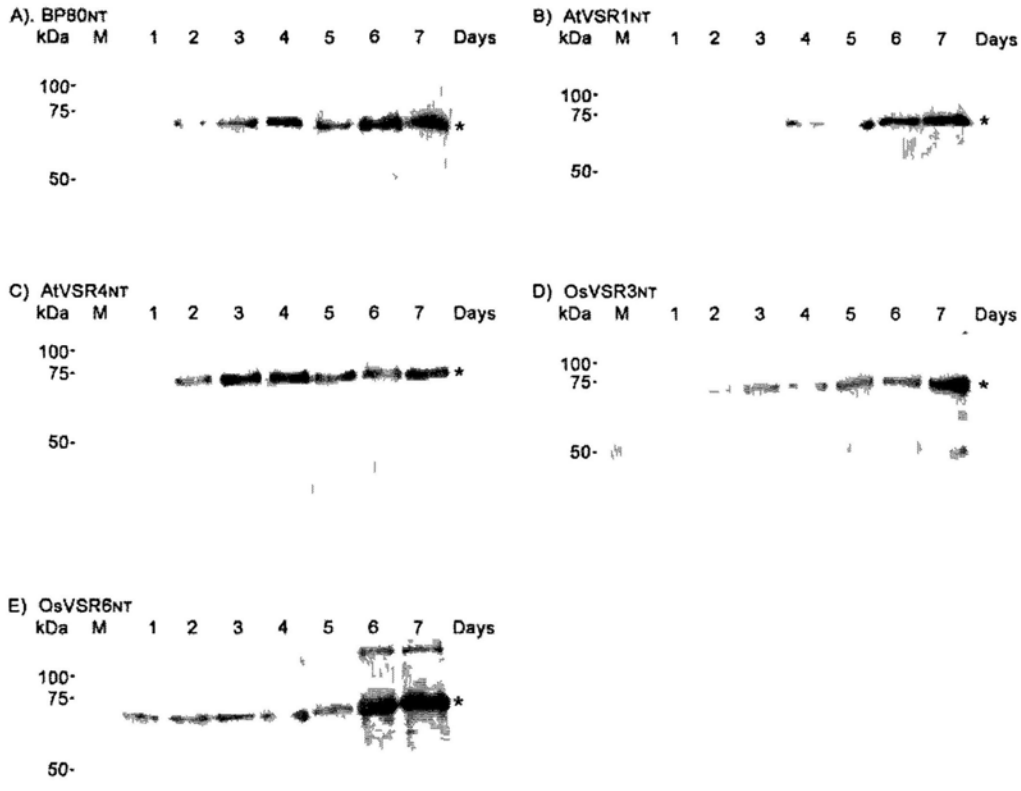

Figure 5 Expression profile of VSRNT in transgenic tobacco BY-2 cell lines over the 7-Days period.

Secreted protein from (A). BP80NT, (B). AtVSR1NT, (C). AtVSR4NT, (D). OsVSR3NT and (E). OsVSR6NT, Day1 to Day7 (lane 1-7) after subculture were collected from the culture medium of transgenic tobacco BY-2 cells, followed by SDS-PAGE and western blot detection by VSR antibodies. M, molecular mass in kliodaltons. Asterisk, truncated VSRNT proteins.

3.2 The culture media of transgenic BY-2 cells expressing VSRNT is free of intracellular proteins

To further rule out the possibility that the detection of VSRNT were be due to the leakage of the transgenic BY-2 cells, I next carried out western blot analysis on intracelluar and secreted proteins of both WT and transgenic VSRNT BY-2 cells using antibodies against various known marker proteins, including the ER markers anti-BIP (Figure 6A) and anti-calreticulun (Figure 6B) (Jia et al., 2009), the Golgi marker anti-Man1 (Figure 6C) (Tse et al., 2004; Saint-Jore-Dupas et al., 2006), the vacuole marker anti-alpha-TIP (Figure 6D) (Jauh et al., 1999), the PM/TGN marker anti-SCAMP1 (Figure 6E) (Lam et al., 2007b) and cytoskeleton marker anti-tubulin (Figure 6F) (Yoneda et al., 2007). As shown in Figure 6, protein bands corresponding to the marker proteins were only detected in the intracellular fraction (I) rather than in the secretion fraction (S) from both WT and transgenic BY-2 cells. These results clearly indicate that there is no leakage of the cellular components into the secreted fraction. Therefore the method of collecting secreted fraction can retain integrity of the culture cells and VSRNT in the culture medium is secreted from the cells rather than owing to leakage of damaged or disrupted cells.

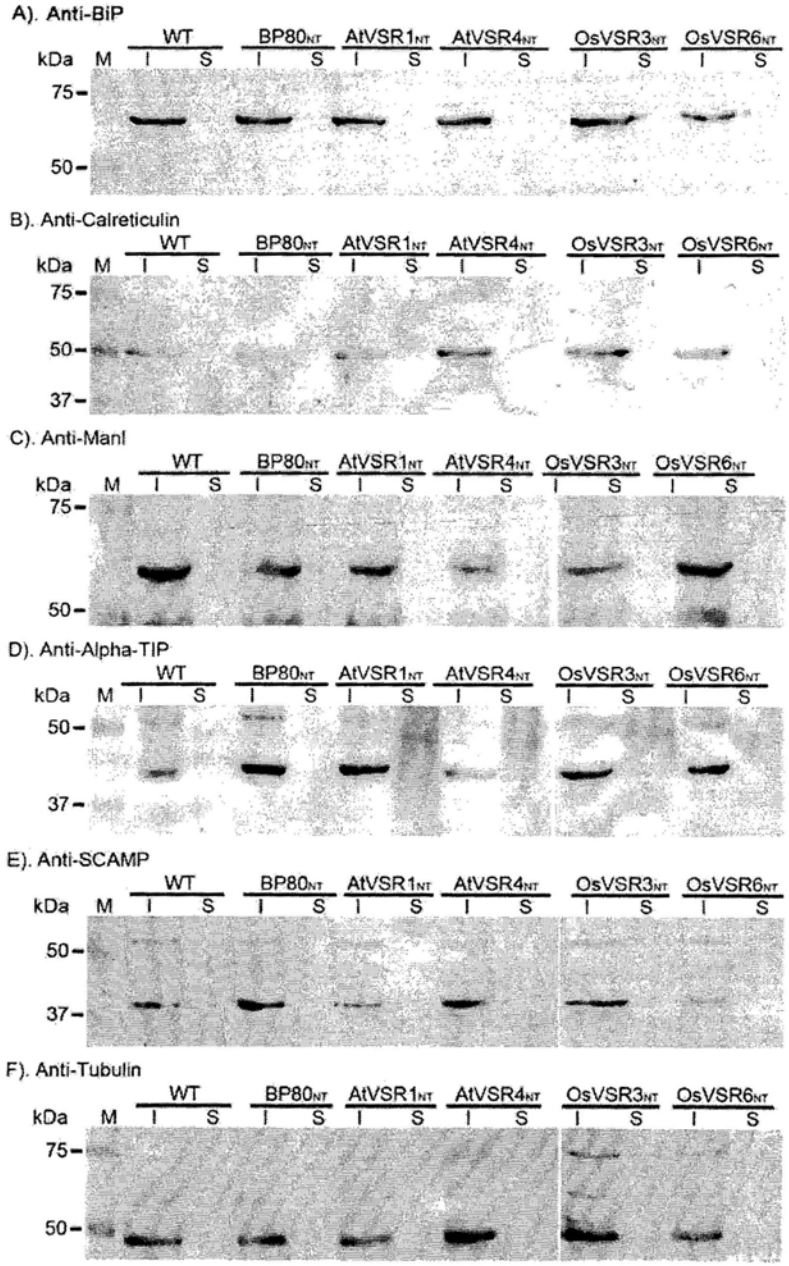

Figure 6 The cultured media of transgenic BY-2 cell line expressing VSRNT is free of intracellular proteins.

Intracellular proteins (I) and secreted protein (S) fractions were collected from wild type (WT) and transgenic tobacco BY-2 cells expressing VSRNT proteins. Western blot analysis using different organelle markers were used to detect contaminants in the secreted protein fractions, including the ER resident protein BiP (A) and calreticulin (B), Golgi marker Mannosidase I (C), endosome and plasma membrane marker SCAMP1 (D), vacuolar membrane marker alpha TIP (E) and cytoskeleton protein tublin (F). M, molecular mass in kliodaltons.

3.3 VSRNT interaction with peptides

I next want to find out if the secreted VSRNT are functional proteins by testing theirs binding ability and specificity to various known or putative vacuolar sorting determinants (VSDs) and their mutants (Figure 3 and Table 2). To do this, synthetic peptides corresponding to various VSDs and their mutants (Table 2) are conjugated CNBr-activated sepharose prior to binding with the secreted VSRNT from the Day-7 culture media of transgenic BY-2 cells under different conditions (Figure 3). The bound proteins are then released from the agarose for further SDS-PAGE separation and western blot analysis using VSR antibodies (Figure 7 and Figure 8).

Several known VSDs and their mutants peptides are used: the NPIR motif of aleurain and sporoamin and the IAGF motif on Brazil nut 2S albumin (BN2S) that known to interact with VSRs (Kirsch et al., 1994; Kirsch et al., 1996; Saalbach et al., 1996), and the putative VSDs of AFVY found in other vacuolar proteins such as the common bean phaseolin, jackbean pro-concanavalin A (proConA), soybean beta-conglycinin, 2S albumin, vicilin and vicilin-like storage protein (VLSP) (Table 2) (Shimada et al., 1997; Frigerio et al., 1998; Nishizawa et al., 2003; Saint-Jore-Dupas et al., 2005; Nishizawa et al., 2006), even though their interaction with BP-80 remains to be demonstrated.

As shown in Figure 7A, BP80NT did not interact with the sepharose matrix because no protein band was detected from the blank column (lane 1). In contrast, BP80NT not only interacts with VSD peptides containing the NPIR motif from aleurain and sporamin (lane 2 peptide a and lane 4 peptide c), but also interacts with putative VSD peptides containing the AFVY motif from phaseolin (lane 5, peptide d) and the known VSDs from other storage proteins including the pro2S albumin (lane 7, peptide f), beta-conglycinin (lane 9, peptide h) and pro-concanavalin A (lane 11, peptide j), but has very weak interaction with the VSDs of 2S albumin and vicilin (lanes 8 peptide g and 10 peptide i). In addition, such BP80NT-VSD interaction are specific because mutated VSDs of aleurain and phaseolin no longer interact with BP80NT under the identical binding condition (compare lanes 2 and 3, peptide a and b, and lane 5 and 6, peptide d and e, respectively). Interestingly, mutation on the WSIV rather than the AFVY-like (AVFV) motif of the VLSP abolished the BP80NT-binding (lane 13 peptide L vs. lane 14 peptide m), likely representing a new VSD interacting with BP80NT. Taken together, these results demonstrate that the secreted BP80NT from transgenic BY-2 cells specifically bind to the known VSDs but not their mutants of aleurain, sporamin and several other storage proteins. Generally, AtVSR1NT (Figure 7B), AtVSR4NT (Figure 7C), OsVSR3NT (Figure 7D) and

OsVSR6NT (Figure 7E) showed similar binding specificity against the same synthetic peptides and the mutant peptides as BP80NT (Figure 7A), except for weaker interaction with VSD for phaseolin and pro-concanavalin A and stronger interaction with VSD from 2S albumin for AtVSR1NT (compare lane 5 peptide d, lane 11 peptide j and lane 8 peptide g for Figure 7A and 7B); weaker interaction with VSD from phaseolin and stronger with interaction with VSD from 2S albumin and vicillin for AtVSR4NT (compare lane 5 peptide d, lane 8 peptide g and lane 10 peptide i for Figure 7A and 7C); weaker interaction with VSD from phaseolin for OsVSR3NT (compare lane 5 peptide d for Figure 7A and 7D); and weaker interaction with VSD from phaseolin and stronger with interaction with VSD from 2S albumin for OsVSR6NT (compare lane 5 peptide d and lane 8 peptide g for Figure 7A and 7E). These results suggested that VSRNTs are alike, yet has their own specificity in terms of VSD peptide affinity, indicating that each of the VSRNT protein may have its own group of interacting cargo proteins (through interacting to the VSD presence in the cargo proteins), but these cargo proteins may partially overlaps with other groups of cargo protein from other VSRNT proteins.

In a further step, BP80NT was shown able to interact with novel, functional VSD from an *Arabidopsis* chitinase (At3g12500, Figure 8A, lane 4). Chitinase is

a plant defense protein that cleaves the glycosidic bonds in chitin, found on the cell wall of fungi or exoskeleton of insects. Plants contain two types of chitinases, intracellular (vacuolar) chitinase and extracellular (secreted) chitinase (Neuhaus et al., 1991; Neuhaus et al., 1994). Trafficking of chitinases depend on the presence of VSD in the C-terminal of chitinase (Neuhaus et al., 1991; Neuhaus et al., 1994). The tobacco basic chitinase is a vacuolar enzyme that contains the C-terminal VSD, LLVDTM, while tobacco acidic chitinase, lacking the C-terminal VSD, is secreted (Table 2, peptide n and o) (Neuhaus et al., 1991; Neuhaus et al., 1994). *Arabidopsis* has 5 chitinases that similar to tobacco chitinases, At3g12500, At1g05850, At1g02360, At3g16920 and At4g01700, and have C-terminal that may potentially act as VSDs, however, whether these *Arabidopsis* chitinases are targeted to vacuole is unknown. Figure 8A showed that BP80NT interact with VSD from tobacco basic chitinase but not with tobacco acidic chitinase (Figure 8A, lane 2 and 3, respectively), indicated that BP80NT also has the ability to binds with VSD from chitinase, and confirmed that the crucial motif for the interaction is LLVDTM (Table 2). For those synthetic VSDs from *Arabidopsis* chitinases, only At3g12500, with a C-terminal motif (LLEAAI) similar to that of the crucial motif (LLVDTM) found tobacco basic chitinase, interacts with BP80NT strongly (Figure 8A, lane 4), while such

interaction is absent for the At1g05850, At1g02360, At3g16920 and At4g01700 (Figure 8A. lane 5, 6, 7 and 8, respectively). Similarity, all other VSRNTs interacted with VSD from tobacco basic chitinase and *Arabidopsis* At3g12500 (lane 3 and lane 4 respectively, AtVSR1NT, Figure 8B; AtVSR4, Figure 8C; OsVSR3, Figure 8D and OsVSR6, Figure 8E). These suggested that *Arabidopsis* At3g12500 contains functional VSDs that able to interact with VSRs and should be transported to vacuoles. Taken together, the secreted VSRNTs not only specifically bind with known VSDs, but also bind to novel potential VSDs, indicating that these secreted VSRNTs may carry novel cargo proteins into the culture media.

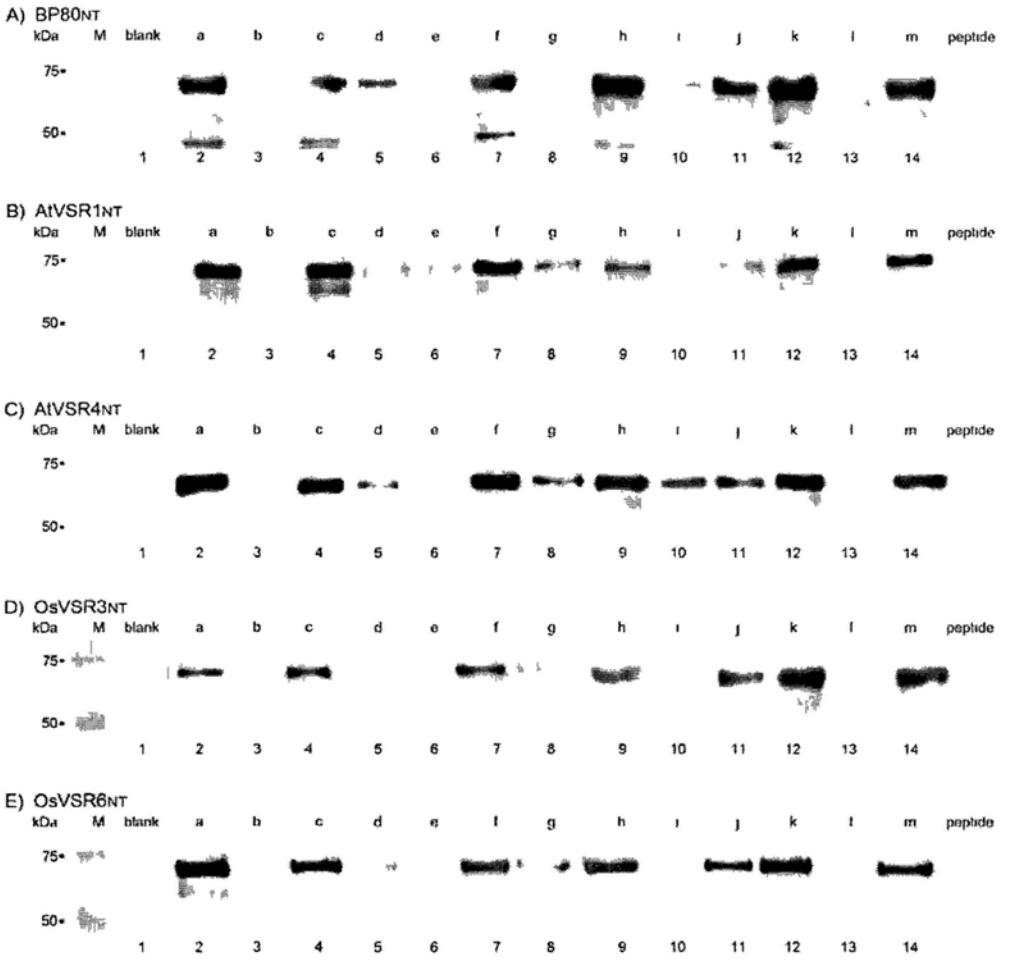

Figure 7 Interaction of VSRNT proteins with synthetic peptides containing VSDs.

Culture media from transgenic tobacco BY-2 culture cells expressing A). BP80NT, B). AtVSR1NT, C). AtVSR4NT, D). OsVSR3NT and E). OsVSR6NT were collected and pull down assay with synthetic peptides containing known VSDs and their mutants (Table 2) were performed as described (Figure 3). VSR antibodies were used in western blot analysis. M, molecular mass in kilodalton. Blank, blank sepharose column without peptide conjugation. Synthetic peptides containing VSD are from a, aleurain; b, mutated VSD of aleurain; c, sporamin; d, phaseolin; e, mutated VSD of phaseolin; f, pro2S albumin; g, 2S albumin; h, beta-conglycinin; i, vicillin; j, ProConcanavalinA; k, villicin like storage protein (VLSP); l, WSIV-mutated VLSP and m, AVFV-mutated VLSP.

Figure 8 Interaction of VSRNT proteins with synthetic peptides containing VSDs from various tobacco and *Arabidopsis* chitinases.

Culture media from transgenic tobacco BY-2 culture cells expressing A) BP80NT, B). AtVSR1NT, C) AtVSR4NT, D). OsVSR3NT and E). OsVSR6NT were collected and pull down assay with synthetic peptides containing known VSDs from tobacco and *Arabidopsis* chitinases (Table 2) were performed as described (Figure 3) VSR antibodies were used in western blot analysis M, molecular mass in kilodalton. Blank, blank sepharose column without peptide conjugation. Synthetic peptides containing VSD or potential VSDs of chitinases are from n, tobacco acidic chitinase; o, tobacco basic chitinase; p, *Arabidopsis* At3g12500; q, At1g05850; r, At1g02360; s, At3g16920 and t, At4g01700.

3.4 VSRNT-VSD interaction is calcium-dependent

Both pea BP-80 and pumpkin PV72 contains EGF repeats in their N-terminal, prior to their TMD region (Cao et al., 2000; Paris and Neuhaus, 2002; Watanabe et al., 2002). These EGF repeats are shown to be responsible for regulating the VSR-ligand binding ability of both BP-80 and PV72, in a calcium dependent manner (Cao et al., 2000; Watanabe et al., 2002). Similar to BP-80 and PV72, AtVSR1, AtVSR4, OsVSR3 and OsVSR6 also contains the EGF repeats (Figure 9), thus these VSRNT should also interact with ligands (VSDs in this study) in a calcium dependent manner. To further find out if these VSRNT-VSDs interaction depends on calcium, we next carry out binding study with selective VSD synthetic peptide of aleurain under different calcium concentrations. As shown in Figure 10A, the interaction of BP80NT with the aleurain VSD is peaked at 1000μM calcium, but such interaction was gradually reduced with decreasing calcium concentrations (Figure 10A). At 100μM calcium, only 45% of BP80NT is still bounded with the aleurain VSD, with further decrease of calcium concentration, the affinity of BP80NT become weaker and weaker, only 5% of BP80NT is bounded in the absence (0μM) of calcium ion (Figure 10F, black bars).

Similarly, AtVSR1NT, AtVSR4NT and OsVSR3NT also interacted with the

synthetic aleurain peptide in a calcium dependent manner from identical binding assay, the affinity of these VSRNTs with synthetic aleurain peptide decreases with decreasing calcium concentration (Figure 10F), except that OsVSR6NT interacted stronger with synthetic aleurain peptide at 100μM and 50μM compare with 1000μM of calcium ion, yet the interaction of OsVSR6NT and synthetic aleurain peptide dropped from 125% at 50μM to 0% in the absence (0μM) of calcium ion (Figure 10F, cross-hatched bars).

These results support the notion that the interaction of VSRNTs and aleurain VSD is calcium dependent, and that the presence of calcium ion generally enhance the interaction between the VSRNTs and the aleurain VSD. The differences in the affinity between the VSRNT to synthetic aleurain peptide (for example, at 10μM of calcium ion, around 20% of BP80NT is bounded, compare with around 55% of AtVSR4NT is bounded) is possibility due to the differences in the amino acid sequences in their EGF repeats (Figure 9) as the EGF repeats of the VSR proteins are thought to regulated the VSR-VSD interaction in response to calcium ion concentration (Watanabe et al., 2002).

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AtVSR1  CLENNGGCWQDKAANITACRDTFRGRLCECPTVQGVKFVGDGYTHCKASGALHCGINNGG
PV72    CLTNNGGCWFDKEANISACRDTFRGRVCECPTVGGVKFVGDGYTHCEASGALRCEINNGG
OsVSR6  CLENNGGCWQDKAANISACKDTFRGRVCECPVVKGVKFVGDGYTHCEASGSGHCEINNGG
OsVSR3  CLESNGGCWQDKTNNFTACKDTFRGRVCECPIARGVKFVGDGYTHCEASGVGRCQINNGG
BP-80   CLTNNGGCWQDKTANIAACKDTFRGRVCECPLVDGVQFKGDGYTTCEVSGHGRCKINNGG
AtVSR4  CLDNNGGCWQDKSANITACKDTFRGKVCVCPIVDGVRFKGDGYSHCEPSGPGRCTINNGG
        ** .***** **  *::**:*****::* ** . **:* ****: *: **  :* *****

AtVSR1  CWRESRGGFTYSACVDDHSKDCKCPLGFKGDGVKNCEDVDECKEKTVCQCPECKCKNTWG
PV72    CWKGTHDGKTYSACSDDHTKGCKCPPGFKGDGVHTCEDVDECKEKLACQCPECKCKNTWG
OsVSR6  CWKDSRHGRTYSACTND---GCKCPDGFKGDGVHKCEDIDECKERTACQCKECKCKNTWG
OsVSR3  CWKETKNGKTVSACSNEESKGCKCPPGFKGDGIKSCEDIDECKDKLFCQCKDCSCENTWG
BP-80   CWHDARNGHAFSACLDDGGVKCQCPAGFKGDGVKNCEDIDECKDKKACQCPECSCKNTWG
AtVSR4  CWHEERDGHAFSACVDKDSVKCECPPGFKGDGVKKCEDINECKEKKACQCPECSCKNTWG
        **:  : * : *** :.    *:** ******::.***::***::  *** :*.*:****

AtVSR1  SYECSCSNG-LLYMREHDTCIGSGKVGTTKL
PV72    SYECSCRNG-LLYMHEHDTCIGN--IGSTVT
OsVSR6  SYECGCSGG-LLYMKEHDTCISKN--AATE-
OsVSR3  SYECSCGGSNMLYMREHDTCISK--VASSS-
BP-80   SYNCSCSGD-LLYIKDQDTCISKT--ASQAK
AtVSR4  SYECSCSGD-LLYMRDHDTCISKT--GSQVK
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Figure 9 Epidermal growth factor (EGF) like repeats from VSR proteins

Multiple alignment on the amino acid sequences from VSR proteins (pea BP-80, pumpkin PV72, *Arabidopsis* AtVSR1 and AtVSR4, rice OsVSR3 and OsVSR6) was performed on ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Only the EGF repeats were shown in this figure. Asterisk, colons and dots indicate identical amino acids, conserved amino acids and partially conserved amino acids respectively

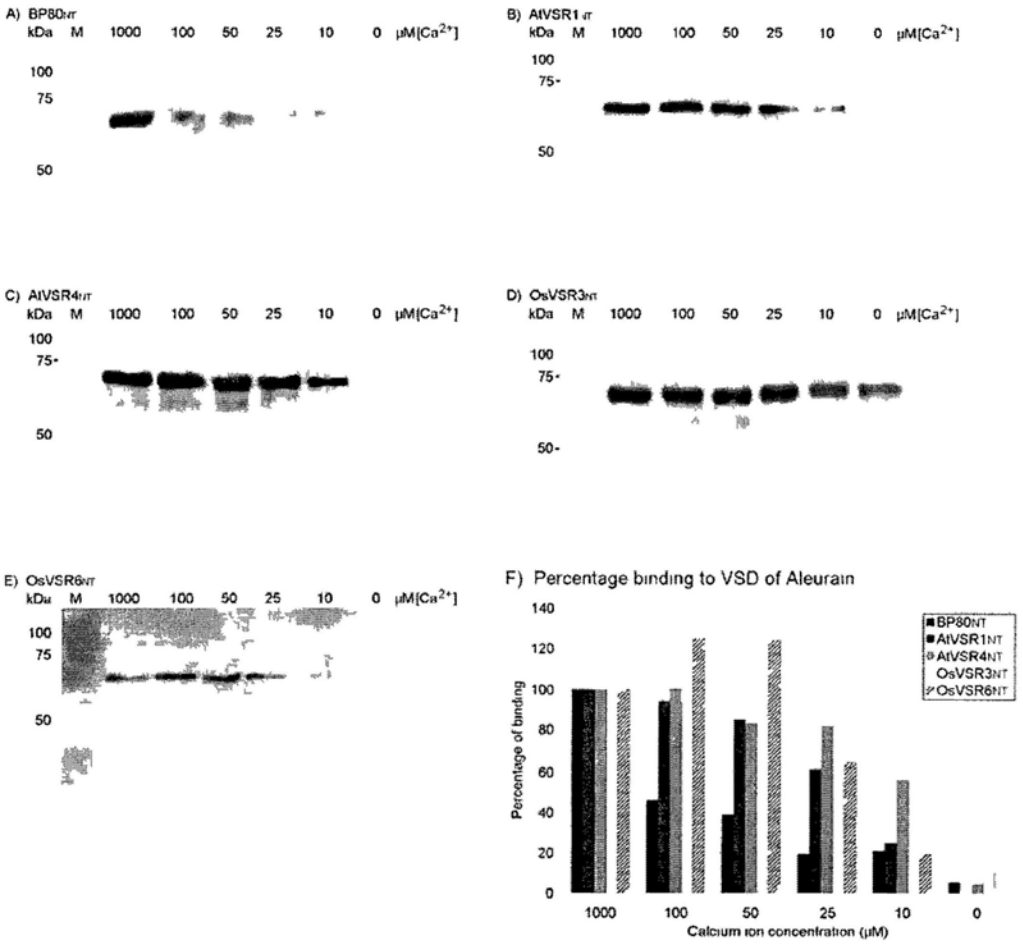

Figure 10 The interaction between the VSD-containing aleurain synthetic peptide and VSRNT is calcium-dependent.

Sepharose conjugated with synthetic peptide of aleurain was incubated with BP80NT (A), AtVSR1NT (B), AtVSR4NT (C), OsVSR3NT (D) or OsVSR6NT (E) in the presence of various calcium ion (Ca^{2+}) concentrations (from 0 to 1000μM as indicated). After washing with binding buffer with the same Ca^{2+} concentration, VSRNT proteins in sepharose were released by boiling in SDS loading buffer, followed by SDS-PAGE and western blot analysis with VSR antibodies. Percentage of binding for VSRNT with synthetic aleurain peptide under different Ca^{2+} concentrations was calculated by comparing the protein band intensity among different Ca^{2+} concentrations, where the signal obtained from binding at 1000μM Ca^{2+} concentration is considered as 100% binding. Black bars, BP80NT; dark gray bars, AtVSR1NT; light gray bars, AtVSR4NT; white bars, OsVSR3NT; cross hatched bars, OsVSR6NT

3.5 Generation and characterization of transgenic *Arabidopsis* PSB-D culture cells expressing truncated VSR proteins

I have thus far generated and characterize transgenic tobacco BY-2 cells expressing various truncated VSR proteins (BP80NT, AtVSR1NT, ATVSR4NT, OsVSR3NT and OsVSR6NT). The obtained results showed that these VSRNT proteins interact with various known VSD peptides in a calcium dependent manner. To further use such expression system for the identification of VSR cargo proteins in *Arabidopsis*, I have also generated transgenic *Arabidopsis* PSB-D cells expressing various truncated VSR proteins including the pea BP-80, *Arabidopsis* AtVSR1 and AtVSR4 and rice OsVSR3 and OsVSR6. Based on the hypothesis that expression of the truncated VSR (VSRNT) proteins in plant cells will lead to the secretion of these VSRNT proteins along with their specific cargoes into the culture media (Figure 2), putative cargo proteins can thus be identified.

These truncated VSRs (BP80NT, AtVSR1NT, AtVSR4NT, OsVSR3NT and OsVSR6NT) constructs were successfully transformed into *Arabidopsis* PSB-D culture cells via *Agrobacterium*-mediated transformation. Culture media of transgenic PSB-D cells were collected for western blot analysis to confirm the expression of VSRNT proteins (Figure 11). Similar to transgenic tobacco BY-2 cells, truncated VSRs expressed in transgenic *Arabidopsis* PSB-D cells were also

found to be presented and secreted into the culture media (BP80NT, lane 1 to 3; AtVSR1 lane 4 to 6; AtVSR4NT, lane 7 to 9; OsVSR3NT, lane 10 to 12; OsVSR6NT, lane 13 and 14, as indicated by asterisk) but missing from the medium of wild type PSB-D culture cells (lane 15). Thus, all these truncated VSR (BP80NT, AtVSR1NT, AtVSR4NT, OsVSR3NT and OsVSR6NT) have been successfully expressed and secreted into the culture media of transgenic *Arabidopsis* PSB-D cells, which will facilitate the identification of potential cargo proteins in the culture media via LC-MS/MS and FT-MS analysis.

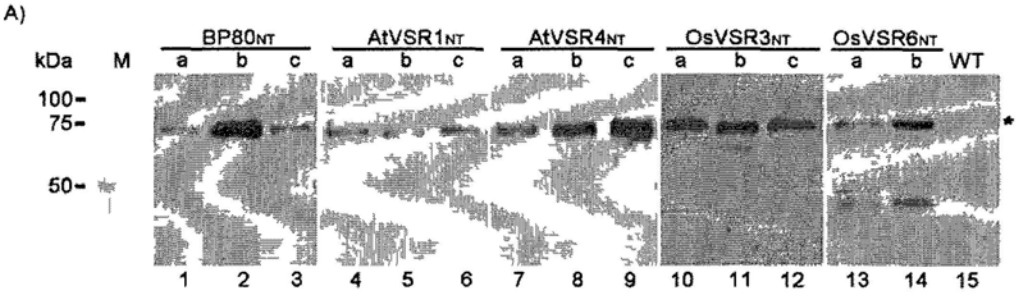

Figure 11 Expression of truncated BP-80 (BP80NT), AtVSR1 (AtVSR1NT), AtVSR4 (AtVSR4NT), OsVSR3 (OsVSR3NT) and OsVSR6 (OsVSR6NT) in transgenic *Arabidopsis* PSB-D cells

Secreted proteins were prepared from the culture media of various transgenic *Arabidopsis* PSB-D cell lines expressing BP80NT (Cell lines a, b and c, Lane 1 to 3), AtVSR1NT (Cell lines a, b and c, Lane 4 to 6), AtVSR4NT (Cell lines a, b and c, Lane 7 to 9), OsVSR3NT (Cell lines a, b and c, Lane 10 to 12) and OsVSR6NT (Cell lines a and b, Lane 13 and 14), followed SDS-PAGE and Western blot analysis using VSR antibodies. Asterisk indicated the secreted truncated VSR (VSRNT) proteins detected in the culture media from transgenic cells but misses in wild type (WT) cells (Lane 15). Transgenic cell lines with high levels of accumulation of VSRNT from BP80NT (Cell line b, Lane 2), AtVSR1NT (Cell line a, Lane 4), AtVSR4NT (Cell line c, Lane 9), OsVSR3NT (Cell line a, Lane 10) and OsVSR6NT (Cell line b, Lane 14) were chosen for further analysis. M, molecular mass in kilodaltons.

3.6 Identification of putative VSR cargo proteins

In this study, I also used transgenic *Arabidopsis* culture cells expressing VSRNT proteins as a platform to identify soluble cargo proteins secreted into the culture media as a consequence of VSRNT secretion. To prepare protein samples, day 4 culture media from wild type and transgenic PSB-D culture cells expressing BP80NT, AtVSR1NT, AtVSR4NT, OsVSR3NT and OsVSR6NT were filtered through filter papers, secreted proteins were precipitated by methanol-chloroform for protein identification via LC-MS/MS and FT-MS analysis.

A total of seventeen potential VSR cargo proteins was identified (Table 4), including three vacuolar proteases (At1g11910, At3g52500 and At5g67360), five glycosyl hydrolases (At4g34480, At5g10560, At5g11720, At5g45280 and At5g58090), three oxireductases (At1g30740, At5g06720 and At5g11540) of which two of them contains a flavin adenine dinucleotide (FAD)-binding domain and a peroxidase, two chitinases (At2g43570 and At3g54420), one curculin-like (mannose-binding) lectin family protein (At1g78830) that was able to interact with polysaccharides (Barre et al., 1997), a cysteine protease inhibitor family protein (At4g16500), an endoribonuclease RNS1 (At2g02990) that may link to phosphate starvation (Bariola et al., 1994) and one protein with unknown function (At4g34260). Among these seventeen identified proteins, none of them

has been described as VSR cargo protein, and only four of them (At1g11910, At5g10560, At4g16500 and At5g11720) are known to be vacuolar proteins (Carter et al., 2004).

Further analysis on these proteins indicated that individual truncated VSR proteins secreted different kinds of proteins, and yet shared common cargo proteins. For example, two unique proteins were only found in BP80NT media: endoribonuclease RNS1 (At2g02990) and cystine protease inhibitor (At4g16500). Five unique proteins were identified in AtVSR1NT media, including a FAD-binding domain-containing oxireductase (At1g30740), two glycosyl hydrolase family 17 proteins (At4g34480 and At5g58090), an unknown protein (At4g34260) and a pectin acetylesterase (At5g45280). A single unique protein, aspartic proteinase (At1g11910) was identified in OsVSR6NT media. However, no unique proteins were found in the media of AtVSR4NT and OsVSR3NT cell lines, whereas aspartyl protease family protein (At3g52500) was identified in media of both AtVSR4NT and OsVSR3NT cells.

Common proteins found in the media of all transgenic *Arabidopsis* cell lines included enzymes that are related to glycosyl hydrolyase such as GH3 (At5g10560), chitinases (At2g43570 and At3g54420) and alpha-glucosidase 1 (At5g11720), and other proteins such as subtilase (At5g67360), peroxidase

(At5g06720) and curculin-like (mannose-binding) lectin family protein (At1g78850).

Three putative vacuolar proteases, including two aspartic proteases (At1g11910 and At3g52500) and subtilase (At5g67360), were found in the culture media of the transgenic PSB-D cell lines, which could be caused by the secreted VSRNT proteins.

Table 4: Putative VSR cargo proteins identified by LC-MS/MS and FT-MS

Protein Name	Accession	Locus	SP	TMD	Vacuole?	FT-MS (Score/Peptide)	LC-MS/MS (Score/Peptide)	BP80NT	AtVSR1NT	AtVSR4NT	OsVSR3NT	OsVSR6NT
aspartic proteinase	gi|1354272	At1g11910	Y	N	**Y**	Y (58/5)						Y
FAD-binding domain-containing protein	gi|15221497	At1g30740	Y	N	N		Y (106/3)		Y			
curculin-like (mannose-binding) lectin family protein	gi|15219200	At1g78850	Y	N	N		Y (166/3)	Y	Y	Y		
RNS1 (RIBONUCLEASE 1)	gi|15227068	At2g02990	Y	N	N		Y (68/2)	Y				
putative chitinase	gi|15224308	At2g43570	Y	N	N	Y (137/3)	Y (100/2)		Y		Y	
aspartyl protease family protein	gi|18409620	At3g52500	Y	N	N		Y (98/2)			Y	Y	
class IV chitinase	gi|2597826	At3g54420	Y	N	N	Y (107/2)	Y (73/2)	Y	Y			Y
cystatin (Cystine protein inhibitor family protein)	gi|15235771	At4g16500	Y	N	**Y**		Y (90/20)	Y				
unknown protein	gi|30689979	At4g34260	Y	N	N		Y (64/3)		Y			
glycosyl hydrolase family 17 protein	gi|30690053	At4g34480	Y	N	N	Y (57/3)	Y (92/2)		Y			
putative peroxidase	gi|21593262	At5g06720	Y	N	N		Y (151/4)	Y	Y	Y		
glycosyl hydrolase family 3 protein	gi|15238197	At5g10560	Y	N	**Y**	Y (150/4)	Y (74/4)	Y	Y	Y	Y	Y
FAD-binding domain-containing protein	gi|15239081	At5g11540	Y	N	N		Y (75/3)	Y			Y	Y
alpha-glucosidase 1	gi|2323344	At5g11720	Y	N	**Y**	Y (122/5)		Y	Y			
pectin acetylesterase	gi|21593445	At5g45280	Y	N	N		Y (73/2)		Y			
glycosyl hydrolase family 17 protein	gi|22327934	At5g58090	Y	N	N		Y (63/2)		Y			
ARA12, subtilase	gi|18425181	At5g67360	Y	N	N		Y (410/7)	Y	Y			

The secreted proteins from wild type PSB-D cells and transgenic PSB-D cells expressing BP80NT, AtVSR1NT, ATVSR4NT, OsVSR3NT and OsVSR6NT were precipitated by methanol/chloroform and digested by typsin. Peptides mixture was analyzed by LC-MS/MS and FT-MS. Three independent repeats were performed, and for each of the repeats, the digested peptides are injected twice. Only those proteins that can be repeatly found in transgenic PSB-D cell lines but not in wild type cell, with MASCOT ion score above 50, at least 2 peptides, and contain a signal peptide but no TMD were accepted as potential cargo proteins. Score, MASCOT ion score. Peptides, number of peptides identified. Locus ID was retrieved from TAIR database (http://www.arabidopsis.org) SP, presence of signal peptide as predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). TMD, presence of transmembrane domain predicted by TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0). Vacuole, identified as *Arabidopsis* vacuolar proteins as described by Carter (2004).

3.7 Confirmation of the PVC Localization and VSR Interaction of Selected Proteins

As a proof-of-concept principle, I next selected one of the putative VSR cargo proteins for further characterization. The *Arabidopsis* glycosyl hydrolase family 3 protein At5g10560 (GH3) was found in the culture media of all the transgenic *Arabidopsis* PSB-D cell lines and thus was chosen for study of subcellular localization and interaction. GH3 consists of 792 amino acids with an N-terminal signal peptide (Figure 12A). GH3 is a beta-xylosidase found in the plant vacuole (Carter et al., 2004) but not in cell wall (Borderies et al., 2003; Charmont et al., 2005).

GFP fusion approach was first used to study the subcellular localization of GH3. As shown in Figure 12B, when transiently expressed in *Arabidopsis* protoplasts for 13hr, GH3-GFP localized in both ER and punctate dots containing the PVC marker mRFP-AtVSR2 (Figure 12B). The ER pattern of GH3-GFP may be due to the slow or inefficient transport of GH3-GFP out of the ER, because during the same time interval, both aleurain-GFP and 2S albumin were already reached the PVC and vacuole (Figure 13A and B). Indeed, GH3-GFP reached the vacuole after 24hrs of incubation (Figure 12C).

To find out if GH3 also interacts with BP80NT in vivo, I next use the tool of the BP80NT fusion with red fluorescent protein (RFP) and an ER retention sequence, the TMD/CT region of an ER resident protein calnexin (CNX), which trapped both

GFP-CNX (daSilva et al., 2005; daSilva et al., 2006; Pimpl et al., 2006) and BP-80-CNX (Niemes et al., 2010) in the ER membrane. The hypothesis was that, if GH3 interacts with BP80NT in vivo, co-expression of GH3-GFP and BP80-CNX-RFP together in the same cell would trap the GH3-GFP within the ER. Indeed, as shown in Figure 12D, when co-expression of GH3-GFP and BP80-CNX-RFP were transiently co-expressed in *Arabidopsis* protoplasts, both reporters were found to colocalize within the ER after 13hrs of incubation, whereas aleurain-GFP was partially retained in the ER (Figure 13C). These results indicated that GH3-GFP, a soluble protein reaching PVC and vacuole (Figure 12B and 12C), was retained in the ER by the ER-localized BP80-CNX-RFP (Figure 12D). Taken together, GH3 is likely a true VSR cargo.

3.8 Deletion mapping studies of GH3

To find out the region of VSD responsible for PVC targeting of GH3, two truncated GH3-GFP constructs (Figure 12A) were made and tested for subcellular localization in *Arabidopsis* protoplast. The obtained results indicated that GH3-277-GFP remained in ER (Figure 12F) while GH3-534-GFP showed similar pattern as GH3-GFP (Figure 12E), indicating that the region responsible for PVC targeting of GH3 lies within the region of amino acid 278 to 792.

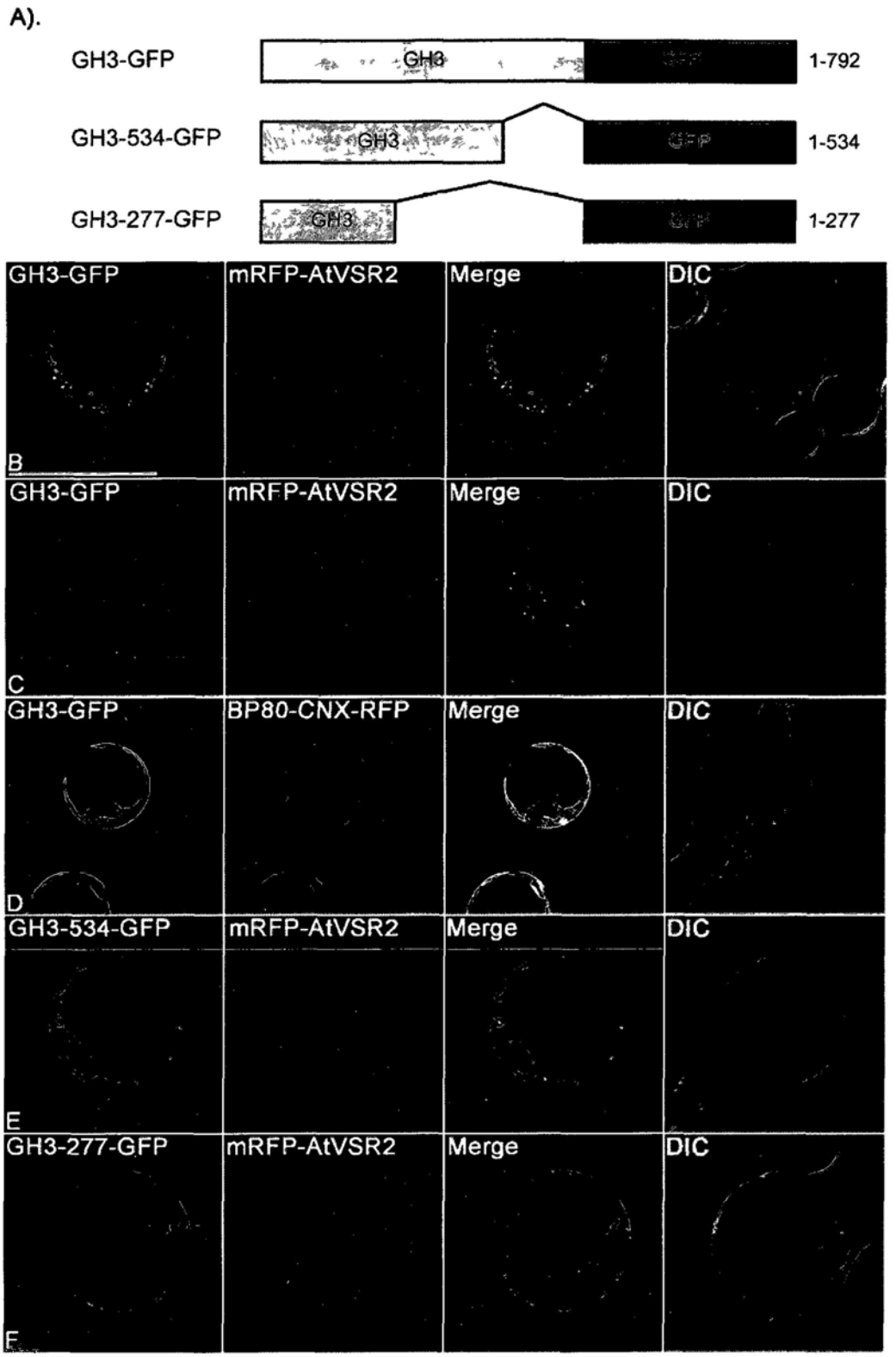
A).
GH3-GFP
GH3
1-792
GH3-534-GFP
GH3
1-534
GH3-277-GFP
GH3
1-277
GH3-GFP
mRFP-AtVSR2
Merge
DIC
B
GH3-GFP
mRFP-AtVSR2
Merge
DIC
C
GH3-GFP
BP80-CNX-RFP
Merge
DIC
D
GH3-534-GFP
mRFP-AtVSR2
Merge
DIC
E
GH3-277-GFP
mRFP-AtVSR2
Merge
DIC
F

Figure 12 Characterization of GH3-GFP

A). Full length GH3 and its two deletion constructs were fused with GFP protein and transiently expressed in *Arabidopsis* PSB-D culture cells. B). GH3-GFP (green) localized ER and co-localized to punctuate PVCs with PVC marker (mRFP-AtVSR2, red) in *Arabidopsis* protoplasts after 13hrs of incubation and C). eventually accumulated in vacuole after 24hrs of incubation. D). in vivo interaction of GH3-GFP and BP80-CNX-RFP, by retaining GH3-GFP (green) in the ER in the presence of the BP80NT domain on the BP80-CNX-RFP (red). Note the disappearance of the punctuated PVC signals. Deletion mapping of GH3, GH3-277-GFP consists of first 277 amino acids of GH3 and fused with GFP, GH3-534-GFP consists of first 534 amino acids of GH3 and fused with GFP. Transient expression in *Arabidopsis* PSB-D protoplast showed that punctuated PVC signal is observed in E). GH3-534-GFP and only ER signal is observed in F). GH3-277-GFP. Bar = 50μm.

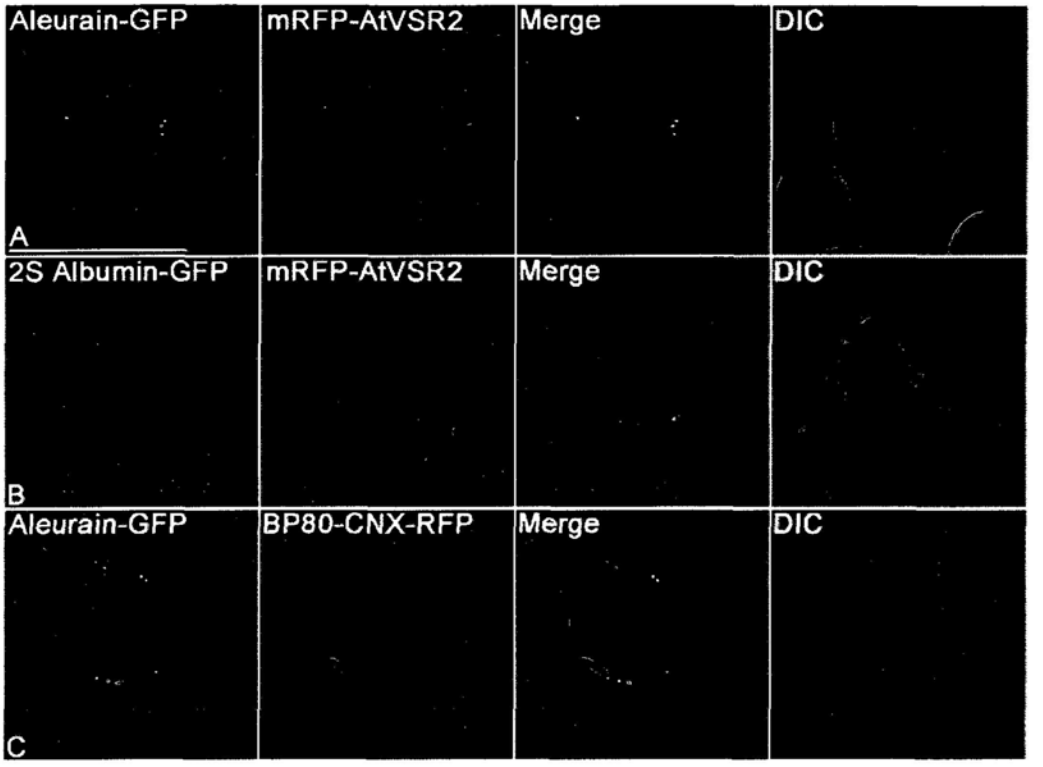

Figure 13 Subcellular localization aleurain-GFP and 2S albumin-GFP and in vivo interaction of Aleurain-GFP and BP-80 in *Arabidopsis* PSB-D culture cells. Known cargo protein, aleurain-GFP (A, green) and 2S albumin-GFP (B, green) were used as positive control to indicate the PVC and vacuolar accumulation of cargo proteins. C). in vivo interaction of aleurain-GFP (green) and BP80-CNX-RFP (red). Aleurain-GFP is partially retained in ER by BP80-CNX-RFP. Bar = 50μm.

Chapter 4 Discussion

4.1 Plant cell as an expression platform for functional studies of VSR proteins

VSR isoforms have been found in many plant species including pea, pumpkin, mung bean, *Arabidopsis* and rice (Kirsch et al., 1994; Paris et al., 1997; Shimada et al., 1997; Ahmed et al., 2000; Paris and Neuhaus, 2002; Shimada et al., 2002; Laval et al., 2003; Wang et al., 2007). The *Arabidopsis* genomes contains seven VSR isoforms with different spatial and temporal expression profiles (Wang et al., 2009), suggesting their possible functional diversity and redundancy throughout the plant developmental stages, even though all these AtVSRs are found in the same PVC populations in *Arabidopsis* cells (Laval et al., 2003; Shimada et al., 2003a; Miao et al., 2006; Fuji et al., 2007; Avila et al., 2008; Craddock et al., 2008; Miao et al., 2008) as well as in trans-Golgi network (TGN) in *Arabidopsis* seeds (Craddock et al., 2008). The important roles of VSR proteins in plants have been demonstrated by several recent studies. For example, the seeds of the *Arabidopsis* AtVSR1 knock-out mutant was shown to have poor germination ability (Shimada et al., 2003a; Craddock et al., 2008), VSRs were shown to play role during seed germination of mung bean (Wang et al., 2007), whereas both VSRs and SCAMPs (secretory carrier membrane proteins) were demonstrated to be essential for pollen tube growth (Wang et al., 2009). In addition, overexpression of GFP-AtVSR fusion proteins (with GFP replacing the lumenal

ligand binding domain of AtVSRs) in plant cells had been shown to cause secretion of vacuolar cargo proteins likely because this GFP-AtVSRs competed with the endogenous VSR proteins for cargo binding (daSilva et al., 2005; daSilva et al., 2006). However, in these studies, the native cargo proteins that are supposed to be transported by VSR proteins in plants remain largely unknown. It is possible that individual VSR protein may mediate the vacuolar transport of its specific cargo proteins.

Heterologous expression of VSR proteins in an eukaryotic cell system is essential for the production of functional VSR proteins because of its ability in correct protein folding and post-translational modification. Several systems have been tested for the expression and purification of VSR proteins for functional characterization. For example, various recombinant forms of the truncated BP-80 derived from expressing in Drosophila S2 cells were used to study BP-80-proaleurain peptide interaction, which has identified two separate proaleurain peptide ligand-binding sites within the N-terminus of BP-80 (Cao et al., 2000). However, large purification of truncated BP-80 proteins from Drosophila S2 cells was not feasible because chromatographic purification resulted in large losses and low yield (Cao et al., 2000). A further study using the same expression system for a His-tagged version of a truncated *Arabidopsis* VSR has allowed purification of the truncated AtBP80b in relatively large amount for

crystallization and initial diffraction studies (Rogers et al., 2004). However, comparing to other expression systems for producing recombinant proteins including *E. coli* and yeast, using Drosophila S2 cells as an expression for producing large amounts of properly-folded VSR proteins to be used in functional study has been technically challenging and economically expensive due to the animal cell culture nature.

With such scenario, it has recently proposed that the truncated VSR proteins expressed in suspension cultured cells will be secreted into the cultured media along with their native cargo proteins (Miao et al., 2007). In fact, a recent study using reporters transiently expressed in tobacco protoplasts suggests that the VSR-cargo interaction or selection may have first occurred within the ER lumen (Niemes et al., 2010). In this study, I have first developed and tested an expression system using transgenic tobacco BY-2 cells to express truncated VSRs. The obtained results demonstrate that the truncated VSRs are properly expressed and secreted into the cultured media of transgenic BY-2 cells, and these secreted VSRNT bind known VSDs of vacuolar proteins in a calcium-dependent manner. Thus, this BY-2 cell expression and secretion system may represent an attractive alternative for the heterologous expression and purification of truncated VSR proteins for their functional characterization in future studies.

Next, I have also generated transgenic *Arabidopsis* cultured cells expressing individual truncated VSR proteins for the analysis of additional secreted proteins in the cultured media of transgenic versus wild type cell lines so that putative VSR cargo proteins in the media of transgenic cell lines can be identified via LC-MS/MS and FT-MS analysis.

4.2 Expression of functional truncated VSR proteins in transgenic tobacco BY-2 culture cells

Pea BP-80 has been well-known for its binding ability to the NPIR-containing peptides of aleurain and sporamin (Kirsch et al., 1994; Paris et al., 1997), but it is not known if BP-80 and other AtVSR proteins would interact with other VSDs of vacuolar proteins. As a first step to test various VSD-receptor interactions, I have expressed various truncated VSR proteins, including the well-studied BP-80, its most closely related *Arabidopsis* VSR AtVSR4, another well studied *Arabidopsis* VSR AtVSR1, and two rice VSRs (OsVSR3 and OsVSR6), in tobacco BY-2 culture cells. These secreted VSRNT from transgenic BY-2 cells highly accumulated in the culture media (Figure 4), thus facilitate downstream analysis, such as the VSRNT-VSD interaction assay (Figure 7 and Figure 8). All the tested VSRNTs have similar binding nature to various known VSDs (Table 2, Figure 7 and Figure 8), such binding is calcium-dependent (Figure 10). VSRNTs also share similar binding ability towards

different types of VSD, but the affinity of different VSRNT versus VSD of aleurain is different under the same calcium concentration. For example, there is around 20% of BP80NT interact with VSD of aleurain at 10μM of calcium, whereas there is 60% of AtVSR4NT and OsVSR3NT interact with VSD of aleurain in this concentration (Figure 10). The difference in affinity of VSR to VSD is likely due to the difference in their EGF repeats (Figure 9), which are thought to regulate the confirmation of N-terminal of VSR proteins in response to environmental calcium concentration (Watanabe et al., 2002), and thus regulate the affinity of VSRNT to aleurain VSD. Although the exact calcium ion concentration is unknown in the organelles along the secretory pathway, it was reported that the calcium ion concentration in ER is up to 1500μM (Bygrave and Benedetti, 1996; Montero et al., 1997; Ashby and Tepikin, 2001; Watanabe et al., 2002), which is sufficient for supporting the association of VSRNT and aleurain VSD at ER. Besides, the acidicity increases along the secretory pathway towards vacuole (Grabe and Oster, 2001), which favors the dissociation of cargo proteins from VSR in vacuole (Kirsch et al., 1994; Paris et al., 1997).

Also, VSRNTs can interact with various functional VSDs but not their mutants, for example, BP80NT not only interact with VSDs of aleurain and sporamin, but also interact with known VSDs of storage proteins including phaseolin, procanavalin A and beta-conglycinin, but not interacted with VSDs of mutated aleurain and mutated

phaseolin (Figure 7A), therefore, such BY-2 cells-derived secreted VSRs are useful tools for studying specific ligand-receptor interaction. Furthermore, it is observed that different VSRNT interacts with different VSDs (Figure 7A to 7E). These results suggested that VSRNT are alike, yet has their own specificity in terms of VSD peptide affinity, indicating that VSR proteins are functionally diverse and redundant in carrying cargo proteins into the plant vacuole.

In conclusion, transgenic tobacco BY-2 cells can be used not only as bioreactors for large-scale production of active pharmaceutical recombinant proteins into the cultured media for easy down streaming purification (Fu et al., 2009), but also as an expression system for producing properly-folded functional truncated VSR proteins into the media to be used for ligand-receptor binding study. Such proof-of-principle demonstration shows great promise in using this expression system to express individual truncated AtVSR and other VSR proteins for studying ligand-receptor interaction and for possible cargo identification in further study. In addition, further protocol development in large-scale purification of truncated VSR proteins from the media of these transgenic BY-2 or *Arabidopsis* cultured cells will allow VSR crystal formation for future structural study of receptor as well as ligand-receptor interaction, which was limited in other systems (Cao et al., 2000; Rogers et al., 2004).

4.3 Proteomic approach of functional studies of VSR proteins in *Arabidopsis* PSB-D culture cells

Culture media from transgenic *Arabidopsis* PSB-D culture cells containing both VSRNT and its interacting cargo proteins were analyzed by LC-MS/MS and FT-MS. Results indicated that, potential cargo proteins are uniquely found in the culture media of the transgenic *Arabidopsis* PSB-D cells including glycosyl hydrolases and proteases (Table 4). Each truncated VSR secreted its own group of interacting cargo proteins, while share common cargo proteins, indicating that they have overlapping functions yet diversity in the vacuolar transport of cargo proteins (Table 4). In addition, all these potential cargo proteins have never been reported as interacting partners of VSR proteins.

The results on cargo proteins identification indicated that the major types of VSR-interacting proteins in the PSB-D cells are related to carbohydrate modification, such as the glycosyl hydrolases, chitinases and the carbohydrate interacting curculin-like (mannose-binding) lectin family protein, which is similar to previous report (Carter et al., 2004). However, the functional role of these glycosyl hydrolases remains to be determined.

The other glycosyl hydrolyases, chitinase is a defense protein that is able to digest chitin found in fungal cell wall and exoskeleton of insects. Chitinase contains

both vacuolar chitinases and secreted chitinases. Tobacco contains 2 types of chitinases, the vacuolar basic chitinase and the secreted acidic chitinase, The ctVSD (LLVDTM) is present in the tobacco basic chitinase, but not in the tobacco acidic chitinase, leading the vacuolar accumulation of the tobacco basic chitinase. In fact, the ctVSD on tobacco basic chitinase interacted with VSRNT (Figure 8), indicated that both *Arabidopsis* chitinase At2g43570 and At3g54420 may be a VSR cargo. However, both At2g43570 and At3g54420 lacks the ctVSD as the tobacco basic chitinase or the NPIR motif presence on the aleurain and sporamin, therefore the VSR interacting region of At2g43570 and At3g54420 remains to be determined.

Putative vacuolar proteases were also found to co-secreted with VSRNT in the culture media of the transgenic PSB-D cell lines. Proteases are involved in protein turnover and degradation in the plant vacuole. Previously, AtVSR1 was shown able to interact with the synthetic peptide of the *Arabidopsis* aleurain (AtALEU) (Shimada et al., 2003a), however AtALEU was not found in the media of transgenic cell lines expressing these VSRNTs, which could be due to the intense competition between VSRNT with the endogenous VSR proteins for the vacuolar transport of AtALEU thus result in undetectable of AtALEU in the culture media.

4.4 Characterization of an *Arabidopsis* glycosyl hydrolyases family 3 protein, At5g10560 (GH3)

Glycosyl hydrolases, function as to hydrolase and modify molecules that consists of the glycosidic bond, such as polysaccharide or glycoprotein. Due to the function diversity, glycosyl hydrolases can be classified in more than 80 families (Faure, 2002). Among them, glycosyl hydrolase family 3 proteins comprises enzymes such as beta-glucosidase, beta-xylosidase and beta-N-acetylhexosaminidases (Faure, 2002). Glycosyl hydrolase family 3 protein can be found in wide range of plants, including barley and alfalfa (Lee et al., 2003; Xiong et al., 2007). Glycosyl hydrolase family 3 protein play an important role in plant growth and development, such as modifying plant cell wall during tobacco pollen development or during root growth in alfalfa (Xiong et al., 2007) and mobilizing starchy endosperm during barley grain germination (Lee et al., 2003). In *Arabidopsis*, glycosyl hydrolase family 3 protein contains 14 to 15 members (Hruba et al., 2005) (http://www.arabidopsis.org/browse/genefamily/GlycosideHydrolase.jsp,).

Glycosyl hydrolases play an important role in plant, such as modifying cell wall components. However its role in plant vacuole remains elusive. The *Arabidopsis* glycosyl hydrolase family 3 protein (At5g10560), found in the culture media of all the transgenic *Arabidopsis* PSB-D cell lines among all the potential cargo proteins found, was chosen for further study. The full length At5g10560 consists of 792 amino acids,

including the N-terminal signal peptide (Figure 12A). At5g10560 is a beta-xylosidase found in plant vacuole (Carter et al., 2004), but not in cell wall (Borderies et al., 2003; Charmont et al., 2005). However, the function of At5g10560 and the glycosyl hydrolase family 3 proteins in plant vacuole remains unknown.

Transient expression of the GH3-GFP fusion in *Arabidopsis* PSB-D protoplast revealed the PVC localization and vacuolar deposition of At5g10560 (Figure 12B and 12C). Furthermore, when co-expressed with of the ER resident version of BP80NT, the BP80-CNX-RFP, GH3-GFP was trapped in the ER of the *Arabidopsis* PSB-D protoplast (Figure 12D), indicating an *in vivo* interaction between GH3 and BP80NT. Further experiments are required to find out whether GH3 is directly interacted with BP80NT or not. In addition, deletion studies of GH3 indicated that GH3 may contain a novel VSD.

Chapter 5 Conclusion

Plant contains multiple VSR proteins that are functionally diverse and overlapping, making the functional characterization of individual VSR proteins difficult and challenging. Through expression of the N-terminal cargo protein interacting domain of individual VSR proteins (the truncated VSR proteins), lacking the TMD and CT for VSR targeting, in tobacco BY-2 and *Arabidopsis* PSB-D culture cells, functions of individual VSR protein can be elucidated. The obtained results indicated that each of the secreted truncated VSR proteins bring along both common and different kinds of cargo proteins into the cultured media. Seventeen potential cargo proteins were identified through LC-MS/MS and FT-MS analysis. These newly identified proteins include vacuolar proteases, glycosyl hydrolases, and chitinases, along with proteins of unknown function. The *Arabidopsis* glycosyl hydrolases family 3 protein (At3g10560, GH3) was chosen for further study. GFP-tagged CH3 was found to colocalize with the PVC marker mRFP-AtVSR2 in *Arabidopsis* protoplasts. Furthermore, when coexpressed with an ER-localized BP80-CNX-RFP, CH3 was trapped in the ER, indicating a possible *in vivo* interaction of GH3 and the truncated BP-80. Loss-of-function analysis demonstrated that GH3 contained vacuolar sorting determinant for PVC targeting. Thus, the newly identified CH3 is a VSR cargo.

To conclude, in this study, I have developed a system to study the functions of

individual VSR proteins, through transgenic tobacco BY-2 and Arabidopsis PSB-D culture cells expressing the truncated VSR proteins (VSRNT) lacking the TMD/CT. The truncated VSRs, functionally indistinguishable from the native VSRs in term of VSD interaction, are secreted into the culture media, and thus VSR interacting cargo proteins are likely co-secreted into the cultured media along with the truncated VSRs, which allow the identification of these VSR cargo proteins from the cultured media of transgenic cells through proteomic approaches. So far, seventeen putative VSR cargo proteins unique to the cultured media of transgenic *Arabidopsis* PSB-D cell line have been identified. Studies on selective protein (At5g10560) demonstrated that it contains vacuolar sorting determinant (VSD) recognized by VSRs for PVC targeting. Thus, the current system should be useful for further study of receptor-cargo interaction in plants.

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Appendix

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Expression and characterization of two functional vacuolar sorting receptor (VSR) proteins, BP-80 and AtVSR4 from culture media of transgenic tobacco BY-2 cells

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ABSTRACT

Vacuolar sorting receptors (VSRs) are type I integral membrane family proteins that mediate protein transport from late Golgi or trans Golgi network (TGN) to vacuole in plant cells The N terminus of VSR is believed to be important for cargo binding while its transmembrane domain (TMD) and cytoplasmic tail (CT) are essential for its correct subcellular localization In this study we have developed and tested an expression system using transgenic tobacco BY-2 cells to produce truncated VSR (VSRNT) proteins lacking the TMD/CT into the culture media The expressed truncated VSRs (BP80NT and AtVSR4NT) are properly secreted into the culture media and bind specifically to the known vacuolar sorting determinants (VSDs) of various vacuolar proteins in a calcium-dependent manner *in vitro* Therefore, since VSR cargo proteins are likely secreted into the culture media along with the truncated VSRs, potential applications of such expression system include identification of native VSR cargo proteins from the culture media of transgenic cells via LC–MS/MS analysis and large-scale purification of truncated VSR proteins for structural study to understand cargo–receptor interaction mechanisms in plants

1. Introduction

All eukaryotic cells contain a secretory pathway, an endomembrane system that is comprised of several distinct organelles that are defined by specific proteins on or within their membranes [1–5] Although the secretory pathway is responsible for secretion of soluble proteins to the outside of the cell, it also includes membrane-bound organelles that are the final destination of soluble proteins that do not leave the cell, these are the endosome or prevacuolar compartment (PVC) [6,7] and the lysosome or vacuole [8] Soluble proteins that are destined for the lysosome or vacuole contain positive information that causes them to be sorted away from the flow of proteins to be secreted from the cell [1,7]

In plant cells, receptor-mediated sorting is one of the major mechanisms responsible for sorting soluble proteins to vacuoles in plant cells [9–11] Soluble vacuolar proteins such as storage proteins and hydrolytic enzymes reach vacuoles because they contain vacuolar sorting determinants (VSDs) that can be recognized by specific vacuolar sorting receptor (VSR) proteins [10] or receptor-like proteins termed RMR [12] BP-80 was the first VSR protein identified in pea (*Pisum sativum*) that recognized the VSD Asn-Pro-Ile-Arg (NPIR) of the barley cysteine protease aleurain [13 14] Similarly, the major seed storage protein 2S albumin and 12S globulin were transported to protein storage vacuole (PSV) by AtVSR1 in *Arabidopsis* seed [15], whereas the transport of phaseolin to a PSV-like compartment was achieved by the AtRMR1 receptor protein [12] in *Arabidopsis* cells [16] and tobacco cells [17] However, the native cargo proteins for most of the studied sorting receptors in plants remain unknown because either reporters or heterologous vacuolar proteins with known VSDs were used in most of these studies [7]

VSRs are type I integral membrane protein containing a single transmembrane domain (TMD) and a short cytoplasmic tail (CT) [9,10,14] The N-terminus of all VSRs contains both cargo-binding domain and epidermal growth factor (EGF)-like repeats [18–20] The cargo-binding domain of VSR is believed to be responsible for sorting cargo proteins at late Golgi or *trans-Golgi network* (TGN) via specific cargo–receptor interaction [19], even though a recent study using transient expression system with tobacco protoplasts suggested that such cargo–receptor interaction and protein sorting could occur at the ER [21] Eventually, the receptor–cargo complex assembled in clathrin coated vesicles (CCVs) is delivered to an intermediate prevacuolar compartment (PVC) prior to reaching the vacuoles [5,6,10] In addition, the TMD and CT of a VSR have been shown to be both essential and sufficient for its PVC targeting

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in plant cells [1,22]. Using VSR antibodies [23] and the YFP-BP-80 reporter [22] as PVC markers, multivesicular bodies (MVBs) were identified as being PVCs in plant cells [22,24–26].

Calcium also plays an important role in protein–protein interaction, especially in regulating the protein structure [27]. The EGF-like repeat of VSRs has been shown to be important for VSR–cargo interaction [18–20], likely due to its function as a calcium binding domain that induces conformational change of VSR structure upon its interaction with cargo proteins [18]. Such scenario is supported by the resolved 3D structure of the calcium-bound form of EGF motif in animal cells [28]

The genome of *Arabidopsis thaliana* contains seven VSR isoforms (AtVSR1-7) with highly conserved amino acid sequences especially at their N-terminal domains [20,25,29]. When the seven green fluorescent protein (GFP) fusions with the transmembrane domain (TMD) and cytoplasmic tail (CT) of individual AtVSR1-7 were expressed in transgenic tobacco BY-2 cells, the seven GFP-AtVSR fusions were found to localize to the same PVCs [25], where these same PVCs mediated the transport of vacuolar reporters for both hydrolytic enzymes and storage proteins in both Arabidopsis and tobacco BY-2 cells [30,31] However, the function of most individual AtVSRs remains elusive

Several approaches have been used to study the possible functions of individual VSR proteins in plants. BP-80 may function in transporting hydrolytic enzymes to lytic vacuole (LV) because it recognized the NPIR motif of barley aleurain [13,14]. AtVSR1/AtELP also bound to the NPIR motif for vacuolar transport of proteases in *Arabidopsis* cells [32] In T-DNA insertional *Arabidopsis* atvsr1 mutant, PSV transport of two storage proteins 12S globulin and 2S albumin was impaired, resulting in secretion of these two vacuolar proteins into the extracellular space, which demonstrated the *in vivo* function of AtVSR1 as a sorting receptor in transporting storage proteins to the protein storage vacuole (PSV) in *Arabidopsis* seeds [33] It thus seems that AtVSR1 may function as a sorting receptor in transporting both cysteine protease and storage proteins in *Arabidopsis* [32–34]. Another study using RNAi technology demonstrated that AtVSR3 were important for proper physiological function of the guard cell, because the guard cells in *atvsr3 RNAi knowk-down* mutant *Arabidopsis* plant did not close properly in response to abscisic acid treatment [35]. More recently, both VSRs and SCAMPs (secretory carrier membrane proteins) were shown to be essential for pollen tube growth [36]. However, except for AtVSR1 and AtVSR3, we know little about the function of most *Arabidopsis* AtVSRs and VSR proteins in other plants such as rice. It is possible that different VSRs bind different cargo proteins for their vacuolar transport in plants

As a first step to address these questions about the possible functions of VSRs in plants, and as proof-of-principle, we have expressed the soluble truncated forms or N-terminus of two closely related VSR proteins (the pea BP-80 and the *Arabidopsis* VSR4) lacking their TMD and CT in transgenic tobacco BY-2 cells Both of these expressed VSR N-terminus (BP80NT and AtVSR4NT) are properly secreted into the culture media of transgenic BY-2 cells We have used ligand binding studies with known VSDs of vacuolar proteins to study the specific ligand binding ability of the truncated VSRs and demonstrated that both BP80NT and AtVSR4NT had a ligand binding specificity that was indistinguishable from the previously described full-length BP-80 [13] or the CHO cells-derived truncated BP-80 [19]. In addition, such receptor–ligand interaction is calcium-dependent *in vitro*. Therefore, BY-2 cells-derived truncated VSR proteins may represent a useful and reliable tool for studying specific receptor–ligand interaction for other members of VSR proteins of *Arabidopsis* and rice. This study will also service as a first essential step for the identification and characterization of new cargo proteins with vacuolar sorting determinants (VSDs) in plant cells

2. Materials and methods

General methods for the construction and characterization of recombinant plasmids, the maintenance of suspension culture tobacco (*Nicotiana tabacum*) BY-2 cells, and the preparation and characterization of antibodies have been described previously [1,12,22,37,38].

2 1 Plasmid construction

For the construction of truncated BP-80, full-length BP-80 from plasmid NP472 [14] was used as template for PCR amplification Two primers (forward, 5′-CGGTCTAGAATGAAGTGTTGGAGATTGT CGGCG-3′; reverse, 5′-GCCGA GCTCTCAACCCATCTGCTGAC-CACCAGTCATCGAAGCCATGGATTTTGC CTGACTGGCAGTTTTAC-3′) were used to amplify BP80NT with the T7 epitope tag added at its C-terminus. For the construction of the truncated AtVSR4, full-length AtVSR4 cDNA from Riken was used as template Two primers (forward, 5′-CGGGGATCCATGAAGCAGCTT CTATGTTATCTTCC-3′, reverse, 5′-GCCGAGCTCTCAACCCATCTGCTGA CCACCAGTCATCGAAG CCATTGATTTCACTTGTGAACCCGTCTTG-3′) were used to amplify AtVSR4NT with T7 epitope tag added at the C-terminus The PCR-amplified fragments were digested with XbaI/SacI for BP80NT and BamHI/SacI for AtVSR4NT and cloned into the plant expression vector pBI121 via replacing the GUS reporter gene The construct was checked and verified by restriction mapping and DNA sequencing.

2 2 Agrobacterium-mediated transformation of tobacco BY-2 culture cells

Plasmid containing BP80NT and AtVSR4NT was first introduced into the *Agrobacterium* strain LBA4404 by electroporation prior to transfecting wild-type tobacco BY-2 culture cells as described previously [22,38]. Kanamycin-resistance calli were transferred into MS liquid medium containing kanamycin to initiate suspension culture. Culture media of transgenic BY-2 cell lines were collected and concentrated by methanol–chloroform for SDS-PAGE and Western blot analysis of BP80NT or AtVSR4NT using VSR antibodies

2.3. Protein precipitation by methanol–chloroform

Culture media were collected by filtering the cultured cells through filter paper. The filtrates with secreted proteins were collected Protein precipitation was carried out by adding 4 volume of methanol and 1 volume of chloroform, mixed thoroughly and then added 3 volume of double-distilled water After centrifuge for 5 min at 14 000 rpm, the upper aqueous phase was discarded and 2 volume of methanol were added and mixed with the organic phase. The protein pellet was collected by centrifuge for 5 min at 140 00 rpm SDS-PAGE sample loading buffer (1×) was used to dissolve the pellet and the protein samples were boiled for 10 min before performing SDS-PAGE.

2 4 Construction of peptide sepharose for pull down assay

Synthetic peptides containing vacuolar sorting determinants (VSDs) or their mutated forms of vacuolar proteins were synthesized by Genescript. Amino acid sequences and other related information about the synthetic peptides used in this study were listed in Fig. 3D. The synthetic peptides were conjugated into CNBr-activated sepharose (Sigma) according to the manufacturer's protocol. Different synthetic peptides are designed to test the specific interaction of truncated BP-80 and AtVSR4 versus different types of known or putative VSDs of vacuolar proteins, including the BP-80-binding NPIR VSD of aleurain and sporamin, the AFVY

VSD of phaseolin VSDs from other storage proteins such as pro2S albumin, BN2S, beta-conglycinin, vicilin and proConA were also used Besides, mutation analysis was also performed on VSD of vicilin-like storage protein (VLSP) containing one AFVY-like motif (AVFV) and one WSIV motif, found to be important for correct targeting of phaseolin to protein storage vacuole A "blank column" without peptides was constructed as the same way and used as control

2 5 Pull down assay

Day-7 culture media were collected by filter the cell culture through a filter paper The filtrates containing secreted proteins were collected and dialyzed against double-distilled water at 4 °C overnight, followed by lyophilization The lyophilized proteins were resuspended in binding buffer (25 mM HEPES, pH 7 1, 150 mM NaCl, 1 mM $MgCl_2$) with 1000 μM $CaCl_2$ in the BP80NT–peptide interaction assay, or with 0, 10, 25, 50, 100, 1000 μM $CaCl_2$ in the calcium-dependent assay [13,18] One milliliter of the resuspended proteins was mixed with 10 μL of sepharose conjugated with peptides at 4 °C overnight After washing with binding buffer for three times, the sepharose containing the BP80NT– or AtVSR4NT–peptide complex was boiled in 20 μL SDS-PAGE sample loading buffer for 10 min and subject to Western blot by VSR antibodies (Fig 3A)

2 6 Calculation of percentage of binding of BP80NT and AtVSR4NT to synthetic aleurain peptide

The percentage of binding of BP80NT and AtVSR4NT to VSD of aleurain is calculated from comparing the image intensity of the correspond protein bands in Western blot analysis, as determined by ImageJ (available in http //rsbweb nih gov/ij/index html), using the signal obtained at 1000 μM concentration of calcium as 100%

2 7 Western blot analysis of marker proteins of various cellular compartments

Intracellular and secreted proteins were collected, followed by SDS-PAGE and Western blot analysis using antibodies against different marker proteins including ER, Golgi, vacuolar membrane, plasma membrane, endosome and cytoskeleton marker BiP and calreticulin are the two ER chaperones that assist protein folding [39] Mannosidase I (ManI) is a Golgi resident protein and is responsible for protein glycosylation along the secretory pathway [22,40] Tonoplast marker α-tonoplast intrinsic protein (α-TIP) is an aquaporin present in the tonoplast membrane of plant cell [41] Plasma membrane and early endosome marker SCAMP (secretory carrier membrane protein) is a protein cycling between TGN and PM [38], and cytoskeleton marker tubulin is a cytosolic proteins and is the building blocks of microtubules [42]

2 8 Bioinformatics analysis

Multiple alignment of the pea BP-80 (GI 1737222), the pumpkin PV72 (GI 2943792) and the *Arabidopsis* AtVSR4 (GI.30725454) amino acids was performed using the ClustalW2 tool with default parameters (available in http //www ebi ac uk/Tools/clustalw2/index html) [43]

3. Results

3 1 Generation and characterization of transgenic tobacco BY-2 cells expressing truncated BP-80 (BP80NT) and AtVSR4 (AtVSR4NT)

The pea VSR BP-80 consists of a signal peptide, N-terminal cargo-binding domain (NT), a single transmembrane domain (TMD) and cytoplasmic tail (CT) The BP-80 NT is believed to be responsible for cargo binding while its TMD/CT is essential for its correct targeting to prevacuolar compartments (PVCs) in plant cells [1] Similarly, the seven *Arabidopsis* VSRs are also localized to the same PVC populations in plant cells [23,25] It has been suggested that individual VSRs might bind to specific cargo(es) for vacuolar targeting in plants

As a first step to study the cargo binding of individual VSR proteins in plants, we tested a secretion expression system using transgenic tobacco BY-2 cells [44] Since the native full-length VSR is concentrated on PVCs [23] whereas its TMD/CT is important for PVC targeting [1,22] in plant cells, we have thus hypothesized that the truncated VSR (VSRNT), lacking its TMD and CT, will be secreted into the culture media when expressed in transgenic tobacco BY-2 cells (Fig 1A and B) In fact, in wild-type (WT) tobacco BY-2 cells anti-VSR antibodies only detected the endogenous VSR proteins in the intracellular (I) fraction (Fig 1C, lane 1, double-asterisks) but missing from the secreted (S) media fraction (Fig 1C, lane 2) In contrast, in transgenic tobacco BY-2 cells expressing the truncated BP80NT, in addition to the presence of the endogenous VSR proteins in the intracellular fraction (Fig 1C, lane 3, as indicated by double-asterisk), a protein band corresponding to the truncated BP80NT was detected in both the intracellular fraction (I) and the secretion fraction (S) (Fig 1C, lanes 3 and 4, as indicated by single asterisk) Similar results were obtained from transgenic BY-2 cells expressing the truncated AtVSR4NT (Fig 1C lanes 5 and 6), even though the amount of the truncated AtVSR4NT in the intracellular fraction (I) was below the detectable level (Fig 1C, lane 5, asterisk) In addition, the detectable amounts of the truncated BP80NT and AtVSR4NT in the secretion fraction (S) (Fig 1C, lanes 4 and 6 respectively) are much higher than that of intracellular fraction (I) (Fig 1C, lanes 3 and 5 respectively), indicating the stable accumulation of secreted VSRNT in the culture media Furthermore, when the culture media of these two transgenic BY-2 cell lines expressing either BP80NT or AtVSR4NT were collected from Day 1 to Day 7 after subculture and used for Western blot analysis with VSR antibodies, the detectable amounts of the truncated BP80NT and AtVSR4NT in the culture media were found to be increased gradually from Day 1 to Day 4 and peaked at Day 4, but the signals for the secreted VSRNT proteins remained similar thereafter (Fig 1D and E, asterisk) Taken together, these results demonstrate that transgenic tobacco BY-2 cells expressing BP80NT and AtVSR4NT have resulted in proper expression and secretion of the truncated VSR proteins in the culture media

3 2 The culture media of transgenic BY-2 cells expressing VSRNT is free of intracellular proteins

To further rule out the possibility that the detection of VSRNT was due to the leakage of the transgenic BY-2 cells, we next carried out Western blot analysis on intracelluar and secreted proteins of both WT and transgenic VSRNT BY-2 cells using antibodies for known marker proteins, including the ER markers anti-BIP and anti-calreticulun [39], the Golgi marker anti-Man1 [22,40], the vacuole marker anti-alpha-TIP [41] the PM/TGN marker anti-SCAMP1 [38] and cytoskeleton marker anti-tubulin [42] As shown in Fig 2, protein bands corresponding to the

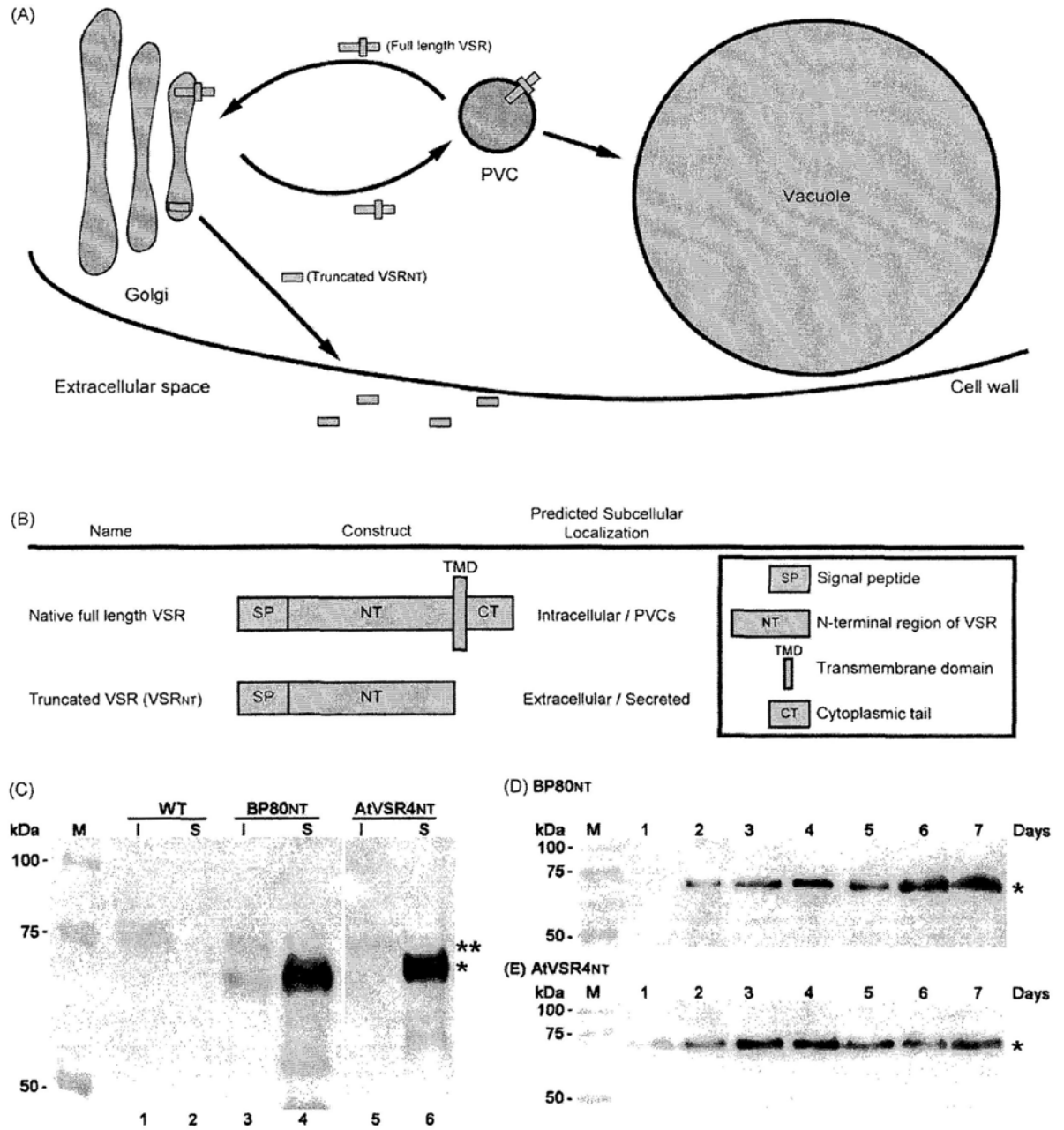


Fig. 1. Expression and characterization of transgenic tobacco BY-2 cell lines expressing the truncated N-terminus of the pea BP-80 (BP80NT) and the *Arabidopsis* AtVSR4 (AtVSR4NT). (A) Working model of protein trafficking of full-length and truncated VSR in the plant secretroy pathway. The full-length VSR is believed to traffic from late Golgi or trans-Golgi network (TGN) to prevacuolar compartment (PVC), while the truncated VSR will be secreted into the culture media of transgenic tobacco BY-2 cells. (B) Structures and predicted subcellular localization of native VSR and truncated VSR proteins. The native VSR contains an N-terminal signal peptide (SP), an N-terminal domain (NT) responsible for binding of cargo protein, a single transmembrane domain (TMD) and a short cytoplasmic tail (CT). The truncated VSR, lacking its TMD and CT, is expected to secrete outside of the cells when expressed in tobacco BY-2 cells. (C) Western blot analysis of transgenic tobacco BY-2 cells expressing truncated BP-80 (BP80NT) and AtVSR4 (AtVSR4NT). The intracellular (I) and secreted (S) proteins were isolated from either wild-type (WT, lanes 1 and 2) or transgenic tobacco BY-2 cells expressing truncated BP80NT (lanes 3 and 4) or AtVSR4NT (lanes 5 and 6), followed by protein separation via SDS-PAGE and Western blot analysis using VSR antibodies. Double-asterisks and asterisk indicate the full-length endogenous VSR and the expressed truncated BP80NT or AtVSR4NT proteins respectively. (D and E) Expression and secretion profiles of BP80NT (D) and AtVSR4NT (E) in transgenic tobacco BY-2 cell lines. Secreted proteins from Day 1 to Day 7 (lanes 1–7) culture cells after subculture were collected from the culture medium of transgenic tobacco BY-2 cells expressing BP80NT or AtVSR4NT, followed by protein separation via SDS-PAGE and Western blot analysis using VSR antibodies. M, molecular mass in kliodaltons.

marker proteins were only detected in the intracellular fraction (I) rather than in the secretion fraction (S) from both WT and transgenic BY-2 cells. These results clearly indicate that there is no leakage of the cellular components into the secreted fraction. Therefore, the method of collecting secreted fraction can retain the integrity of the culture cells, and that both BP80NT and AtVSR4NT in the culture medium are secreted from the cell s rather than owing to leakage of damaged or disrupted cells.

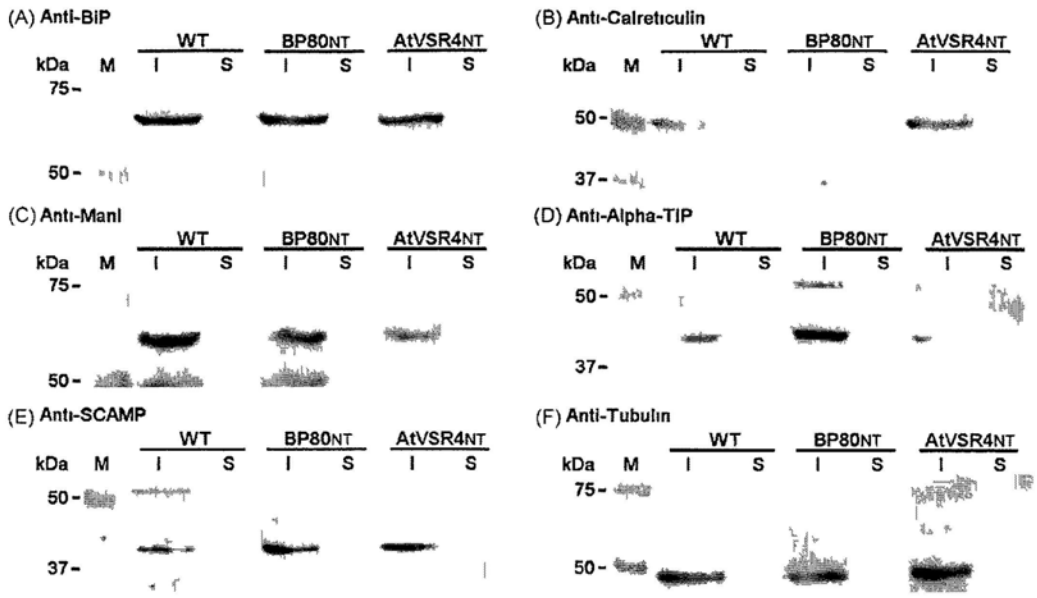


Fig 2 The culture media of transgenic BY 2 cell line expressing truncated BP80NT and AtVSR4NT are free of intracellular proteins Intracellular proteins (I) and secreted protein (S) fractions were collected from wild-type (WT) and transgenic tobacco BY 2 cells expressing BP80NT and AtVSR4NT followed by SDS PAGE and Western blot analysis using various antibodies including anti BiP (A) anti-calreticulin (B) anti Mannosidase I (C) anti SCAMP1 (D) anti-alpha TIP (E) and anti tublin antibodies (F) M molecular mass in kliodaltons

3 3 VSRNT interaction with peptides

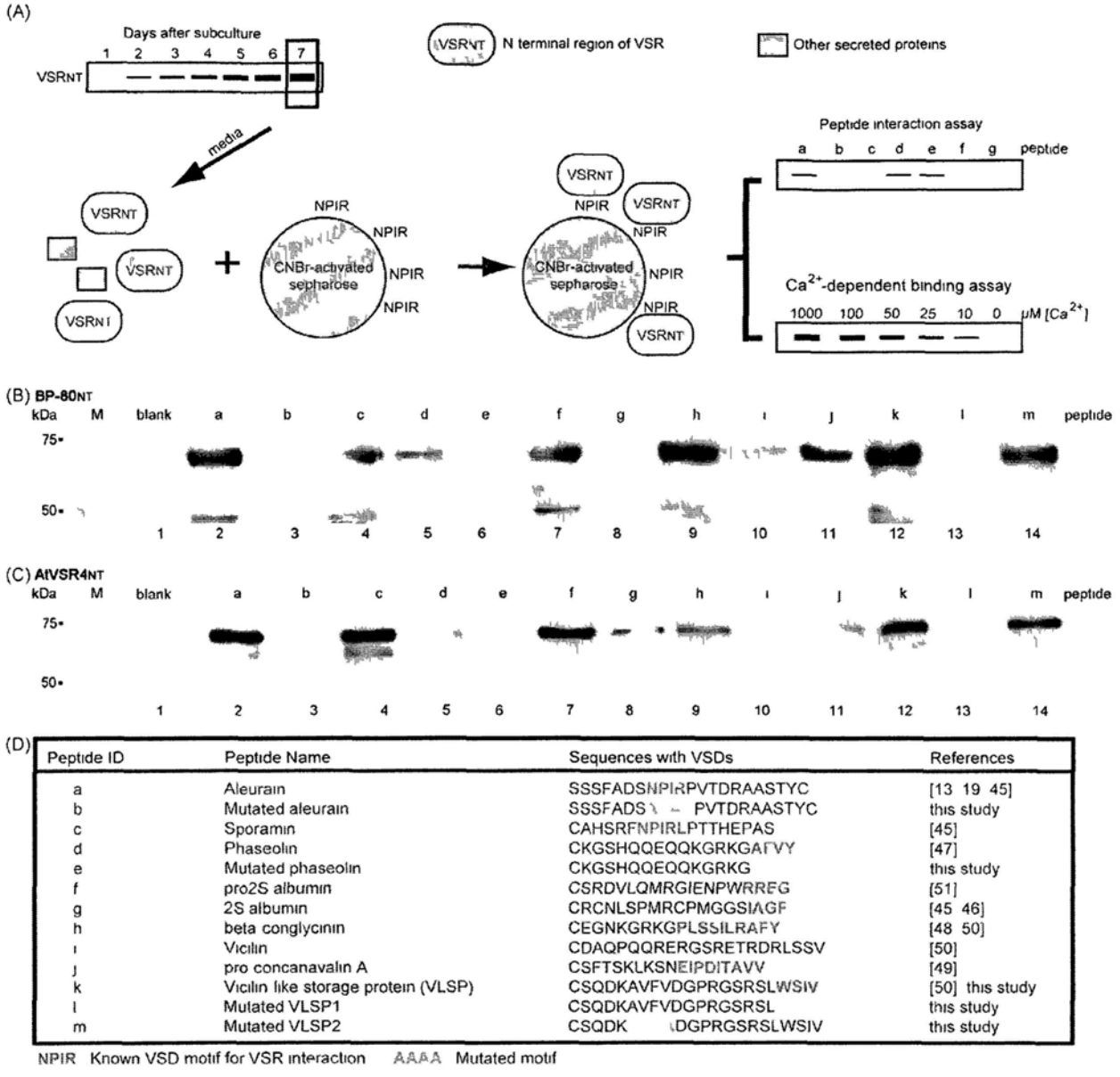
We next want to find out if the secreted BP80NT and AtVSR4NT are functional proteins by testing their binding ability and specificity to several known or putative vacuolar sorting determinants (VSDs) and their mutants of vacuolar proteins (Fig 3) To do this, synthetic peptides corresponding to the VSDs and their mutants (Fig 3D) are conjugated onto the CNBr-activated sepharose prior to binding with the secreted VSRNT from the Day-7 culture media of transgenic BY-2 cells under different conditions (Fig 3A) The bound proteins are then released from the agarose for further SDS-PAGE protein separation and Western blot analysis using VSR antibodies (Fig 3B and C)

Several known VSDs and their mutants peptides are used the NPIR motif of aleurain, sporoamin and BN2S known to interact with VSRs [13,45 46], and the putative VSDs of AFVY found in other vacuolar proteins such as the common bean phaseolin, jackbean pro-concanavalin A (proConA), soybean beta-conglycinin, 2S albumin vicilin and vicilin-like storage protein (VLSP) [47–51] (Fig 3D), even though their interaction with BP-80 remains to be demonstrated

As shown in Fig 3B, BP80NT did not interact with the control sepharose matrix because no protein band was detected from the blank column (lane 1) In contrast, BP80NT not only interacted with VSD peptides containing the NPIR motif from aleurain and sporamin (lane 2 peptide a and lane 4 peptide c), but also interacted with the putative VSD peptides containing the AFVY motif from phaseolin (lane 5, peptide d) and the known VSDs from other storage proteins including the pro2S albumin (lane 7, peptide f) beta-conglycinin (lane 9, peptide h) and pro-concanavalin A (lane 11, peptide j), but had very weak interaction with the VSDs of 2S albumin and vicilin (lane 8 peptide g and lane 10 peptide i) In addition, this BP80NT–VSD interaction was specific because mutated VSDs of aleurain and phaseolin no longer interacted with BP80NT under the identical binding condition (e g Fig 3B, lanes 3, 6, 8) Interestingly, mutation on the WSIV rather than the AFVY-like (AVFV) motif of the VLSP abolished the BP80NT-binding (lane 13 peptide l vs lane 14 peptide m), likely representing a new VSD interacting with BP80NT Taken together, these results demonstrate that the secreted BP80NT from transgenic BY-2 cells specifically bind to the known VSDs but not their mutants of aleurain, sporamin and several other storage proteins Generally, AtVSR4NT (Fig 3C) showed similar binding specificity against the same synthetic peptides and the mutant peptides as BP80NT (Fig 3B), except for weaker interaction with the VSDs of phaseolin, beta-conglycinin and pro-concanavalin A (compare lane 5 peptide d, lane 9 peptide h and lane 11 peptide i for Fig 3B and C) These results suggest that BP80NT and AtVSR4NT are alike, yet has its own specificity in terms of VSDs affinity

3 4 VSRNT–VSD interaction is calcium-dependent

Both the pea VSR BP-80 and the pumpkin VSR PV72 contain the EGF repeats in the N-terminus in front of the TMD region [18–20] These EGF repeats are responsible for regulating the VSR–ligand binding ability of both BP-80 and PV72 in a calcium-dependent manner [18 19] Similarly, AtVSR4 may interact with its ligands (or the VSD peptides used in this study) in a calcium-dependent manner because AtVSR4 also contains the EGF repeats at its N-terminus (Supplementary Fig 1) To further find out if these BP80NT–VSDs and AtVSR4NT–VSDs interactions depend on the presence of calcium, we next carry out binding study with selective VSD synthetic peptides of aleurain under different calcium concentrations As shown in Fig 4A, the interaction of BP80NT with the aleurain VSD was peaked at 1000 μM calcium, but such interaction was gradually reduced with decreasing calcium concentrations (Fig 4A) At 100 μM calcium, only 45% of BP80NT was still bounded with the aleurain VSD peptide, with further decrease of calcium concentrations, the affinity of BP80NT became weaker and only 5% of BP80NT was bounded with the peptide at 0 μM calcium ion (Fig 4C black bars) When identical binding assay was carried out, AtVSR4NT was found to interact with the aleurain VSD peptide in a similar calcium-dependent manner, because the affinity of AtVSR4NT with the aleurain VSD peptide gradually decreased as the calcium concentrations were reduced from 1000 to 0 μM (Fig 4B and C cross-hatched bars) These results support the notion that the interaction of BP80NT and AtVSR4NT with the aleurain VSD peptide is calcium-dependent and that the presence of calcium ion enhances



(D)

Peptide ID	Peptide Name	Sequences with VSDs	References
a	Aleurain	SSSFADSNPIRPVTDRAASTYC	[13 19 45]
b	Mutated aleurain	SSSFADS\ – PVTDRAASTYC	this study
c	Sporamin	CAHSRFNPIRLPTTHEPAS	[45]
d	Phaseolin	CKGSHQQEQQKGRKGAFVY	[47]
e	Mutated phaseolin	CKGSHQQEQQKGRKG	this study
f	pro2S albumin	CSRDVLQMRGIENPWRREG	[51]
g	2S albumin	CRCNLSPMRCPMGGSIAGF	[45 46]
h	beta conglycinin	CEGNKGRKGPLSSILRAFY	[48 50]
i	Vicilin	CDAQPQQRERGSRETRDRLSSV	[50]
j	pro concanavalin A	CSFTSKLKSNEIPDITAVV	[49]
k	Vicilin like storage protein (VLSP)	CSQDKAVFVDGPRGSRSLWSIV	[50] this study
l	Mutated VLSP1	CSQDKAVFVDGPRGSRSL	this study
m	Mutated VLSP2	CSQDK \DGPRGSRSLWSIV	this study

NPIR Known VSD motif for VSR interaction AAAA Mutated motif

Fig 3 Secreted BP80NT and AtVSR4NT specifically bind to vacuolar sorting determinants (VSDs) in pull down assay (A) Procedures of pull down assay and Ca^{2+} dependent binding assay Day 7 culture media of transgenic BY-2 cells expressing truncated VSR (VSRNT) were collected After dialysis the media were lyophilized and resuspended in binding buffer (25 mM HEPES pH 7 1 150 mM NaCl 1 mM $MgCl_2$) with 1000 μM $CaCl_2$ in the BP80NT–peptide interaction assay or with 0 10 25 50 100 1000 μM $CaCl_2$ in the calcium dependent assay The resuspended proteins were mixed with sepharose conjugated with various synthetic peptides of VSDs or mutated VSDs (D) and washed with binding buffer prior to SDS PAGE and Western blot analysis using VSR antibodies (B and C)

the interaction between the two receptor proteins and the aleurain VSD peptides Interestingly, BP80NT and AtVSR4NT show different degrees of binding ability to the same aleurain VSD peptide when the binding was performed at the same calcium ion concentration (Fig 4C) For example, at 10 μM calcium ion, only 20% of BP80NT while around 55% of AtVSR4NT binded to the aleurain VSD peptide, which could be due to the differences of amino acids in the EGF repeats between BP-80 and AtVSR4 (Supplementary Fig 1) Such scenario can be tested in future experiments

4. Discussion and conclusions

Heterologous expression of VSR proteins in an eukaryotic cell system is essential for the production of functional VSR proteins because of its ability in correct protein folding and post-translational modification Several systems have been tested for the expression and purification of VSR proteins for functional characterization For example, various recombinant forms of the truncated BP-80 derived from expressing in Drosophila S2 cells were used to study BP-80–proaleurain peptide interaction which has identified two separate proaleurain peptide ligand binding sites within the N-terminus of BP-80 [19] In this study, large purification of truncated BP-80 proteins from Drosophila S2 cells was not feasible because chromatographic purification resulted in large losses and low yield [19] A further study using the same expression system for a His-tagged version of a truncated *Arabidopsis* VSR has allowed purification of the truncated AtBP80b in relatively large amount for crystallization and initial diffraction studies [52] However, comparing to other expression systems for pro-

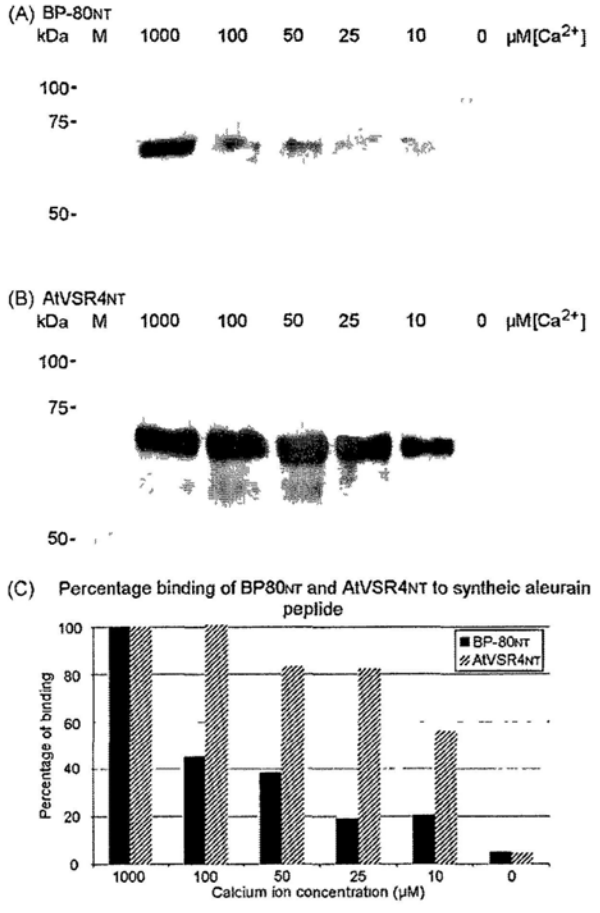


Fig. 4. The interaction between the VSD-containing aleurain synthetic peptide and BP80NT or AtVSR4NT is calcium-dependent. Sepharose conjugated with synthetic peptide of aleurain was incubated with BP80NT (A) or AtVSR4NT (B) in the presence of various calcium ion (Ca^{2+}) concentrations (from 0 to 1000 μM as indicated). After washing with binding buffer with the same Ca^{2+} concentration, VSRNT proteins in sepharose were released by boiling in SDS loading buffer, followed by SDS-PAGE and Western blot analysis with VSR antibodies. Percentage of binding for BP80NT and AtVSR4NT with synthetic aleurain peptide under different Ca^{2+} concentrations was calculated by comparing the protein band intensity among different Ca^{2+} concentrations, where the signal obtained from binding at 1000 μM Ca^{2+} concentration is considered as 100% binding (C). Black bars, BP80NT. Cross-hatched bars, AtVSR4NT.

ducing recombinant proteins including *E. coli* and yeast, using *Drosophila* S2 cells as an expression for producing large amounts of properly-folded VSR proteins to be used in functional study has been technically challenging and economically expensive due to the animal cell culture nature. To address some of these disadvantages, in this study, we have developed and tested an expression system using transgenic tobacco BY-2 cells to express truncated BP-80 and AtVSR4. The obtained results demonstrate that the truncated VSRs are properly expressed and secreted into the culture media of transgenic BY-2 cells, and these secreted VSRNT (both BP80NT and AtVSR4NT) bind to known VSDs of vacuolar proteins in a calcium-dependent manner. Thus, this BY-2 cell expression and secretion system may represent an attractive alternative for the heterologous expression and purification of truncated VSR proteins to be used for their functional characterization in future studies.

VSR isoforms have been found in many plant species including pea, pumpkin, mung bean, *Arabidopsis* and rice [13,14,20,26,32,51,53,54]. The *Arabidopsis* genomes contain seven VSR isoforms with different spatial and temporal expression profiles [36], suggesting their possible functional diversity and redundancy throughout the plant developmental stages, even though all these AtVSRs are found in the same PVC populations in *Arabidopsis* cells [15,25,31,35,54–56] as well as in trans-Golgi network (TGN) in *Arabidopsis* seeds [56]. The important roles of VSR proteins in plants have been demonstrated by several recent studies. For example, the seeds of the *Arabidopsis* AtVSR1 knock-out mutant were shown to have poor germination ability [15,56], VSRs were shown to play role during seed germination of mung bean [26], whereas both VSRs and SCAMPs (secretory carrier membrane proteins) were demonstrated to be essential for pollen tube growth [36]. Interestingly, overexpression of GFP-AtVSR fusion (in which the lumenal ligand binding domain of VSR N-terminus was replaced by GFP) in plant cells had been shown to cause secretion of vacuolar cargo proteins likely because this GFP-AtVSR competed with the endogenous VSR proteins for cargo binding [57,58]. However, in most of these studies, the native cargo proteins that are supposed to be transported by VSR proteins in plants remain largely unknown. It is possible that individual VSR protein may mediate the vacuolar transport of its specific cargo proteins. With such scenario, we have recently proposed that the truncated VSR proteins expressed in suspension culture cells will be secreted into the culture media along with their native cargo proteins [59]. In fact, a recent study using reporters transiently expressed in tobacco protoplasts suggests that the VSR–cargo interaction or selection might have first occurred within the ER lumen [21]. Therefore, in future study, transgenic *Arabidopsis* culture cells expressing individual truncated AtVSR proteins can be established for the analysis of additional proteins in the culture media of transgenic versus wild-type cell lines so that putative VSR cargo proteins in the media can be identified via LC–MS/MS analysis. In fact, we are currently carrying out research in this direction.

BP-80 has been well-known for its binding ability to the NPIR-containing peptides of aleurain and sporamin [13,14], but it is not known if BP-80 and other AtVSR proteins would interact with other VSDs of vacuolar proteins. As a first step to test various VSD–receptor interactions, we have expressed truncated BP-80 and its most closely related *Arabidopsis* VSR (AtVSR4) in this study and shown that the secreted BP80NT and AtVSR4NT from transgenic BY-2 cells have similar binding nature to various known VSDs (Fig. 3), and most importantly, such binding is calcium-dependent (Fig. 4). For example BP80NT or AtVSR4NT not only interact with VSDs of aleurain and sporamin, but also interact with known VSDs of storage proteins including phaseolin, procanavalin A and beta-conglycinin (Fig. 3). In addition, these interactions are specific for the tested VSDs because VSD mutations abolish such interaction (Fig. 3). Furthermore, using the VSD from vicilin-like storage protein (VLSP) and its mutated form, we discovered that WSIV motif, but not the AVFV (AFVY-like) motif, is important for VLSP interaction with BP80NT (Fig. 3). Thus, such BY-2 cells-derived truncated VSRs in the culture media are useful tools for studying specific ligand–receptor interaction.

In conclusion, transgenic tobacco BY-2 cells can be used not only as bioreactors for large-scale production of active pharmaceutical recombinant proteins into the culture media for easy down streaming purification [44], but also as an attractive expression system for producing properly-folded functional truncated VSR proteins into the media to be used for ligand–receptor binding study. Such proof-of-principle demonstration shows great promise in using this expression system to express individual truncated AtVSR and other VSR proteins such as rice VSRs for studying ligand–receptor interaction as well as for possible cargo identification via LC–MS/MS analysis of culture media in future study. In addition, further protocol development in large-scale purification of truncated VSR proteins from the culture media of these transgenic BY-2 or *Arabidopsis* cells will allow VSR crystal formation for future structural study of sorting receptor as well as ligand–receptor interaction in

plant cells, which is limited in other systems [19,52] Indeed, some of our on-going studies are going toward these directions with promising and exciting results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi.10 1016/j.plantsci.2010.04 008.

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