A SPX domain vacuolar transporter links phosphate sensing to homeostasis in *Arabidopsis*

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**ABSTRACT**

Excess phosphate (Pi) is stored into the vacuole through Pi transporters so that cytoplasmic Pi levels remain stable in plant cells. We hypothesized that the vacuolar Pi transporters may harbor a Pi-sensing mechanism so that they are activated to deliver Pi into the vacuole only when cytosolic Pi reaches a threshold high level. We tested this hypothesis using Vacuolar Phosphate Transporter 1 (VPT1), a SPX domain-containing vacuolar Pi transporter, as a model. Recent studies have defined SPX as a Pi-sensing module that binds inositol polyphosphate signaling molecules (InsPs) produced at high cellular Pi status. We showed here that Pi-deficient conditions or mutation of the SPX domain severely impaired the transport activity of VPT1. We further identified an auto-inhibitory domain in VPT1 that suppresses its transport activity. Taking together the results from detailed structure-function analyses, our study suggests that VPT1 is in the auto-inhibitory state when Pi status is low, whereas at high cellular Pi status InsPs are produced and bind SPX domain to switch on VPT1 activity to deliver Pi into the vacuole. This thus provides an auto-regulatory mechanism for VPT1-mediated Pi sensing and homeostasis in plant cells.

**Key words:** VPT1, Pi signaling, autoinhibition, transport activity, inositol phosphates

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**INTRODUCTION**

Phosphate (Pi) is an essential component of nucleic acids, ATP, and cell membranes shared in all life forms. Plants acquire Pi from the soil and supply the food chain with this essential nutrient. As a result, plants have evolved a large number of transport proteins responsible for the uptake, translocation, and distribution of Pi throughout all cells in the whole plant and among subcellular compartments (Gu et al., 2016; Luan et al., 2017; Wang et al., 2021). To maintain Pi homeostasis at the cellular and whole plant levels, the transport activities must be coordinated by signaling networks to balance the supply and demand according to metabolic and environmental cues. When the external supply is low, Pi enters the cells through the high affinity transporters, including the Pi transporter 1 (PHT1) family members whose expression is often induced by Pi starvation (Rubio et al., 2001; Shin et al., 2004). When external Pi levels are high, high-affinity transport is not needed and PHT1-type transporters are degraded through a ubiquitin-mediated mechanism (Bayle et al., 2011; Huang et al., 2013; Lin et al., 2013; Park et al., 2014). At the same time, the excessive Pi in the cytosol is sequestered into the vacuole that serves as a major Pi pool in plant cells (Yang et al., 2017).

Recent studies identified vacuolar Pi transporter (VPT, also named PHT5-type) family proteins as responsible for Pi sequestration into the vacuole (Liu et al., 2015; Liu et al., 2016b). The VPT family consists of three members in Arabidopsis, i.e., VPT1, VPT2, and VPT3 (PHT5; 1, PHT5; 2 and PHT5; 3). Loss of function of these transporters inhibits vacuolar Pi accumulation and renders plants hypersensitive to high Pi conditions (Liu et al., 2015; Liu et al., 2016b; Luan et al., 2019). Furthermore, double knockout of VPT1...
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and VPT3 results in defects in the systemic Pi allocation vital for reproductive development (Luan et al., 2019). While the VPTs load Pi into the vacuolar lumen under high Pi condition, the vacuolar Pi efflux (VPE-type) transporters are believed to mediate retrieval of Pi from vacuoles, contributing to Pi remobilization from the vacuole under Pi-deficient conditions (Xu et al., 2019). Consistently, the expression of VPEs is regulated by PHR1, a central transcription factor related to Pi starvation response.

Although the VPTs and VPEs are transcriptionally responsive to Pi status in the environment (Liu et al., 2015; Liu et al., 2016b; Xu et al., 2019), it is not known if the activities of these transporters are regulated at the post-translational level by Pi-sensing pathways. In this context, VPTs, but not VPEs, contain an SPX domain in their N-terminal region preceding transmembrane domains (Luan and Lan, 2019), indicating that their activities may be regulated by different mechanisms. In particular, the SPX was defined as a conserved sensory domain in eukaryotic organisms, and it binds inositol polyphosphates (InsPs), signaling molecules produced in response to elevated cellular Pi status (Wild et al., 2016; Dong et al., 2019; Zhu et al., 2019). For example, the SPX domain in Vtc3 and Vtc4, two subunits of vacuolar transporter chaperone complex for catalyzing polyP synthesis and transport into yeast vacuoles, binds InsP$_7$ and thereby stimulates inorganic polyphosphate synthesis (Wild et al., 2016). In higher plants, InsP$_8$ is defined as the major InsPs signal that binds to the SPX domain (Dong et al., 2019; Zhu et al., 2019). When cellular Pi is sufficient, InsP$_8$ is synthesized and facilitates the interaction between the SPX1 protein and PHR1 transcription factor, blocking the Pi starvation response (Ried et al., 2021; Wild et al., 2016). In contrast, as Pi status is lowdecreased, the level of InsP$_8$ is decreased, resulting in the release of PHR1 from SPX1, followed by activated expression of Pi starvation induced (PSI) genes (Dong et al., 2019; Zhu et al., 2019). Loss of function of VIH1 and VIH2, two diphosphoinositol pentakisphosphate kinases required for InsP$_8$ synthesis, or mutation of SPX1, constitutively activates expression of PSI genes in Arabidopsis, demonstrating that InsP$_8$ binding to SPX1 is critical for sensing Pi availability to regulate Pi response genes (Dong et al., 2019; Zhu et al., 2019). In addition, mutations in the SPX domain of PHO1, a transport protein responsible for root to shoot Pi translocation (Poirier et al., 1991), would affect its function in Pi long distance transport (Wild et al., 2016). These studies illustrate that the SPX domain is vital for Pi homeostasis. Therefore, we hypothesize that VPT-type transporters with the SPX domain may be capable of sensing and linking cellular Pi status to transport activity.

In this study, we used VPT1 protein as a model to unravel the regulatory function of the SPX domain in controlling the transport activity of VPT1. We started with the finding that VPT1 activity depends on cellular Pi status in plants, underscoring the possibility of this vacuolar Pi transporter being regulated by Pi availability. We further showed that InsPs-binding sites of its SPX domain are important for high transport activity of VPT1, whereas the SPX domain and the adjacent two $\alpha$ helices together serve as an auto-inhibitory structure that suppress VPT1 activity. Our results suggest that the high Pi status induces the production of InsPs that in turn bind to the SPX domain, switching on VPT1 and initiating Pi sequestration into the vacuole. This finding reveals an autoregulatory mechanism for Pi status to control Pi homeostasis and distribution among cellular compartments.

RESULTS

VPT1-mediated Pi accumulation depends on cellular Pi status

Previous studies identified VPT1 as the major transporter for vacuolar Pi sequestration: while loss of function impairs Pi accumulation, overexpression of the gene efficiently enhances Pi accumulation in Arabidopsis (Liu et al., 2015; Liu et al., 2016b). When we measured vacuolar Pi influx currents using patch-clamp recording, we found that the vacuolar Pi current was diminished in the vpt1 mutant, suggesting that VPT1 was largely responsible for the vacuolar Pi influx (Liu et al., 2015). During the patch-clamp experiment, we found that the large Pi currents generated by VPT1 were observed when VPT1 overexpressing seedlings were cultured under sufficient Pi conditions. When plants were grown under Pi-deficient conditions, however, the VPT1 Pi currents were remarkably decreased (Figure 1A and 1B), indicating that the activity of VPT1 was repressed under low Pi status. As the VPT1 protein level was not changed in plants grown under variable Pi (Supplemental Figure 1), the results above indicate that the activity of VPT1 must be regulated by the intracellular Pi status. Recent studies identified InsPs as signaling molecules whose concentrations change with Pi availability and cellular Pi status (Wild et al., 2016; Dong et al., 2019; Zhu et al., 2019; Riemer et al., 2021). InsPs bind SPX, the Pi sensor domain, to regulate Pi-responsive components governing Pi homeostasis in plants and other species. In Arabidopsis, InsP$_8$ levels tend to vary depending on Pi status (Dong et al., 2019; Zhu et al., 2019). Because VPT1 contains a SPX domain, our finding of VPT1 regulation by the cellular Pi status supports a hypothesis that cellular Pi levels may be sensed by the SPX domain of VPT1 that in turn controls the transport activity for vacuolar Pi accumulation.

Mutations in the SPX domain affect the transport activity of VPT1

If, as we proposed, VPT1 senses cellular Pi status for vacuolar Pi sequestration, mutations of the InsPs-binding sites in the SPX domain should suppress the activity of VPT1. As previously reported (Wild et al., 2016), SPX domain consists of six $\alpha$ helices in which three amino acids are defined as the Pi-binding clustering (PBC) sites vital for efficient ligand binding. Through sequence alignment, Y23, K27 and K130 found to be clustered (PBC) sites binding InsPs, suggesting that SPX VPT1PBC bound InsP$_8$ as a generic ligand. The microscale thermophoresis assay (MST) presented in Supplemental Figure 3 suggested that SPX$_{\text{vpt1pbc}}$ bound InsP$_8$ with a much higher $K_d$ (98.6 $\mu$M) than SPX$_{\text{vpt1}}$ (4.69 $\mu$M), indicating that the mutation of PBC sites dramatically decreased the ligand affinity of VPT1 to InsPs.

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To ensure that such mutations did not alter the subcellular localization of the mutated versions, which is critical for function, we examined the subcellular localization of the VPT1PBC mutant protein. After crossing the VPT1eGFP-expressing line with a VPT1PBCmCherry-expressing line, we examined the fluorescent signals in the F1 seedlings and found that the eGFP and mCherry fluorescent signals overlapped exactly, suggesting that VPT1PBC, like the wild-type VPT1, was localized in the tonoplast (Supplemental Figure 4).

We then measured the Pi transport activities of VPT1 versus VPT1PBC using a patch-clamp recording of whole vacuoles. As shown in Figure 1C, overexpression of VPT1 but not VPT1PBC enhanced the currents from N. benthamiana vacuoles.Shown are representative whole-vacuole Pi current density traces. (D) The current-voltage curves were derived from whole-vacuole Pi currents as in (C). Results are means ± standard deviation from three independent experiments, n = 12.

To identify such an inhibitory domain within the VPT1 protein, we first analyzed the structure of VPT1. Previous study indicated that VPT1 consists of two major domains, the N-terminal SPX and the C-terminal MFS (Liu et al., 2015). As shown in Supplemental Figure 5A, MFS is the transmembrane transporter domain, while SPX domain is harbored in a long

The N-terminal region is responsible for auto-inhibition of VPT1 activity

The activities of many transporters are often inhibited by a structural domain and activated by conformational changes as a result of covalent modifications and/or binding of ligands (Lee and Boron, 2018; Siddiqui et al., 2019; Xie et al., 2020; Rodenas et al., 2021). Such a regulatory mechanism enables the transporters to rapidly respond to changes in external conditions by a signaling process. In the case of VPT1, we showed earlier that its function was evident under high Pi but not under low Pi conditions (Figure 1A and 1B). This indicated that VPT1 was inhibited by an auto-inhibitory domain under low Pi conditions.
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cyttoplasmic region that may contribute as a regulatory entity, and more specifically may contribute to the auto-inhibition of VPT1. Using Iterative Threading ASSEMBly Refinement (I-TASSER; Yang et al., 2015), an online structure prediction service, we identified eight α helices in the long N-terminal region (Supplemental Figure 5A). The first six α helices constituted the SPX domain and the seventh and eighth α helices are not defined previously (Figure 2A). We then predicted the structure of full-length VPT1 protein. As shown in Figure 2B, the α helices of the MFS domain are annularly arranged to form the channel pore. The SPX domain and seventh and eighth helices may contribute to two conformational states of VPT1. In the first VPT1 conformation (VPT1con.1), the SPX domain and seventh and eighth helices are adjacent to the side wall of the channel domain, allowing permeability. In the second VPT1 conformation (VPT1con.2), the SPX domain and seventh and eighth helices block the channel pore, representing an inhibition state. When the SPX and seventh and eighth helices are all deleted, as presented in DN protein, the channel should be fully open (Figure 2A and 2B).

To test this model, we made a construct to delete the entire N-terminal region (DN protein) and examined the Pi transport activity of this truncated VPT1. After confirming the vacuolar localization of DN (Supplemental Figure 5B), we measured the transport activity of DN protein using patch-clamp recording. The Pi currents recorded from DN-expressing vacuoles were much larger than those from the vacuoles expressing VPT1PBC, the version defective in ligand binding (Figure 2C and 2D). The deletion of the cytoplasmic N-terminal region (in the DN version) activated Pi transport activity of VPT1 despite loss of the ligand-binding domain, suggesting that ligand-binding and autoinhibition of VPT1 may be connected and both are located in the N-terminal region. Furthermore, DN protein was also more active than the wild-type VPT1, suggesting that the removal of the N-terminal region made VPT1 hyperactive (Figure 2C and 2D).

We showed that ligand (InsPs) binding sites in the SPX domain are important to VPT1 activity and the deletion of the N-terminus containing the SPX domain activates transport (Figures 1 and 2). This can be explained by the possibility that the SPX domain may function as an auto-inhibitory structure. To test this model, we made two deletions in the SPX domain of VPT1PBC. In the first deletion (D1 protein), we removed the first four α helices where the PBC sites are located. In the second deletion (D2 protein), we truncated the entire SPX domain (Supplemental Figure 6A). We then tested whether these deletions affected the subcellular localization of VPT1. With the wild-type VPT1 protein as a tonoplast marker, we found that the D1 and D2 deletions did not alter the subcellular localization of VPT1 (Supplemental Figure 6B). Subsequently, we tested the activity of D1 and D2 through patch-clamp recording of Pi currents across isolated vacuoles from Nicotiana benthamiana leaves expressing different VPT1 proteins. The data illustrated that those vacuoles expressing wild-type VPT1 generated large Pi currents, but Pi currents from D1- or D2-expressing vacuoles were as small as the negative control (VPT1PBC), displaying low activities (Figure 3A and 3B). Furthermore, we performed a complementation assay using a vpt1-mutant background expressing either wild-type or truncated versions of the VPT1 protein. Our data suggested that, unlike the VPT1 protein, D1 and D2 did not complement the phenotype of vpt1 mutant plants (Figure 3C). Consistently, overexpression of VPT1 in the vpt1 mutant robustly increased the Pi content in the vacuole, whereas the overexpression of D1 or D2 did not upregulate the Pi content in vacuoles of vpt1 mutant (Figure 3D). We further analyzed the structure of D1 and D2 in silico and found that the

**Figure 2. Deletion of the N-terminal domain renders high activity of VPT1.**

(A) Schematic structure of VPT1. The red letters Y23, K27, and K130 are PBC sites. DN protein is from truncation of 229 amino acids in N-terminal of VPT1.

(B) Predicted structures of VPT1 and DN. The protein structures were predicted online at https://zhanggroup.org/I-TASSER (Yang et al., 2015). Two rationally high scored structures of VPT1 were presented (VPT1con.1 and VPT1con.2). The white parts represent SPX domain; yellow parts represent the α7 and α8 helices; white double arrows indicate channel holes.

(C) Representative Pi current density traces from vacuoles isolated from N. benthamiana overexpressing truncated VPT1PBC.

(D) Current-voltage curves of steady state currents recorded as in (C). Symbols and error bars denote mean and standard error; n = 9 for all.
The channel-forming domain was covered by the remaining α helices (Figure 3E). Thus, deletion of the SPX domain in the D1 or D2 protein was not sufficient to remove the inhibition. Taken together, these data suggested that removal of the SPX domain did not activate the transport activity of VPT1PBC. The entire N-terminal region, including the SPX domain and the seventh and eighth α helices, might be responsible for inhibition of VPT1, and we thus named this N-terminal region an “inhibitory domain” (ID) (Figure 2A).

To further test the function of DN protein, we generated Arabidopsis transgenic plants overexpressing DN. We found that DN-overexpressing seedlings were necrotic and wilted under sufficient (130 μM) Pi condition (Figure 4A). This was not expected if DN showed higher activity to sequester Pi into the vacuole. We then measured the Pi content in the plants and found that DN-overexpressing seedlings accumulated nearly three-fold more Pi than WT seedlings under Pi-sufficient condition (130 μM), higher than the Pi content in VPT1-overexpressing seedlings (Figure 4B). If DN-overexpressing seedlings overaccumulate Pi into the vacuole, it may cause a decrease in cytosolic Pi. To test this possibility, we performed a quantitative PCR analysis of Pi starvation response (PSR) genes, including PHT1;1, PHT1;4, miR399b, and IPS1 in the WT and various transgenic lines. Indeed, expression levels of these PSR genes were highest in DN overexpressors, followed by VPT1-overexpressing seedlings and WT plants. These data implied that DN-overexpressing cells contained a lower level of cytosolic Pi (Figure 4C), and this conclusion was validated by assays using plants expressing a Pi sensor (cpFLiPi-5.3 m) (Mukherjee et al., 2015; Luan et al., 2019). However, lower cytosolic Pi levels may not account for cell death phenotype in DN plants. We further isolated intact vacuoles of WT and transgenic plants for Pi content measurement and found that DN vacuoles contained at least twice as much Pi than VPT1-overexpressing vacuoles (Figure 4F). Taken together, these results suggest that the Pi toxicity-like phenotype of DN plants may result from excessive vacuolar Pi accumulation, which may affect the integrity and stability of the vacuole membrane, and that this transduction is mediated by an auto-inhibitory Pi sensor-transporter.
that DN plants contained much more Pi than WT (Figure 4B), this of this region constitutively activated VPT1. The ID region of VPT1 are responsible for inhibition and that the deletion together with the seventh and eighth statuses. Taken together, we concluded that the SPX domain constitutively and highly activated under both low and high Pi deficiency in the cytoplasm, especially when external Pi is limited. All these results imply that the Pi transport activity of DN is severe growth retardation and accumulated more plants were hypersensitive to deficient Pi. DN seedlings (white and yellow-colored parts in Figure 2B) seem to directly display severe growth retardation and accumulated more anthocyanidin as compared with WT or VPT1-overexpressing seedlings (Figure 4F). However, DN plants were hypersensitive to deficient Pi. DN seedlings displayed severe growth retardation and accumulated more anthocyanidin as compared with WT or VPT1-overexpressing seedlings under a 2-\textmu M Pi condition (Figure 4A and 4G). Given that DN plants contained much more Pi than WT (Figure 4B), this phenotype also supports the idea that the DN protein constantly interact with the MFS domain to block the channel pore in the closed conformation. In the open conformation, however, the SPX domain seems to contact the side of channel forming domain, bending the entire N-terminal region away from the channel pore. If ligand binding is required for the formation of this open conformation, the SPX domain may bind ligands and in turn physically interacts with MFS domain that would contribute to the release of ID from the pore (Figure 2B). We then tested the hypothesis that direct interaction of the SPX domain with the MFS domain may require ligand binding for the activation of the VPT1. We conducted the protein-protein interaction assay using a cCFP/nVenus system transiently expressed in N. Benthamiana leaves. Constructs containing truncated VPT1 proteins were built as shown in Figure 5A. VPT1-1, VPT1-4, and VPT1-1PBC proteins were tested for their interaction with DN (MFS domain) by co-expression in N. Benthamiana leaves. As shown in Figure 5B, VPT1-1PBC proteins were tested for their interaction with DN (MFS domain) by co-expression in N. Benthamiana leaves. As shown in Figure 5B, VPT1-1PBC or VPT1-1PBC interacted with the DN protein. We tested the SPX/MFS interactions using an alternative procedure, the yeast split-ubiquitin system (Figure 5C), and the results were consistent with the data from the BIFC assay. As the level of InsPs fluctuates depending on cellular Pi status (Wild et al., 2016; Dong et al., 2019), we further tested the interaction between SPX domain and MFS will, in turn, lead to cell death. We further examined Pi accumulation in seedlings grown under very low Pi condition (2 \textmu M). Interestingly, the DN-overexpressing seedlings retained much more Pi than WT or VPT1-overexpressing seedlings (Figure 4F). However, DN plants were hypersensitive to deficient Pi. DN seedlings displayed severe growth retardation and accumulated more anthocyanidin as compared with WT or VPT1-overexpressing seedlings under a 2-\textmu M Pi condition (Figure 4A and 4G). Given that DN plants contained much more Pi than WT (Figure 4B), this phenotype also supports the idea that the DN protein constantly transfer cytosolic Pi into the vacuole, contributing to severe Pi deficiency in the cytoplasm, especially when external Pi is limited. All these results imply that the Pi transport activity of DN is constitutively and highly activated under both low and high Pi statuses. Taken together, we concluded that the SPX domain together with the seventh and eighth \alpha helices in the N-terminal region of VPT1 are responsible for inhibition and that the deletion of this region constitutively activated VPT1. SPX domain physically interacts with the transmembrane domains to activate VPT1 The predicted VPT1 protein structures indicated that the ID region (white and yellow-colored parts in Figure 2B) seem to directly interact with the MFS domain to block the channel pore in the closed conformation. In the open conformation, however, the SPX domain seems to contact the side of channel forming domain, bending the entire N-terminal region away from the channel pore. If ligand binding is required for the formation of this open conformation, the SPX domain may bind ligands and in turn physically interacts with MFS domain that would contribute to the release of ID from the pore (Figure 2B). 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Figure 4. Overexpression of DN contributes to the hypersensitivities to high and low Pi. (A) DN overexpressing seedlings are hypersensitive to high (130 \mu M) and low (2 \mu M) Pi conditions. Red arrows indicate necrotic leaves; bar, 2 cm. (B) Pi contents of WT and different overexpressing Arabidopsis lines under various Pi conditions. Five DAG old seedlings are transferred to various Pi conditions and cultured for another 12 days, then the seedlings were used for Pi content measurements. (C) The expression levels of representative PSR genes in various genotype seedlings. WT seedlings were used as control, and the values were normalized to UBQ10. (D) Pi sensor (cpFLIPPi-5.3 m) (Mukherjee et al., 2015) was used for detecting cytosolic Pi concentration in various genotypes, including WT, OV1-1, DN-1, and DN-2 Arabidopsis seedlings. We crossed the Pi sensor Arabidopsis seedling with the variable transgenic lines for cytoplasm Pi detection. The F1 seedlings (5 DAG) were cultured in hydroponic solution with 130 \mu M Pi for 2 days and the leaves were collected for fluorescence detection. The fluorescence ratio of FRET/CFP indicates cytosolic Pi concentration, and the representative FRET ratio images were presented. Bars, 25 \mu M. Pseudocolor was used to display the FRET/CFP ratio according to the color scale bar at bottom right in (D). (E) The FRET/CFP ratio index analyses were performed using Leica software (LASAFWPF). (F) Vacuolar Pi contents of various Arabidopsis seedlings grown under 130 \mu M Pi as in (A). (G) The anthocyanin contents of various plants grown in 2 \mu M Pi as in (A). From (B) to (G), different letters above each bar under the same Pi condition indicate statistically significant differences (p \leq 0.01, Tukey’s honestly significant difference test). Error bars indicate ± standard deviation; n = 3 technical replicates × 4 biological replicates. FW = fresh weight.
domain under different Pi condition. As shown in Figure 5D, the SPX/MFS (VPT1a1-a6/DN) interaction was abolished as the plants were transferred to the Pi-deficient medium, but such interactions remained robust under sufficient Pi. Therefore, levels of InsPs may alter the interaction between the SPX and MFS domains.

In the closed conformation of VPT1, the α7-8 domain may also physically interact with MFS domain. We made constructs containing VPT1α7-α8, VPT1α5-α8, VPT1α1-α8, and VPT1PBCα1-α8 proteins fused with indicated fluorescent proteins and tested their interaction with the DN protein (Supplemental Figure 7A). The results indicated that all four of these proteins interacted with DN (Supplemental Figure 7B). Additionally, VPT1α1-α8 constitutively interacted with DN under different Pi conditions (Supplemental Figure 7C). Thus, we proposed that the α7-8 domain may directly interact with the DN to block the Pi permeability of VPT1, whereas the SPX domain (with ligand binding) physically interact with DN to pull the ID away from the transport path and activate VPT1 transport.

A previous study in yeast revealed that, while mutations of PBC residues inhibit polyP synthesis activity of the VTC proteins, substitutions at a K residue (K126/129 in VTC3/4) near the PBC sites contribute to the constitutive activation of polyP synthesis into a vacuole even in the absence of InsPs signaling molecules (Wild et al., 2016). We expected that mutations of the conserved K site in the SPX domain of VPT1 may activate its transport activity without ligand binding sites. Through sequence alignment, the K site of VPT1 was found to be the K126 in the fourth α helix of the SPX domain (Supplemental Figure 8A). We generated a K126A mutation in the VPT1PBC protein, a mutant version of VPT1 that lacks ligand binding and transport activity (Figure 1 and Supplemental Figure 3). We referred to this new mutant version of VPT1 as VPT1PBCK126A. After confirming the vacuolar localization of the VPT1PBC K126A protein (Supplemental Figure 8B), we tested whether the K126A mutation could recover transport activity of VPT1PBC using a whole vacuole patch-clamp recording. The Pi current density traces recorded from vacuoles overexpressing VPT1PBCK126A was much higher than that from VPT1PBC-overexpressing...
vacuoles (Figure 6A and 6B), similar to that from VPT1 overexpressing vacuoles, indicating that K126A restored Pi transport activity of VPT1PBC.

To examine whether the K126A-mediated activation of VPT1PBC may result from the recovery of its ligand binding, we cloned and purified the SPX domain from VPT1PBCK126A and conducted a ligand-binding assay using MST procedure. The data suggested that the SPX domain of VPT1PBCK126A (SPX PBCK126A) bound InsP6 with a $K_d$ of 106.2 μM, which was comparable with the SPX protein (Supplemental Figure 8C and 8D), indicating that the K126A mutation did not enhance ligand binding of the VPT1PBC protein.

To further examine the function of the VPT1PBCK126A protein in planta, we generated VPT1PBCK126A-overexpressing Arabidopsis seedlings and conducted Pi content measurement. The data shown in Figure 6C indicate that the overexpression of VPT1PBCK126A enhanced Pi accumulation in Arabidopsis. Moreover, VPT1PBCK126A overexpressors, similar to DN plants, accumulated more Pi, even under low Pi conditions (Figure 6C), leading to hypersensitivity to deficient Pi (Supplemental Figure 8G and 8H), consistent with a constitutive vacuolar Pi influx transport activity of VPT1PBCK126A. However, unlike DN overexpressors, the VPT1PBCK126A overexpressors did not show the Pi toxicity-like phenotype under sufficient Pi condition (Supplemental Figure 8G), consistent with less Pi accumulation as compared with DN plants when Pi is sufficient (Figures 4B and 6C). These data suggest that K126A in the SPX domain is a gain-of-function mutation for VPT1 activation in a Pi-independent manner, consistent with the mechanism underlying signal-independent activation of SPX proteins in yeast (Wild et al., 2016).

We proposed that the SPX domain interacts with MFS domain for VPT1 activation. As K126A mutation recovered the transport activity of VPT1PBC protein, it may do so by acquiring a ligand-free interaction between the SPXPBC domain and the MFS domain. Indeed, we detected a strong interaction between VPT1PBCK126A and DN (Figure 6D and 6E), which supports the conclusion that K126A mutation activates VPT1PBC through restoring the SPX/MFS interaction, indicating that K126 is a key residue for the activated state of VPT1.

**DISCUSSION**

Vacuoles are the primary intracellular compartments for Pi storage in plants. Transport of Pi across vacuolar membrane plays a pivotal role in buffering the cytoplasmic Pi level against the changing external Pi supply and metabolic activities (Yang et al., 2017). However, it remains unclear how a vacuole senses the cellular Pi status and activates Pi sequestration in response to an elevated Pi. Our study here revealed that VPT1 (also named PHT5; 1), the major vacuolar Pi influx transporter, may sense the cellular Pi status by its SPX domain, which in turn interacts with and activates the transport domain to initiate vacuolar Pi sequestration. Indeed, the mutation of ligand-binding sites in the SPX domain dramatically impaired the transport activity of VPT1, whereas removal of the entire N-terminal domain constitutively activated the transport. We, therefore, conclude that the N-terminal region serves as an auto-inhibitory domain that keeps VPT1 in an
inactive state under low Pi conditions. Elevated Pi produces InsPs that bind to SPX domain and release the transport activity of VPT1. This may provide a critical mechanism for coupling Pi status to vacuolar sequestration and thus maintaining Pi homeostasis in plant cells.

Recent studies suggest that the InsPs-SPX signaling module may be evolutionarily conserved mechanism for Pi sensing across eukaryotes (Wild et al., 2016; Dong et al., 2019; Zhu et al., 2019; Zhou et al., 2021; Guan et al., 2022). For the metabolism of InsP_8, the major Pi signaling molecule in plants, two VIH kinases catalyze reversible reactions to synthesize and hydrolyze InsP_8, depending on the cellular Pi status (Zhu et al., 2019; Riemer et al., 2021; Whitfield et al., 2020). When the cellular Pi status is high as cells are supplied with sufficient Pi, the VIH kinases phosphorylate InsP_7 to generate InsP_8. In contrast, VIHs display phosphatase activity for InsP_8 hydrolysis when the cellular Pi level decreases upon Pi starvation (Zhu et al., 2019; Riemer et al., 2021; Whitfield et al., 2020). When the cellular Pi status is high as cells are supplied with sufficient Pi, the VIH kinases phosphorylate InsP_7 to generate InsP_8. In contrast, VIHs display phosphatase activity for InsP_8 hydrolysis when the cellular Pi level decreases upon Pi starvation (Zhu et al., 2019; Riemer et al., 2021; Whitfield et al., 2020). Thus, InsP_8 abundance reflects the cellular Pi status in plants. The InsP_8 molecules directly bind to SPX domains to regulate multiple components for governing Pi homeostasis. For example, InsP_8 synthesis promotes the formation of a PHR-SPX complex to block PHR function and suppresses the expression of PSR genes (Dong et al., 2019). In parallel, VPT1 perceives InsP_8 through SPX domain and, thus, activates transport activity for the storage of excessive Pi into vacuole (this study). Under Pi-deficient conditions, the decreased cellular Pi initiates a decrease in InsP_8 levels, resulting in the release of PHR1 from SPX1 to activate PSR genes, including those encoding high-affinity Pi transporters for efficient Pi uptake. Meanwhile, low InsP_8 levels would lock the VPT1 transporter in the auto-inhibitory state and execute low transport activity, leaving more Pi in the cytosol for metabolic use.

Although VPT1 is identified as the major vacuolar Pi import transporter that contains a SPX domain, the regulation mechanism of its transport activity remains unknown until this study that identified VPT1 as a sensor-transporter molecular switch on the vacuole that can sense Pi status and translate that into transport activity. According to the current data, we propose that SPX domain may bind InsP_8 to facilitate a structural conformational change of VPT1 and hence its activity. As shown in Figure 7, under sufficient cellular Pi status, the interaction between SPX and MFS domains is induced by InsP_8 signals, and the ID (including SPX domain and seventh and eighth α helices) would be pulled away from the channel pore of VPT1 for its permeability. When cellular Pi is deficient, limited InsP_8-binding abolishes the interaction of SPX/MFS and contributes to the blockage of the Pi path by ID region that represents an inhibition state of VPT1, and that also occurred in a PBC mutation version of VPT1. Truncation of the SPX domain cannot switch on VPT1, whereas, decapitation of the whole ID region results in constitutively enhanced transport activity of VPT1 (E). Mutation at the K126 leads to the constitutive interaction of SPX/MFS in a ligand-binding independent manner, thereby restoring the transport activity of VPT1 protein.

![Figure 7: Proposed models deciphering the auto-regulatory mechanism of VPT1 activity.](image)

We propose that SPX domain may bind InsPs-type molecules (InsP_8 here) to facilitate a structural conformational change of VPT1 and hence its activity. (A) InsP_8 mediates the interaction between SPX and MFS domains under sufficient condition, and the ID (including SPX domain and seventh and eighth α helices here) will be pulled away from the channel pore of VPT1 for its permeability. (B and C) When cellular Pi is deficient, limited InsP_8-binding abolishes the interaction of SPX/MFS and contributes to the blockage of the Pi path by ID region that represents an inhibition state of VPT1, and that also occurred in a PBC mutation version of VPT1. (D) Truncation of the SPX domain cannot switch on VPT1, whereas, decapitation of the whole ID region results in constitutively enhanced transport activity of VPT1 (E). (F) Mutation at the K126 leads to the constitutive interaction of SPX/MFS in a ligand-binding independent manner, thereby restoring the transport activity of VPT1 protein.
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the channel pore (model D), consistent with the findings that deletion or mutation of SPX domain suppresses the transport activity of Pi transporters, as described in this and previous studies (Wild et al., 2016; Potapenko et al., 2018). Nevertheless, decapitation of the whole ID region consisting of eight alpha helices results in constitutively and extremely high transport activity of VPT1 (model E). Finally, we found K126 is the key residue that, when mutated, can render a gain-of-function effect for the ligand-independent SPX/MFS interaction, thereby restoring the transport activity of VPT1〈PBC protein (model F). This finding may also explain why substitutions at K126/129 in VTC3/4 in VTC3/4 lead to constitutive polyP synthesis into the vacuole in the absence of InsPs signals in yeast (Wild et al., 2016). Thus, the interaction of SPX with other functional domains (such as VTC and MFS) may be evolutionarily conserved among eukaryotes for the activation of SPX-containing proteins. These models should also be applicable to VPT-type proteins in other eukaryotes besides plants.

Interestingly, patch-clamp and genetic assays suggested that transport activity of DN was higher than the wild-type VPT1 and VPT1〈PBC K126A (Figures 2D and 6B). The Pi toxicity-like phenotype occurred in DN-overexpressing seedlings, but not in VPT1 or VPT1〈PBC K126A-overexpressing transgenic lines when Pi is sufficient (Figure 4A and Supplementary Figure 8G), indicating that the N-terminal region of VPT1 may harbor more than one regulatory mechanism. Furthermore, these data also imply that the overaccumulation of Pi in the vacuole may affect the integrity and stability of the vacuole, leading to cell death and necrosis in DN overexpressing plants. This vacuolar Pi toxicity may be different from the cytoplasmic Pi toxicity that typically occurred in the vpt1 single mutant (Figure 1E). Thus, the activity of plant vacuolar Pi influx transporters may be fine tuned by multiple mechanisms, including intracellular Pi status, as shown in this study and possibly by post-translational modifications, such as phosphorylation, which requires further investigation in the future.

VPT-type transporters play prominent physiological roles in Pi homeostasis in plants. Our previous work demonstrated that lack of VPTs impairs Pi accumulation in vacuoles under replete Pi conditions and, thus, compromises their ability to cope with deficient Pi (Liu et al., 2015; Luan et al., 2019). Given that Pi levels in most farm land soils are limited and constantly changing, manipulating the Pi transport activity of VPTs may enhance the adaptability of crops to variable Pi conditions for sustainable production. Moreover, VPTs, through their function in vacuolar Pi sequestration, contribute to fine tune the systemic Pi homeostasis during the reproductive stage (Luan et al., 2019). Loss of function of VPT1 and VPT3 leads to more Pi allocation into the reproductive organs, providing a new way to control the long-distance transport of Pi through regulating vacuole transporters. Recent studies revealed that the downregulation of Pi accumulation into the grain would contribute to decreasing phytate in seeds and enhanced grain filling for rice production (Yamaji et al., 2017; Ma et al., 2021). Therefore, maintaining a low content of Pi in the reproductive organ is important, especially in crops. Genetically engineering the transport activities of VPT-type proteins in crops may provide a new solution for low-Pi-containing grains because of their roles in systemic Pi distribution, which should be explored in the future work.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (Col-0) seedlings were used in this research. The vpt1-1 (SAIL_96_H01) T-DNA insertion mutant was obtained from the Arabidopsis Stock Center. The hydroponic culture solutions were prepared as previously described (Liu et al., 2016a). The tobacco used in the study is N. benthamiana. The Pi sensor genetic seedlings (cpFLPiPi-S.3 m) were kindly provided by Prof. Wayne Versaw. The stable transgenic Arabidopsis lines were used for phenotyping and Pi content measurements. The transient expression in N. benthamiana were mainly used for subcellular localization assay and patch-clamp assays. The stable transgenic Arabidopsis lines were selected depend on the expression of VPT1 that were valued by western blot (Supplementary Figure 9). All the plant materials were grown under normal light conditions (90 μmol/m²/s) with a long day cycle (16 h light/8 h dark) at 22°C.

Plasmid constructs

The cDNA sequences of wild-type VPT1 and N-terminal sequential deletion series including D1 (Δ163), D2(Δ178), DN(Δ229) were cloned (Trans-Start FastPfu DNA Polymerase, AP221) into a modified pCAMBIA1300 vector with an mCherry marker. VPT1〈PBC and VPT1〈PBC K126A sequences were generated from the wild-type VPT1 cDNA through PCR-based site-directed mutagenesis and cloned into the modified pCAMBIA1300 vector. The SPX domain sequences of wild-type VPT1, VPT1〈PBC, and VPT1〈PBC K126A were cloned into pET28a vector for protein expression. For the BIFC assay, different parts of VPT1 sequences were cloned into modified pCAMBIA1300 vectors with a cCFP or nVenus tag. All vectors were constructed through homologous recombination system. Primers for vector constructs are listed in supplementary Table 1.

Subcellular localization analyses

Wild-type VPT1-GFP and various truncated VPT1 mCherry constructs were transformed into GV3101 agrobacterium tumefaciens strain. One colony was selected from each transformation strain and cultured in liquid YEB medium at 28°C overnight. The mid-exponential phase cells of each strain were collected and resuspended to an OD600 of 1.0 in infiltration buffer (10 mM MgCl₂, 10 mM MES, 0.2 μM acetosyringone, pH 5.8). We mixed a VPT1-eGFP strain with different truncated VPT1 mCherry strain with equal volumes, then the mixers were infiltrated in 4-week-old N. benthamiana leaves. After 2 days in culture, the transformed leaves were spotted for fluorescent signal detection with a confocal laser scanning microscope (Leica TCS SP8). For excitation wavelengths, 488 nm is for GFP and 587 nm is for mCherry.

Vacuole extraction

The vacuole extraction procedure followed a previous method with modification (Robert et al., 2007). Briefly, modified protoplast enzyme solution (1% cellulase, 1% macerozyme, 25 mM CaCl₂, 0.4 M mannitol, 100 μM NaH₂PO₄, 10 mM MES, pH 5.6) was used to disrupt protoplasts. Then the purified protoplasts were corrected for vacuole isolation as previously described (Robert et al., 2007) with small modifications. The number and purity of vacuoles were monitored under the light microscopy.

Immunoblot

Briefly, plant materials were homogenated and lysed in a protein extraction buffer containing 50 mM HEPES (NaOH, pH 7.5), 150 mM NaCl, 50 mM β-glycerophosphate, 2 mM DTT, 1% Triton X-100 and 10% glycerol, with EDTA free protease inhibitor cocktails tablet for three hours. The 120 μl extracted proteins were eluted by adding 30 μl SDS-PAGE loading buffer and boiled for 5 min. The VPT1 proteins were detected by immunoblotting using anti-GFP or anti-mCherry antibodies, respectively.

Purification of fusion proteins in Escherichia coli

We induced 6×His-tagged SPX〈PBC, SPX〈PBC K126A, and SPX〈PBC K126A. Proteins by 0.5 mM isopropyl-β-D-thiogalactoside in E. coli strain BL21

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(De) and purified the proteins by a standard procedure. Briefly, bacteria were lysed in lysis buffer containing 50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 8 M urea, 0.05% Tween 20, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail, and His-fusion proteins were purified using Ni-NTA Magnetic Agarose Beads (Qiagen). Then the purified proteins were refolded in a renaturation buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, pH 8.0).

Microscale thermophoresis assay

Binding of InsP3 by different SPX domains was measured by MST as previously described (Lata et al., 2006; Wild et al., 2016). We pre-incubated 60 nM protein with 60 nM trisNTA-OG488 on ice for 5×His tag staining in 1 mL renaturation buffer (50 mM Tris-Cl, 250 mM NaCl, 1 mM DTT, 10% glycerol, pH 8.0). Then the stained proteins were mixed with the serial dilutions of ligand in the same renaturation buffer. The reaction mixture was loaded into Monolith NT Premium Coated Capillaries (NanoTemper Technologies GmbH) and thermophoresis was measured using a Monolith NT.115 instrument (NanoTemper Technologies GmbH) at an ambient temperature with 5 s/30 s/5 s laser off/on/off times, respectively. The MST laser power was set to 30%. No ligand-dependent changes in the fluorescence signal were observed.

Transport activity assay in N. benthamiana

Wild-type or truncated VPT1m Cherry constructs were transformed into GV3101 strain for transient expression. Four-week-old N. benthamiana leaves were selected for infiltration. At 36 hours after infiltration, the transformed leaves were spotted for fluorescent signal checking for determination of the expression levels of various VPT1 proteins. Then the seedlings were further cultured under sufficient Pi condition (130 μM) for another 2 or 3 days; thereafter, the transformed leaves were harvested for Pi contents measurements.

Measurements of Pi contents

Arabidopsis seedlings grown in the hydroponic culture system were collected and washed three times in distilled water. We used 50 mg of each tissue samples or 10° extracted vacuoles from each genotype seedlings for Pi content measurement following the ascorbate-molybdate-antimony method (John, 1970).

Patch-clamp recordings on isolated vacuoles

Patch-clamp recordings follow the protocol previously described (Liu et al., 2015). The mesophyll vacuoles were isolated from N. benthamiana and Arabidopsis plants grown in hydroponic culture solution. The recording was performed with the Axon Multiclamp 700B Amplifier (Molecular Devices), and the current-voltage relationships were expressed as current density (pA/pF).

BifC experiments

The indicated pairs of expression constructs were co-transformed into N. benthamiana leaves through infiltration transfection method. The fluorescent signals were detected with excitation wavelength 488 nm.

Yeast two hybrid assays

Yeast two hybrid analysis was performed using a DUAL membrane kit3 (Du-alysystems Biotech) according to the manufacturer’s instructions. The coding sequences of MFS domain (DN) and truncated/mutated VPT1 proteins (VPT1s, VPT1sD, VPT1PBC, VPT1K126A, VPT1K126A58) were cloned into the vectors pBTN3 and pPR3-N, respectively. The vectors were transformed into the yeast strain Y2HGold. Interactions were shown on SD/-Leu/-Trp and SD/-Leu/-Trp/-His medium supplemented with 2 mM 3-AT.

Statistical analysis

The data in this study are averages from at least three independent experiments, and the values were subjected to statistical analysis through ANOVA followed by the Student’s t-test or Tukey’s honestly significant difference test.

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

M.L. and S.L. designed the research, M.L., F.Z., and G.S. carried out the experiments. M.L. and S.L. analyzed the data. M.X., A.F., and W.L. provided technical assistance. M.L. and S.L. wrote the manuscript.

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Sequence data for genes and proteins presented in this article can be found in the Arabidopsis Genome Initiative of GenBank/EMBL database under the following accession number: VPT1 (AT1G63010), PHT1;1 (AT5G43350), PHT1;4 (AT2G38940), IPS1 (AT3G09922), mIR399b (AT1G63005), and UBO10 (AT4G05320).
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