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REVIEW PAPER

# Role of vacuoles in phosphorus storage and remobilization

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## Abstract

**Vacuoles play a fundamental role in storage and remobilization of various nutrients, including phosphorus (P), an essential element for cell growth and development. Cells acquire P primarily in the form of inorganic orthophosphate (Pi). However, the form of P stored in vacuoles varies by organism and tissue. Algae and yeast store polyphosphates (polyPs), whereas plants store Pi and inositol phosphates (InsPs) in vegetative tissues and seeds, respectively. In this review, we summarize how vacuolar P molecules are stored and reallocated and how these processes are regulated and co-ordinated. The roles of SYG1/PHO81/XPR1 (SPX)-domain-containing membrane proteins in allocating vacuolar P are outlined. We also highlight the importance of vacuolar P in buffering the cytoplasmic Pi concentration to maintain cellular homeostasis when the external P supply fluctuates, and present additional roles for vacuolar polyP and InsP besides being a P reserve. Furthermore, we discuss the possibility of alternative pathways to recycle Pi from other P metabolites in vacuoles. Finally, future perspectives for researching this topic and its potential application in agriculture are proposed.**

**Key words:** Autophagy, inositol phosphate, orthophosphate, phosphate transporter, phosphorus, polyphosphate, SPX domain, vacuole.

## Introduction

Phosphorus (P) is essential for all living organisms. It is the structural element of nucleic acids and phospholipids, and is also required for energy metabolism, signaling transduction cascades, and protein modification. Inorganic orthophosphate ($PO_4^{3-}$ or Pi) is the major form of P taken up by most organisms, but its availability in the environment is often a limiting factor for the growth of algae and plants (Smith *et al.*, 2003; Juneja *et al.*, 2013). For example, the free Pi content in soil solution is low, ranging from 1 µM to 10 µM, because it is easily chelated by cations or converted into organic phosphate by microbial immobilization (Holford, 1997). However, the concentration of Pi in cells must be maintained within the millimolar range to sustain basic cellular function (Bieleski, 1973; Ticconi and Abel, 2004). As a consequence, organisms use multiple strategies to absorb Pi efficiently from the environment and store and remobilize Pi inside cells.

In fungal and plant cells, vacuoles play crucial roles in nutrient storage and recycling, detoxification, and maintenance of turgor pressure (Klionsky *et al.*, 1990; Marty, 1999; Martinoia *et al.*, 2012). Vacuoles are the storage compartments for a wide range of molecules such as amino acids, ions, heavy metals, proteins, carbohydrates, secondary metabolites, and signaling molecules. In addition, vacuoles are the major sites for macromolecule degradation because most of the hydrolytic activities are localized inside vacuoles, which indicates a role for vacuoles in nutrient recycling. Thus, vacuoles serve as safeguards to maintain the cytosolic homeostasis of various metabolites.

Vacuoles are the well-documented primary intracellular compartments for P storage and remobilization (Bieleski, 1973; Marty, 1999; Martinoia *et al.*, 2012). The main storage forms of P include Pi, inositol phosphate (InsP), and polyphosphate (polyP), depending on the organism and tissue. In this review, we summarize the critical role of vacuoles in storage and remobilization of P in different organisms, the importance of vacuolar P storage and remobilization, and the corresponding molecular components mediating these processes.

## Vacuoles as a major storage compartment of P

The storage forms of P in vacuoles vary in different organisms and tissues. In prokaryotes, fungi, and algae, inorganic polyP, a linear chain of three to thousands of Pi residues linked by high-energy phosphoanhydride bonds, is the main storage form of P inside vacuoles. An early study detected 40% of acid-soluble P compounds, largely present as polyP, in the isolated vacuolar fraction in the yeast *Saccharomyces carlsbergensis* (Indge, 1968). In *Saccharomyces cerevisiae*, most if not all of the polyP is located in isolated vacuoles, and the level of polyP inside cells could reach 120 mM (Urech *et al.*, 1978; Kornberg, 1999). In arbuscular mycorrhizal fungi, transmission electron microscopy and energy dispersive X-ray analysis found electron-dense granules containing a high level of P and calcium similar to the polyP granules within vacuoles (White and Brown, 1979). Unicellular algal cells, *Chlamydomonas eugametos*, for example, also store polyP inside vacuoles (Siderius *et al.*, 1996).

In higher plants, Pi is the major storage form of P inside vacuoles of vegetative cells. Under sufficient Pi supply, ~70% to 95% of the intracellular Pi is stored in vacuoles (Bieleski, 1973). $^{31}$P-NMR analysis clearly demonstrated that most of the cellular Pi is present inside vacuoles and its concentration can fluctuate according to the external Pi supply (Rebeille *et al.*, 1983; Brodelius and Vogel, 1985; Mathieu *et al.*, 1989). In plant seeds, P is predominantly stored in specialized protein storage vacuoles as phytate rather than Pi. Upon germination, phytate is hydrolyzed to release Pi and inositol to support seedling growth (Bewley and Black, 1978).

Methods such as $^{31}$P-NMR, X-ray microanalysis, non-aqueous fractionation, and isolation of intact vacuoles have been used to measure Pi content inside vacuoles and/or other subcellular compartments of plants (Kanno *et al.*, 2016). *In vivo* $^{31}$P-NMR analysis is commonly used because it can trace the dynamic changes of Pi and other P-containing compounds in intact living organisms. However, it is limited by its inability to differentiate Pi clearly in subcellular compartments having similar pH conditions (e.g. vacuolar Pi versus apoplastic Pi and cytosolic Pi versus organellar Pi). In addition, it is expensive and the experimental set-up is complicated. When coupled with electron microscopy, X-ray microanalysis of different emission spectra can be used to resolve and quantify many elements, including P, in different subcellular compartments (Macklon *et al.*, 1996; Shane *et al.*, 2004). Non-aqueous fractionation can be used to separate different metabolites or subcellular solutes into different subcellular fractions after centrifugation (Dietz and Heber, 1984), but cross-contamination of Pi among different compartments and the specificity of subcellular markers are concerns (Mimura *et al.*, 1990). Isolated intact vacuoles can be used to provide direct measurement of vacuolar P (Cocking, 1960), albeit with possible perturbation during isolation. Nonetheless, the results from different analyses all agree that vacuoles are the primary intracellular compartments for P. Vacuolar Pi concentrations are estimated to range from micromolar to millimolar depending on the extracellular Pi concentrations. However, the exact Pi concentration in the cytosol is still controversial.

In the following sections, we discuss the storage and remobilization of different forms of vacuolar P and their regulation. The pathways are outlined and presented in Fig. 1.

## Polyphosphate: a major storage P in vacuoles of fungi and algae

PolyP synthesis, transport, and utilization have been well characterized in the model yeast, *S. cerevisiae*. The yeast vacuolar transporter chaperone (VTC) complex, consisting of Vtc1, Vtc2, Vtc3, and Vtc4 (also referred as Phm4, Phm1, Phm2, and Phm3, respectively), was found to be essential for vacuole membrane fusion, V-ATPase stability, membrane trafficking, and microautophagy before their function as polyP polymerases was confirmed (Cohen *et al.*, 1999; Müller et al., 2002, 2003; Uttenweiler *et al.*, 2007). Whether the VTC complex participates in these processes directly or as a result of polyP accumulation is unclear. When wild-type yeast accumulated a huge amount of polyP in vacuoles under high Pi supply following Pi starvation, *vtc1* and *vtc4* single mutants showed completely abolished polyP accumulation, and loss of both Vtc2 and Vtc3 also significantly reduced total polyP accumulation (Ogawa *et al.*, 2000). Recently, Vtc5 was found to interact with the VTC complex, and deletion of Vtc5 also reduced polyP accumulation, so Vtc5 may be a new subunit of the VTC complex (Desfougères *et al.*, 2016). The five members of the VTC complex all possess three transmembrane helixes. Except for Vtc1, they have two hydrophilic N-terminal domains facing the cytosol: the N-terminal SYG1/PHO81/XPR1 (SPX) domain and the central domain (Müller *et al.*, 2003; Desfougères *et al.*, 2016).

The exact function of the VTC complex was resolved by X-ray crystallography, which revealed that Vtc4 is the catalytic component to synthesize polyP, and Vtc1/2/3 are the accessory partners probably regulating polyP synthesis or transfer (Hothorn *et al.*, 2009; Gerasimaitė et al., 2014). The central domain but not the SPX domain of Vtc4 creates a tunnel and is required for the catalytic activity of polyP synthesis by using ATP as a substrate. The catalytic activity of the VTC complex requires metal ions (with $Mn^{2+}$ most effective) and is highly stimulated by pyrophosphate (PPi) and moderately by Pi and inorganic triphosphate. PPi is speculated to be the primer to initiate synthesis of the polyP chain (Hothorn *et al.*, 2009). After synthesis, polyP is subsequently translocated into the vacuole lumen by the VTC complex. PolyP synthesis is tightly coupled with its transport into the lumen and depends on the proton gradient established by

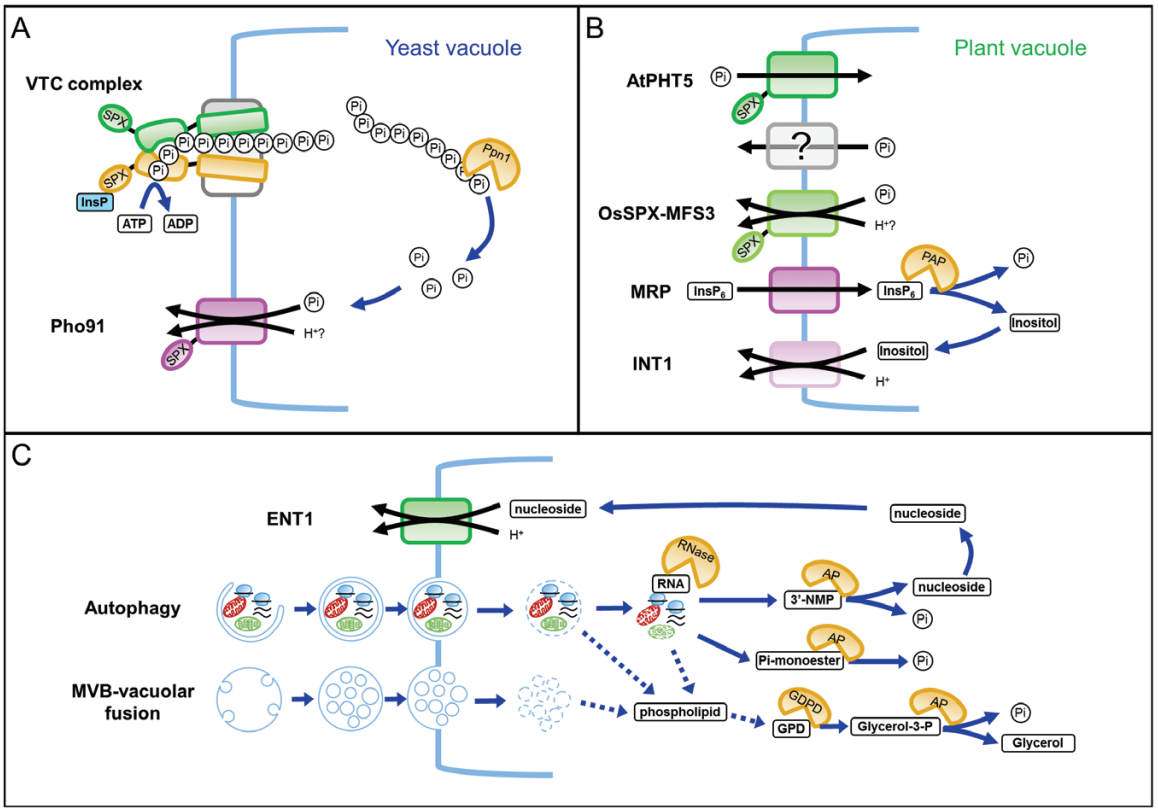


**Fig. 1.** Storage and remobilization of vacuolar phosphorus (P). (A) In yeast, polyP is synthesized and transported into vacuoles via the vacuolar transporter chaperone (VTC) complex. Pi is released from the hydrolysis of polyP by the polyphosphatase Ppn1 and subsequently exported out of the vacuole by the Pho91 Pi transporter. Inositol phosphates (InsPs) or inositol pyrophosphates (PP-InsPs) may regulate the activity of VTC complexes by their interaction with the SYG1/PHO81/XPR1 (SPX) domain. (B) In plant vacuoles, AtPHT5 mediates Pi influx in Arabidopsis, whereas OsSPX-MFS3 mediates Pi efflux in rice. In seeds, storage of *myo*-inositol hexakisphosphates ($InsP_6$) inside vacuoles is mediated by multidrug resistance-associated protein (MRP)-type ABC transporters. During seed germination, $InsP_6$ is hydrolyzed by purple acid phosphatases (PAPs) into Pi and inositol, which are released into the cytosol by Pi transporters and inositol transporters (presumably INT1), respectively. An additional unidentified Pi export system is expected and indicated. (C) Potential pathways for recycling P from RNA or phospholipids via autophagy and multivesicular body (MVB)–vacuolar fusion are shown. Inside the vacuoles, RNA is degraded by RNase, then phosphatases (AP, yeast alkaline phosphatase or plant acid phosphatase) to release Pi and nucleosides, which are exported by Pi transporters and equilibrative nucleoside transporter 1 (ENT1), respectively. Pi is also liberated from phospholipid catabolism via a series of enzymatic reactions. 3'-NMP, 3'-nucleotide monophosphate; GPD, glycerophosphodiester; GDPD, glycerophosphodiester phosphodiesterase.

V-ATPase (Gerasimaitė *et al.*, 2014). Of note, homologs of Vtc proteins are found only in protists, fungi, and algae, and are absent in plants and animals (Secco *et al.*, 2012).

Acidocalcisomes, also known as polyP granules, are the acidic electron-dense specialized vacuoles rich in polyP complexed with calcium and other cations present in both prokaryotes and eukaryotes (Wiame, 1947; Docampo *et al.*, 2010; Docampo and Huang, 2016). Likewise, the VTC complex plays an important role in accumulation of polyP inside acidocalcisomes. In the green algae *Chlamydomonas reinhardii*, loss of Vtc1 affects formation and viability of the acidocalcisome under sulfur deprivation and post-sulfur recovery, respectively, thereby suggesting a role for Vtc proteins in acidocalcisome formation and adaptation to nutrient stress in algae (Aksoy *et al.*, 2014).

Two types of VTC subcomplexes occur with different localizations. Vtc4/Vtc3/Vtc1 is found on the vacuolar membrane. Vtc4/Vtc2/Vtc1 is located in the endoplasmic reticulum (ER) and nuclear envelope, but relocates to the vacuole upon Pi starvation (Gerasimaitė and Mayer, 2016). The presence of different subcomplexes might be a strategy to regulate multiple polyP pools with distinct functions. In addition, the Vtc genes in yeast and the diatom *Thalassiosira pseudonana* are up-regulated in response to limited Pi (Ogawa *et al.*, 2000; Dyhrman *et al.*, 2012).

Diphosphoinositol phosphates, PP-InsPs (commonly called inositol pyrophosphates), which contain one or more highly energetic diphospho moieties on InsPs, regulate polyP synthesis. Mutations in PP-InsPs synthesis, such as phospholipase C (Δ*plc1*), inositol polyphosphate multikinase (Δ*arg82*), and *myo*-inositol hexakisphosphate ($InsP_6$) kinase (Δ*kcs1*), significantly reduce intracellular polyP levels (Auesukaree *et al.*, 2005). This finding was further confirmed by a study showing that *kcs1* mutation, with ablated PP-$InsP_4$ or PP-$InsP_5$ synthesis, blocked polyP synthesis (Lonetti *et al.*, 2011). Thus, PP-InsPs and polyP are metabolically linked. The target of the polyP synthesis system that is under PP-InsP regulation has yet to be identified, but a recent study involving crystallography and biochemical analyses demonstrated a strong binding affinity (at the submicromolar range) of PP-InsP for the SPX domains of various proteins across species, including yeast Vtc4 and Vct2 subunits (Wild *et al.*, 2016).

Inside vacuoles, polyP is digested by polyphosphatase Ppn1 (also called Phm5), which is induced by Pi starvation (Ogawa

et al., 2000). Deletion of Ppn1 does not strongly affect the level of polyP but causes the accumulation of longer polyP chains, reduced cytosolic Pi content, and subtle defects in the Pi starvation signaling pathway (*PHO* regulon) (Ogawa et al., 2000; Sethuraman et al., 2001; Auesukaree et al., 2004; Thomas and O'Shea, 2005). Ppn1 was discovered as an endopolyphosphatase which can hydrolyze long polyP chains to yield Pi and triphosphate as the end-products, and its activity requires metal ions such as $Mn^{2+}$ and $Mg^{2+}$ (Kumble and Kornberg, 1996). Later on its exopolyphosphatase activity was also revealed (Andreeva et al., 2006). The Pi hydrolyzed from polyP is subsequently exported from the vacuole to the cytosol. The low-affinity Pi transporter Pho91 localized on tonoplasts is thought to mediate this activity (Hürlimann et al., 2007), although its transport activity has not been revealed. Like Vtc2/3/4/5, Pho91 contains an N-terminal SPX domain, but whether PP-InsPs also regulate the activity of Pho91 via the SPX domain remains to be studied.

In animals, in addition to serving as an energy source, polyP can be delivered by blood platelets and act as a signaling molecule to amplify energy production in mitochondria or trigger metabolic pathways in different cell types (Wang et al., 2016). The diverse roles of polyP await discovery.

## Orthophosphate: a major storage P in vacuoles of vegetative plant cells

Unlike in yeast, higher plants store Pi inside vacuoles, the same form as acquired (Bieleski, 1973). Because Pi is the most abundant P molecule in vacuoles of vegetative plant cells, where its concentration can change quickly in response to fluctuating external Pi (Rebeille et al., 1983, 1985; Brodelius and Vogel, 1985; Mimura et al., 1990), a Pi transport machinery that mediates the efflux and/or influx of Pi across the tonoplast of plant cells has long been speculated. However, the molecular identity of a Pi transport system was not discovered until recently.

In *Arabidopsis thaliana*, members of the PHT5 family were demonstrated to be the Pi transporters responsible for vacuolar Pi storage and essential for Pi adaptation (Liu et al., 2015; Liu et al., 2016). The members are localized to the tonoplast and possess an SPX domain and a major facilitator superfamily (MFS) transporter domain at the N- and C-termini, respectively. Among three members in the family, PHT5;1 (also called VPT1) plays a predominant role. Disruption of *PHT5;1* reduced the Pi content, and overexpression of any one of the PHT5 members augmented Pi accumulation. *In vivo* $^{31}$P-NMR analysis further revealed that the altered Pi accumulation in these plants resulted from the change in Pi content inside the vacuole (Liu et al., 2016). Patch-clamp analysis of isolated vacuoles suggested that PHT5;1 mediates vacuolar influx of Pi depending on external Pi concentrations (Liu et al., 2015). Transport kinetics further suggested that PHT5;1 is a channel-like transporter (Liu et al., 2015). In addition, Pi uptake activity was detected in yeast vacuoles expressing a rice homolog of PHT5, OsSPX-MFS1 (Liu et al., 2016). The activity was independent of ATP and $H^+$, and was probably mediated by facilitated diffusion along the electrochemical gradient, which agrees with the channel-like property of PHT5;1. An ATP-independent Pi influx activity into the vacuoles isolated from Pi-replete barley leaves was also reported (Mimura et al., 1990). However, the Pi influx activity of vacuoles isolated from suspension-cultured cells of *Catharanthus roseus* was stimulated by ATP and pyrophosphate (Massonneau et al., 2000), suggesting the requirement for a proton motive force. Reasons for causing this discrepancy are currently unclear, but may be partly due to the differences in cell property and growth conditions. Nevertheless, how Pi transport into vacuoles is energized and how it is regulated certainly require further studies.

Because OsSPX-MFS1 possesses Pi influx activity when expressed in yeast vacuoles and also complements the low Pi phenotype of Arabidopsis *pht5;1* mutants, the activity of SPX-MFS homologs and PHT5;1 may be similar. However, one rice homolog, OsSPX-MFS3, was proposed to mediate Pi efflux out of the vacuole rather than influx (Wang et al., 2015). Heterologous expression of OsSPX-MFS3 at the plasma membrane of *Xenopus* oocytes showed Pi uptake activity, analogous to vacuolar Pi efflux activity, with no Pi-dependent electric current detected (Wang et al., 2015). Therefore, the Pi efflux out of the vacuole mediated by OsSPX-MFS3 may be coupled with $H^+$, thereby resulting in electroneutral flux. Of note, a Pi-dependent inward current was detected under high pH conditions when oocytes were pre-loaded with Pi, which hints at the potential of OsSPX-MFS3 to transport Pi bi-directionally. In contrast to the overexpression of PHT5 in Arabidopsis, overexpression of OsSPX-MFS3 results in a reduced Pi level in the rice vacuole (Wang et al., 2015), which suggests its Pi efflux activity out of the vacuole *in planta*. Because Arabidopsis and rice SPX-MFS homologs share 68–72% amino acid identity, what contributes to their functional differences in Pi export versus Pi import is intriguing. Phylogenetic analysis revealed that the eudicot and monocot SPX-MFS homologs fall into two different clades, so the monocot and eudicot *SPX-MFS* genes may have evolved independently and diverged before their speciation (Liu et al., 2016). Functional characterization of the orthologs among plant species and paralogs within a species should provide further insights.

The SPX-MFS members show different transcriptional levels in response to changes in Pi supply. Under Pi starvation, the transcript level of *OsSPX-MFS1* and *OsSPX-MFS3* is reduced, whereas that of *OsSPX-MFS2* is induced (Lin et al., 2010; Wang et al., 2012). In Arabidopsis, the *PHT5;1* transcript is expressed primarily in younger tissues under Pi sufficiency but strongly induced by Pi starvation in older tissues (Liu et al., 2015). Under Pi starvation, the transcript level of *PHT5;1* is increased and that of *PHT5;3* reduced in roots; both levels remain relatively constant in shoots (Liu et al., 2016). The expression of *PHT5;2* is very low and present in guard cells, pollen, and root vascular tissue (Liu et al., 2016). Thus, SPX-MFS proteins might play distinct roles in different tissues and developmental stages in response to a change in Pi availability.

In rice, *OsSPX-MFS1* and *OsSPX-MFS2* transcripts are the targets of miR827, a Pi starvation-induced miRNA (Lin et al., 2010). In Pi-starved rice, up-regulation of miR827 is

associated with down-regulation of *OsSPX-MFS1*. However, *OsSPX-MFS2* is up-regulated by Pi starvation, which suggests the involvement of other regulatory mechanisms in addition to miR827. In Arabidopsis, miR827 is induced by Pi starvation, but it primarily cleaves the transcripts of *NITROGEN LIMITATION ADAPTATION* (*NLA*), and its cleavage on the *PHT5;1* transcript is much less effective (W.-Y. Lin, L-C. Hsieh, Y.-Y. Lin, and T.-J. Chiou, unpublished data). NLA functions to regulate the degradation of plasma membrane-localized Pi transporter PHT1 proteins by ubiquitination-mediated endocytosis (Lin *et al.*, 2013). How miR827 target sites evolved in different genes and regulate Pi activities at different membrane compartments is fascinating. Moreover, Arabidopsis *PHT5;1* and *OsSPX-MFS2* have several transcript variants with different lengths of their 5'-untranslated region, some lacking the miR827 target site. Differential targeting of miR827 may fine-tune the regulation of Pi homeostasis.

## Phytic acid: a major storage P in vacuoles of plant seeds

InsPs present in eukaryotic organisms are a group of metabolites with various numbers of Pi groups covalently bound to a *myo*-inositol molecule. In plant seeds, the predominant InsP inside vacuoles is $InsP_6$, also known as phytic acid (PA), or phytate (mixed salts of $InsP_6$ with cations such as $Ca^{2+}$, $Fe^{3+}$, $K^+$, $Mg^{2+}$, $Mn^{2+}$, and $Zn^{2+}$). Phytate commonly represents a substantial proportion of seed dry weight and up to 80% of total P in seeds (Raboy, 2009). Plants utilize a set of evolutionarily conserved enzymes for PA synthesis (Suzuki *et al.*, 2007; Raboy, 2009; Kim and Tai, 2011). PA synthesis uses the 6-carbon *myo*-inositol as a backbone, which can be acquired externally (Klepek *et al.*, 2005; Schneider *et al.*, 2007) or synthesized *de novo* via conversion of glucose-6-phosphate to D-inositol-3-phosphate mediated by D-*myo*-inositol-3-phosphate synthases (MIPSs) in plants (Loewus and Loewus, 1983). According to the proponents of a lipid-dependent pathway to PA (Raboy, 2001), the latter step of PA synthesis involves sequential phosphorylation of $Ins(1,4,5)P_3$ released from phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] by phospholipase C. Several, rarely cited, early studies proposed pathways to PA not involving $PI(4,5)P_2$ (Asada *et al.*, 1969; Mandal and Biswas, 1970; Bollmann *et al.*, 1980), results supported by later studies of PA synthesis in duckweed (Brearley and Hanke, 1996*b*, *c*). In addition to PA, a number of PP-InsPs of unknown stereoisomerism have been recently described in Arabidopsis (Desai *et al.*, 2014; Laha *et al.*, 2015), confirming much earlier work in guard cells of *Commelina communis* (Parmar and Brearley, 1993) and barley aleurone (Brearley and Hanke, 1996*a*).

PA biosynthesis initiates shortly after flowering, and the acid accumulates during development until seed maturation and desiccation (Asada *et al.*, 1969; Raboy and Dickinson, 1987). Although the site where phytate is synthesized and accumulated in seeds varies among plant species, it is predominantly present in the embryo (dicots and maize) and endosperm cells (aleurone cells of cereal grains such as wheat, barley, and rice) (O'Dell *et al.*, 1972), where it is mainly stored in protein storage vacuoles as electron-dense inclusions called globoids (Pfeffer, 1872; Lott and Ockenden, 1986; Raboy, 1997). The intracellular compartments where PA synthesis occurs in plants are probably the cytoplasm and nucleus, as implied by the subcellular localization of several InsP kinases (Xia *et al.*, 2003; Kuo *et al.*, 2014; Zhan *et al.*, 2015). PA may be synthesized in the cytoplasm in association with the ER and accumulated initially on the ER cisternae before being transported into the developing protein bodies following the same path as storage protein (Greenwood and Bewley, 1984; Lott *et al.*, 1995). A multidrug resistance-associated protein (MRP) belonging to the ATP-binding cassette ABC transporter family was identified to mediate PA transport into vacuoles (Nagy *et al.*, 2009), and its loss-of-function mutation caused significantly reduced seed phytate content in maize, soybean, and Arabidopsis (Raboy *et al.*, 2000; Shi *et al.*, 2007; Nagy *et al.*, 2009; Becker *et al.*, 2014). AtMRP5, now known as AtABCC5, and its plant homologs are the only InsP transporters identified in any kingdom, though vectorial transport of InsPs to the extracellular matrix is implied by accumulation of massive quantities of $InsP_6$ in the parasitic cestode *Echinococcus granulosus* (Irigoín *et al.*, 2004; Casaravilla *et al.*, 2006).

During germination, phytate is hydrolyzed sequentially by phytase(s) to release various minerals, Pi, and a series of lower phosphoric esters of *myo*-inositol (Bewley and Black, 1978; Raboy, 1997; Loewus and Murthy, 2000). Phytase is a class of phosphatases that initiate the dephosphorylation of PA at different positions on the inositol ring and produce different isomers of the lower InsP (Lim and Tate, 1971; Greiner *et al.*, 2002). In plants, phosphatases known to display phytase activity include histidine acid phosphatase and purple acid phosphatase (PAP) (Mullaney and Ullah, 2007). Phytase activity increases during germination (Graf *et al.*, 1987; Egli *et al.*, 2002; Greiner, 2007). Roles of gibberellic acid in controlling phytate degradation during germination of cereals have been investigated (Eastwood and Laidman, 1971), but its effects on the association between phytate degradation and phytate-degrading activity among different plant species are inconclusive (Greiner, 2007). In addition, Pi was reported to inhibit the activities of phytate-degrading enzymes *in vitro* and to repress their transcription (Sartirana and Bianchetti, 1967; Eastwood and Laidman, 1971; Konietzny and Greiner, 2002). While the Pi released from phytate is presumably recycled back to the cytosol by an unidentified Pi transporter, a tonoplast-localized *myo*-inositol exporter (INOSITOL TRANSPORTER 1; INT1) identified in Arabidopsis, which is higly expressed during seed germination, and its orthologs are likely to be involved in recycling inositol yielded from full dephosphorylation of PA in seeds (Schneider *et al.*, 2008).

## Recycling of organic P via alternative pathways

In addition to polyP, Pi, and InsPs, P can be recycled from other organic P of metabolites (monoester- or diester-bound P)

in vacuoles, as suggested by the localization of phosphatases inside vacuoles (Kaneko *et al.*, 1982; Veljanovski *et al.*, 2006; Hurley *et al.*, 2010). Furthermore, the expression or activity of many vacuolar phosphatases is induced in Pi-starved cells, and their hydrolytic activity is strongly inhibited by Pi *in vitro* (Veljanovski *et al.*, 2006; Hurley *et al.*, 2010), which demonstrates the importance of vacuoles in scavenging and remobilizing organic P. For example, vacuolar-localized PAP26 is up-regulated in Pi-starved Arabidopsis (Veljanovski *et al.*, 2006), and loss of PAP26 delays leaf senescence and impairs P remobilization (Robinson *et al.*, 2012). Although a cargo for delivery of organic P into vacuoles probably exists, its role in P recycling remains largely elusive. Here, we take RNA catabolism as an example and discuss this possibility.

RNA comprises up to 60% of organic P in the cell (Klionsky and Emr, 1989; Shane *et al.*, 2014), which potentially can be an important source of P during Pi deprivation (Lambers *et al.*, 2015). Vacuoles are considered one of the subcellular compartments for RNA degradtion. RNase and its activity were found in the vacuole of fungi and plants (Abel and Glund, 1986; Unno *et al.*, 2005). An estimated 70–80% of RNase activity resides inside plant vacuoles (Abel and Glund, 1987), and the activity is increased by Pi starvation (Löffler *et al.*, 1992). Consistently, RNA oligonucleotides representing the degradative intemediates of RNA were detected in purified vacuoles of tomato cells (Abel *et al.*, 1990). Nucleosides and Pi are liberated from RNA by the activities of both RNase and acid phosphatase. The released Pi can be recycled to the cytosol, and nucleosides are presumably exported by equilibrative nucleoside transporter 1 (ENT1) (Bernard *et al.*, 2011).

The delivery of RNA into vacuoles is believed to be via autophagy (Hillwig *et al.*, 2011; Floyd *et al.*, 2015), an intracellular self-degradative process that engulfs cytoplasmic materials (e.g. misfolded proteins or damaged organelles) into double membrane-bound vesicles (autophagosomes) for delivery to vacuoles or lysosomes for degradation (Klionsky and Emr, 2000; Mizushima, 2007). Autophagy has a survival and also a housekeeping role in the balance and re-allocation of energy sources during cell development and in response to nutrient stresses. Previous studies in plants and yeast cells showed that rRNA is degraded by an autophagy-like process, termed ribophagy, which involves a selective or non-selective turnover of ribosomes or ribosome–RNA complexes (Thumm *et al.*, 1994; Kraft *et al.*, 2008; Huang *et al.*, 2015). Inside yeast vacuoles, RNA is first processed by a T2-type RNase, Rny1, into 3'-nucleotide monophosphate, followed by the phosphatase Pho8 to release nucleosides and Pi (Huang *et al.*, 2015). Rny1- and Pho8-dependent RNA turnover is induced by nitrogen starvation (Huang *et al.*, 2015), but its role in Pi starvation is unclear. Autophagy-dependent RNA degradation was also observed in plants (Hillwig *et al.*, 2011; Floyd *et al.*, 2015). Arabidopsis RNS2, an RNase T2 enzyme, has a similar role to yeast Rny1. Loss of RNS2 activity results in a longer half-life of rRNA, increased total RNA amount, and enhanced formation of autophagosomes under normal growth conditions (Floyd *et al.*, 2015), which underlines the requirement of autophagy and RNS2 activity for rRNA turnover. Although Pi is released from RNA catabolism in vacuoles, the contribution of such a Pi pool to the cytoplasmic Pi metabolism and its biological relevance remain to be studied. The autophagy-mediated degradation of other organelles, such as mitochondria and chloroplasts, was also observed (Minibayeva *et al.*, 2012; Ishida *et al.*, 2014); yet its role in Pi recycling is not known.

The vacuole is also an active place for vesicle trafficking, and phospholipids derived from internalized membrane vesicles are expected to be decomposed inside vacuoles. Phospholipids are first hydrolyzed into fatty acids and glycerophosphodiester (GPD) by lipase. GPD is degraded by glycerophosphodiester phosphodiesterase (GDPD) to generate glycerol 3-phosphate which is further hydrolyzed by acid phosphatase to release Pi. Plant GDPD activities were identified in the cell wall and vacuoles, and the overall GDPD activity was found to be induced by Pi starvation (van der Rest *et al.*, 2002, 2004). However, to what extent phospholipid catabolism in vacuoles contributes to recycling Pi is unknown.

Of note, long-term Pi starvation triggers senescence, which promotes Pi recycling and remobilization. Genes responding to Pi starvation and senescence considerably overlap in expression (Stigter and Plaxton, 2015), including genes coding for RNase and acid phosphatases, which suggests a crosstalk between senescence and Pi-deficient regulatory networks. During senescence, release of RNase and PAP enzymes from the breakdown of tonoplasts may be important for scavenging Pi from organic P substances in the cytosol (Robinson *et al.*, 2012).

## Significance of storage and remobilization of vacuolar P

The major role of vacuolar P is to buffer the cytoplasmic Pi concentration to maintain cellular homeostasis when the external Pi supply fluctuates. Under Pi starvation, wild-type yeast cells continue to grow for 2–3 generations. The cytosolic Pi level remains constant, but the vacuolar P pool decreases significantly. In contrast, *slp1* (*small lysine pool*) mutants with no detectable vacuolar compartments stop growing immediately under limited Pi (Shirahama *et al.*, 1996). These results suggest that yeast vacuoles could be a Pi reservoir and mobilize Pi into the cytosol to sustain cell growth during Pi starvation stress. In addition, vacuoles have a role in buffering the cytoplasmic Pi level in plants. When *Acer pseudoplatanus* cells were transferred to Pi-deficient or -sufficient medium, the cytoplasmic Pi content remained constant (Rebeille *et al.*, 1983). Pi-starved *Catharanthus roseus* cells fed Pi showed a strong accumulation of Pi in the vacuole after a short transient increase in cytoplasmic Pi content (Mathieu *et al.*, 1989). In barley mesophyll cells grown under different Pi supply, the cytoplasmic Pi concentration was regulated within a narrow range, whereas the vacuolar Pi concentration varied widely (Mimura *et al.*, 1990). Pi uptake by *C. roseus* cells did not show an obvious feedback control to avoid Pi toxicity, which highlighted the importance of the vacuole in maintaining cytoplasmic Pi homeostasis (Sakano *et al.*, 1995).

Manipulating the expression of tonoplast Pi transporters would indeed affect the relatively constant cytoplasmic Pi level. For example, the level is decreased in the Arabidopsis *pht5;1-2 pht5;2 pht5;3* triple mutant and increased in *PHT5;2* overexpression lines. In mutants lacking PHT5;1, cytoplasmic Pi was accumulated to a toxic level on re-feeding with Pi after starvation because of failure to sequester surplus Pi into vacuoles; in contrast, the mutants are susceptible to Pi deficiency due to shortage of P stored in vacuoles (Liu *et al.*, 2015; Liu *et al.*, 2016). Moreover, altered PHT5 expression disturbs the expression of many Pi starvation-responsive genes (Liu *et al.*, 2016), which suggests that vacuolar P storage can regulate Pi starvation responses by modulating cellular Pi status. When yeast cells are transferred to low Pi medium, polyP synthesis and import to the vacuole are activated because of the up-regulation of Vtc genes and increased VTC complex abundance in the tonoplast (Hothorn *et al.*, 2009; Vardi *et al.*, 2014). This fast incorporation of Pi into the storage compartment may be adopted to maintain a low cytosolic Pi level to sustain the Pi starvation responses.

Vacuolar polyP has additional roles other than being a P reservoir. It plays a role in osmoregulation by reducing the osmotic pressure of free Pi and osmotic activity of vacuolar amino acids. Vacuolar polyP could also retain amino acids and cations (Klionsky *et al.*, 1990; Kornberg, 1999). For example, $Ca^{2+}$ chelated in yeast vacuoles acts as a $Ca^{2+}$ sink (Dunn *et al.*, 1994), and polyP accumulation contributes to $Mn^{2+}$ detoxification (Andreeva *et al.*, 2013). In addition, polyP could be the energy source and a substitute for ATP (Kornberg, 1999). Proton released by the hydrolysis of polyP inside algal vacuoles could neutralize amines under stressed alkaline pH conditions (Pick and Weiss, 1991). Acidocalcisomes also have functions in osmoregulation, autophagy, and calcium signaling, and interact with the contractile vacuole complex in eukaryotic microbes (Docampo *et al.*, 2010; Docampo and Huang, 2016). Vacuolar polyP accumulation may be associated with the pathogenesis of the parasitic protozoan *Trypanosoma brucei* and biotrophic fungus *Ustilago maydis* because defects in their Vtc4 weakened their virulence (Boyce *et al.*, 2006; Lander *et al.*, 2013).

In addition to the provision of P, phytate is considered to furnish inositol and metallic monovalent and divalent cations for seed germination (Bewley and Black, 1978). Phytate can also function as an antioxidant to protect seeds against iron-mediated oxidative reactions (Graf *et al.*, 1987; Doria *et al.*, 2009), and its synthesis is co-ordinated to maintain the Pi homeostasis required for cellular metabolism, such as starch biosynthesis in developing seeds (Matheson and Strother, 1969; Strother, 1980; Raboy and Dickinson, 1987; Iwai *et al.*, 2012). Besides being synthesized in seeds, PA is also synthesized in vegetative tissues, such as roots, stems, and leaves (Roberts and Loewus, 1968; Bollmann *et al.*, 1980; Campbell *et al.*, 1991; Brearley and Hanke, 1996*c*), but at a much lower level (at micromolar concentrations) (Alkarawi and Zotz, 2014; Hadi Alkarawi and Zotz, 2014; Phillippy *et al.*, 2015), for a questionable role as a storage form of P during vegetative growth. However, a wealth of studies demonstrate that PA and its synthetic intermediates/derivatives play important roles in a number of cellular, developmental, and signaling pathways required for plant growth and reproduction (Carland and Nelson, 2004; Gillaspy, 2011; Kuo *et al.*, 2014; Williams *et al.*, 2015; Zhan *et al.*, 2015). PA *per se* is involved in multiple biological functions in plants, such as facilitating mRNA export, controlling stomatal aperture by remobilizing endoplasmic $Ca^{2+}$, and acting as a structural cofactor of auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Lemtiri-Chlieh *et al.*, 2003; Tan *et al.*, 2007; Lee *et al.*, 2015). Defects in transporting PA into the vacuoles in vegetative tissues caused aberrant stomatal aperture and auxin-mediated root phenotypes (Gaedeke *et al.*, 2001; Klein *et al.*, 2003; Suh *et al.*, 2007; Nagy *et al.*, 2009), which suggests that vacuolar compartmentalization plays an important role in sequestration of the cytoplasmic signaling roles of PA.

## Perspectives for future studies

The recent discoveries of SPX-MFS proteins as vacuolar Pi transporters of plants (Liu *et al.*, 2015; Wang *et al.*, 2015; Liu *et al.*, 2016) has shed light on their crucial role in the adaptation of plants to Pi fluctuations and also opened up a new direction in exploring how plants modulate cellular Pi homeostasis. Since vacuoles serve as a dynamic temporary storage compartment, further identifying and characterizing the components involved in influx and efflux of Pi across the tonoplasts is needed. In addition, the role of autophagy as an alternative cellular mechanism in recycling Pi requires more attention. Finally, understanding how the expression and activity of these transport machineries are regulated in response to extracellular Pi and how they are co-ordinated spatially and temporally should be emphasized.

Pi *per se* has been shown to regulate transcriptional reprogramming during Pi starvation by modulating the activity of the transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1) in association with SPX-domain proteins (Puga *et al.*, 2014; Wang *et al.*, 2014). Whether Pi can directly regulate the activity of vacuolar P allocation is unknown, but emerging evidence has demonstrated the important roles of InsPs and PP-InsP in regulating Pi homeostasis via their interaction with multiple SPX-domain proteins associated with polyP synthesis, Pi transport, and Pi signaling (Wild *et al.*, 2016). These findings highlight an intricate co-ordination between cellular P homeostasis and the InsP metabolic pathway, which deserves more investigation.

To better understand the dynamics of cellular Pi homeostasis in the physiological context, we need non-destructive quantitative measurements or visualization of Pi and relevant organic P compounds (such as InsPs) in different subcellular compartments (i.e. cytosol, vacuole, and chloroplasts); hence, improving current methods or developing new techniques with high detection sensitivity is highly desired. The Pi biosensor of Fluorescence indicators for Pi (FLIPPi) was recently developed to monitor dynamic changes in Pi concentration in the cytosol or plastids of Arabidopsis roots (Mukherjee *et al.*, 2015; Banerjee *et al.*, 2016), and this method could be used to

monitor vacuolar Pi concentration in real time. In addition, Raman spectroscopy was used to detect PA in the aleuronic cell layer and to demonstrate the absence of polyP in wheat seeds (Kolozsvari *et al.*, 2015). Further development of these techniques is anticipated to provide visualization of PA or polyP in different cell types and organisms.

Because of the storage feature of vacuoles, an understanding of reallocation and recycling of vacuolar P has potential applications in agriculture. Modulation of these systems may help improve crop nutritional value and assist in crop adaptation to a low P environment. Indeed, low-PA seeds have been proposed as a means to provide better P nutrition for livestock and reduce environmental pollution (Raboy, 2001). Given the non-renewable nature of global P reserves, research on this topic will have significant impact on the development of sustainable agriculture.

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