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Long Wang[1,2,6], Xianqing Jia[1,6], Yuxin Zhang[1], Lei Xu[1], Benoit Menand[3], Hongyu Zhao[1], Houqing Zeng[4], Liam Dolan[5], Yiyong Zhu[2,*] and Keke Yi[1,*]

[1]Key Laboratory of Plant Nutrition and Fertilizers, Ministry of Agriculture and Rural Affairs, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[2]Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing 210095, China

[3]Aix Marseille Univ, CEA, CNRS, BIAM, Luminy Plant Genetics and Biophysics Team, Marseille 13009, France

[4]College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China

[5]Gregor Mendel Institute of Molecular Plant Biology GmbH, Dr. Bohr-Gasse 3, 1030 Vienna, Austria

[6]These authors contributed equally to this article.

***Correspondence: Yiyong Zhu (yiyong1973@njau.edu.cn), Keke Yi (yikeke@gmail.com)**

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ABSTRACT

Phosphorus is an essential nutrient for plants. It is stored as inorganic phosphate (Pi) in the vacuoles of land plants but as inorganic polyphosphate (polyP) in chlorophyte algae. Although it is recognized that the SPX-Major Facilitator Superfamily (MFS) and VPE proteins are responsible for Pi influx and efflux, respectively, across the tonoplast in land plants, the mechanisms that underlie polyP homeostasis and the transition of phosphorus storage forms during the evolution of green plants remain unclear. In this study, we showed that *CrPTC1*, encoding a protein with both SPX and SLC (permease solute carrier 13) domains for Pi transport, and *CrVTC4*, encoding a protein with both SPX and vacuolar transporter chaperone (VTC) domains for polyP synthesis, are required for vacuolar polyP accumulation in the chlorophyte *Chlamydomonas reinhardtii*. Phylogenetic analysis showed that the SPX-SLC, SPX-VTC, and SPX-MFS proteins were present in the common ancestor of green plants (Viridiplantae). The SPX-SLC and SPX-VTC proteins are conserved among species that store phosphorus as vacuolar polyP and absent from genomes of plants that store phosphorus as vacuolar Pi. By contrast, SPX-MFS genes are present in the genomes of streptophytes that store phosphorus as Pi in the vacuoles. These results suggest that loss of SPX-SLC and SPX-VTC genes and functional conservation of SPX-MFS proteins during the evolution of streptophytes accompanied the change from ancestral polyP storage to Pi storage.

Key words: *Chlamydomonas*, polyphosphate, SPX-SLC, SPX-VTC, vacuolar phosphate, plant evolution

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INTRODUCTION

Phosphorus is a finite non-renewable resource essential for life on earth. It is a critical component of nucleic acids, phospholipids, and ATP, and participates in numerous biochemical pathways, including gene expression and signal transduction. Consequently, phosphorus deficiency can impair a wide range of biological processes, ultimately affecting plant growth and development (Raghothama, 1999). To cope with the stress caused by phosphorus deficiency, green plants have evolved adaptive mechanisms for phosphorus acquisition and conservation that enhance survival where available phosphorus is limiting.

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Extant green plants (Viridiplantae) comprise two monophyletic lineages, Chlorophyta and Streptophyta (land plants and their closest algal relatives, a grade collectively known as streptophyte algae) (Becker and Marin, 2009). Vascular land plants take up phosphorus as the oxyanion phosphate (Pi) from soil through roots and associated fungal symbionts. However, nearly 70% of global arable land is deficient in Pi, and land plants have evolved multiple strategies to adapt to this stress. A class of proteins that exclusively contain an SPX (SYG1/Pho81/XPR1) domain (SPXs) are the main negative regulators of Pi signaling. They can modulate the activity of central transcription factors, the PHOSPHATE STARVATION RESPONSE (PHR) proteins, in plants (Lv et al., 2014; Puga et al., 2014; Wang et al., 2014). Other proteins with conserved SPX domains also regulate Pi transport and signaling. PHOSPHATE1 (PHO1), an SPX-EXS protein with both SPX and EXS (ERD1, XPR1, SYG1) domains, is a transporter required for Pi loading into root xylem vessels that facilitates Pi translocation from roots to shoots (Poirier et al., 1991; Hamburger et al., 2002). The SPX-MFS (major facilitator superfamily) proteins, which contain both SPX and MFS domains, transport Pi into the vacuole (Liu et al., 2015, 2016; Xu et al., 2019). A class of E3 ligases (NLAs) with both SPX and RING (Really Interesting Gene) domains are involved in the degradation of Phosphate Transporter 1 proteins (Park et al., 2014). Taken together, these lines of evidence demonstrate that SPX-domain-containing proteins are involved in phosphate transport and phosphorus signaling in land plants, but nothing is known about the function of SPX-containing proteins in algae.

In *Chlamydomonas reinhardtii* (Chlorophyta), phosphorus is stored in vacuoles in the form of dense, calcium-associated polymers of phosphate (polyP) bodies that are called acidocalcisomes (Ruiz et al., 2001). PolyP is a linear, unbranched polymer of three to several hundred Pi residues linked by phosphoanhydride bonds. PolyP has been observed in the vacuole of the red alga *Cyanidioschyzon merolae* and in a number of other chlorophyte algae (Yagisawa et al., 2009; Lorenzo-Orts et al., 2020). Vacuolar polyP is synthesized in the cytoplasm and transported into the vacuole by the VTC (vacuolar transporter chaperone) complex (Hothorn et al., 2009). Consistent with the role of the VTC complex in polyP synthesis, a *Chlamydomonas* mutant with a *VTC1* deletion accumulates less polyP and has fewer acidocalcisomes than the wild type (WT) (Aksoy et al., 2014). Vtc4p (a protein with both SPX and VTC domains), one of the VTC subunits, is a polyP polymerase in yeast (Hothorn et al., 2009), and its *Chlamydomonas* homolog (VTC4) was suggested to be required for polyP synthesis (Aksoy et al., 2014). However, the regulation of vacuolar polyP accumulation in chlorophyte algae remains unclear. Unlike chlorophytes, land plants store phosphorus as Pi in their vacuoles (Lorenzo-Orts et al., 2020). However, the evolution of vacuolar phosphorus storage forms in the Viridiplantae is still unknown.

To examine the evolution of vacuolar phosphorus storage forms in Viridiplantae, we showed that phosphorus is stored as polyphosphate (polyP) in chlorophyte vacuoles; furthermore, we characterized a new vacuolar Phosphate Transporter C in *C. reinhardtii* (termed CrPTC1), one of the previously uncharacterized SPX-SLC proteins, which has both SPX and SLC (permease solute carrier 13) domains. Our results suggest that CrPTC1 catalyzes Pi transport out of acidocalcisomes and is involved in polyP accumulation and Pi starvation-dependent signaling in *Chlamydomonas*. We further showed that CrVTC4, a protein with both SPX and VTC domains, is essential for polyP synthesis. We performed a phylogenetic analysis to identify which SPX domain-containing proteins were present in the last common ancestor of chlorophytes and streptophytes, which existed approximately 1200 million years ago (Nie et al., 2020). We showed that the SPX-SLC and SPX-VTC proteins are ancient and conserved in the genomes of plants that store phosphorus as vacuolar polyP. However, they are not present in the genomes of later-diverging streptophytes that store phosphorus as vacuolar Pi but not as polyP. The conservation of SPX-MFS and the loss of SPX-VTC and SPX-SLC in streptophyte algae accompanied the change in vacuolar phosphorus storage from polyP to Pi and may have been a pre-adaptation for land colonization.

RESULTS AND DISCUSSION

The *Chlamydomonas* SPX-SLC protein CrPTC1 is a Pi transporter involved in polyP storage

Given the function of proteins with SPX domains in Pi signaling and transport in land plants, we searched the *C. reinhardtii* genome for sequences that encoded proteins with SPX domains. One of these proteins, CrPTC1, also harbored an anion–permease SLC domain, suggesting that it may have a function in ion transport. Topology prediction showed that CrPTC1 had 14 transmembrane segments with an N-terminal SPX domain that was predicted to be located in the cytoplasm (Figure 1A). To test whether CrPTC1 transported Pi across membranes, we expressed *CrPTC1* in a yeast mutant strain (YP100) that is defective in Pi transport and does not grow unless supplemented with galactose (Figure 1B). *CrPTC1* restored growth on a medium that contained 20 mM Pi, where it grew almost as well as yeast that expressed the high-affinity Pi transporter PHO84. Some growth occurred on medium that contained 100 μM Pi, although it was less than that of yeast that expressed PHO84 (Figure 1B). The growth of YP100 transformed with *CrPTC1* indicated that net Pi uptake occurred in yeast when *CrPTC1* was expressed, consistent with the hypothesis that CrPTC1 is a Pi transporter. Further analysis showed that oocytes expressing CrPTC1 took up Pi at ~0.1 nmol h^{-1} per oocyte, whereas a water-injected control exhibited lower Pi uptake of ~0.01 nmol h^{-1} per oocyte (Supplemental Figure 1). This result verified the role of CrPTC1 as a Pi transporter.

To investigate the function of CrPTC1, we characterized two mutant alleles (*Crptc1-1* and *Crptc1-2*) (Supplemental Figure 2A) isolated from a library of *Chlamydomonas* indexed insertional mutants (Li et al., 2019). We mainly used *Crptc1-1* for analysis because it is a complete loss-of-function (null) mutant, whereas *Crptc1-2* is a hypomorph (incomplete loss-of-function) mutant (Supplemental Figure 2B–2E). Our results showed that total phosphorus content was higher in the *Crptc1-1* mutant than in the WT (Figure 1B). The defect in total phosphorus accumulation of *Crptc1-1* was suppressed in the CrPTC1 complementation line (*Crptc1-1:PTC1-13*) and was partially suppressed in the line *Crptc1-1:PTC1-16*, confirming that the *Crptc1* mutant phenotype was caused by the disruption of

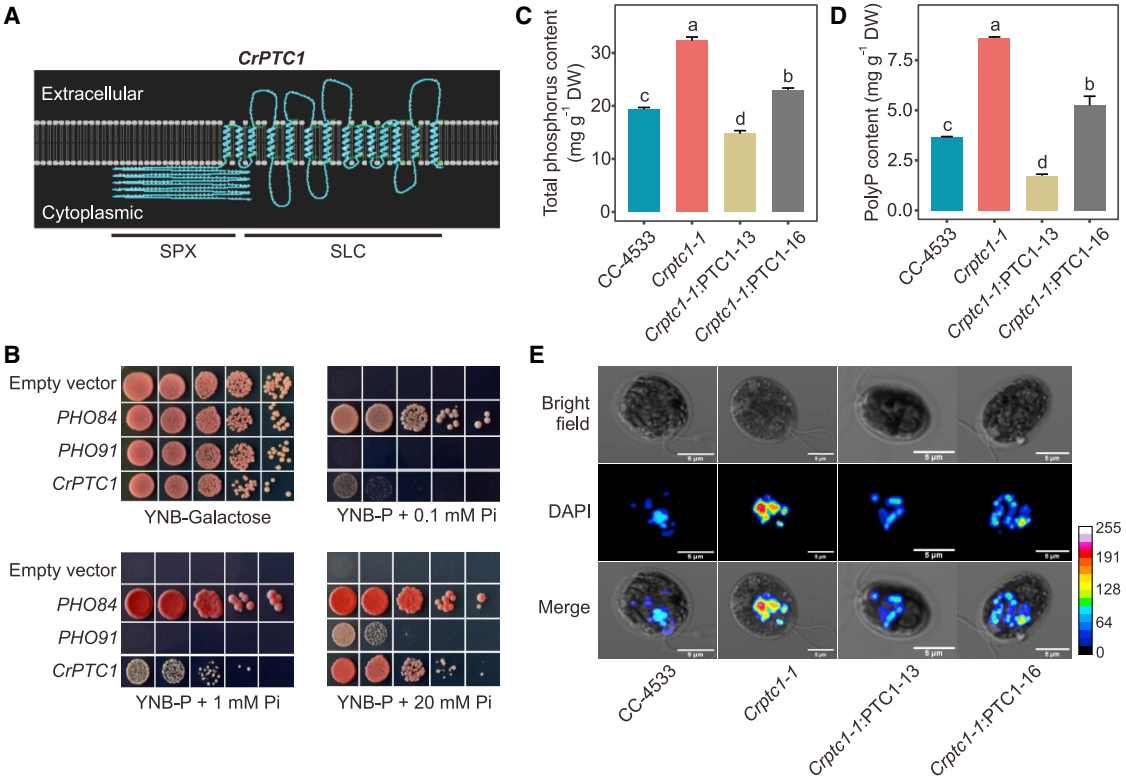

Figure 1. The *Chlamydomonas Crptc1* mutant over-accumulates total phosphorus and polyP.
(A) Predicted topological structure of CrPTC1 showing the transmembrane SLC domain and the SPX domain.
(B) CrPTC1 (SPX-SLC in *C. reinhardtii*) mediates Pi membrane transport in yeast. Complementation of yeast mutant YP100 by yeast *ScPHO84*, *ScPHO91*, and *Chlamydomonas PTC1* driven by the ADH1 promoter.
(C) Total phosphorus content of the WT CC-4533, the *Crptc1-1* mutant, and two complementation strains.
(D) PolyP content of the WT CC-4533, the *Crptc1-1* mutant, and two complementation strains. Error bars indicate SE. Differences were tested by ANOVA using the least significant difference (LSD) method with a Bonferroni correction at $\alpha = 0.05$. Means with the same letters are not significantly different.
(E) Representative pseudo-color images of cellular polyP stained with DAPI. DAPI was excited at 405 nm and an emission spectrum of 532–632 nm was collected using a ZEISS LSM 880 scanning confocal microscope. Bar, 5 μm. Experiments were repeated three times with similar results.

CrPTC1 gene function (Figure 1C and Supplemental Figure 2D and 2E). We further noticed that polyP content in both *Crptc1-1* and *Crptc1-2* mutants was significantly higher than in WT; in particular, the *Crptc1-1* mutant accumulated approximately three times more polyP than the WT (Figure 1D and Supplemental Figure 2F). The higher accumulation of polyP in the mutant was significantly suppressed in the complementation lines (Figure 1D).

To determine whether vacuolar polyP accumulation is defective in the *Crptc1-1* mutant, we stained polyP in *Chlamydomonas* cells. Consistent with the report that most of the polyP in the *Chlamydomonas* cell accumulates in acidocalcisomes (Komine et al., 2000), we detected a punctate polyP fluorescence signal that colocalized with acidic compartments indicated by LysoTracker Green (Supplemental Figure 3). DAPI fluorescence was higher in the *Crptc1-1* mutant than in the WT, and this enhanced fluorescence signal was suppressed by the introduction of a WT copy of CrPTC1 (Figure 1E). This result suggests that CrPTC1 is a tonoplast Pi transporter that facilitates Pi release from acidocalcisomes. To test this hypothesis, we attempted to express a CrPTC1-GFP fusion in *Chlamydomonas*. However, after 10 attempts at transformation and screening of more than 2000 zeocin-resistant clones, we failed to isolate GFP-positive colonies. Although we could not determine the subcellular localization of CrPTC1 in *Chlamydomonas*, we were able to detect the vacuolar membrane localization of CrPTC1-GFP in yeast cells (Supplemental Figure 4). Together, these findings indicated that CrPTC1 may be an acidocalcisomal membrane protein that facilitates Pi release from acidocalcisomes.

Disruption of *CrPTC1* causes defective Pi starvation-dependent signaling

The observation that levels of total phosphorus and polyP are higher in *CrPTC1* loss-of-function mutants than in the WT is consistent with the hypothesis that CrPTC1 is involved in Pi starvation-dependent signaling. To test this hypothesis, we first measured the activity of alkaline phosphatase, which is secreted by *Chlamydomonas* under Pi starvation but not in Pi-replete conditions (Wykoff et al., 1999). Under Pi-sufficient conditions, extracellular phosphatase activity was barely detectable in the WT, the *Crptc1-1* mutant, and the complementation strains (Figure 2A and Supplemental Figure 5). However, under phosphate starvation, the *Crptc1-1* mutant accumulated higher levels of extracellular phosphatase than the WT and the complementation strains, as indicated by the intense blue precipitate surrounding

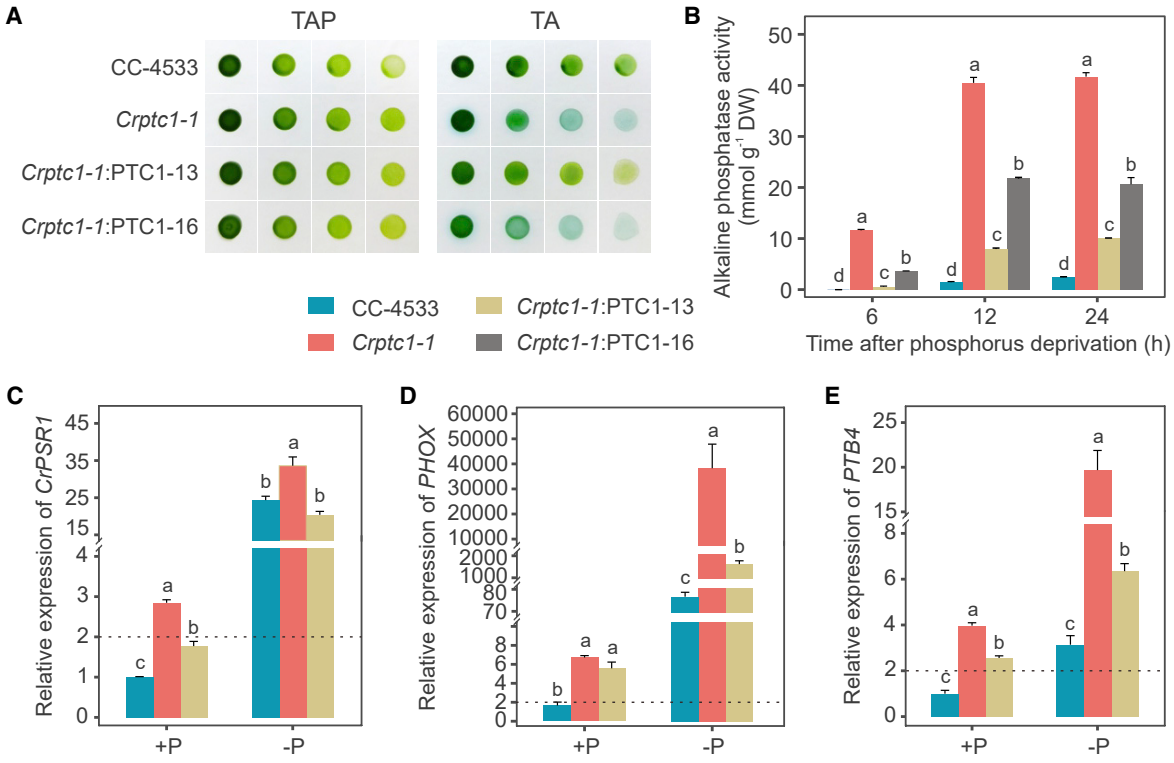

Figure 2. Disruption of *CrPTC1* caused an alteration in Pi starvation-dependent signaling.
(A) Qualitative analysis of phosphatase activity of WT CC-4533, the *Crptc1-1* mutant, and complementation strains. Cells were streaked onto TAP and TA ([tris-acetate-phosphate] medium without phosphate) solid medium prior to spraying the plates with the colorimetric phosphatase substrate 5-bromo-4-chloro-3-indolyl-P, which turns blue after cleavage by alkaline phosphatase. The plates were allowed to develop for 2 days before recording the results.
(B) Alkaline phosphatase activity of WT CC-4533, the *Crptc1-1* mutant, and complementation strains after 6, 12, or 24 h of phosphorus deprivation. Alkaline phosphatase activity is represented as absorbance at 410 nm of p-nitrophenol produced per gram of algal dry weight.
(C–E) Quantification of *PSR1* **(C)**, *PHOX* **(D)**, and *PTB4* **(E)** mRNA by qRT–PCR over a period of 6 h of phosphate deprivation. *CBLP* was used as the internal control. The data are presented relative to the values of CC-4533 under +P conditions. Error bars indicate SE. Differences were tested by ANOVA using the LSD method with a Bonferroni correction at $\alpha = 0.05$. Means with the same letters are not significantly different.

the colonies (Figure 2A and Supplemental Figure 5). We next measured the phosphatase activity of these strains after Pi deprivation. Although phosphatase activity was barely detected in the WT after 6 h of Pi starvation, it increased after 12 h or 24 h of starvation (Figure 2B). Phosphatase activity was higher in the *Crptc1-1* mutant than in the WT after 6 h of Pi starvation (Figure 2B), and this activity increased further after 12 or 24 h of Pi deprivation, when levels were 40-fold higher than those observed in the WT (Figure 2B). Both the high extracellular phosphatase activity and the enhanced phosphatase activity in *Crptc1-1* were fully or partially suppressed in the complementation strains (Figure 2A and 2B). Together, these data suggest either that Pi starvation signaling is more active in the *Crptc1-1* mutant than in the WT under Pi starvation stress or that *Crptc1* mutants are more sensitive to Pi starvation stress than the WT.

We next measured steady-state transcript levels of genes that encode PHOX phosphatase, the PTB4 phosphate transporter, and the PSR1 transcription factor in the WT, the *Crptc1-1* mutant, and a complementation strain (*Crptc1-1:PTC1-13*) grown with sufficient Pi. Expression of *PSR1*, *PHOX*, and *PTB4* was higher in the *Crptc1-1* mutant than in the WT under Pi-replete conditions (Figure 2C–2E), indicating that Pi starvation signaling was activated in the mutant under these conditions; steady-state levels of *PHOX* and *PTB4* mRNA were much higher in the *Crptc1-1* mutant than that in WT in Pi-replete conditions. Furthermore, steady-state levels of *PSR1* mRNA were slightly but significantly higher in the *Crptc1-1* mutant than in the WT. Higher levels of *PSR1*, *PHOX*, and *PTB4* expression in *Crptc1* were largely suppressed in the CrPTC1 complementation line (Figure 2C–2E). These data indicate that a Pi-stress response is constitutively active in the *Crptc1-1* mutant, whereas this response is inducible in the WT. These data suggest that CrPTC1 represses the low Pi-stress response.

Together, these data suggest that the SPX-SLC protein CrPTC1 modulates polyP accumulation by facilitating Pi release from the acidocalcisome and that loss of CrPTC1 function alters Pi starvation-dependent signaling in *Chlamydomonas*.

SPX-SLC and SPX-VTC are ancient and have been lost during the evolution of Streptophyta

To trace the phylogenetic history of SPX-containing proteins, we carried out a phylogenetic analysis of all SPX-containing proteins from 49 species of Archaeplastida, including 41 Viridiplantae species, three Glaucophyta algae, and five Rhodophyta algae (red algae). We generated alignments for the SPX domains and

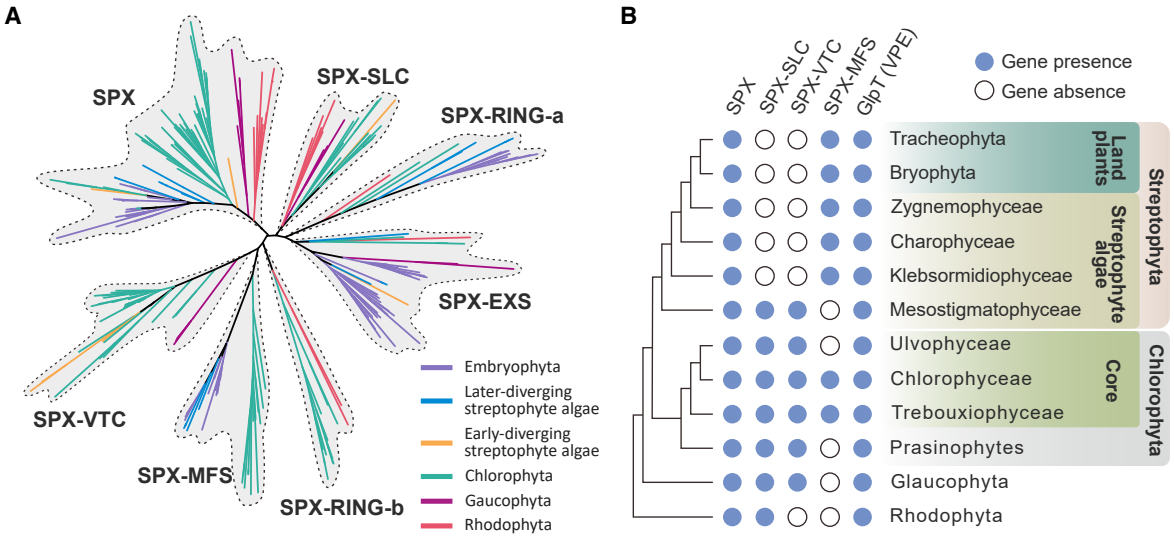

Figure 3. Evolution and presence of SPX domain-containing genes in the plant kingdom.

(A) Phylogeny of SPX domain-containing proteins in 49 species of plantae. *M. viride* was designated as an early-diverging streptophyte alga, and *Klebsormidium nitens*, *C. braunii*, and *S. muscicola* were late-diverging streptophytes. The tree was built from SPX domain sequences of all SPX domain-containing proteins. Multiple sequence alignment was performed using MAFFT, and the tree was constructed using IQ-Tree with the maximum likelihood method. It has been submitted here: https://itol.embl.de/tree/20211940236266511594463324.

(B) Presence of SPX domain-containing genes and GlpT (VPE) genes in different phyla. SPX, SPX-SLC, SPX-VTC, SPX-MFS, SPX-EXS, SPX-RING, and GlpT (VPE) orthologs were retrieved from OneKP or genome database searches (Supplemental Table 1).

constructed trees using maximum likelihood statistics (Supplemental Data 1 and Supplemental Figures 6–11). The topology of the tree shows that SPX, SPX-SLC, SPX-VTC, SPX-MFS, and SPX-EXS proteins each form distinct monophyletic clades. The SPX-RING proteins fall into two different monophyletic clades: one SPX-RING clade (designated SPX-RING-a) is sister to the SPX-EXS clade, and the second (designated SPX-RING-b) is sister to the clade comprising SPX-MFS and SPX-VTC proteins (Figure 3A). SPX-MFS may be the most recently evolved clade because it contains no members in either Rhodophyta or Glaucophyta, whereas the presence of SPX-MFS proteins in both Chlorophyta and Streptophyta suggests that they were present in the common ancestor of these lineages (Supplemental Figure 12). SPX-VTC proteins are present in Glaucophyta, Chlorophyta, and the early-diverging streptophyte algae Mesostigmatophyceae, indicating that they existed in the Archaeplastida lineage after the divergence of Rhodophyta. SPX-SLC proteins are present in Rhodophyta, Glaucophyta, Chlorophyta, and Mesostigmatophyceae, suggesting that they appeared earlier than the SPX-VTC proteins and were present in the common ancestor of the Archaeplastida. Interestingly, both SPX-SLC and SPX-VTC proteins are absent in the later-diverging streptophytes, including streptophyte algae and Embryophyta (Figure 3B). This is consistent with the hypothesis that SPX-SLC and SPX-VTC proteins were present in the ancestors of the early-diverging streptophytes but lost in more derived groups of streptophytes. These data suggest that SPX-SLC and SPX-VTC proteins perform a function in the Archaeplastida that was lost in later-diverging streptophytes.

SPX-VTC is essential for polyP synthesis

VTC4, an SPX-VTC protein, is a polyP polymerase that is essential for vacuolar polyP synthesis in yeast (Hothorn et al., 2009). Because genes encoding SPX-VTC proteins are present in the genomes of chlorophytes but not late-diverging streptophytes, we hypothesized that SPX-VTC proteins participate in acidocalcisomal polyP synthesis in *Chlamydomonas* and that neither *Klebsormidium* nor *Chara braunii* store acidocalcisomal polyP. To test this hypothesis, we first identified two CrVTC4 loss-of-function *Chlamydomonas* mutants and measured their polyP accumulation (Supplemental Figure 13). Our data showed that polyP was undetectable in the *Crvtc4* mutants, indicating that CrVTC4 is essential for polyP synthesis. Furthermore, our *in vitro* synthesis assay showed that truncated CrVTC4 with the putative kinase domain could synthesize polyP, thereby verifying that CrVTC4 is a polyP polymerase (Supplemental Figure 13F and 13G).

We then examined vacuolar phosphorus storage forms in early-diverging *Mesostigma viride* and later-diverging *Klebsormidium* and *C. braunii*. PolyP staining and data from the published literature (Pick and Weiss, 1991; Ruiz et al., 2001) showed that, like chlorophytes, *Mesostigma* accumulated polyP in vacuoles, whereas neither *Klebsormidium* nor *C. braunii* accumulated polyP in vacuoles (Figure 4A and 4B). These data suggest that storage of Pi in the vacuole evolved in the streptophyte lineage after the divergence of *Mesostigma* but before the divergence of *Klebsormidium*.

Evolution of vacuolar phosphate storage forms in plants

SPX-SLC and SPX-VTC proteins are not encoded in the genomes of later-diverging streptophytes, and SPX-MFS and GlpT/VPE catalyze vacuolar Pi influx and efflux, respectively, in land plants (Liu et al., 2015, 2016; Xu et al., 2019). We therefore hypothesized that the absence of SPX-SLC and SPX-VTC genes is correlated with the presence of SPX-MFS and GlpT/VPE proteins in the Archaeplastida lineage. Accordingly, we determined the

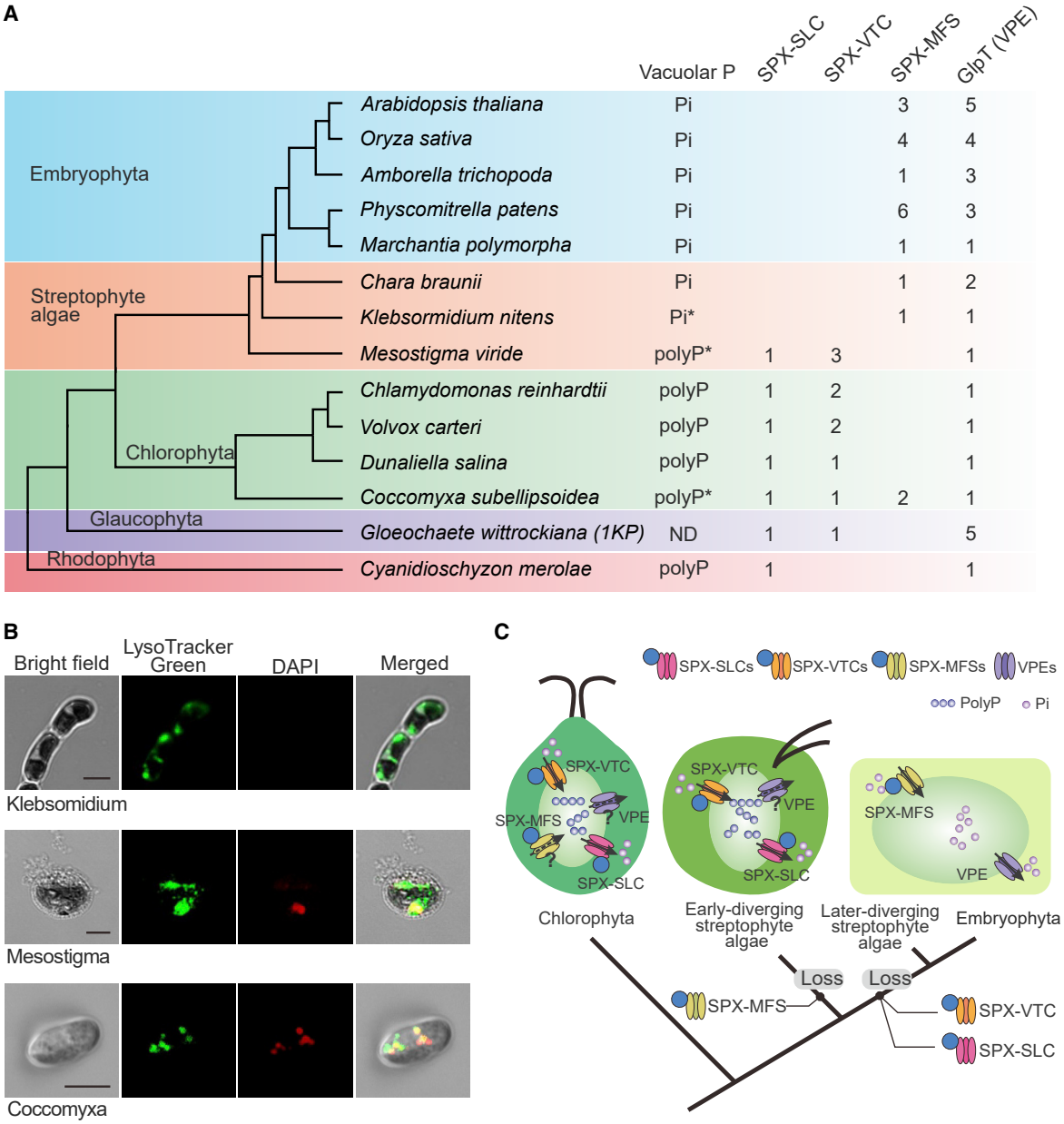

Figure 4. Co-evolution of phosphate transporter and vacuolar phosphorus storage forms in plants.

(A) Identification of the vacuolar phosphorus storage forms and related vacuolar phosphate transporter proteins (SPX-SLC, SPX-VTC, SPX-MFS, and GlpT [VPE]) in representative species from the plant kingdom. The species phylogeny was reconstructed from a concatenation of 154 genome-wide low-copy orthologous genes. ND, not determined. Asterisk (*) indicates data from this study.

(B) Staining of acidic compartments (LysoTracker Green) and PolyP (DAPI) in algae. Colocalization can be observed in *Coccomyxa* and *Mesostigma* but not in *Klebsormidium*. Scale bars, 5 μm.

(C) Proposed scenario of the stepwise evolution of SPX-containing genes and vacuolar phosphorus storage forms. The SPX-VTC and SPX-SLC proteins were present in the ancestor of chlorophyte algae and participate in vacuolar polyP synthesis and accumulation, respectively. It is unknown whether GlpT/VPE and SPX-MFS proteins mediate vacuolar Pi accumulation in the chlorophyte algae. SPX-MFS proteins were lost in early-diverging streptophyte algae. The later-diverging streptophyte algae, which use GlpT/VPE and SPX-MFS for vacuolar Pi homeostasis, have lost SPX-VTC and SPX-SLC proteins, and their vacuolar phosphorus storage form has changed to Pi. Question marks and dashed arrows indicate transporters that lack solid functional identification.

presence of SPX-MFS and GlpT/VPE in species of the green plant lineage (Figure 4A). Genes encoding GlpT/VPE proteins are present in all groups of Viridiplantae, indicating that GlpT/VPE proteins are ancient and conserved among the green plant lineage, but their number increased during the course of embryophyte (land plant) evolution. There are five GlpT/VPE genes in *Arabidopsis* compared with one in *Klebsormidium*. SPX-MFS genes are present in the genomes of embryophytes and later-diverging streptophyte algae but absent in the early-diverging unicellular streptophyte *Mesostigma*. These

data are consistent with functions of SPX-MFS and GlpT/VPE proteins in vacuolar Pi homeostasis in late-diverging streptophyte algae and Embryophyta. However, SPX-MFS genes were also found in the genomes of the chlorophytes *Asterochloris*, *Coccomyxa*, and *Uronema* (Supplemental Table 1), which also contain SPX-SLC and SPX-VTC genes. To determine whether the presence of SPX-MFS and GlpT/VPE affects vacuolar phosphorus storage forms, we also examined the presence of vacuolar polyP in *Coccomyxa*. We showed that *Coccomyxa* accumulates polyP (Figure 4B). Therefore, these data suggest that polyP is the vacuolar phosphorus storage form in chlorophyte algae that possess SPX-SLC and SPX-VTC genes, despite the presence of GlpT/VPE genes and in some cases the presence of SPX-MFS genes.

These data are consistent with the hypothesis that polyP was the ancestral form of phosphorus storage among the Viridiplantae and that polyP storage required SPX-VTC and SPX-SLC protein functions in the last common ancestor of the Viridiplantae. SPX-VTC and SPX-SLC genes were then lost during early streptophyte evolution, leading to a transition in vacuolar phosphorus storage form from polyP to Pi.

In contrast to polyP, whose production requires ATP synthesis from Pi (Hothorn et al., 2009), Pi is an energetically efficient form of vacuolar phosphorus storage in land plants. The fulfillment of cytosolic Pi demand by vacuolar Pi is much quicker than by polyP, which must be degraded to Pi before release from the vacuole to the cytosol (Lorenzo-Orts et al., 2020). Given that SPX-MFS and GlpT/VPE genes evolved among later-diverging streptophytes, together with our finding that SPX-VTC and SPX-SLC function in chlorophyte vacuolar polyP homeostasis, we propose an evolutionary hypothesis relating the presence of these genes to vacuolar phosphorus storage forms in green plants (Figure 4C). The SPX-VTC and SPX-SLC proteins were present in the ancestor of Chlorophyta algae and participate in vacuolar polyP synthesis and accumulation, respectively. It is unknown whether GlpT/VPE and SPX-MFS proteins mediate vacuolar Pi accumulation among chlorophyte algae. Although GlpT/VPE and SPX-MFS are both present in certain chlorophytes, their presence is not sufficient to change the major vacuolar phosphorus storage form from polyP to Pi (Figure 4). The later-diverging streptophyte algae have retained GlpT/VPE and SPX-MFS proteins but have lost SPX-VTC and SPX-SLC proteins, and their vacuolar phosphorus storage form has concomitantly changed to Pi. Given that phosphorus storage in the form of Pi evolved in the aquatic ancestors of land plants and that polyP has not been identified in land plant vacuoles, this transition may be considered a pre-adaptation to life on land. It is formally possible that the transition in vacuolar phosphorus storage form from polyP to Pi in Streptophyta may have been adaptive for land colonization.

The gain and loss of genes are two important sources of genetic variation that can cause adaptive phenotypic diversity. There are abundant examples in which increases in gene number are associated with evolutionary changes. For example, *Spirogloea muscicola* acquired additional genetic capacity through horizontal gene transfer from soil bacteria (Cheng et al., 2019). The genome sequence of *Penium margaritaceum* reveals an expanded repertoire of genes for cell wall biosynthesis that limit water loss and protect against UV radiation (Jiao et al., 2020). By contrast, our results suggest that the loss of SPX-SLC and SPX-VTC among later-diverging streptophyte algae and the consequent change in phosphorus storage form from polyP to Pi may have been a pre-adaptation to land colonization.

METHODS

C. reinhardtii strains and growth conditions

The *C. reinhardtii* WT strain CC-4533 and the mutant strains *Crptc1-1* (LMJ.RY0402.181899), *Crptc1-2* (LMJ.RY0402.121135), *Crvptc4-1* (LMJ.RY0402.195991), and *Crvptc4-2* (LMJ.RY0402.216999) were obtained from the Chlamydomonas Resource Center (Li et al., 2019). Cells were cultured in standard Tris-acetate-phosphate (TAP) medium at pH 7.0 under continuous illumination (50 mmol photons m^{-2} s^{-1}) on a rotating platform (150 rpm) at 24°C (Harris, 1989). For transformation, *CrPTC1* was cloned into pJM43Ble and transformed into *Crptc1-1* by electroporation as previously described (Shimogawara et al., 1998).

Transport activity assay in yeast and oocytes

The coding regions of *PHO84*, *CrPTC1*, and *PHO91* were introduced into the PRS426–ADH1 vector. These constructs and the empty vector were transformed into the yeast strain YP100 (Popova et al., 2010). To determine the localization of CrPTC1, the GFP tag was fused to the N terminus of CrPTC1 and transformed into yeast K699 (Popova et al., 2010). For the transport activity assay in oocytes, full-length cDNA of CrPTC1 was cloned into the oocyte expression vector pT7Ts. The transport activity assay was performed as described previously (Xu et al., 2019).

Quantitative real-time PCR analysis

RNA extraction and expression analysis were performed as described in our previous study (Wang et al., 2020). Primers used for qRT–PCR are listed in Supplemental Table 2.

Measurement of total phosphorus and polyP

Five milliliters of cells were harvested and dried to measure the total phosphorus concentration as described previously (Chen et al., 2007). PolyP was purified with PCR purification columns (Werner et al., 2005). Purified polyP solution was mixed with an equal volume of 2 M HCl and heated at 95°C for 30 min for acid degradation, and released Pi was quantified by the malachite green assay (Ohno and Zibilske, 1991).

Staining PolyP with DAPI

PolyP within cells was stained with DAPI and imaged using a ZEISS LSM 880 scanning confocal microscope following a previous report (Aschar-Sobbi et al., 2008). Cells were counterstained with 10 μM LysoTracker Green for colocalization analysis.

Identification of SPX-containing proteins and construction of the phylogenetic tree

Genome and gene annotation data of 49 selected plant species were obtained from the Phytozome database (Goodstein et al., 2012), NCBI Assembly, PLAZA (Van Bel et al., 2018), and OneKP (One Thousand Plant Transcriptomes Initiative, 2019) (Supplemental Table 1). SPX-containing proteins were identified by InterProScan 5 (Jones et al., 2014) and validated by BLASTP with an e-value cutoff of $1e^{-10}$ (Supplemental Table 3). Details of the phylogenetic analysis are provided in Supplemental Document 1. All sequences and information on SPX-containing proteins have been submitted to the Figshare database (https://doi.org/10.6084/m9.figshare.12431861).

Protein expression and polyP synthesis

Truncated *CrVTC4* (amino acids 1–550, CrVTC4p*) was cloned into pET29b (Promega). The 6×His-CrVTC4p* proteins were purified according to the manufacturer's instructions (Qiagen) and used for polyP synthesis (Hothorn et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

K.Y. and Y.Z. conceived and supervised the project. L.W., L.X., Y.Z., and K.Y. designed the research. L.W., YX.Z., and X.J. performed the experiments. L.W., L.X., B.M., X.J., H.Z., HQ.Z., L.D., Y.Z., and K.Y. analyzed the data. L.W., X.J., B.M., L.D., and K.Y. wrote the paper with input from all the authors.

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