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Functional PTB phosphate transporters are present in streptophyte algae and early diverging land plants

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Summary

- Two inorganic phosphate (Pi) uptake mechanisms operate in streptophytes and chlorophytes, the two lineages of green plants. PHOSPHATE TRANSPORTER B (PTB) proteins are hypothesized to be the Na⁺/Pi symporters catalysing Pi uptake in chlorophytes, whereas PHOSPHATE TRANSPORTER 1 (PHT1) proteins are the H⁺/Pi symporters that carry out Pi uptake in angiosperms. PHT1 proteins are present in all streptophyte lineages. However, Pi uptake in streptophyte algae and marine angiosperms requires Na⁺ influx, suggesting that Na⁺/Pi symporters also function in some streptophytes.
- We tested the hypothesis that Na⁺/Pi symporters exist in streptophytes.
- We identified PTB sequences in streptophyte genomes. Core PTB proteins are present at the plasma membrane of the liverwort *Marchantia polymorpha*. The expression of *M. polymorpha* core PTB proteins in the *Saccharomyces cerevisiae* *pho2* mutant defective in high-affinity Pi transport rescues growth in low-Pi environments. Moreover, levels of core PTB mRNAs of *M. polymorpha* and the streptophyte alga *Coleochaete nitellorum* are higher in low-Pi than in Pi-replete conditions, consistent with a role in Pi uptake from the environment.
- We conclude that land plants inherited two Pi uptake mechanisms – mediated by the PTB and PHT1 proteins, respectively – from their streptophyte algal ancestor. Both systems operate in parallel in extant early diverging land plants.

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Introduction

Phosphorus is an essential macronutrient required for structural and metabolic functions in all living organisms. Phosphorus is absorbed largely as inorganic phosphate (Pi), through Pi transporters located at the plasma membrane of cells at the interface with the external environment. High-affinity and low-affinity Pi transporters catalyse Pi uptake from environments containing micromolar and millimolar concentrations of Pi, respectively (Werner *et al.*, 1998; Harris *et al.*, 2001; Werner & Kinne, 2001; Bucher, 2006; Dick *et al.*, 2014). Chlorophytes and streptophytes are two monophyletic clades that constitute the green plant lineage. The land plants (also known as embryophytes) are a monophyletic group of streptophytes and the angiosperms are the most derived group of land plants (Ruhfel *et al.*, 2014). Angiosperms absorb Pi through PHOSPHATE TRANSPORTER 1 (PHT1) plasma membrane H⁺/Pi symporters (Nussaume *et al.*, 2011). The kinetics of Pi uptake in yeast mutants and plant protoplasts expressing plant PHT1 proteins have demonstrated that PHT1 genes encode either high-affinity or low-affinity H⁺/Pi symporters (Leggiewie *et al.*, 1997; Mitsukawa *et al.*, 1997; Daram *et al.*, 1998; Harrison *et al.*, 2002; Rae *et al.*, 2003; Liu *et al.*,

2008). Consistent with a role in Pi absorption from low-Pi environments, the steady state level of mRNA of most members of the PHT1 gene family is higher in plants grown in low-Pi than in Pi-replete environments, and in roots than in shoots (Mudge *et al.*, 2002; Nussaume *et al.*, 2011; Qin *et al.*, 2012). The PHT1 proteins are the only plasma membrane Pi transporters identified in angiosperms (Ayadi *et al.*, 2015). Furthermore, Pi uptake is 87% lower in the *Atph1;1 Atph1;2 Atph1;3 Atph1;4* quadruple mutant than in wild-type (WT) *Arabidopsis thaliana*, suggesting that the PHT1 family is the major plasma membrane Pi transporter family in angiosperms (Ayadi *et al.*, 2015).

Physiological evidence suggests that Na⁺/Pi symporters are involved in Pi uptake in algae and in some marine angiosperms. Na⁺ uptake is necessary for Pi influx in the streptophyte alga *Chara corallina* (Reid *et al.*, 2000; Mimura *et al.*, 2002) and stimulates Pi uptake in the marine angiosperm *Zostera marina* (Rubio *et al.*, 2005) when grown in low-Pi conditions. This suggests that Na⁺/Pi symporters may also function as plasma membrane Pi transporters in some groups of streptophytes. Physiological analysis of membrane transport has demonstrated that Pi uptake involves an Na⁺/Pi symport mechanism in at least four chlorophytes (*Dunaliella salina*, *Ankistrodesmus braunii*, *Chlorella*

pyrenoidosa, *Hydrodictyon africanum*) (Raven, 1984; Weiss *et al.*, 2001). It has been suggested that PHOSPHATE TRANSPORTER B (PTB) proteins, members of the Pi TRANSPORTER (PiT) protein family, are the Na⁺/Pi symporters involved in chlorophyte Pi uptake because of the sequence conservation and structural similarity of these proteins to the members of two other PiT subfamilies – the metazoan PiT and fungal PiT – known to be functional plasma membrane Na⁺/Pi symporters (Chung *et al.*, 2003; Kobayashi *et al.*, 2003; Li *et al.*, 2006, 2012; Moseley *et al.*, 2006; Guan *et al.*, 2008; Djouani-Tahri *et al.*, 2011). Consistent with the hypothesized Na⁺/Pi symporter activity is the observation that PTB proteins of the chlorophyte *Dunaliella viridis* increase the Na⁺ sensitivity of a yeast mutant with no plasma membrane Na⁺ export pumps (Li *et al.*, 2006). Moreover, the higher steady state level of most PTB mRNAs in low-Pi relative to Pi-replete conditions in the chlorophyte algae *D. viridis* (Li *et al.*, 2006; Guan *et al.*, 2008), *Ostreococcus taurii* (Djouani-Tahri *et al.*, 2011), *Tetraselmis chuii* (Chung *et al.*, 2003) and *Chlamydomonas reinhardtii* (Kobayashi *et al.*, 2003; Moseley *et al.*, 2006) supports the hypothesis that PTB proteins are involved in Pi uptake from the environment in chlorophytes. However, the Pi transport function of the PTB proteins has not been demonstrated and PTB sequences have not been identified in streptophytes.

As Pi uptake in chlorophyte and streptophyte algae and some aquatic angiosperms depends on the activity of Na⁺/Pi symporters, we set out to determine whether Na⁺/Pi symporters were encoded in the genomes of streptophytes whose life cycle is dependent on aquatic or semi-aquatic environments (streptophyte algae, early diverging land plants and aquatic angiosperms). Here, we report that genes encoding PTB proteins are present in the genomes of streptophytes (both algae and land plants), and that core PTB proteins pass through the secretory system to the plasma membrane in *Marchantia polymorpha*. We show that heterologous expression of *M. polymorpha* core PTB proteins is sufficient to restore Pi uptake in a *Saccharomyces cerevisiae* mutant with defective high-affinity Pi transport (Pinson *et al.*, 2009). Furthermore, the increased expression of streptophyte algae and early diverging land plant core PTB genes in low-Pi growth conditions supports the hypothesis that these proteins are involved in Pi uptake from the environment.

Materials and Methods

Sequence collection

The genome and transcriptome databases used to retrieve the nucleic and amino acid sequences are specified in Supporting Information Table S1. The GenBank accession numbers for the coding sequence (CDS) and corresponding proteins reported in this article are as follows: KX495367 (MpPTB1), KX495368 (MpPTB2), KX495369 (MpPTB3), KX495370 (MpPTB4), KX495371 (MpPTB5), KX495372 (MpPTB6), KX495373 (MpPTB7), KX495374 (MpPTB8), KX495375 (MpPHT2-1), KX495376 (CnPTB) and KX495377 (CnPHT2-1).

The amino acid sequences of ScPho89 (YBR296C) and NcPho4 (NCU09564) were used as queries in TBLASTN searches (Altschul *et al.*, 1990) for PiT sequences in the genomes and transcriptomes of 23 green plant species (species list in Table S1) and in the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database. No *E*-value threshold was used; all hit sequences were systematically analysed as described hereafter. For each species, the retrieved transcripts were aligned using Multalin (Corpet, 1988) and compared with each other to eliminate duplicates and to assemble partial transcripts (annotated in Table S1). Amino acid sequences were predicted from transcripts using ExPASy translate (ExPASy, University of Geneva, Geneva, Switzerland) and from genomic sequences with Softberry FGENESH⁺ (Solovyev, 2007) using *AtPHT2-1* and *CrPTB2* as gene models. Reciprocal BLAST searches (Altschul *et al.*, 1990) were conducted on NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to verify whether the sequences belonged to the clade of interest. Nucleic acid and amino acid sequences were aligned (Figs S1, S2) using the iterative refinement strategy E-INSI (Katoh *et al.*, 2005) in MAFFT version 7 (Katoh & Standley, 2013). The alignments were manually adjusted with BIOEDIT v.7.1 (Ibis Biosciences, Carlsbad, CA, USA). Comparison of the nucleic acid and amino acid alignments allowed the identification of putative pseudogenes with interrupted open reading frames, and partial proteins resulting from partial transcripts or gene prediction errors (Table S1).

Phylogenetic analyses and sequence nomenclature

To increase the resolution of the phylogenies, the pseudogenes and partial sequences were included in the analyses (Jiang *et al.*, 2014). The PiT amino acid alignments were trimmed manually with BIOEDIT v.7.1 (Ibis Biosciences) to remove non-conserved regions (Figs S1, S2). The LG amino acid substitution model (Le & Gascuel, 2008) was estimated to be the best fit for the analysis of the PTB phylogenies using PROTTEST v.2.4 (Abascal *et al.*, 2005). ATGC PhyML was used to generate maximum-likelihood phylogenetic trees (Guindon *et al.*, 2010). Statistical support values of the embranchments were calculated with an approximate likelihood ratio test (aLRT) (Guindon *et al.*, 2010). Trees were visualized using FIGTREE (<http://tree.bio.ed.ac.uk/software/figtree/>). For each gene, the name used in the study and the name according to the Commission of Plant Nomenclature are given in Table S1.

PiT amino acid sequence analysis

Percentages of similarity and identity between plant PiT proteins and ScPho89 were calculated using Sequence Identity And Similarity (SIAS) (<http://imed.med.ucm.es/Tools/sias.html>). Hydropathy profiles and subcellular localizations were predicted using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and TARGETP (Emanuelsson *et al.*, 2007). Protein motifs were identified using MEME (Multiple Em for Motif Elicitation) (Bailey & Elkan, 1994) with a constraint of 30 conserved motifs. The presence of the motifs in streptophyte core PTB and PTB clade 4 proteins was assessed using MEME and the alignments.

Plant growth conditions

Coleochaete nitellarum: 1 g of WT strain culture (accession provided by the Skidmore Algal Collection of Professor David Domozych), obtained from a single colony and grown in cell culture flasks (cell star, 250 ml, filter cap; Greiner Bio-One, Kremsmünster, Austria) in 75 ml of liquid NaNO_3 -BBM (Bold Basal Medium (Nichols, 1973) supplemented with 1 g l^{-1} NaNO_3 , renewed every 6 wk), was transferred in cell culture flasks (cell star, 50 ml, filter cap; Greiner Bio-One) in 25 ml of liquid Pi-modified 3N-BBM + V (CCAP; Culture Collection of Algae and Protozoa; Dunstaffnage Marine Laboratory, Oban, UK) for 2 wk. 3N-BBM + V media with high- and low-Pi content were obtained by mixing the stock solutions according to the manufacturer's instructions, except for K_2HPO_4 and KH_2PO_4 , which were added in identical amounts to the media to a final Pi concentration of 0, 50, 500 or 1600 μM . K_2SO_4 was added to each Pi-modified 3N-BBM + V medium to restore the final concentration of potassium to 2.15 mM. Algae were grown under a cycle of 8 h : 16 h, dark : light ($38 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at 23°C at 45 rpm.

Marchantia polymorpha: male (Takaragake-1) and female (Takaragake-2) (Ishizaki *et al.*, 2008) WT gemmae were propagated on sterile half-strength Johnson medium (Johnson *et al.*, 1957), pH 5.6, supplemented with 0.6% agar. One-week-old plants were transferred on low- or high-Pi modified half-strength Johnson medium (Saint-Marcoux *et al.*, 2015) for 2 wk. Plants were grown under continuous illumination ($38 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at 23°C .

Coleochaete nitellarum de novo transcriptome assembly

The whole-plant *C. nitellarum* draft transcriptome was generated by the sequencing of a strand-specific cDNA library with an insert size of 500 bp using a paired-end read length of 2×100 bp on Illumina HiSeq2000. Both the library preparation from 2 μg RNA and the sequencing were performed by GATC Biotech Ltd (Konstanz, Germany). For the assembly, raw reads were quality trimmed using TRIMMOMATIC-0.32 (Bolger *et al.*, 2014), BAYESHAMMER (SPADES-3.5.0) (Nikolenko *et al.*, 2013) and ALLPATHS-LG-4832 (Butler *et al.*, 2008). Ribosomal RNA was removed using SORTMERA-1.9 (Kopylova *et al.*, 2012). The quality trimmed reads were then normalized with a khmer size of 21 in KHMER-0.7.1 (Crusoe *et al.*, 2015). Normalized reads were then assembled and scaffolded using SGA (Simpson & Durbin, 2012), SSPACE-v.3 (Boetzer *et al.*, 2011) and CAP3 (Huang & Madan, 1999). Assembled scaffolds then underwent a final correction step using PILON-1.6 (Walker *et al.*, 2014).

RNA extraction, cDNA synthesis, sequencing and expression analysis

Marchantia polymorpha rhizoids and thalli were collected from the same plants and flash frozen in liquid nitrogen. Total RNA was extracted from ground frozen tissue with an RNeasy plant mini kit (#74904; Qiagen, Venlo, the Netherlands) and DNase

treated on-column using DNase I (#79254; Qiagen). *Coleochaete nitellarum* samples were collected on 0.2- μm nylon sterile filters and flash frozen in liquid nitrogen. Total RNA was extracted from ground frozen tissue with a Zymo Research Direct-Zol RNA Miniprep kit (#R2051; ZymoResearch, Irvine, CA, USA) and DNase treated using Turbo DNase (#AM1907; Thermo Fisher Scientific, Waltham, MA, USA). cDNAs from *M. polymorpha* and *C. nitellarum* samples were synthesized from 1 μg of total RNA in a 20- μl reaction using 200 U of Protoscript II reverse transcriptase (#M0368S; NEB, Ipswich, MA, USA) and dT₁₇ oligos at 2 μM , and diluted in 180 μl of nuclease-free water. *Marchantia polymorpha* and *C. nitellarum* sequences were verified by PCR amplification (primers listed in Table S2) and Sanger sequencing (Source Bioscience, Nottingham, UK).

Quantitative PCR experiments were performed as described previously (Saint-Marcoux *et al.*, 2015) for *M. polymorpha* and using 20 μl of reaction volume containing 1 μM of forward and reverse primers, 10 μl of $2 \times$ SensiFAST Sybr-Green mHi-ROX master mix (#BIO-92020; Bioline, London, UK) and 5 μl of diluted cDNA for *C. nitellarum*. Amplification experiments were carried out using a 7300 Applied Biosystems thermocycler (Thermo Fisher Scientific). Data analysis was performed as described previously (Saint-Marcoux *et al.*, 2015). Reference genes were MpACT and MpSAND for *M. polymorpha* (Saint-Marcoux *et al.*, 2015), and CnEF1 α for *C. nitellarum* (primers listed in Table S2).

Vector construction

The CDSs encoding the yellow fluorescent protein (YFP) and MpPTB6 proteins were amplified from a published construct (Breuninger *et al.*, 2016) and from cDNA, respectively, for construction of the *pOsAct1:MpPTB6-YFP:Term* and *pOsAct1:YFP:Term* binary vectors using the high-fidelity polymerase Phusion (#F-530L; Thermo Fisher Scientific) with the primers listed in Table S2. The reverse primer for MpPTB6 CDS amplification and the forward primer for YFP CDS amplification for the translational fusion are partially complementary and contain a sequence encoding a peptide linker between MpPTB6 and YFP. The assembly of the amplicons of the MpPTB6 and YFP CDSs in one MpPTB6-YFP CDS was carried out by PCR using the high-fidelity polymerase Phusion (#F-530L; Thermo Fisher Scientific) with the forward primer for MpPTB6 amplification and the reverse primer for YFP amplification. MpPTB6-YFP and YFP CDSs were cloned into *pCR8-GW-TOPO* (#K2500-20; Thermo Fisher Scientific) to establish Gateway™ entry vectors. These were subsequently recombined, using the LR reaction (#12538-200; Thermo Fisher Scientific), with the destination vector *pCambia pOsACT1:GW:Term* (Proust *et al.*, 2016) containing the rice ACTIN1 promoter, *pOsAct1*, driving the constitutive expression in *M. polymorpha* (Breuninger *et al.*, 2016).

MpPTB4, MpPTB6 and MpPTB7 CDSs were amplified from cDNA for the construction of the yeast expression/shuttle vectors using the high-fidelity polymerase Phusion (#F-530L; Thermo Fisher Scientific) with the adaptor primers listed in Table S2. CDSs were cloned into the *pGEM[®]-T* easy vector (Promega, Madison, WI, USA) and subcloned into the *pCM189* vector behind a

tetracycline repressible promoter (Gari *et al.*, 1997). Subclonings of MpPTB6 and MpPTB7 in pCM189 were performed between BamHI and PstI restriction sites, using the BamHI and PstI restriction sites designed at the 5' end of the adaptor primers. Subcloning of MpPTB4 in pCM189 was performed between NotI and PstI restriction sites using the NotI and PstI restriction sites of the Multi-cloning-site of the PGemT-easy vector.

Saccharomyces cerevisiae strains, transformation and culture

The *S. cerevisiae* strain $\Delta pho2$ Y2092 (Mat α his3 Δ 1 leu2 Δ 0 ura3 Δ 0 *pho2::KanMX6*; B. Pinson, unpublished) (Sc $\Delta pho2$) was transformed (Chen *et al.*, 1992) with pCM189 vectors expressing the MpPTB4, MpPTB6 or MpPTB7 CDS or the *S. cerevisiae* ScPho84 or ScPho89 CDS (Pinson *et al.*, 2004). The $\Delta pho2$ Y2092 and ScPHO2 WT Y178 (Mat α his3 Δ 1 leu2 Δ 0 ura3 Δ 0 derived from BY4742; Euroscarf, Frankfurt, Germany) strains were transformed with the cognate pCM189 empty vector. A drop test was used to compare the growth of the different transformed strains, as described previously (Pinson *et al.*, 2004). Drops of cell dilutions were spotted onto freshly prepared plates of a modified SD medium containing 0.57% of yeast nitrogen base with (NH₄)₂SO₄, but without Pi and NaCl (#114027812; MP Biomedical, Santa Ana, CA, USA), 2% glucose, 2 mM NaCl and 2.5% agar, buffered at pH 6 with 50 mM phthalic acid and supplemented with tryptophan (0.2 mM) and adenine (0.3 mM). The Pi concentration was adjusted using KHPO₃. When required, doxycycline (10 mg l⁻¹) was used to repress the expression of the various CDSs under the tetracycline-repressible promoter (Gari *et al.*, 1997). Cultures were grown at 30°C for 11 d before scanning.

Marchantia polymorpha transformation and confocal microscopy imaging

Marchantia polymorpha sporelings were transformed with one of four binary vectors: pMpSYP12A:mCitrine-MpSYP12A:Term (Kanazawa *et al.*, 2016), p35S:YFP:HDEL:Term (Teh & Moore, 2007), pOsAct1:YFP:Term and pOsAct1:MpPTB6-YFP:Term using a published protocol (Proust *et al.*, 2016). Images were captured using an upright confocal microscope (Leica DM6000CS; Leica, Wetzlar, Germany) and Leica Advance Fluorescence Leica application suite software (LAS AF). Excitation of the fluorophores and chlorophyll was performed with an argon laser (514 nm) at 29% power. Emissions were acquired at 525–571 nm for YFP and mCitrine, and at 696–761 nm for chlorophyll. Pinhole $m = 111.5 \mu\text{m}$ and pinhole airy = 1. Cell and tissue images were obtained with $\times 20$ and $\times 50$ lenses.

Results

Identification of PiT-encoding genes in chlorophytes and streptophytes

Plant PiT proteins, which comprise members of both the PTB and PHOSPHATE TRANSPORTER 2 (PHT2) subfamilies, are

homologues of the fungal PiT Pi transporters ScPho89 of *S. cerevisiae* and NcPho4 of *Neurospora crassa*. The PTB and PHT2 protein subfamilies are distinct phylogenetically (Versaw & Harrison, 2002; Chung *et al.*, 2003; Kobayashi *et al.*, 2003; Zhao *et al.*, 2003; Moseley *et al.*, 2006; Tyra *et al.*, 2007; Djouani-Tahri *et al.*, 2011; Li *et al.*, 2012). Moreover, members of the PHT2 protein subfamily are H⁺/Pi symporters located at the chloroplast envelope with no role in Pi uptake to the cell (Versaw & Harrison, 2002; Zhao *et al.*, 2003). There are no published amino acid or nucleic motifs specific to the PHT2 or PTB subfamilies. Therefore, to identify new chlorophyte and streptophyte PTB sequences, we first identified the PiT (PHT2 and PTB) sequences in the genomes and transcriptomes of 23 taxa representative of the green plant lineage by searching for protein sequences similar to ScPho89 and NcPho4 using the TBLASTN algorithm (Altschul *et al.*, 1990). We identified 74 sequences (Fig. 1; Table S1). Their predicted amino acid sequences were 42–87% similar and 15–36% identical to ScPho89 (Table S1) and contained the PiT signature motifs: the PiT N- and C-terminal GANDVAN domains (G-A/S/F-N/H-D-V/T-A-N-A/S and G-A/G-N/G-D-V-x-x-x) and the two glutamate (E⁵⁵ and E⁵⁷⁵ in HsPiT2) and serine (S¹¹³ and S⁵⁹³ in HsPiT2) PiT conserved residues (Salaün *et al.*, 2001, 2004; Böttger & Pedersen, 2002, 2005; Böttger *et al.*, 2006) (Fig. S1). These data suggest that all 74 sequences identified are either PiT-encoding genes or PiT-related pseudo-genes in the case of 14 partial sequences. They include 60 new PiT sequences, the 10 published *C. reinhardtii* PTB proteins (Kobayashi *et al.*, 2003; Moseley *et al.*, 2006) and four published PHT2 proteins from the angiosperm *A. thaliana* (Versaw & Harrison, 2002), *Solanum lycopersicum* (Chen *et al.*, 2007), *Medicago truncatula* (Zhao *et al.*, 2003) and *Oryza sativa* (Liu *et al.*, 2011) (Figs 1, S1). Taken together, these data indicate that PiT proteins are present in both chlorophytes and streptophytes, including streptophyte algae and land plants.

PTB proteins are present in both chlorophytes and streptophytes

We used maximum-likelihood statistics (Guindon *et al.*, 2010) to determine the phylogenetic relationships between the 60 new PiT sequences identified in this survey and the PHT2 and PTB PiT subfamilies. The topology of the phylogenetic tree showed that the plant PiT sequences form two monophyletic groups corresponding to the PHT2 and PTB subfamilies (Figs 2, S3). The PHT2 subfamily is sister to the bacterial PiT proteins and, together with the animal PiT proteins, constitutes a sister group to the PTB subfamily (Figs 2, S3) (Tyra *et al.*, 2007). The PHT2 subfamily (supported by an aLRT value of 1) includes 22 of the new PiT sequences and the four published angiosperm PHT2 proteins (Versaw & Harrison, 2002; Zhao *et al.*, 2003; Chen *et al.*, 2007; Liu *et al.*, 2011) (Figs 2, S3). PHT2 sequences were identified in all the streptophyte taxa used in this study, but not in chlorophytes, suggesting that PHT2 proteins are streptophyte specific.

The remaining 38 new PiT sequences are members of the monophyletic PTB subfamily (supported by an aLRT value of

0.75) which form four different monophyletic clades (Figs 2, S3). The largest monophyletic clade (supported by an aLRT value of 0.99) comprises 34 sequences that include the 12 published chlorophyte PTB proteins present in our data (the *C. reinhardtii* CrPTB1–CrPTB9 and CrPTB12, and the *D. viridis* DvSPT1 and DvSPT2) (Kobayashi *et al.*, 2003; Li *et al.*, 2006; Moseley *et al.*, 2006; Guan *et al.*, 2008). This clade was therefore designated the core PTB clade. Core PTB proteins were identified in both chlorophytes and streptophytes, including the streptophyte

algae *Klebsormidium flaccidum* and *Coleochaete nitellarum*, and the early diverging land plants *M. polymorpha* (a liverwort), *Physcomitrella patens* and *Encalypta streptocarpa* (two mosses), and *Nothoceros vincentianus* and *Phaeoceros carolinianus* (two hornworts). There are no tracheophyte members of the core PTB clade. The second monophyletic PTB clade (PTB clade 2) forms an algal-specific clade (supported by an aLRT value of 1) and comprises sequences from the chlorophyte *Coccomyxa subellipsoidea* and the streptophyte *K. flaccidum*. The third

	Chlorophytes algae		Streptophyte algae		Bryophytes		Lycophytes		Monilophytes		Gymnosperms		Angiosperms											
	<i>O. lucimarinus</i>	<i>C. subellipsoidea</i>	<i>C. reinhardtii</i>	<i>K. flaccidum</i>	<i>C. nitellarum</i>	<i>M. polymorpha</i>	<i>P. patens</i>	<i>E. streptocarpa</i>	<i>N. vincentianus</i>	<i>P. carolinianus</i>	<i>S. moelendorffii</i>	<i>H. lucidula</i>	<i>D. conjugata</i>	<i>C. acrostichoides</i>	<i>P. abies</i>	<i>P. taeda</i>	<i>S. polytricha</i>	<i>Z. marina</i>	<i>Z. mays</i>	<i>O. sativa</i>	<i>M. truncatula</i>	<i>S. lycopersicum</i>	<i>A. thaliana</i>	Total
PIT identified	2	3	11(1)	8(2)	2	9	4(1)	4(1)	3(2)	8(4)	2	2(1)	3	3(1)	2	1(1)	1	1	1	1	1	1	1	74(14)
Published PTB	–	–	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10
Published PHT2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	1	1	4
New PIT (PTB and PHT2)	2	3	1(1)	8(2)	2	9	4(1)	4(1)	3(2)	8(4)	2	2(1)	3	3(1)	2	1(1)	1	1	1	0	0	0	0	60 (14)

Fig. 1 Number and type of PiT sequences identified in 23 representative species of green plant. The total number of PHOSPHATE (Pi) TRANSPORTER (PiT) sequences identified (PiT identified) in the genomic and transcriptomic databases of 23 green plant taxa can be divided into three categories: (1) the PHOSPHATE TRANSPORTER B (PTB) sequences that have been published previously (Published PTB); (2) the PHOSPHATE TRANSPORTER 2 (PHT2) sequences that have been published previously (Published PHT2); (3) the new sequences identified (New PiT). The number of putative pseudo-genes or partial sequences among the sequences is given in parentheses. The taxa are arranged according to the green plant phylogeny. The dashed branches of the tree indicate the paraphyletic clades (charophyte and bryophyte).

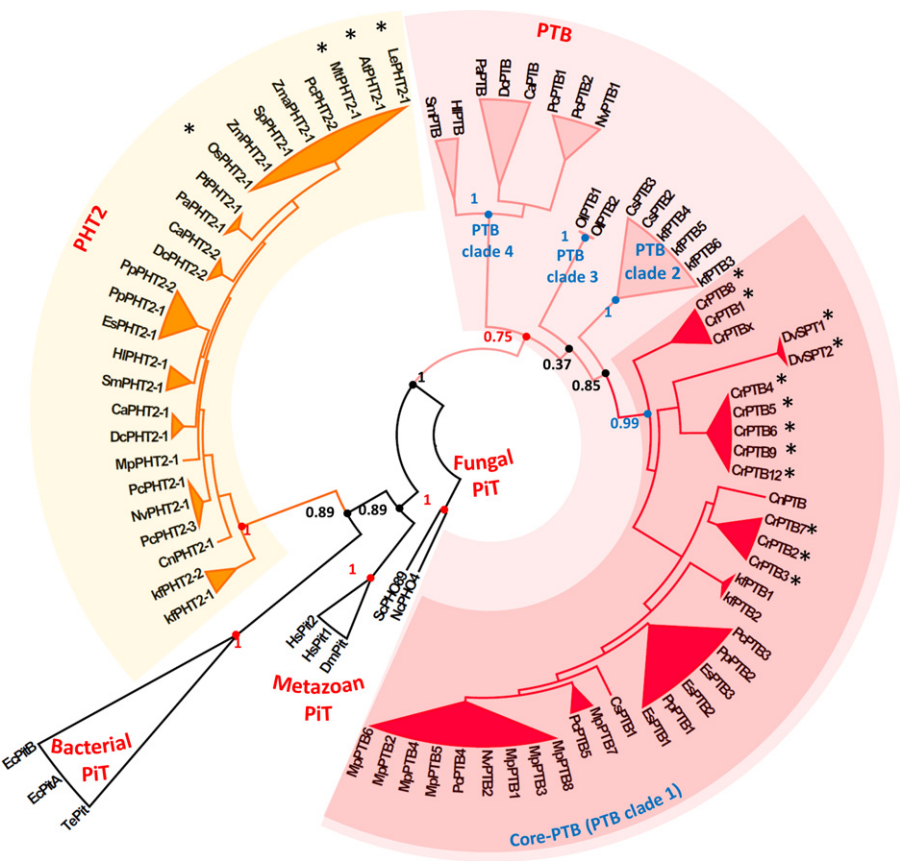


Fig. 2 Schematic representation of the maximum-likelihood phylogeny of the green plant PiT sequences. The phylogeny was produced using the multiple amino acid alignment of the plant PHOSPHATE (Pi) TRANSPORTER (PiT) sequences. Three metazoan PiT (DmPiT, HsPiT1 and HsPiT2), three bacterial PiT (TePiT, EcPiT1 and EcPiT2) and two fungal PiT (ScPho89 and NcPho4) proteins were used as outgroups. Sequence names consist of the species code (first letter of the genus and first letter of the species name, or first letter of the genus and two first letters of the species name for *Zostera marina*) and gene name (as published or given according to the published sequences with which they grouped in this phylogenetic analysis). The major nodes of the tree are marked by a point (red for the PiT subfamilies, blue for the PHOSPHATE TRANSPORTER B (PTB) clades and black for others). The approximate likelihood ratio test (aLRT) support values are given for the major nodes. *The published PHOSPHATE TRANSPORTER 2 (PHT2) or PTB sequences.

monophyletic PTB clade (PTB clade 3; supported by an aLRT value of 1) comprises a pair of paralogous sequences from the chlorophyte *Ostreococcus lucimarinus*. Its separation from PTB clade 2 is poorly resolved (supported by an aLRT value of 0.37). The fourth monophyletic clade (PTB clade 4, supported by an aLRT value of 1) is land plant specific and sister to all other PTB clades. It comprises sequences from the hornworts *N. vincentianus* and *P. carolinianus*, the lycophytes *Selaginella moelendorffii* and *Huperzia lucidula*, the monilophytes *Dipteris conjugata* and *Cryptogramma acrostichoides*, and the gymnosperm *Picea abies*. No PTB clade 4 sequences were identified in liverworts, mosses or angiosperms (Figs 2, S3).

An analysis of the PiT sequences that included all plant PiT proteins (89 sequences) identified in the NCBI non-redundant nucleotide database confirmed all four monophyletic clades and the topology of the PTB subfamily (Fig. S4). This analysis did not identify new core PTB members among these sequences, which confirmed that core PTB proteins do not exist in tracheophytes. Seventeen new PTB clade 4 members were identified in several angiosperm lineages, including basal angiosperms, monocots and eudicots, demonstrating that secondary losses can explain the absence of PTB clade 4 members in some gymnosperm (*P. taeda*) and angiosperm species. This also suggests that secondary loss is responsible for the absence of PTB clade 4 proteins in liverworts, mosses and streptophyte algae. Together, these data demonstrate that PTB proteins are present in both chlorophytes and streptophytes and that core PTB proteins are present in streptophyte algae and early diverging land plants (liverworts, mosses and hornworts).

The structure of core PTB proteins is conserved between streptophytes and chlorophytes

To evaluate the degree of structural and functional conservation between chlorophyte and streptophyte core PTB proteins, we compared their predicted topology, subcellular location and conserved motifs (Tables 1–3). The published core PTB proteins of chlorophytes have 12 transmembrane domains (TMDs) and a large hydrophilic loop between the seventh and eighth TMDs (Chung *et al.*, 2003; Kobayashi *et al.*, 2003; Li *et al.*, 2006, 2012). TMPRED hydropathy profile prediction software (Hofmann & Stoffel, 1993) predicted a large hydrophilic loop between the seventh and eighth TMDs in all streptophyte core PTB proteins, as in the chlorophyte core PTB proteins (Table 1). A large glycine- and glutamine-rich region inserted in the hydrophilic loop of two core PTB proteins (CrPTB1 and CrPTB8) of the chlorophyte *C. reinhardtii* (Fig. S1) is responsible for the difference in their predicted hydropathy profile from all other core PTB proteins (Kobayashi *et al.*, 2003; Moseley *et al.*, 2006). These data indicate that the topology of core PTB proteins is conserved between streptophytes and chlorophytes.

An N-terminal signal peptide targets proteins to the secretory pathway; proteins are inserted into the endoplasmic reticulum (ER) and either remain in the ER or move to the plasma membrane, vacuole or endomembrane compartments (Chrispeels & Raikhel, 1992). There is a signal peptide in core PTB proteins of

the chlorophyte *C. reinhardtii* (Moseley *et al.*, 2006). Using TargetP software (Emanuelsson *et al.*, 2007), which predicts subcellular location by detecting subcellular targeting motifs, we discovered a signal peptide at the N-terminus of all streptophyte core PTB proteins (Table 2). Therefore, streptophyte core PTB proteins are probably targeted to the secretory pathway. In contrast, TargetP did not identify a signal peptide in any members of the PTB clade 4, except for the *S. moelendorffii* PTB (SmPTB) (Table S3). This suggests that, although core PTB proteins traffic to the secretory pathway, the PTB clade 4 proteins do not.

To test the hypothesis that core PTB proteins traffic to the plasma membrane through the secretory pathway, we expressed an MpPTB6-YFP fusion protein in *M. polymorpha*. YFP fluorescence location was identical to that of the plasma membrane marker mCitrine-MpSYP12A (Kanazawa *et al.*, 2016) and the ER marker YFP-HDEL (Teh & Moore, 2007) in 25% and 75%, respectively, of plants expressing MpPTB6-YFP (Fig. S5). This is consistent with the hypothesis that MpPTB6-YFP moves through the secretory pathway to reach the plasma membrane.

Table 1 TMPRED predictions of the hydrophilic loop position of core PHOSPHATE TRANSPORTER B (PTB) proteins

	Species	Protein	TM number	Hydrophilic loop after
Chlorophytes	<i>Chlamydomonas reinhardtii</i>	CrPTB1*	14	TMD10
		CrPTB2	12	TMD7
		CrPTB3	12	TMD7
		CrPTB4	11	TMD7
		CrPTB5	11	TMD7
		CrPTB6	12	TMD7
		CrPTB7	12	TMD7
		CrPTB8 *	12	TMD8
		CrPTB9	11	TMD7
		CrPTB12	12	TMD7
	<i>Dunaliella viridis</i>	DvSPT1	11	TMD7
		DvSPT2	11	TMD7
Streptophytes	<i>Coccomyxa subellipsoidea</i>	CsPTB1	12	TMD7
	<i>Klebsormidium flaccidum</i>	KfPTB1	13	TMD7
		KfPTB2	13	TMD7
	<i>Coleochaete nitellarum</i>	CnPTB	12	TMD7
	<i>Marchantia polymorpha</i>	MpPTB1	12	TMD7
		MpPTB2	11	TMD7
		MpPTB3	12	TMD7
		MpPTB4	12	TMD7
		MpPTB5	12	TMD7
		MpPTB6	12	TMD7
		MpPTB7	12	TMD7
		MpPTB8	11	TMD7
	<i>Physcomitrella patens</i>	PpPTB1	12	TMD7
		PpPTB2	11	TMD7
	<i>Encalypta streptocarpa</i>	EsPTB1	12	TMD7
		EsPTB2	11	TMD7

The total number of predicted transmembrane domain (TM number) and the transmembrane domain after which TMPRED predicts the long hydrophilic loop (Hydrophilic loop after) is given for each core PTB protein with a complete sequence.

*Proteins with a glycine- and glutamine-rich region inserted in the hydrophilic loop.

There are no ER retention signals (KDEL or HDEL) (Chrispeels & Raikhel, 1992) at the N- or C-termini of any core PTB proteins (Figs S1, S6), suggesting that the retention of MpPTB6-YFP at the ER is an artefact caused by the over-expression or misfolding of the fusion protein in transgenic plants. Nevertheless, the absence of MpPTB6-YFP at the tonoplast supports the hypothesis that core PTB proteins are not located in this membrane.

We used MEME (Bailey & Elkan, 1994), a protein family motif finder software, to identify protein motifs that distinguish the core PTB proteins from other members of the PiT family. By analysing the sequence of the four published angiosperm PHT2 proteins (Versaw & Harrison, 2002; Zhao *et al.*, 2003; Chen *et al.*, 2007; Liu *et al.*, 2011), 12 published chlorophyte core PTB proteins (Kobayashi *et al.*, 2003; Li *et al.*, 2006; Moseley *et al.*, 2006; Guan *et al.*, 2008), fungal PiT proteins ScPho89 and NcPho4 (Mann *et al.*, 1989; Martinez & Persson, 1998), metazoan PiT proteins DmPit (from *Drosophila melanogaster*) (Bergwitz *et al.*, 2012), HsPit1 and HsPit2 (from *Homo sapiens*) (O'Hara *et al.*, 1990; Van Zeijl *et al.*, 1994), and bacterial PiT proteins TePit (NK55_00390 from *Thermosynechococcus elongatus*) (Böttger & Pedersen, 2005), EcPitA and EcPitB (from *Escherichia coli*) (Harris *et al.*, 2001), MEME identified 18 conserved motifs distributed among the PiT subfamilies (Table 3; Fig. S6). Five motifs (motifs 1–5) were present in all PiT proteins. They contain the PiT signature GANDVAN domains

(motifs 1 and 4) and the PiT signature residues (motifs 2 and 5). One motif (motif 6) was common to the fungal PiT and core PTB proteins, but absent from the metazoan PiT, bacterial PiT and PHT2 proteins. Seven motifs (motifs 7–13) were specific to the core PTB proteins and five motifs (motifs 14–18) were specific to the PHT2 proteins. The analysis of the streptophyte core PTB amino acid sequences showed that all motifs identified in the sequence of the chlorophyte core PTB proteins (motifs 1–13) were present in the streptophyte core PTB proteins (Table 3; Fig. S6). None of the motifs specific to the PHT2 subfamily was found in streptophyte core PTB proteins. The conservation of the protein motifs between core PTB proteins of chlorophytes and streptophytes suggests that the core PTB proteins are structurally similar in the two lineages of green plants. By contrast, of the five motifs present in the PTB clade 4 proteins (motifs 1, 3, 4, 6 and 10), four were absent in the seed plant PTB clade 4 sequences (Table S4; Fig. S6), including the GANDVAN domains (motifs 1 and 4) and the glutamate PiT signature residues (E⁵⁵ and E⁵⁷⁵ in HsPit2), both necessary for the Pi transport function of fungal and metazoan PiT proteins (Böttger & Pedersen, 2002, 2005; Böttger *et al.*, 2006; Andersson *et al.*, 2012). The loss of these motifs suggests that the function of PTB clade 4 proteins changed during the course of land plant evolution.

Taken together, these results imply that there has been little sequence divergence of the core PTB proteins since chlorophytes

Table 2 TargetP prediction of subcellular targeting of core PHOSPHATE TRANSPORTER B (PTB) proteins

			TARGETP score				
	Species	Protein	Chloroplast	Mitochondrion	Secretory pathway	Other	Prediction
Chlorophytes	<i>Chlamydomonas reinhardtii</i>	CrPTB1	0.008	0.019	0.953	0.06	Secreted (RC1)
		CrPTB2	0.004	0.008	0.913	0.395	Secreted (RC3)
		CrPTB3	0.004	0.007	0.894	0.475	Secreted (RC3)
		CrPTB4	0.002	0.012	0.979	0.309	Secreted (RC2)
		CrPTB5	0.002	0.013	0.982	0.28	Secreted (RC2)
		CrPTB6	0.011	0.015	0.943	0.339	Secreted (RC2)
		CrPTB7	0.011	0.006	0.899	0.304	Secreted (RC3)
		CrPTB8	0.011	0.029	0.688	0.108	Secreted (RC3)
		CrPTB9	0.008	0.013	0.958	0.264	Secreted (RC2)
		CrPTB12	0.008	0.014	0.957	0.243	Secreted (RC2)
Streptophytes	<i>Klebsormidium flaccidum</i>	KfPTB1	0.004	0.026	0.909	0.117	Secreted (RC2)
		KfPTB2	0.006	0.224	0.687	0.098	Secreted (RC3)
	<i>Coleochaete nitellarum</i>	CnPTB	0.016	0.019	0.889	0.114	Secreted (RC2)
		<i>Marchantia polymorpha</i>	MpPTB1	0.015	0.011	0.916	0.085
	MpPTB2		0.034	0.008	0.801	0.259	Secreted (RC3)
	MpPTB3		0.022	0.008	0.872	0.187	Secreted (RC2)
	MpPTB4		0.02	0.009	0.875	0.179	Secreted (RC2)
	MpPTB5		0.017	0.008	0.895	0.102	Secreted (RC2)
	MpPTB6		0.031	0.009	0.796	0.265	Secreted (RC3)
	MpPTB7		0.004	0.011	0.956	0.156	Secreted (RC1)
	MpPTB8		0.012	0.01	0.902	0.143	Secreted (RC2)
	<i>Physcomitrella patens</i>	PpPTB1	0.008	0.011	0.932	0.23	Secreted (RC2)
		PpPTB2	0.006	0.011	0.934	0.154	Secreted (RC2)
	<i>Encalypta streptocarpa</i>	EsPTB1	0.011	0.010	0.829	0.285	Secreted (RC3)
		EsPTB2	0.026	0.009	0.743	0.310	Secreted (RC4)
	<i>Phaeoceros carolinianus</i>	PcPTB4*	0.022	0.007	0.868	0.195	Secreted (RC2)

TARGETP prediction is given with a reliability class (RC) indicated, from 1 to 5, where '1' indicates the strongest prediction for streptophyte core PTB proteins with a complete sequence or with a partial sequence* if the N-terminus of the protein is present.

and streptophytes last shared a common ancestor. By contrast, PTB clade 4 proteins diverged considerably during the course of land plant evolution and the loss of the GANDVAN domains and conserved PiT residues suggests that they may not be Pi transporters.

Expression of *M. polymorpha* core PTB proteins in the *S. cerevisiae* *pho2* mutant rescues the *ScΔpho2* growth defect

The *S. cerevisiae pho2* (*ScΔpho2*) mutant encodes a defective *ScPHO2* transcription factor required for the expression of the two *S. cerevisiae* high-affinity Pi transporters: *ScPho84* (homologue of plant PHT1 Pi transporters) and *ScPho89* (homologue of plant PTB Pi transporters) (Bun-Ya *et al.*, 1991; Martinez & Persson, 1998; Pinson *et al.*, 2009). The inability of *ScΔpho2* mutants to express high-affinity Pi transporters (*ScPho84* and *ScPho89*) means that growth is impaired in low-Pi environments (< 500 μM), but normal at Pi concentrations higher than 500 μM because of the constitutive expression of yeast low-affinity Pi transporters (Fig. 3). To determine whether the *M. polymorpha* core PTB proteins have a Pi transporter function, we tested the ability of MpPTB4, MpPTB6 and MpPTB7 proteins to rescue the *ScΔpho2* growth defect on low-Pi media.

Transformation of *ScΔpho2* mutants with constructs expressing MpPTB4, MpPTB6 or MpPTB7 rescued the *ScΔpho2* growth defect to varying degrees (Fig. 3). No growth was observed in any strain in the absence of Pi. At Pi concentrations equal to or greater than 250 μM, the expression of MpPTB4 partially rescued the *ScΔpho2* growth defect. At 500 μM of Pi, the expression of MpPTB6 and MpPTB7 partially rescued the

ScΔpho2 growth defect. This suggests that the expression of MpPTB4, MpPTB6 and MpPTB7 proteins in the *ScΔpho2* mutant restores Pi transport. As controls, expression of the yeast high-affinity Pi transporters *ScPho84* or *ScPho89* in *ScΔpho2* completely rescued the mutant growth defect at a Pi concentration equal to or greater than 250 μM. The addition of doxycycline – a repressor of the tetracycline-responsive promoter used to express the transporter genes (Gari *et al.*, 1997) – blocked the growth rescue by MpPTB4, MpPTB6, MpPTB7, *ScPho84* and *ScPho89*. This indicates that the restoration of *ScΔpho2* growth is a result of the expression of the Pi transporters introduced on the expression plasmid. Taken together, these data are consistent with the hypothesis that MpPTB4, MpPTB6 and MpPTB7 are functional Pi transporters.

The expression of the core PTB genes is induced in low-Pi environments in the streptophyte alga *C. nitellarum* and the liverwort *M. polymorpha*

In angiosperms, the steady state level of most PHT1 mRNAs is higher in low-Pi than in high-Pi environments, and in roots than in shoots, consistent with their role in Pi uptake from low-Pi environments (Mudge *et al.*, 2002; Nussaume *et al.*, 2011; Qin *et al.*, 2012). Similarly, the steady state level of most chlorophyte PTB mRNAs is also higher in low-Pi than in high-Pi environments (Chung *et al.*, 2003; Kobayashi *et al.*, 2003; Li *et al.*, 2006; Moseley *et al.*, 2006; Djouani-Tahri *et al.*, 2011). To determine whether the expression of genes encoding the core PTB proteins is regulated by Pi availability in streptophyte algae and early diverging land plants, we measured the steady state levels of core PTB mRNAs in the streptophyte alga *C. nitellarum* and the

Table 3 Conserved motifs of PHOSPHATE (Pi) TRANSPORTER (PiT) subfamilies identified by MEME (Multiple Em for Motif Elicitation)

Motif	AA	Sequence	PHT2	Bacterial PiT	Metazoan PiT	Fungal PiT	Chlorophyte core PTB	Streptophyte core PTB
Motif 1	14	GaNDVANafgTSVg	Yes	Yes	Yes	Yes	Yes	Yes
Motif 2	15	lpvStTHxixGaxxG	Yes	Yes	Yes	Yes	Yes	Yes
Motif 3	6	SWfxSP	Yes	Yes	Yes	Yes	Yes	Yes
Motif 4	14	FaHGaNNDVxnaxGP	Yes	Yes	Yes	Yes	Yes	Yes
Motif 5	34	TPxrGfxaElaxASxxGLPxStTG	Yes	Yes	Yes	Yes	Yes	Yes
Motif 6	12	HaxAEVfDPxtE	–	–	–	Yes	Yes	Yes
Motif 7	8	FpyxxGxV	–	–	–	–	Yes	Yes
Motif 8	12	kaAWVxaxxaaG	–	–	–	–	Yes	Yes
Motif 9*	8	KxaaxxGx	–	–	–	–	Yes	Yes
Motif 10	12	AAxFAxGayaPS	–	–	–	–	Yes	Yes
Motif 11	7	kdxkYe	–	–	–	–	Yes	Yes
Motif 12	5	INxxI	–	–	–	–	Yes	Yes
Motif 13	8	nxSxxtxG	–	–	–	–	Yes	Yes
Motif 14	27	MAxAFHISsRtAxalSixlAfAALtxP	Yes	–	–	–	–	–
Motif 15	19	KxKxLsYxTLLfGFYMAWNI	Yes	–	–	–	–	–
Motif 16	29	KCIRRFVYSaPnPGQAAAAAIAVFXGV	Yes	–	–	–	–	–
Motif 17	11	GPTGtQLxIVY	Yes	–	–	–	–	–
Motif 18	15	FARGINxVRAETVRE	Yes	–	–	–	–	–

For each motif, the amino acid length (AA), the sequence and the presence/absence (Yes/–) in proteins of the PiT subfamilies (PHOSPHATE TRANSPORTER 2 (PHT2), bacterial PiT, metazoan PiT, fungal PiT, chlorophyte core PHOSPHATE TRANSPORTER B (core PTB) and streptophyte core PTB) are given. Upper case, lower case and 'x' indicate residues of the motifs conserved in 80%, 60% or < 60%, respectively, of the concerned proteins.

*Motif found in all core PTB proteins except *Dunaliella viridis* DvSPT1 and DvSPT2.

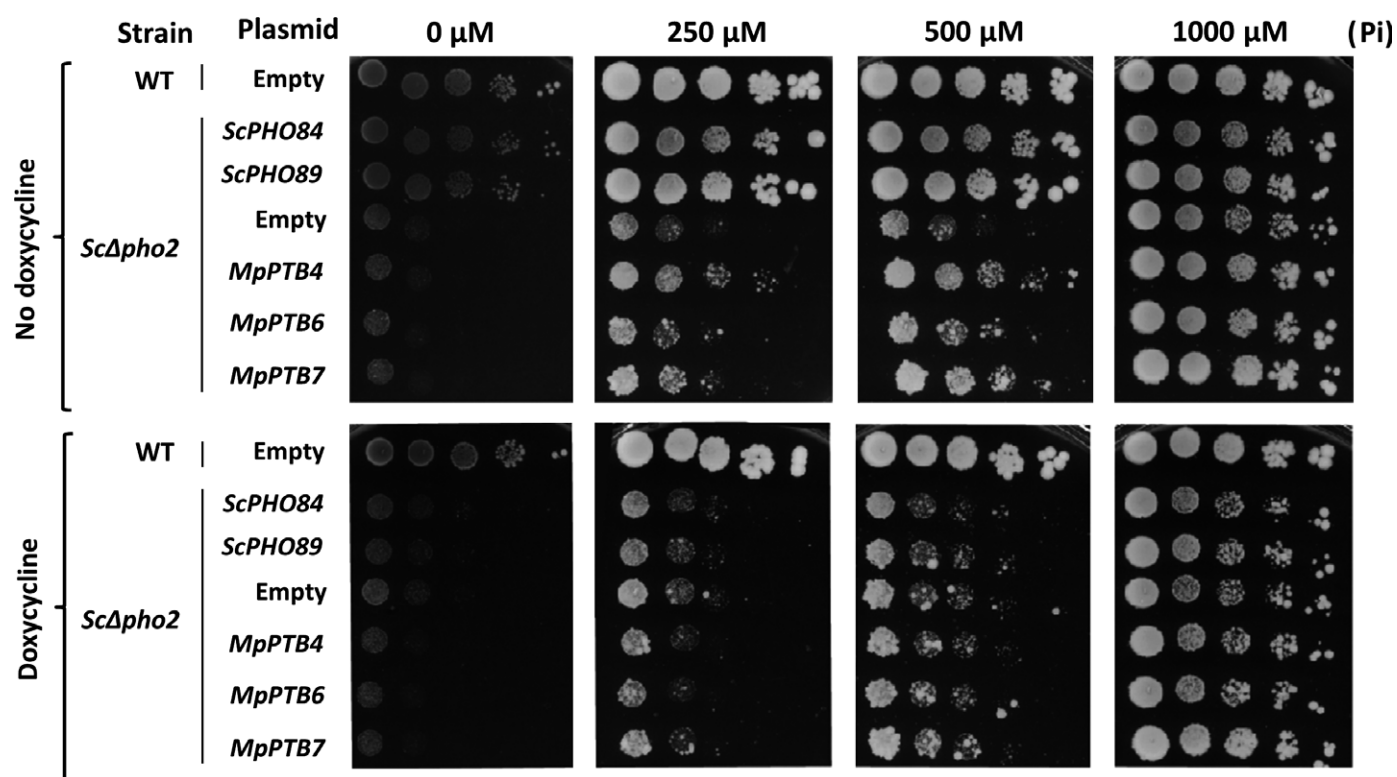


Fig. 3 Complementation of the inorganic phosphate (Pi) uptake-deficient yeast mutant *ScΔpho2* by expression of *Marchantia polymorpha* core PHOSPHATE TRANSPORTER B (PTB)-encoding genes. The yeast strain Y2092 lacking the *ScPHO2* gene (*ScΔpho2*) was transformed with the *pCM189* vector expressing, under a tetracycline-repressible promoter, the coding sequence (CDS) of MpPTB4, MpPTB6 or MpPTB7, the CDS of the yeast high-affinity Pi transporter *ScPHO84* or *ScPHO89* (positive controls), or the *pCM189* vector without insertion (wild-type (WT) Empty; negative control). The *PHO2* wild-type yeast strain (WT, Y178), isogenic to Y2092, was transformed with the *pCM189* empty vector and used as a positive control. To compare the growth of the different strains, 10 times serial dilutions of exponentially growing cells were spotted and grown for 11 d at 30 °C on medium containing the concentrations of Pi indicated and 10 mg l⁻¹ of doxycycline to repress the expression of the CDS, or no doxycycline.

liverwort *M. polymorpha* grown in different concentrations of Pi. We found that the expression of the single *C. nitellarum* core PTB-encoding gene and of seven of the eight *M. polymorpha* core PTB-encoding genes was higher in low-Pi than in Pi-replete conditions (Fig. 4). Steady state levels of *CnPTB* mRNAs were three-fold higher in low-Pi (0 mM) than in Pi-replete (1.6 mM) medium (Fig. 4a). In *M. polymorpha*, the steady state levels of the MpPTB2, MpPTB3, MpPTB4, MpPTB5 and MpPTB7 mRNAs were between 12.5 times higher (MpPTB7) and 730 times higher (MpPTB4) in the rhizoids – the filamentous rooting cells of early diverging land plants – of plants grown in low-Pi than in Pi-replete conditions (Fig. 4b). In addition, MpPTB1 and MpPTB8 mRNAs were only detected in the rhizoids of plants grown in low-Pi conditions, indicating that they are exclusively expressed in low-Pi conditions (Fig. 4b). Only one *M. polymorpha* core PTB gene – MpPTB6 – was transcriptionally insensitive to low Pi in our conditions; the steady state levels of MpPTB6 mRNA were identical in low- and Pi-replete conditions (Fig. 4b). The increased expression of the only *C. nitellarum* and of the majority of the *M. polymorpha* core PTB genes – *CnPTB*, MpPTB1, MpPTB2, MpPTB3, MpPTB4, MpPTB5, MpPTB7 and MpPTB8 – in low-Pi growth conditions is typical of plasma membrane Pi transporter families catalysing Pi uptake from low-Pi environments (Mann *et al.*, 1989; Bun-Ya *et al.*, 1991; Versaw,

1995; Daram *et al.*, 1998; Martinez & Persson, 1998; Harris *et al.*, 2001; Rae *et al.*, 2003). The higher steady state mRNA levels of *M. polymorpha* MpPTB1, MpPTB2, MpPTB3, MpPTB4, MpPTB5, MpPTB6 and MpPTB8 in rhizoids than in thallus support the hypothesis that these genes are involved in Pi uptake from the environment. Taken together, these data suggest that core PTB genes encode plasma membrane Pi transporters involved in Pi uptake in *C. nitellarum* and *M. polymorpha*.

Discussion

The uptake of Pi is necessary for plant growth and development, and here we report that members of the PTB family function as Pi transporters in streptophytes. PTB sequences have been identified previously in chlorophytes and hypothesized to be involved in Pi uptake on the basis of their sequence and structural similarity to the metazoan and fungal PiT Na⁺/Pi symporters (Chung *et al.*, 2003; Kobayashi *et al.*, 2003; Li *et al.*, 2006; Moseley *et al.*, 2006; Guan *et al.*, 2008; Djouani-Tahri *et al.*, 2011; Bergwitz *et al.*, 2012) and the ability of *D. viridis* core PTB proteins to increase the Na⁺ sensitivity of a yeast mutant defective in Na⁺ export (Li *et al.*, 2006). We demonstrate that core PTB genes from the liverwort *M. polymorpha* encode functional Pi transporters that complement a yeast mutant defective in high-affinity

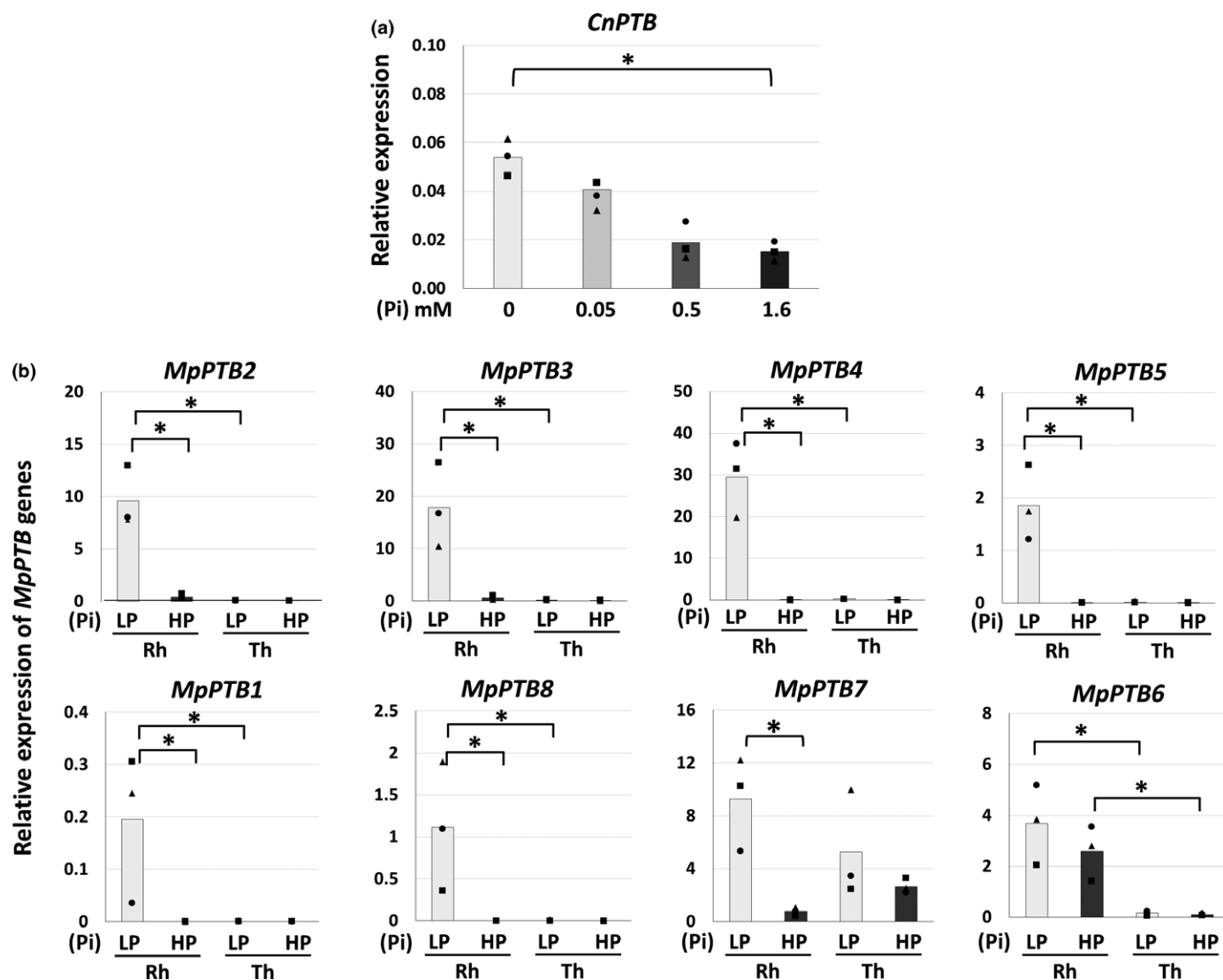


Fig. 4 Transcript level of core *PHOSPHATE TRANSPORTER B* (*PTB*)-encoding genes in *Marchantia polymorpha* and *Coleochaete nitellarium* grown in various inorganic phosphate (Pi) conditions. (a) Histograms indicating the mean transcript level ($n = 3$) of *CnPTB* in *C. nitellarium* under different Pi concentrations. Transcript levels were normalized with *CnEF1* expression. *The transcript level measured for each growth condition is significantly different from that measured in all other conditions ($P < 0.05$, Kruskal–Wallis test). Each biological replicate is represented by a circle, a square and a triangle for replicate 1, replicate 2 and replicate 3, respectively. (b) Histograms showing the mean transcript level ($n = 3$) of *MpPTB1*, *MpPTB2*, *MpPTB3*, *MpPTB4*, *MpPTB5*, *MpPTB6*, *MpPTB7* and *MpPTB8* in rhizoids (Rh) and thallus (Th) in low-Pi (LP) and high-Pi (HP) conditions in *M. polymorpha*. Transcript levels were normalized with the geometric mean of the *MpACT* and *MpSAND* expression levels. *The transcript levels measured in the conditions indicated by the ends of the brackets are significantly different from each other ($P < 0.05$, Kruskal–Wallis test). Each biological replicate is represented by a circle, a square and a triangle for replicate 1, replicate 2 and replicate 3, respectively.

plasma membrane Pi transport. The presence of an N-terminal signal peptide on core PTB proteins suggests that core PTB Pi transporters are targeted to the secretory pathway and may be located at the plasma membrane. This hypothesis is supported by the location of the core PTB protein, *MpPTB6*, at the plasma membrane of *M. polymorpha*. Furthermore, the observation of higher steady state levels of most core *PTB* genes of *M. polymorpha* and the streptophyte alga *C. nitellarium* in low-Pi than in Pi-replete growth conditions, and of most core *PTB* genes of *M. polymorpha* in rhizoids than in thallus, resembles the expression of members of the PHT1 family (Mudge *et al.*, 2002; Nussaume *et al.*, 2011; Qin *et al.*, 2012) and is characteristic of

Pi-responsive genes, including genes encoding Pi transporters involved in the uptake of Pi from low-Pi environments (Mann *et al.*, 1989; Bun-Ya *et al.*, 1991; Versaw, 1995; Daram *et al.*, 1998; Martinez & Persson, 1998; Harris *et al.*, 2001; Rae *et al.*, 2003). Taken together, these data are consistent with core PTB proteins being plasma membrane Pi transporters in streptophytes.

In angiosperms, proteins in the PHT1 family catalyse the uptake of Pi from the environment through H^+/Pi symport (Leggiewe *et al.*, 1997; Mitsukawa *et al.*, 1997; Daram *et al.*, 1998; Rae *et al.*, 2003; Liu *et al.*, 2008; Nussaume *et al.*, 2011). Although members of the PHT1 Pi transporter family have been

reported in streptophyte algae and early diverging land plants, their function has not been demonstrated in these taxa (Wang *et al.*, 2008; Delaux *et al.*, 2015; Saint-Marcoux *et al.*, 2015). However, the higher steady state level of one *M. polymorpha* and two *P. patens* PHT1 mRNAs in plants grown on low-Pi relative to Pi-replete conditions (Wang *et al.*, 2008; Saint-Marcoux *et al.*, 2015) is consistent with the hypothesis that PHT1 proteins are active in Pi uptake in early diverging land plants. The presence of functional core PTB Pi transporters in early diverging land plants and streptophyte algae suggests that two families of plasma membrane Pi transporters – core PTB and PHT1 – act alongside each other in Pi uptake in these species. Such a dual plasma membrane Pi transporter system has not been described previously in streptophytes. Two high-affinity Pi uptake systems act in fungi; H⁺/Pi transporters homologous to PHT1 proteins and Na⁺/Pi transporters homologous to PTB proteins are active in Pi uptake in low external Pi conditions in *N. crassa*, *S. cerevisiae* and *Yarrowia lipolytica*. Differences in pH optima and counter-ion selectivity of the fungal Na⁺/Pi and H⁺/Pi symporters have led to the hypothesis that different transporters are active in different environmental conditions, providing the organisms with a wide adaptive range (Versaw, 1995; Zvyagil'skaya & Persson, 2003; Dick *et al.*, 2014). This could also be true in streptophyte algae and early diverging land plants, where both PHT1 and core PTB proteins are probably active in Pi uptake.

H⁺/Pi symport by PHT1 proteins in angiosperms depends on the generation of an H⁺ gradient through the activity of the plasma membrane H⁺-ATPase (Leggewie *et al.*, 1997; Mitsukawa *et al.*, 1997; Daram *et al.*, 1998; Rae *et al.*, 2003; Liu *et al.*, 2008; Nussaume *et al.*, 2011). Such an H⁺ gradient cannot be generated in an aquatic environment because of the rapid dispersion and reaction of H⁺ with hydroxide ions in the water (Agmon *et al.*, 2016). Moreover, both marine and fresh water habitats occupied by charophyte and chlorophyte algae are alkaline (Raven, 1984; Walker & Sanders, 1991; Rubio *et al.*, 2005; Moseley *et al.*, 2006; Chan *et al.*, 2012). The hypothesis that Na⁺ could be used as a driving force by organisms occupying environments in which the production of an H⁺ gradient is not possible, such as an alkaline or aquatic environment, was originally proposed after physiological studies showed that the uptake of several nutrients, including Pi, amino acids and glucose, was dependent on Na⁺ uptake in algae, but on H⁺ uptake in terrestrial angiosperms (Ullrich-Eberius & Yinchol, 1974; Raven, 1984; Walker & Sanders, 1991; Bush, 1993; Reid *et al.*, 2000; Weiss *et al.*, 2001; Rubio *et al.*, 2005; Kuzyakov & Jones, 2006; Svennerstam *et al.*, 2011; Chan *et al.*, 2012). Consistent with this model is the observation that *D. viridis* core PTB proteins catalyse Na⁺ transport, suggesting that these proteins are probably Na⁺/Pi symporters in aquatic chlorophytes (Li *et al.*, 2006). Chlorophytes, streptophyte algae, liverworts, mosses and hornworts require moist habitats. Thus, the activity of core PTB in these lineages could facilitate Na⁺/Pi uptake from water. Our report that proteins involved in Na⁺- and H⁺-dependent Pi uptake co-exist in streptophyte algae and early diverging land plants suggests that Na⁺ and H⁺ symport systems co-existed in streptophytes before plants colonized the land. Early diverging land plants inherited the dual Pi uptake system

comprising both core PTB Na⁺/Pi and PHT1 H⁺/Pi symporters from streptophyte algae, where it is conserved in extant liverworts, mosses and hornworts, whose life cycle depends on the presence of free water.

There are PTB proteins in tracheophytes (lycophytes, monilophytes, gymnosperms and angiosperms). These tracheophyte proteins belong to PTB clade 4, a PTB clade that is phylogenetically distinct from the core PTB proteins (Figs 2, S2, S3). During tracheophyte evolution, members of PTB clade 4 lost the N-terminal signal peptide (Table S3), the GANDVAN signature domains and the signature glutamates that are required for Pi transport in metazoan and fungal PiT proteins (Böttger & Pedersen, 2002, 2005; Böttger *et al.*, 2006; Andersson *et al.*, 2012) (Table S4; Fig. S6). This suggests that PTB clade 4 proteins are not Pi transporters and are not located at the plasma membrane. Therefore, the absence of core PTB proteins in tracheophytes suggests that land plants lost the core PTB Na⁺/Pi transport function as plants radiated into drier habitats, leading to the situation in angiosperms in which PHT1 is the major Pi uptake system (Nussaume *et al.*, 2011; Ayadi *et al.*, 2015). These data suggest that there was a gradual transition from a dual H⁺/Pi and Na⁺/Pi uptake system in early diverging land plants to a Pi uptake system entirely dependent on H⁺/Pi uptake in late diverging groups during the course of land plant evolution as they adapted to drier habitats. PTB proteins are not encoded in the genome of *Z. marina* (Olsen *et al.*, 2016) despite physiological evidence for Na⁺/Pi uptake (Rubio *et al.*, 2005). This suggests that a PTB-independent Na⁺/Pi uptake mechanism evolved secondarily in angiosperms that re-adapted to aquatic habitats.

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Author contributions

C.B. designed the research. C.B. and L.D. wrote the paper. C.B., F.P.L.C and A.L.V. identified the plant PiT sequences. H.P. performed the expression analysis in *M. polymorpha*. C.B., H.P. and B.P. performed the yeast complementation test. C.B. and H.B. produced the RNA sample for *C. nitellarum* RNAseq. C.C., A.J.H. and S.K. performed the transcriptome assembly. C.B. and C.C. captured and analysed the confocal images. C.B. performed all other experiments and analysed all the data.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Multiple amino acid alignment sequences used for the Pi TRANSPORTER (PiT) phylogeny of green plants.

Fig. S2 Multiple amino acid alignment sequences used for the enlarged Pi TRANSPORTER (PiT) phylogeny of green plants.

Fig. S3 Maximum-likelihood tree of the Pi TRANSPORTER (PiT) sequences of green plants.

Fig. S4 Maximum-likelihood tree of the enlarged set of Pi TRANSPORTER (PiT) sequences of green plants.

Fig. S5 The MpPTB6-YFP fusion protein is located at the plasma membrane and endoplasmic reticulum.

Fig. S6 Location of conserved motifs in the Pi TRANSPORTER (PiT) amino acid sequences.

Table S1 Cross-reference information for the *PiT* sequences used for the Pi TRANSPORTER (PiT) phylogeny of green plants

Table S2 List of primers for cloning, vector construction and expression analysis

Table S3 TARGETP prediction of subcellular targeting of PHOSPHATE TRANSPORTER B (PTB) clade 4 proteins

Table S4 Conserved motifs of Pi TRANSPORTER (PiT) sub-families identified by MEME (Multiple Em for Motif Elicitation) in PHOSPHATE TRANSPORTER B (PTB) clade 4

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