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Increased phosphate transport of Arabidopsis thaliana Pht1;1 by site-directed mutagenesis of tyrosine 312 may be attributed to the disruption of homomorphic interactions

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ABSTRACT

Members of the Pht1 family of plant phosphate (Pi) transporters play vital roles in Pi acquisition from soil and in planta Pi translocation to maintain optimal growth and development. The study of the specificities and biochemical properties of Pht1 transporters will contribute to improving the current understanding of plant phosphorus homeostasis and use-efficiency. In this study, we show through split in vivo interaction methods and in vitro analysis of microsomal root tissues that Arabidopsis thaliana Pht1;1 and Pht1;4 form homomeric and heteromeric complexes. Transient and heterologous expression of the Pht1;1 variants, Pht1;1Y312D, Pht1;1Y312A and Pht1;1Y312F, was used to analyse the role of a putative Pi binding residue (Tyr 312) in Pht1;1 transporter oligomerization and function. The homomeric interaction among Pht1;1 proteins was disrupted by mutation of Tyr 312 to Asp, but not to Ala or Phe. In addition, the Pht1;1Y312D variant conferred enhanced Pi transport when expressed in yeast cells. In contrast, mutation of Tyr 312 to Ala or Phe did not affect Pht1;1 transport kinetics. Our study demonstrates that modifications to the Pht1;1 higher-order structure affects Pi transport, suggesting that oligomerization may serve as a regulatory mechanism for modulating Pi uptake.

Key-words: Arabidopsis; oligomerization; phosphate transporters; transporter activity.

INTRODUCTION

All life requires phosphorus (P), a mineral nutrient crucial for energy storage, signalling, and the synthesis of nucleotides and lipids. Inorganic phosphate (Pi) is the primary form of P available for plant uptake, and its concentration is often low and heterogeneously distributed in soils (Raghothama 2000). As a result, agricultural systems introduce large quantities of non-renewable Pi fertilizers to increase crop yields, but the majority of the applied Pi becomes immobilized in the soil or is carried away into adjacent waterways during heavy rain events, which can lead to eutrophication. Understanding plant mechanisms of Pi acquisition and translocation may lead to the enhancement of uptake- and usage-efficiencies of Pi, which will help to ameliorate Pi deficiency, over-fertilization and resource depletion challenges.

Plant Pht1 transporters comprise a subfamily of major facilitator superfamily (MFS) transporters that catalyse proton-Pi symport (Pao et al. 1998; Reddy et al. 2012). Individual plant species have a number of Pht1 members that collectively are expressed in diverse tissues and exhibit high- and low-affinity Pi transport, functioning in Pi uptake and distribution (Schulze et al. 2003; Nussaume et al. 2011; Fan et al. 2013). Of the nine members of the Arabidopsis thaliana Pht1 family (Mudge et al. 2002), five have been functionally characterized: Pht1;1 and Pht1;4 are expressed at relatively high levels in the root and contribute significantly to Pi uptake (Misson et al. 2004; Shin et al. 2004; Catarecha et al. 2007; LeBlanc et al. 2013), Pht1;8 and Pht1;9 are also root-expressed and contribute to Pi acquisition (Remy et al. 2012), and Pht1;5 is predominantly expressed in shoots where it plays a key role in Pi distribution in accordance with Pi status and development (Nagarajan et al. 2011).

The mechanisms that regulate distinct Pht1 transporters are not completely understood, but a complex and multi-tiered regulatory programme is emerging. For example, transcription of Arabidopsis Pht1 transporters is regulated by WRKY transcription factors (e.g. WRKY45 and WRKY75), and PHR1, a MYB transcription factor recognized as a major low-Pi responsive transcriptional activator (Bari et al. 2006; Devaiah et al. 2007; Wang et al. 2014). In addition to transcriptional control, Pht1 transporters are regulated by a number of post-translational mechanisms, all of which are modulated by Pi availability. Proper targeting of Pht1 proteins to the plasma membrane (PM) requires PHF1, an ER localized chaperone and is influenced by a phosphorylation event (Gonzalez et al. 2005; Bayle et al. 2011). Pht1 proteins at the PM can be internalized to endosomes where they can be recycled or sent to lytic vacuoles for degradation (Bayle et al. 2011). Two Pi-responsive ubiquitination components, PHOSPHATE 2 (PHO2) and NITROGEN LIMITATION ADAPTATION (NLA), have been revealed to play an important role in Pht1 regulation. PHO2 is an E2 conjugase shown to target Pht1;1, and possibly other Pht1 proteins, for
degradation via physical interactions within ER compartments (Huang et al. 2013). NLA is an E3 ligase that may act in cooperation with PHO2 to target Pht1 proteins, with an apparent preference for AtPht1;4 (Lin et al. 2013; Park et al. 2014). Notably, both PHO2 and NLA transcript abundance is negatively regulated by microRNAs (miR399 and miR827, respectively), highlighting a post-transcriptional mechanism that influences Pht1 accumulation (Aung et al. 2006; Bari et al. 2006; Kant et al. 2011; Huang et al. 2013; Lin et al. 2013; Rojas-Triana et al. 2013; Liu et al. 2014b; Park et al. 2014).

Many membrane transporters can oligomerize into higher-order structures, which acts to confer structural and functional variations distinct from monomers, such as changes in affinity, catalysis and stability (Marianayagam et al. 2004; Clarke & Gulbis 2012; Matthews & Sunde 2012). Several membrane nutrient transporters and channels have been reported to form oligomeric structures, including Arabidopsis high-affinity sulphate transporters (Kataoka et al. 2004; Rouached et al. 2008), Solanaceae and Arabidopsis sucrose transporters (Reinders et al. 2002a,b; Schulze et al. 2003), ammonium transporters from tomato and Arabidopsis (Ludewig et al. 2003; Graff et al. 2011; Yuan et al. 2013), Arabidopsis high-affinity nitrate transporters (Okamoto et al. 2006; Orsel et al. 2006; Wirth et al. 2007; Sun et al. 2014), and Arabidopsis potassium channels (Obrdlik et al. 2004; Duby et al. 2008). Evidence for the oligomerization of Pi transporters stems from studies of the Medicago truncatula Pht1 transporter MtP1T1, in which immunoblot analysis showed high molecular weight bands coinciding with possible oligomeric structures (Chiou et al. 2001). Additional immunoblot analysis of other Pht1 family members (Nussaume et al. 2011) and a dominant-negative phenotype for ectopic expression of an AtPht1;1 mutant (Catarecha et al. 2007) further suggests the formation of Pht1 oligomeric complexes. Recently, the crystal structure of PiPT, a Pht1 transporter from the endophytic fungus Piriformospora indica, shows the packing of two crystallographic threefold units that may be biologically relevant to a trimeric complex (Pedersen et al. 2013). However, oligomerization of Pht1 transporters has not previously been demonstrated.

Herein, we show homomeric and heteromeric oligomerization of Arabidopsis Pht1;1 and Pht1;4 via split in vivo assays and in vitro analysis. To better understand the underlying mechanisms involved in Pht1 oligomerization, site-directed mutagenesis was performed on a highly conserved Pht1 transporter motif, now recognized to play a role in Pht1 transporter substrate-binding (Pedersen et al. 2013). We show that the substitution of Tyr 312 to Asp in Pht1;1 impedes oligomerization and confers an increased Pi transport capacity in yeast. These results suggest an active site-oligomerization relationship in which oligomerization may serve as a mechanism to regulate transporter activity.

MATERIALS AND METHODS

Generation of constructs

Clones for use in SLC, mbSUS, and localization studies were generated using a PCR-based Gateway® system (Invitrogen, Carlsbad, CA, USA). Amplified AtAMT1;1, AtPHT1;1, AtPHT1;4 and AtPht1;1 mutant cDNAs containing appropriate attB flanking regions were cloned into pDONR™/Zeol via recombination (Invitrogen, Carlsbad, CA, USA). Constructs verified by sequencing were recombined into desired Gateway® destination vectors. Site-directed mutagenesis of Pht1;1 was performed via overlap extension PCR as previously described (Ho et al. 1989). For yeast expression, ScPHO84, AtPht1;1 and AtPht1;1 mutant cDNAs were cloned into the pYES2 vector, which contains a Gα1-inducible promoter, via restriction enzyme/ligation and the resulting plasmids were sequenced for verification. Primer sequences are listed in Supporting Information Table S1.

Split luciferase complementation (SLC) assays

A. thaliana (Col-0) protoplasts were prepared and transformed as described (Kato & Jones 2010). Briefly, 1.2 × 10⁴ protoplasts were transformed with the appropriate pDuExAc6 and pDuExDc6 vectors (2.0 μg each) in a well of a 96-well plate. These vectors constitutively express a protein of interest at the C-terminal end with NLuc- and CLuc-tags, respectively. Four independent transformations were conducted for each protein pair of interest in one experiment. The experiment was then repeated at least three times using protoplasts prepared from different plants. Relative luminescence units (RLU) were measured 16 h after transformation. RLU in the experimental and biological replicates were used to obtain means and SEs for each protein pair.

Immunoprecipitation and immunoblot analysis

Root microsomal proteins were extracted, immunoprecipitated and immunoblotted as previously described (Huang et al. 2013) with Pht1;1/2/3- or Pht1;4-specific antibodies.

Mating-based split ubiquitin and split-ubiquitin yeast-two-hybrid systems

Clones were transformed into yeast haploid strains THY.AP4 (MATa; ade2-, his3-, leu2, trp1-, ura3-; lexA::ADE2, lexA::His3, lexA::lacZ) and THY.AP5 (Matα; ade2-, his3-, leu2-, trp1-) via LiAc transformation as described (Grefen et al. 2009). Cub fusion isolates were tested for background activity using the lacZ reporter gene prior to mating as described (Grefen et al. 2009). Cub and Nuβ isolates were grown overnight in SC and SCnu liquid media, respectively, at 30 °C in an orbital shaker. One millilitre of overnight culture was spun at room temperature for 5 min at 2500 g. Cells were resuspended in 200 mL yeast extract, peptone, dextrose (YPD). 15 μL of bait and prey were mixed and 10 μL each were plated onto YPD and incubated for 10–16 h at 28 °C. Portions of matings were streaked onto SCnu plates and grown 2–3 d at 28 °C. Matings were inoculated in SCnu liquid media and incubated overnight. Cultures were diluted to OD₆₀0 0.1 and grown to OD₆₀₀
-0.6–1.0. Cultures were diluted to OD_{600} 0.1 with water, plated onto SC_{nmm}, SC_{ahltum} and SC_{alhim} + 10 mM 3-AT and grown 3–9 d at 28 °C. Split-ubiquitin yeast two-hybrid analysis of AtPht1;1 and AtPht1;4 was performed using the DUAL membrane Kit (Dual Systems Biotech, Zurich, Switzerland) according to the manufacturer’s instructions. β-galactosidase activity was observed using a chloroform overlay assay (Duttweiler 1996; Lin et al. 2013).

**Yeast complementation assays and RNA extraction**

Sequenced clones were transformed into the *Saccharomyces cerevisiae* yeast strain PAM2 (Δpho89::TRP1 Δpho84::HIS3 ade2 leu2 his3 trp1 ura3) as described (Geitz et al. 1995; Daram et al. 1999). Preparation of cells for growth rate assays was adapted from Muchhal et al. (1996). Isolates were grown in Pi-replete (7.34 mM) SC_{a}, media (pH 5.6) containing 2.0% sucrose until OD_{600} 0.3–1 (log phase). Cells were spun at 6000 g for 5 min at room temperature. Pellets were washed in Pi- and carbohydrate-deficient SC_{a} media. Cells were brought to OD_{600} ~0.025–0.03 in low-Pi (25 μM) SC_{a} media containing 0.5% sucrose with freshly made 2.0% galactose, and growth was monitored at 30 °C in an orbital shaker through log phase. After about 30 h of growth in low-Pi containing media, yeast cells were collected for RNA extraction as described (Collart & Olivierio 2001). Prior to cDNA synthesis, 0.3–0.5 μg of RNA was DNase-treated with RNase-free RNase-free RQ DNase (Promega, Madison, WI, USA). cDNA was synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). RT-PCR reactions of 30 cycles were performed using Ex-Taq polymerase (Takara Bio, Kyoto, Japan). ScActin was used as a reference gene. RT-PCR gene-specific primers are listed in Supporting Information Table S1. Qualitative analysis was determined via EtBr DNA gel and the ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA). Adobe Photoshop CS3 software (San Jose, CA, USA) was used to organize images.

**Subcellular localization**

Plasmids expressing AtPht1;1 and AtPht1;1 Tyr 312 mutants in pEarleyGate101, TAIR stock #CD3-683 (Earley et al. 2006; Karimi et al. 2007) were used to transform *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Nicotiana benthamiana* plants were grown in soil under long-day conditions at 23 °C for 4 weeks prior to transformation. AtPht1;1 and AtPht1;1^{Tyr312X} mutants were co-infiltrated with *A. tumefaciens* p19::pKYLX7 and PHF1::CAMBIA-bar (Voinnet et al. 2003; Gonzalez et al. 2005; Brkljacic et al. 2009). *A. tumefaciens* cultures were grown to OD_{600} ~1.0 at 28 °C in an orbital shaker. The combination ratio of p19, PHF1, and each AtPht1;1 cDNA was 0.5:1:5:1.0, respectively. Cultures were combined and spun for 10 min at 2000 g. Pellets were washed with 1 mL of 10 mM MgCl2 to remove antibiotics and resuspended in 2 mL of a resuspension solution (10 mM MES pH 5.9, 10 mM MgCl2, 15 μM acetoxyringsone). Leaves were infiltrated immediately with 1 mL of the suspension. Plants were grown for 2–3 d after infiltration under the same conditions. Leaves were infiltrated with 50 μM FM4-64 (Setareh Biotech, Eugene, OR, USA) in water immediately preceding microscopy (Liesche et al. 2010; Lin et al. 2013). Imaging was captured at 40× using a Leica DM RXA2 deconvolution upright microscopy system in the Socolofsky Microscopy Center (Louisiana State University). Adobe Photoshop software was used to organize images.

**32P uptake assays**

Cells were prepared as previously described for yeast complementation assays in SCa media (25 μM Pi, 0.5% sucrose, 2.0% galactose) and brought to OD_{600} ~0.05. Once in log phase, cells were collected by repeating washes as previously described (Pi- and carbohydrate-free SCa media). Cells were brought to OD_{600} 4.0–12.0 with Pi-free SCa media containing 3% glucose (Lau et al. 1998; Cataroche et al. 2007; Ai et al. 2009; Lundh et al. 2009). K_{2}HPO_{4}/KH_{2}PO_{4} solutions were prepared using a final K_{2}HPO_{4} concentration of 2.5 μM with the remaining addition of KH_{2}PO_{4} to obtain total Pi concentrations of 25 μM and 10 μM. For total Pi concentrations of 50, 100 and 250 μM, K_{2}HPO_{4}/KH_{2}PO_{4} solutions were prepared using a final K_{2}HPO_{4} concentration of 25 μM with the remaining addition of KH_{2}PO_{4} 500 μM, 750 μM, and 1000 μM Pi concentration were prepared using a final K_{2}HPO_{4} concentration of 50 μM. Two hundred microliters of cells were used per each K_{2}HPO_{4}/KH_{2}PO_{4} solution for a total volume of 250 μL. K_{2}HPO_{4}/KH_{2}PO_{4} solutions containing no cells were used for obtaining K_{2}HPO_{4} background activity. Following the addition of K_{2}HPO_{4}, assays were terminated after 6 min by passing 100 μL of cells through separate filter-stermed syringes (MCE Membrane Filter, 0.45 μM, TISCH Scientific, North Bend, OH, USA), filtering once with 10 mL ice-cold 100 mM KH_{2}PO_{4} followed by two passes of air to dry filter (Wykoff & O’Shea 2001; Cataroche et al. 2007). Filters were placed in scintillation vials containing 4 mL ScintiVerse BD Cocktail (Fisher Scientific) for counting by a Beckman LS 6000IC.

**Arabidopsis genome identifiers**

Genes from the above work include At4g13510 (AtAMT1;1), At5g43350 (AtPht1;1) and At2g38940 (AtPht1;4).

**RESULTS**

AtPht1;1 and AtPht1;4 proteins interact

A SLC assay optimized for *Arabidopsis* protoplasts (Kato & Jones 2010) was used to determine the homomeric and heteromeric assembly capabilities of Pht1;1 and Pht1;4. This method has been used previously to identify interactions among *Arabidopsis* syntaxin proteins (Fujikawa & Kato 2007), as well as ammonium transporters (Lalonde et al. 2010). Pht1;1 and Pht1;4 cDNAs were cloned into plasmids
containing the N-terminal (NLuc) and C-terminal (CLuc) halves of Renilla luciferase, in which the C-terminal end of each cDNA was fused to the corresponding fragment. As positive controls, we also examined the homomeric interaction of the Arabidopsis AMT1;1 ammonium transporter (Ludewig et al. 2003; Lalonde et al. 2010) and H2A and H2B heteromeric interactions (Fujikawa & Kato 2007). As shown in Fig. 1, luciferase activity was observed for homomeric Pht1;1 and heteromeric Pht1;1-Pht1;4 at levels comparable with the AMT1;1 and H2A–H2B controls. The luciferase activity for homomeric Pht1;4 was lower, but was still above that for the AMT1;1-Pht1;4 negative control. Together the data indicate oligomerization capabilities of Pht1;1 and Pht1;4 in vivo.

In vitro analysis using co-immunoprecipitation confirmed the interactions between Pht1;1 and Pht1;4 (Fig. 2). Polyclonal antibodies were raised against Pht1;4 and Pht1;1/Pht1;2/Pht1;3 (because of the high sequence identity among Pht1;1, Pht1;2, and Pht1;3, and thus the unlikelihood of generating Pht1;1-specific antibodies, a peptide identical among these three Pht1 proteins was used to raise the antibodies). Pht1;1/2/3 and Pht1;4 were immunoprecipitated from total microsomal proteins isolated from root tissue of wild type or the pht1;4 mutant (Misson et al. 2004) using anti-Pht1;1/2/3 and anti-Pht1;4 antibodies, respectively. Subsequent immunoblot analysis of bound proteins was performed with the corresponding antibody to confirm homomorphic and heteromeric interactions. As a negative control, total microsomal proteins were immunoprecipitated without the addition of antibodies, resulting in no detection of unbound IP products. The analysis of bound Pht1;4 from pht1;4 fractions served as an additional negative control and resulted in a lack of Pht1;4 signal.

The mating-based split-ubiquitin system (mbSUS) has been adapted to observe interactions of PM-bound proteins (Obredlil et al. 2004; Grefen et al. 2007, 2009; Chen et al. 2012). We used the mbSUS system to further validate interactions between Pht1;1 and Pht1;4. Ubiquitin specific proteases recognize the formation of a full-length ubiquitin protein as a result of interacting proteins. These enzymes allow for the activation of the lexA promoter-driven reporter genes LucZ, ADE2 and HIS3 (Grefen et al. 2009). AMT1;1, Pht1;1 and Pht1;4 cDNAs were cloned into vectors containing the C-terminal half of ubiquitin (X-CUb) fused to the C-terminal end of the target protein and/or the N-terminal mutated half of ubiquitin (NUbG-X) fused to the N-terminus of proteins. The I13G mutation of the N-terminal half of ubiquitin prevents spontaneous reconstitution of ubiquitin (Grefen et al. 2009). Plasmids were mobilized into appropriate Saccharomyces cerevisiae haploid a and α strains and mated. Interactions were measured by observing growth on selective media (SCahleu) with 10 mM 3-aminotriazole.

Figure 1. Pht1;1 and Pht1;4 interact to form homomeric and heteromeric oligomers. Amt1;1 (AmT1), Pht1;1 (Pht1), and/or Pht1;4 (Pht4) were co-expressed in Arabidopsis protoplasts for split luciferase complementation analysis. AmT1:1 homomeric interactions and AmT1:1 co-expressed with Pht1:4 served as positive and negative controls, respectively. Results were obtained from at least two independent experiments carried out in triplicate with evaluation of standard full-length Renilla luciferase activity and NLuc-H2A and H2B-CLuc interaction activities. Data are means ± SD.

Figure 2. Pht1 immunoprecipitation (IP) of root microsomal proteins. Microsomal proteins were isolated from roots of Col-0 and Ws wild type and pht1;4 mutants. Native Pht1;1/2/3 (a) or Pht1;4 (b) and their interacting proteins were immunoprecipitated by specific antibodies. IP products were loaded for detecting Pht1;1/2/3 or Pht1;4 by immunoblot (bound). IP without adding antibodies was used as a negative control (unbound). The levels of Pht1;1/2/3 (a) or Pht1;4 (b) in root microsomal protein fractions are shown in the lower panels.

Figure 3. Split-ubiquitin assays in yeast confirm homomeric and heteromeric interactions of Pht1;1 and Pht1;4. Positive split-ubiquitin interactions were identified on minimal media (SC\textsubscript{ad}ine) containing 10 mM 3-aminotriazole. As a positive control, growth was monitored for matings of each of Amt1;1, Pht1;1, and Pht1;4 with the non-mutated amino fragment of ubiquitin, NUbWT. Amt1;1, Pht1;1, and Pht1;4 mating with NUbG, the mutated N-terminal half of ubiquitin, as well as Amt1;1 mating with Pht1;1 and Pht1;4 served as negative controls.

The role of a conserved protein motif in Pht1 oligomerization

Prior to the availability of the three-dimensional structure of PiPT, a Pi transporter from the endophytic fungus P. indica (Pedersen et al. 2013), we generated a putative Pht1;1 homomeric structure using Phyre2 Fold Recognition and Chimera software to identify potential protein interfaces based on molecular and biochemical similarities of known oligomeric crystal structures (Pettersen et al. 2004; Kelley & Sternberg 2009). Pht1;1 monomeric (Supporting Information Fig. S2a) and dimeric (Supporting Information Fig. S3) models were created by superimposing Pht1;1 to the structure of Escherichia coli multidrug transporter EmrD [RCSB Protein Data Bank (PDB) entry 2GFP], an MFS protein that produced high confidence scores with the Pht1;1 alignment, suggestive of overall protein fold overlap (91% coverage; 99.95% confidence; 13% identity) (Yin et al. 2006). From the dimeric model and sequence similarity among Pi transporters from plants and yeast, we identified a highly conserved motif, WFLLDIAFY (Supporting Information Fig. S4), in the seventh transmembrane alpha helix, which occupied the potential interface between monomers of a hypothetical Pht1;1 dimer (Supporting Information Fig. S3).

Upon release of the PiPT structure (PDB 4J05), a new model of Pht1;1 was generated using the Phyre2 server (Supporting Information Fig. S2b). Interestingly, PiPT was the second highest scoring template and the structure of E. coli XylE, a GLUT1-4 homolog (PDB 4GBZ), was the highest scoring, most likely because the large intracellular loop between helix 6 and 7 (residues 230–298) is not present in the PiPT structure, possibly because of this region being disordered. The structure of XylE was also released after the initial model of Pht1;1 was made. The new model of Pht1;1 is based on both the PiPT and XylE structures with >90% confidence in the model for 96% of the sequence (Supporting Information Fig. S2b). Whether PiPT functions as an oligomer has not been investigated. Trimeric arrangements of PiPT monomers in the same orientation were observed within crystals used to determine the PiPT structure (Pedersen et al. 2013). Although it is not clear how likely this trimer reflects a physiological structure, we aligned our second Pht1;1 model (based on XylE and PiPT; Supporting Information Fig. S2b) with the putative PiPT trimer (Supporting Information Fig. S5). The WFLLDIAFY motif within helix 7 is within the pore and makes contacts with helices 9, 10 and 12, which form the putative trimer interface. Even though GLUT1-4 is known to oligomerize, the crystal structure of XylE did not reveal quaternary structure.

To determine the role of the WFLLDIAFY motif in interaction, we made several mutations (W304R, F305E, D308A, I309E, F311E and Y312D) simultaneously to generate Pht1;1-m, and also generated Pht1;1 proteins containing each individual mutated residue. All mutants were each co-expressed with native Pht1;1 in protoplasts and interaction was measured using SLC (Fig. 4). Protoplasts expressing Pht1;1 and either Pht1;1\textsuperscript{I309E}, Pht1;1\textsuperscript{F305E}, Pht1;1\textsuperscript{D308A}, Pht1;1\textsuperscript{I309E} or Pht1;1\textsuperscript{Y312D} resulted in reconstituted luciferase activity corresponding to 54.4–102.9% of the luciferase activity of the homomeric Pht1;1 interaction. The high relative luminescence (281.3%) observed for the interaction between Pht1;1 and Pht1;1\textsuperscript{W304R} remains unclear, but will be studied further in the future. In contrast, we observed a significant lack of luciferase activity with native Pht1;1 co-expressed with Pht1;1-m, which may be attributed to the Y312D mutation (Fig. 4; see later).

Tyrb 312 plays a role in AtPht1;1 interaction and function

We found that when Pht1;1 was mutated to Pht1;1\textsuperscript{Y312D}, interaction of the Pht1;1 homo-oligomer was disrupted (Fig. 4). RT-PCR of RNA extracted from co-transformed protoplasts confirmed that lack of luminescence in the SLC experiments was not due to a defect in Pht1;1\textsuperscript{Y312D} expression (Supporting Information Fig. S6). To better understand the role of Tyr 312 in homomeric Pht1;1 interaction, we generated two addi-
The native Pht1;1 sequence fused to the amino fragment of luciferase (NLuc) was co-expressed with native Pht1;1, a Pht1;1 variant containing six site-directed mutations (mut), or individual site-directed mutants (as indicated) in Arabidopsis protoplasts for split luciferase complementation analysis. Results were obtained from two independent experiments carried out in triplicate with standard full-length Renilla luciferase activity and NLuc-H2A and H2B-CLuc interaction activity. Data are means ± SD.

Figure 4. Interaction of the Pht1;1 homo-oligomer is abolished by mutation of Tyr 312. The native Pht1;1 sequence fused to the amino fragment of luciferase (NLuc) was co-expressed with native Pht1;1, a Pht1;1 variant containing six site-directed mutations (mut), or individual site-directed mutants (as indicated) in Arabidopsis protoplasts for split luciferase complementation analysis. Results were obtained from two independent experiments carried out in triplicate with standard full-length Renilla luciferase activity and NLuc-H2A and H2B-CLuc interaction activity. Data are means ± SD.

Figure 5. Pht1;1 homomeric interaction is abolished by the Y312D mutation, but not by Y312A or Y312F. Pht1;1 (Pht1) was co-expressed with itself or a site-directed mutant (as indicated) in Arabidopsis protoplasts for split luciferase complementation analysis. Results were obtained from two independent experiments with standard full-length Renilla luciferase activity and NLuc-H2A and H2B-CLuc interaction activity. Data are means ± S.D.

We found reconstituted luciferase activity for the interaction between native Pht1;1 and each mutant at a level similar to the homomeric native Pht1:1 interaction (Fig. 5). Thus, mutation of Tyr 312 to Asp, but not Ala or Phe, abolishes Pht1;1 homo-oligomerization. To determine whether changes in interaction were associated with improper processing (e.g. localization) of Pht1;1Y312D we transiently expressed YFP fusions of the Tyr 312 mutants in N. benthamiana leaves. Because infiltration of Pht1;1 and Tyr 312 mutants alone resulted in little visual detection (data not shown), co-infiltration was performed with p19, an RNAi silencing suppressor previously used to enhance fluorescence in planta (Baulcombe & Molnar 2004; Brkljacic et al. 2009) and AtPHF1 (Gonzalez et al. 2005; Bayle et al. 2011). Prior to microscopy, the PM of epidermal cells was stained with FM4-64, an endocytic marker (Liesche et al. 2010; Bayle et al. 2011; Lin et al. 2013). As shown in Fig. 6, we observed co-localization of FM4-64 and each of the Pht1;1Y312D, Pht1;1Y312A and Pht1;1Y312F proteins, indicating that mutations of Tyr 312 did not disrupt the localization of Pht1;1.

Heterologous expression of plant Pht1 proteins in Pi-uptake defective yeast strains has been used previously to characterize Pi transporter activities (Bun-ya et al. 1996; Yompakdee et al. 1996; Leggewie et al. 1997; Smith et al. 1997; Liu et al. 2008, 2014a; Jia et al. 2011). Herein we used this system to determine the impact of the Tyr 312 mutations on Pht1;1 activity. The mutant S. cerevisiae yeast strain PAM2 (Apho89::TRP1 Δapho84::HIS3 ade2 leu2 his3 trp1 ura3) was transformed with pYES2 plasmids containing Pht1;1, Pht1;1Y312D, Pht1;1Y312A or Pht1;1Y312F under the control of a galactose-inducible promoter. For controls, PAM2 was transformed with the native yeast Pht1 Pi transporter, ScPho84, or empty vector. RT-PCR experiments confirmed comparable transgene expression in the yeast isolates (Supporting Information Fig. S7). Yeast cells were grown on Pi-sufficient media, washed with Pi-free media, and inoculated into media containing galactose and a low level of Pi (25 μM). Cell growth (OD at A660) was monitored, and growth rate coefficients were determined (Supporting Information Fig. S8). Several independently obtained growth rate coefficients (n ≥ 3) and corresponding doubling times were calculated for each cell type (Fig. 7, Supporting Information Table S2). As expected, cells expressing Pho84 exhibited a higher growth rate than cells containing the empty pYES2 vector, consistent with Pho84-complementation of the PAM2 Pi-uptake defect. Expression of Pht1;1, Pht1;1Y312A, or Pht1;1Y312F also complemented this defect, but only moderately. These results suggest that the Y312A and Y312F mutations did not affect Pht1;1 function. In contrast, the growth rate of cells expressing Pht1;1Y312D exceeded that of Pht1;1 by 1.5-fold, a level comparable with that of Pho84-complemented cells, suggesting enhanced Pi transport activity of Pht1;1 by the Y312D mutation.

To determine whether the increased growth rate of yeast expressing Pht1;1Y312D was due to enhanced Pi uptake capabilities, we performed 32P uptake assays to compare the kinetic properties of Pht1;1 and Pht1;1Y312D. Yeast cells were grown in Pi-replete media to log phase, washed with Pi-free media, and subjected to short-term 32Pi uptake assays with varying external Pi concentrations. Representative assays for cells expressing Pht1;1 or Pht1;1Y312D are shown in Supporting Information Fig. S9. Both cell lines showed Michaelis–Menten kinetics and exhibited similar K_m values of 544 μM.
and 581 μM, respectively (Table 1), indicating that Y312D does not affect the Pi affinity of Pht1;1. In contrast, the $V_{\text{max}}$ for Pht1;1 $^{Y312D}$ was sevenfold higher relative to Pht1;1 (Table 1), suggesting that the Y312D mutation enhances the Pi uptake capacity of Pht1;1.

**DISCUSSION**

Oligomerization of Pht1 proteins was implied by observations in some earlier studies, but was not implicitly demonstrated (Chiou et al. 2001; Catarecha et al. 2007; Nussaume et al. 2011). Herein we used *in vivo* and *in vitro* interaction assays to directly show that Pht1;1 and Pht1;4 form homomeric and heteromeric oligomers. Upon observing interaction among Pht1 monomers we sought to investigate the nature of the oligomeric unit. Visual examination of high molecular weight bands on Pht1 immunoblots from previous studies (Chiou et al. 2001; Nussaume et al. 2011) indicated the presence of possible dimers and tetramers. With this information and by searching known protein structures in the RCSB PDB for those sharing similarity to Pht1;1, we generated three-dimensional models of Pht1;1 using EmrD as a template (Supporting Information Fig. S2a) and later using XylE and PiPT (Supporting Information Fig. S2b). EmrD, XylE and PiPT are MFS proteins with standard topology of 12 transmembrane helices (Yin et al. 2006). Structural determination of EmrD revealed two identical molecules present as a dimer in the asymmetric unit of the crystal lattice; although whether this packing arrangement is physiological is unclear (Yin et al. 2006). Using EmrD as a template, we

**Table 1.** Kinetic properties of Pht1;1 and Pht1;1 $^{Y312D}$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol 10$^7$ cells$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM2 + pYES2: Pht1;1</td>
<td>544 ± 95</td>
<td>101 ± 8</td>
</tr>
<tr>
<td>PAM2 + pYES2: Pht1;1 $^{Y312D}$</td>
<td>581 ± 74</td>
<td>707 ± 47</td>
</tr>
</tbody>
</table>

Pht1;1 and Pht1;1 $^{Y312D}$ cDNAs were expressed in the PAM2 yeast mutant using pYES2. Representative transformants expressing each cDNA were used in $^{32}$P uptake assays, measured for nine phosphate concentrations between 0 and 1000 μM for 6 min. The kinetic constants were calculated from Michaelis–Menten plots generated via non-linear regression in KaleidaGraph for results obtained from three independent experiments run in duplicate and are the mean ± SD ($n = 3$).
generated a dimeric model of Pht1;1 and identified the amino acids present in the putative interaction region, WFLLDIAFY, which corresponded to transmembrane helix 7 (Supporting Information Fig. S3). Unlike EmrD, PiPT orients helix 7 towards the centre of the transporter pore, where three amino acids in helix 7, W320, D324 and Y328, are reported to play an important role in Pi binding (Pedersen et al. 2013). Ironically, our data show that mutagenesis of this conserved Tyr (Y312 in Pht1;1) to Asp interferes with oligomerization (Fig. 4). As PiPT presumably serves as a better model for Pht1;1 than EmrD because of higher sequence identity and a shared biological function, it is likely that helix 7 of Pht1;1 occupies the central portion of the cylinder where several of its residues occupy the Pi-binding site, rather than occupying a putative interaction region between two hypothetical Pht1;1 monomers. The asymmetric unit within PiPT crystals used to determine its structure contains trimeric arrangements of PiPT monomers in the same orientation (Pedersen et al. 2013). The interface of this trimer involves interactions between helices 9, 10 and 12, which also border the WFLLDIAFY motif in helix 7. Based on the dynamic nature of MFS proteins during transport, it is possible that activity is linked to oligomerization through allosteric events coupling helix 7 with the interface. Mutations in the active site, like Tyr 312 to an Asp, may be translated to the surface favouring the monomeric or oligomeric state. Additional studies are required to ascertain the oligomeric composition of Pht1 proteins.

The heterologous expression of Pht1 transporters in yeast has previously demonstrated a wide range of kinetic parameters for individual Pht1 proteins (Leggewie et al. 1997; Daram et al. 1998; Rausch et al. 2004; Liu et al. 2008, 2014a; Ai et al. 2009; Jia et al. 2011). Indeed, despite overlapping tissue-specific expression patterns, including the root epidermis, and their presumed high-affinity activities, the Pht1 proteins LePT1, StPT2, MtPT1 and MtPT2 were shown to have $K_m$ values of 31, 130, 587 and 641 μM (Leggewie et al. 1997; Daram et al. 1998; Liu et al. 2008). MtPT1 and MtPT2 share relatively high sequence identity with Pht1;1, and all three genes contain 5′-UTR introns (Liu et al. 2008). Although determining orthology is complicated by differential expansion of Pht1 gene families in individual plant species (Liu et al. 2008), the similarities among MtPT1, MtPT2 and Pht1;1 suggest they are functionally equivalent. Our finding that the $K_m$ of Pht1;1 (544 μM) is very similar to those of MtPT1 (587 μM) and MtPT2 (641 μM) further support this notion.

In addition to affecting oligomerization, our data indicate that the Y312D mutation enhances Pi transport of Pht1;1 in yeast. We found that Pht1;1 partially complemented the PAM2 mutant, whereas Pht1;1Y312D complemented the mutant as well as the native yeast Pho84 transporter (Fig. 7). This is likely due to the sevenfold increase in $V_{\text{max}}$ for Pht1;1Y312D relative to Pht1;1, as shown by short-term $^{32}$Pi uptake analysis (Table 1). These uptake experiments also suggest that Pht1;1 and Pht1;1Y312D exhibit similar Pi affinities, with $K_m$ values of 544 μM and 581 μM, respectively (Table 1). $V_{\text{max}}$ is a function of the enzyme concentration, in this case the abundance of transporter at the PM, and the catalysis rate ($k_{\text{cat}}$), in this case the release of Pi from the transporter into the cytosol. $K_m$ is also a function of $k_{\text{cat}}$, as well as two additional terms: $k_i$, the binding of Pi to the transporter, and $k_e$, the extracellular release of Pi. As $k_{\text{cat}}$ is shared by both $V_{\text{max}}$ and $K_m$, and a change in $k_{\text{cat}}$ would thus affect both parameters, it is possible that the concentration of functional transporters differs between Pht1;1 and Pht1;1Y312D. RT-PCR experiments showed the transgenes were expressed at comparable levels, suggesting an equivalent number of transporter monomers, although the Y312D mutation might alter post-translational regulatory mechanisms, such as PM targeting or degradation/recycling of Pht1 (Bayle et al. 2011). However, our observations from the transient localization of Pht1;1 and Pht1;1Y312D in N. benthamiana leaves (Fig. 6) implies that the Y312D mutation does not have a major effect on PM localization. Pht1;1 as an oligomer may restrict its Pi transport capacity relative to Pht1;1Y312D by allowing only a portion of the oligomer to be receptive to Pi binding at any given time. This would be similar to the MFS lactose transporter, LacS, which, like Pht1 transporters, operates via a ‘rocker-switch’ mechanism where only the intracellular or extracellular side of the transporter is open. LacS functions as a dimer, and the orientation of one monomer (i.e. outside-facing or inside-facing) is coupled to the opposite orientation in the other monomer (Veenhoff et al. 2001). Another explanation for Pht1;1 and Pht1;1Y312D having the same $K_m$, but a different $V_{\text{max}}$ is that substitution of Tyr 312 with Asp still allows for Pi transport (i.e. lower $k_i$) and enhances intracellular release of Pi ($k_e$). Based on the PiPT structure, the conserved Tyr (Y328 in PiPT) is predicted to occupy the Pi-binding site of the transporter where it, along with five additional conserved residues, coordinates Pi (Pedersen et al. 2013). Among the five additional residues is an Asp (D308 in Pht1;1), whose alternating protonation/deprotonation plays a key role in the movement of Pi through the transporter. It is possible that replacing Tyr 312 with an Asp still allows for Pi binding (i.e. does not affect $K_i$), but provides additional negative charge upon deprotonation that results in a faster release of Pi through the transporter (i.e. lower $k_e$ and/or higher $k_{\text{cat}}$).

Environmental conditions, such as Pi availability, could induce Pht1;1 interactions as a means of Pi-uptake regulation, similar to the situation for Arabidopsis nitrate transporter NRT1.1. Previously, NRT1.1 was identified as having two distinct uptake affinities, that is low affinity and high affinity, under the control of a ‘phosphorylation switch’ (Liu et al. 1999; Liu & Tsay 2003; Ho et al. 2009; Gutierrez 2012; Parker & Newstead 2014). Structural analysis revealed that phosphorylation decouples NRT1.1 homeric interactions, leading to the reorganization of functional monomers with high-affinity nitrate uptake capabilities (Sun et al. 2014). Pht1 transporters too have been shown to undergo modifications in response to environmental conditions to maintain Pi homeostasis (Bayle et al. 2011; Huang et al. 2013; Lin et al. 2013). Under Pi-replete conditions, Pht1;1 transporter accumulation is controlled by various transcriptional and post-translational regulatory mechanisms and includes the recycling and/or degradation of Pht1;1 transporters at the
PM in the presence of excess Pi (Bayle et al. 2011; Huang et al. 2013; Lin et al. 2013). While oligomerization does not appear to alter the Pi affinity of Pht1 transporters, a mechanism comparable with the oligomeric function of NRT1.1 could occur with Pht1;1 as an immediate form of regulation prior to the down-regulation, retention and degradation of Pht1 transporters as described by the regulatory roles of NLA, PHO2 and PHF1 (Bayle et al. 2011; Huang et al. 2013; Lin et al. 2013). This may correlate with the temporal and spatial differences recently described between the PHO2- and NLA-mediated pathways despite their abilities to function cooperatively in the degradation of Pht1;1 and Pht1;4 upon Pi-replete conditions (Huang et al. 2013; Lin et al. 2013).

AMTs were previously shown to form phosphorylation-dependent oligomers as a means of providing transporter regulation for rapid transport inactivation to prevent ammonium over-accumulation (Loque et al. 2007; Lanquar et al. 2009; Yuan et al. 2013). Also, the Amt1;1 trimeric complex is proposed to regulate ammonium transport via a gate mechanism (Loque et al. 2007, 2009). Oligomeric forms of Pht1 could act in a regulatory mechanism similar to AMTs to inactivate or down-regulate Pi uptake. If the trimeric form of PiPT represented in Pedersen et al. (2013) has biological significance, this may suggest a higher-order structure for Pht1;1 similar to that of Amt1;1. Alternatively, interactions between Pi transporter monomers may modify the position of transmembrane helices to control substrate access and/or transport through the pore. This perception may be relevant to interlaced models of Pht1;1 based on structures of EmrD and PiP'T, where perhaps the monomeric state positions Tyr 312 within the pore to engage in Pi binding/transport, whereas the oligomeric state may energetically favour Tyr 312 (i.e. helix 7) to shift along the periphery of the protein to trigger interaction and close pore access (Forrest et al. 2011). Future experiments aimed at deciphering the relative impact of the Y312D mutation on transporter activity and oligomerization under fluctuating Pi conditions should shed additional light on these open questions.

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DISCLOSURES

No conflicts of interest declared.

FINANCIAL SOURCES

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AUTHOR CONTRIBUTIONS

A.P.S. and E.B.F. designed the research, performed experiments, analysed data and wrote the paper. S.F.D., N.K., T.J.C., M.A.M., D.M.O., R.D. and W.Y.L. performed experiments and analysed data.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Phr1;1 and Phr1;4 interactions observed using the split-ubiquitin Y2H system.

Figure S2. A model of Phr1;1 based on the structure of (a) EmrD and (b) PiPT and XyIE.

Figure S3. A homodimeric model of Phr1;1 based on the structure of EmrD.

Figure S4. A conserved motif among Phr1 proteins implicated in interaction.

Figure S5. A homotrimer model of Phr1;1 based on the structures of PiPT and XyIE.

Figure S6. RT-PCR of RNA extracted from co-transformed protoplast preparations used for split luciferase complementation assays.

Figure S7. RT-PCR of RNA isolated from yeast shows similar transgene transcript abundance.

Figure S8. Representative growth assays of PAM2 yeast cells expressing empty pYES2 vector, Pho84, Phr1;1 or Phr1;1 Tyr 312 variants.

Figure S9. Representative 32P uptake assays of PAM2 yeast cells expressing Phr1;1 or Phr1;1Y312D via the pYES2 vector.

Table S1. PCR and RT-PCR primer sequences.

Table S2. Growth parameters for yeast cells expressing an empty pYES2 vector, or complemented with Pho84, Phr1;1, Phr1;1Y312A, Phr1;1Y312P, or Phr1;1Y312D.