ABSTRACT

BOICO, IRINA. *Arabidopsis thaliana* High Affinity Phosphate Transporters PHT1;8 and PHT1;9 are Important for Adaptation to Pi Stress. (Under the direction of Dr. Marcela Pierce.)

Phosphorus (P) is a pivotal macronutrient for plant development, but often it is one of the least available in the rhizosphere, having a negative impact on plant productivity. Plants maintain proper P homeostasis by integrating and coordinating nutritional needs and availability and respond by many adaptations. Improving phosphate (Pi) uptake in conditions of Pi insufficiency is one of the critical adaptations to Pi deprivation in *Arabidopsis thaliana,* achieved by the up-regulation of high-affinity phosphate transporters, which are members of the PHOSPHATE TRANSPORTER 1 Family (PHT1). The aim of this study was to decipher the role of the two *PHT1* genes most induced by Pi starvation, *PHT1;8* and *PHT1;9*. Reverse genetics studies show that loss of function mutants for *PHT1;8* and *PHT1;9* have reduced basal Pi levels in the shoots and display a reduced tolerance to Pi shortage, with augmented rhizosphere acidification and anthocyanin accumulation. Conversely, the overexpression of *PHT1;8* or *PHT1;9* results in a higher basal Pi, suggestive of enhanced Pi uptake and possibly translocation. Genetic interactions amongst *PHT1* genes is highly probable, as the *pht1;8* and *pht1;9* mutants have increased *PHT1;1/2/3/4* expression, and *PHT1;8* and *1;9* transcripts accumulate at higher levels in the *pht1;1/4* mutant. Fluorescent-protein translational fusions to PHT1;8 and PHT1;9 driven by a constitutive promoter show that PHT1;8 and PHT1;9 localize predominantly to the endoplasmic reticulum (ER) and slightly to the plasma membrane (PM). PM localization was more prominent in the root apical meristem region, suggestive of a developmental control for the localization of these proteins. Additionally, the PM pool of PHT1;8 and PHT1;9 is sensitive to Brefeldin A (BFA)

suggesting the active role of recycling in regulating the abundance of these proteins at the PM. Despite the coinciding localization of PHOSPHATE2 (PHO2), an ubiquitin E2 conjugating enzyme, and PHT1;8/9 at the ER, the localization of PHT1;8/9 appears to be independent of PHO2, although PHO2 exerts transcriptional control over *PHT1;8*.

Molecular aspects that underlie the antagonistic crosstalk between the iron and phosphate homeostasis were characterized. The Pi-stressed mutants *pht1;8, pht1;9* and *pht1;8 1;9* showed upregulation of Fe uptake-related genes, which suggested an interaction between the Fe and P response pathways. Furthermore, Fe deprivation resulted in the decreased abundance of phosphate starvation markers, hence the Fe availability differentially affects Pi deficiency response. Similarly, mutants with compromised Fe homeostasis displayed upregulation of phosphate starvation markers, indicating that part of the Pi-deprived signaling network is more active in the mutants, in both Pi-replete and low Pi conditions. Altogether, our results indicate that PHT1;8 and 1;9 are distinct, given their unique dual-localization to PM and ER, that these transporters play important roles in the adaptation to Pi deprivation, and that the signaling networks for P and Fe homeostasis interact in a complex manner.

Arabidopsis thaliana High Affinity Phosphate Transporters PHT1;8 and PHT1;9 are Important for Adaptation to Pi Stress

by
Irina Boico

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Biology

Raleigh, North Carolina

2016

APPROVED BY:

Marcela Pierce Committee Chair	Rebecca Boston Vice Chair
Steven Clouse	Imara Perera
Terri Long	

DEDICATION

To my parents, who showed me the value of hard work encouraged me to follow my dreams. To my sister Alina, who has always believed in me and cheered me up. To my son Vincent, who has lighten the burden of tough days with his smile and affection. To my husband Paolo, who supported me throughout my PhD with his unconditional love.

BIOGRAPHY

Irina Boico was born in Chisinau, Republic of Moldova, where she obtained her K-12 education. In 2004 she was awarded with a merit-based full scholarship to the Moldova State University in the Chemical Technology Program. In 2007 she transferred to California where she completed three semesters at San Francisco City College. She then continued her education at NCSU and in 2010 obtained a Bachelor of Science in Biochemistry with a Minor in Economics (*Cum Laude*). In Spring 2010 had a semester long project in Dr. Marcela Rojas-Pierce's Lab and decided to pursue a PhD in Plant Biology. In the Fall of 2010 she entered the PhD program in the Plant Biology Department at NCSU, under the mentorship of Dr. Marcela Rojas-Pierce. During her PhD, Irina was a teaching assistant for several courses: PB200 Plant Life, PB414 Cell Biology and BIT410/510 Manipulations of Recombinant DNA / Core Technologies in Molecular and Cellular Biology.

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere appreciation to my PhD advisor, Dr. Marcela Rojas-Pierce for the continuous guidance, support and mentoring, her hard work and enthusiasm have inspired me throughout the years. I would also like to thank my committee members, Dr. Rebecca Boston, Dr. Imara Perera, Dr. Terri Long and Dr. Steve Clouse for their feedback, helpful suggestions and encouragement.

I would like to express my deepest gratitude to the Rojas-Pierce Lab members: Dr. Sang Won Han, Efraín Rivera Serrano, Dr. Jiameng Zheng, María F. Rodríguez-Welsh, Dr. Ashley Alvarez and Dr. Carla Brillada for their invaluable support and for being like a family to me. A very special thanks goes to all the members of the Plant Genetics Friday Meeting Group also known as "The Awesome Plant Group" for constructive criticism and stimulating discussions.

I am very thankful to Dr. Eva Johannes and the Cell and Molecular Imaging Facility (CMIF) for offering the confocal and lightsheet microscopy training and for the assistance in image processing. I am also forever thankful to all the Plant and Microbial Biology staff, particularly to Sue Vitello, Carol Apperson, Cindy Whitehead and Vicki Lemaster for their administrative support and their kindness.

I am truly grateful to the Plant and Microbial Biology Department for funding and for giving me the opportunity to teach. At the same time, I would like to thank all my students for making my teaching experience extremely rewarding. A very special thanks goes to the instructors that I assisted in teaching, Dr. Chad Jordan, Dr. Jim Mickle, Dr. Marcela Rojas-Pierce, Dr. Mellisa Srougi, Dr. Laura Ott, Dr. Carlos Goller, Dr. Thomas Lentz and Dr. Sabrina Robertson for their remarkable support and guidance. Finally, I would like to thank my family and friends for their unconditional love, understanding and support.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 4

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

ACC-1-Aminocyclopropane-1-Carboxylic Acid

ACP5- Acid Phosphatase 5

AGM- Arabidopsis growth media

AHA2- Arabidopsis H+-ATPase2

Al- Aluminum

AM- arbuscular mycorrhizae

AUX1- AUXIN INSENSITIVE 1

AXR- AUXIN RESISTANT

BES/BRZ2- BRASSINOSTEROID INSENSITIVE1-ETHYL METHANESULFONATE-SUPPRESSOR 1/ BRASSINAZOLE RESISTANT2

BFA- Brefeldin A

bHLH- basic helix-loop-helix

BR- Brassinosteroid

BRI1- BRASSINOSTEROID INSENSITIVE1

BRZ1- BRASSINAZOLE RESISTANT1

BTS- BRUTUS

Ca- Calcium

CaMV35S- Cauliflower Mosaic Virus 35S promoter

EIN- ETHYLENE INSENSITIVE

ER-endoplasmic reticulum

ES- endomembrane system

ESCRT-III- ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT III

ETR- ETHYLENE RESISTANT

FAO- Food and Agriculture Organization of the United Nations

FER1- FERRITIN1

FIT1- FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1

FRET- Förster Resonance Energy Transfer

FRO2- FERRIC REDUCTION OXIDASE2

FW- Fresh weight

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

ILR3 - IAA-LEUCINE RESISTANT3

IPS1- INDUCED BY PHOSPHATE STARVATION 1

IRT1- IRON-REGULATED TRANSPORTER1

MFS- Major Facilitator Superfamily

MVB- Multivesicular bodies

NLA- NITROGEN LIMITATION ADAPTATION

P- Phosphorus

P1BS- PHR1 specific binding sequence

PAP- Purple Acid Phosphatase

PHF1- PHOSPHATE TRAFFIC FACILITATOR 1

PHI- PHOSPHITE

PHL1- PHR1-LIKE

PHO1- PHOSPHATE1

PHO84- PHOSPHATE84

PHR1-PHOSPHATE STARVATION RESPONSE1

PHT-PHOSPHATE TRANSPORTER

Pi- Inorganic Phosphate

PIN- PIN-FORMED proteins

PM- plasma membrane

Poly-P- Polyphosphate

PSI- PHOSPHATE STARVATION INDUCIBLE

PSN- PHOSTIN

PVC- PRE-VACUOLAR COMPARTMENT

PYE- POPEYE

RAM- ROOT APICAL MERISTEM

RHD- ROOT HAIR DEFFECTIVE

RNS- S- like RNases

SDS- PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPX1- SPX motif containing protein

SUMO- small ubiquitin-like modifier

TGN: *trans*-Golgi network

WT- WILD TYPE

CHAPTER 1

Phosphate Transport and Homeostasis

ABSTRACT

Phosphorus (P) is an essential macronutrient involved in an array of metabolic processes critical for plant growth and development. The inorganic phosphate ($H_2PO_4^-$, Pi) is the only form that can be readily used by plants and its uptake occurs via phosphate transporters. Studies have shown that Pi is scarce in many soils worldwide, thus limiting plant productivity. Phosphate fertilizers, which are being used extensively, are costly and have a negative impact on the environment. In addition, phosphate rock, the major source for phosphate fertilizers, is getting exhausted. Understanding how the plants respond and adapt to Pi stress is of great interest for improving Pi uptake and Pi use efficiency and for reducing the dependency on phosphate fertilizers.

Upon low Pi, plants employ a whole array of metabolic and physiological changes that are geared towards a better exploitation of the topsoil, improved uptake and internal re-utilization of the available phosphate. Major changes happen at the root system in order to increase root surface area and allow access to the phosphate outside of the depletion zone. In addition, secretion of acid phosphatases helps to release the bound phosphate, and it is coupled with an enhanced Pi uptake as a result of the increased accumulation of high-affinity *PHOSPHATE TRANSPORTER1 (PHT1)*. Phosphate uptake via specialized PHT1 transporters is one of the major bottlenecks during low Pi conditions, therefore it is important to investigate the regulation of PHT1 transporters and the contribution of individual PHT1, so as to improve the Pi uptake in plants. This review will summarize the existing knowledge on the role of phosphate transporters and their regulation in response to Pi fluctuations.

INTRODUCTION

Water, light and nutrients are indispensable for plant growth and development. Nitrogen, Potassium and Phosphorus (P) are the primary macronutrients involved in the most essential plant cellular processes. Phosphorus in particular, is a structural component of the nucleic acids sugar-phosphate backbone, ATP and phospholipids, as well as a key metabolite required in essential biochemical processes such as photosynthesis, carbon fixation, respiration, glycolysis and many others. Orthophosphate (Pi) is the only form of phosphate that can be readily utilized by plants, and its availability is extremely scarce; even in very fertile soils Pi concentrations fluctuate between 1.5-15 µM (Marschner, 1995m) while in plant tissues it is a thousand fold higher (5-17 mM) (Holford, 1997). The Pi availability is even lower in soil surrounding plant roots resulting in a depletion zone that closely correlates with the root hair length (Marschner, 1995n). The depletion zone, however, can be extended beyond the root hair area due to root- induced changes in the rhizosphere (Marschner, 1995o). Given the low mobility of phosphorus in the soil (Shen et al., 2011) this depletion zone is replenished slowly, thus resulting in an additional constraint on plant productivity.

Soil composition and pH greatly affect the availability of Pi and other key nutrients. Acidic and alkaline soils are the causes of significant micro- and macronutrient imbalances with detrimental effects on plant growth (Marschner, 1995p). Phosphate availability is of particular concern in areas with acidic or alkaline soils, given that at low pH fixation of Pi by aluminum (Al) and iron (Fe) is predominant, while at high pH formation of calcium (Ca) phosphates is favored. The fixation of Pi into insoluble complexes renders these compounds inaccessible for absorption by plants, and therefore, results in a severe Pi-insufficiency in both acidic and alkaline soils (Marschner, 1995m).

In industrialized countries, the phosphate deficiency in soils is compensated by application of Pi fertilizers in order to improve crop yields. According to the Food and Agriculture Organization of the United Nations (FAO) Report (FAO Fertilizer and Plant Nutrition Bulletin, 18; http://www.fao.org), the world's consumption for P in 2015 is predicted to be 45 million tons and it is forecasted to grow annually at about a rate of 1.9%. According to the 2013 United States Department of Agriculture Economic Research Service report (USDA ERS, Fertilizer Use and Price; http://www.ers.usda.gov/data-products/fertilizer-use-and-price.aspx), in 2011 the US alone consumed 4.3 million tons of phosphate fertilizer and 90% of it was produced locally, given that US is the second largest producer of phosphate after China. The cost of phosphate fertilizer has been increasing steadily over the past decades and it is projected to continue growing, posing a significant constraint on agriculture. The main source of Pi fertilizer is phosphate rock, which is a costly and finite resource. The most conservative estimates forecast phosphate rock to be exhausted in approximately 50 years while the least conservative estimates predict depletion in about 500 years at the current rate of consumption (Childers et al., 2011; Cordell et al., 2009; Edixhoven et al., 2013; Gilbert, 2009). The big discrepancy in the estimations are due to variable assessments of the size of phosphate rock reserves.

Excessive Pi fertilizers are a major problem for the environment, being a significant cause of cultural eutrophication in surface waters (Lee and Jones-Lee, 2004). In addition, due to the composition and pH of many soils, more than 80% of the added Pi gets immobilized or washed off, and becomes unavailable for plant uptake (Marschner, 1995p). Thus, it is of critical importance to address this huge problem from a biotechnological perspective by looking into novel and efficient strategies for crop improvement. For example, overexpression of

Arabidopsis *PHT1;1* in tobacco-cultured cells led to increased biomass production during low Pi (Mitsukawa et al., 1997). Thus, enhancing phosphate acquisition by upregulating high-affinity phosphate transporters could be an effective strategy for increasing crop productivity.

Pi stress adaptations

Plants have developed an array of physiological and metabolic adaptations in order to withstand conditions of constant fluctuations of Pi availability. These adaptations comprise modifications of the root morphology through proliferation of the lateral roots and root hairs, Pi acquisition through upregulation of specialized phosphate transporters, Pi recovery through secretion of phosphatases and organic acids and Pi remobilization through catabolism of phospholipids (Karthikeyan et al., 2014; Kobayashi et al., 2009; Lynch, 1995, Peret et al., 2011; Plaxton and Tran, 2011; Ticconi and Abel, 2004; Veljanovski et al., 2006). Altogether, the outcome of Pi deprivation is a drastic adjustment in the developmental program of the plants for the purpose of achieving an adequate fitness. A better understanding of these mechanisms will improve our ability to enhance plants to tolerate low Pi conditions.

The primary root growth arrest and the transition to a shallower root system architecture during Pi deprivation is associated with the brassinosteroid (BR) signaling pathway. BRs are a class of steroidal hormones that control numerous physiological processes, including but not limited to root growth and development (Wei and Li, 2016). Two homologous transcription factors, BRASSINOSTEROID INSENSITIVE1-ETHYL METHANESULFONATE-SUPPRESSOR1/ BRASSINAZOLE RESISTANT2 (BES1/BRZ2) and BRASSINAZOLE RESISTANT1 (BRZ1), regulate transcription of most genes downstream of BRASSINOSTEROID INSENSITIVE1 (BRI1) (Sun et al., 2010), a receptor-like kinase

involved in the BR signal transduction (Clouse et al., 1996; Sun et al., 2010). BRZ1 and BES1/BRZ2 get inactivated by phosphorylation upon low levels of BR (Clouse, 2011; Singh et al., 2014). Interestingly, BRZ1 and BES1/BRZ2 also participate in the regulation of the root growth in response to Pi deprivation, given that constitutively active mutants of these transcription factors result in reduced inhibition of the primary root triggered by low Pi (Singh et al., 2014). Furthermore, upon reduced Pi, BES1 localization shifts from nucleus to the cytoplasm, thus rendering it inactive, and resulting in shallower root architecture (Singh et al., 2014). Altogether, these results are pointing to the existence of a crosstalk between the local Pi sensing and the BR signaling pathway that allows developmental plasticity of the root during Pi-deficiency.

Root hairs have a particular importance in increasing the root surface area. In fact, during Pi deprivation root hair length and density increases more than 5-fold as a result of the increase in the number of the trichoblasts in the cortical cells (Bates and Lynch, 1996; Bates and Lynch, 2000). For this reason, the *ROOT HAIR DEFECTIVE (RHD*) class of mutants have been useful as tools to dissect the role of root hairs during phosphate poor conditions (Schiefelbein and Somerville, 1990). More specifically, *rhd2*, which has lower root hair elongation and *rhd6,* with a reduced root hair density, have been shown to have a lower root surface area, shoot biomass, and overall lower growth rate during Pi stress compared to the wild type (WT) Arabidopsis seedlings (Bates and Lynch, 2000). These results highlight the importance of root hair proliferation in adapting to Pi deficit.

Auxin and ethylene are the phytohormones involved in controlling root hair growth under Pi starvation (Lopez-Bucio et al., 2002; Ma et al., 2003; Nacry et al., 2005; Zhang et al., 2003; Zhang et al., 2014). Ectopic application of auxin or the ethylene precursor 1-

aminocyclopropane-1-carboxylic acid (ACC) promotes an increase in root hair length and density in WT Arabidopsis seedlings and restores the normal root hair growth and density in the *rhd6* mutant (Masucci and Schiefelbein, 1994)**.** However, Pi-induced root hair changes are at least partially independent of auxin and ethylene (Zhang et al., 2003), since Pi-starvation is able to stimulate root hair growth in hairless auxin-resistant (*axr1* and *axr2*) and auxin-insensitive mutants (*aux1*) and in ethylene-resistant (*etr1*) and ethylene-insensitive (*ein2*) mutants (Ma et al., 2003; Schmidt and Schikora, 2001). Taken together, auxin and ethylene collectively, have a substantial contribution to the regulation of root hairs in response to Pi deprivation. However, it is not clear whether the phytohormonal-induced restructuring of the root system actually results in an improved Pi acquisition.

Upon Pi stress, plants acclimate by increasing phosphate acquisition, either by upregulating the high-affinity phosphate transporters or by promoting symbiotic associations with certain fungi. Most vascular flowering plants form symbiotic fungi associations, such as arbuscular mycorrhizae (AM) for the purpose of Pi uptake (Harrison, 1997; Harrison, 2005; Harrison et al., 2002; Vance, 2001). The AM fungi are obligate biotrophs that participate in symbiotic relationships with plants to facilitate phosphate uptake, while the plants provide the necessary carbon supply to the fungi. The mycorrhizae growth adds more surface area to the roots, thus allowing greater access to phosphate, but AM are also able to uptake the least soluble forms of phosphate that plants would not be able to uptake otherwise (Harrison et al., 2002; Marschner, 1995o). While AM have the capacity to store excess phosphate in the polyphosphate (Poly-P) form, however, the AM delivers only inorganic phosphate to the plant via low affinity phosphate transporters across a specialized symbiotic membrane, also known as the periarbuscular membrane (Harrison et al., 2002). Conversely, Arabidopsis and other

Brassicas are incapable of forming symbiosis with AM, thus these plants rely heavily on Pi uptake from the soil via specialized phosphate transporters, which are referred to as the entry points for Pi into roots. Thus, Arabidopsis is a good model system for deciphering the contribution of phosphate transporters to Pi homeostasis.

Many plants respond to Pi starvation by an increased production of various nucleases and phosphatases. Acid phosphatases (APases) and ribonucleases (RNases) are important in scavenging usable Pi, by hydrolysis of intra- and extra-cellular organic P pools, in order to prevent perturbation in growth. APases are a very large and diverse class of proteins, a subclass of which is called purple APases (PAPs). Arabidopsis has 29 PAPs (Li et al., 2002; Tran et al., 2010) and a few of these proteins have been implicated in Pi scavenging. For instance, *At*PAP26 is involved in scavenging Pi from intracellular stores (Veljanovski et al., 2006), while *At*ACP5/PAP17 is important for Pi recovery from older tissues (del Pozo et al., 1999). Other PAPs, such as *At*PAP12 can be secreted to the rhizosphere (Li et al., 2002). Additionally, plants exposed to Pi-starvation exude organic acid, into the rhizosphere for the purpose of releasing of Pi from Al-, Fe- and Ca-P complexes commonly found in soils (Narang et al., 2000). Phosphate-efficient Arabidopsis accessions have been shown to secrete more organic acids than their less efficient counterparts to enhance phosphate availability in the proximate rhizosphere (Narang et al., 2000). Low Pi induces the accumulation of S- like RNases (RNS) which might be involved in remobilization of Pi from intra- and extra-cellular RNA sources (Bariola et al., 1994). Overall, the Pi recycling and Pi mobilization from organic P compounds are very important strategies for adaptation to Pi deprivation, and these are most commonly coupled with the upregulation of phosphate transporters to promote the uptake and redistribution of the newly available Pi.

Lastly, upon extended Pi stress, lipid metabolism is significantly altered in order to conserve the limited phosphate pools. Phospholipid catabolism is increased (Gaude et al., 2008; Kobayashi et al., 2009; Yamaryo et al., 2008) and it is compensated by an enhanced synthesis of sulfolipids (Essigmann et al., 1998) and galactolipids (Benning and Ohta, 2005; Gaude et al., 2008; Kobayashi et al., 2009) in order to repurpose the obtained Pi for other essential processes. Altogether, plants employ an array of adaptations in order to withstand low Pi levels, highlighting their vast plasticity given that all of the processes mentioned above are reestablished when Pi homeostasis is restored.

Signaling under low Pi

Pi accumulation in plants is tightly regulated given that too much phosphate is toxic, while too little P is lethal, thus Pi levels are subject to strict regulation with the integration of both local and systemic sensing and signaling. External Pi availability is sensed in the roots as shown by split-root experiments (Burleigh and Harrison, 1999; Franco-Zorrilla et al., 2007; Liu et al., 1998; Thibaud et al., 2010), while the internal Pi status is monitored in the shoots, provided that P itself acts as a signal. Moreover, external application of phosphite (Phi), a non-metabolizable phosphate equivalent, is able to mimic a state of Pi sufficiency and attenuate most Pi stress responses (Berkowitz et al., 2013; Danova-Alt et al., 2008; Jost et al., 2015b; Ticconi et al., 2001; Ticconi and Abel, 2004; Varadarajan et al., 2002), indicating that Phi compromises phosphate sensing. Phostin (PSN) on the other hand, is a drug that elicits Pi-starvation responses when phosphate is plentiful, thus uncoupling Pi content from Pi homeostasis (Bonnot et al., 2015). Altogether, Phi and PSN have been particularly useful tools for dissecting the local and systemic phosphate starvation responses.

Upon Pi stress, movable signals such as cytokinins, miRNAs, sugars and Pi itself, are employed for the systemic regulation of *PHOSPHATE STARVATION- INDUCED* (*PSI)* genes, being critical components of the Pi signaling (Bari et al., 2006; Doerner, 2008; Hermans et al., 2006; Karthikeyan et al., 2007; Lin et al., 2008; Martin et al., 2000; Pant et al., 2008; Zhou et al., 2008). Cytokinins play an important role in Pi signaling. In fact, exogenous cytokinin application is able to repress certain *PSI* genes in Arabidopsis and rice (Brenner et al., 2005; Martin et al., 2000; Wang et al., 2006). Endogenous levels of cytokinin and its receptors decrease under Pi-starvation; in addition, the reduction in its concentrations is a prerequisite for a proper Pi starvation response (Brenner et al., 2005; Franco-Zorrilla et al., 2007; Karthikeyan et al., 2002; Martin et al., 2000; Shin et al., 2006).

Internal Pi status is controlled by PHOSPHATE STARVATION RESPONSE 1 (PHR1) and by its closest homologue PHR1-LIKE (PHL1), MYB-related transcription factors (Bustos et al., 2010; Nilsson et al., 2007; Puga et al., 2014; Rouached et al., 2011; Rubio et al., 2001). PHR1 is a major constituent of Pi signal transduction given that it controls more than 70% of *PSI* genes and its activity is required for proper Pi starvation response in plants (Bustos et al., 2010; Nilsson et al., 2007; Rubio et al., 2001; Zhou et al., 2008). More specifically, PHR1 binds to a GNATATNC *cis* element common to most *PSI* genes, called the P1BS (PHR1 specific binding sequence) element to activate their expression (Nilsson et al., 2007). Induction of *PSI* genes is important for internal Pi homeostasis, as these genes are involved in reutilization and reallocation of available internal phosphate pools and in enhancement of Pi acquisition from the rhizosphere. PHR1, in turn, is activated by sumoylation via SIZ1 (Figure 1), which is a small ubiquitin-like modifier (SUMO) E3 ligase (Miura et al., 2005). Although, SIZ1 is an activator of PHR1, it acts as a negative regulator of certain components in Pi

starvation-dependent signaling that controls Pi uptake, root architecture modifications and anthocyanin buildup (Miura et al., 2005). This is very intriguing, given that most of the components negatively regulated by SIZ1 are downstream of PHR1. Further investigations are needed to unravel the role of SIZ1in the PHR1-dependent and independent responses to Pi deprivation.

MicroRNAs play a key role in the post-transcriptional regulation of *PSI* genes in response to low Pi. A well-characterized response to low Pi involves the accumulation of microRNA399 (miR399) (Figure 1) in the shoots as early as 24 hours post Pi-starvation (Bari et al., 2006; Fujii et al., 2005). However, PHR1 is required for miR399 induction given that *phr1* is unable to accumulate miR399 (Bari et al., 2006). Upon induction, miR399 is transported via the phloem from shoots to roots (Pant et al., 2008), where it targets *PHOSPHATE2* (*PHO2* or *UBC24)* an ubiquitin E2 conjugating enzyme (Figure 1). Therefore, miR399 is considered to be a systemic signal involved in long distance communication from the shoot to the root (Doerner, 2008; Lin et al., 2008; Pant et al., 2008; Schachtman et al., 1998). The 5'UTR of *PHO2* transcripts contain several miR399 binding sites, which results in the degradation of this transcript via ARGONAUT-mediated cleavage. Both *pho2* mutant and *miR399* over-expressing plants show similar phenotypes including increased Pi uptake, translocation and retention of Pi in the shoots, and therefore, hyper-accumulation of Pi in the shoot (Aung et al., 2006; Chiou et al., 2006). This indicates that *miR399* and *PHO2* act in the same Pi signaling pathway (Bari et al., 2006; Chiou et al., 2006). Under Pi sufficient conditions, high PHO2 activity results in low expression of the high-affinity *PHOSPHATE TRANSPORTER 1* (*PHT1*), since *pho2* mutants accumulate higher levels of *PHT1;8* and *1;9* transcripts when compared to WT (Aung et al., 2006). It is not known, however, how PHO2

function results in transcriptional repression of *PHT1;8* and *PHT1;9*. When the shoot senses limiting Pi, PHO2 is targeted by miR399, this relieves the repression off the high affinity transporter genes, and results in increased Pi transporter expression in the root. In addition, when Pi is limiting, the expression of the *INDUCED BY PHOSPHATE STARVATION1 (IPS1*) and *At4/IPS2* genes is highly induced (Figure 1) (Liu et al., 1997; Shin et al., 2006). *IPS1* and *At4/IPS2* are members of the same family of non-coding RNA, and function as riboregulators thru interfering in the miR399-mediated silencing of *PHO2* by target mimicry (Figure 1) (Franco-Zorrilla et al., 2007; Liu et al., 1997). Therefore, miR399 plays an important role in Pi homeostasis by regulating the proteolysis machinery in a Pi-dependent manner.

Another microRNA, miR827, is induced significantly upon Pi stress. The target of miR827 is *NITROGEN LIMITATION ADAPTATION (NLA),* which encodes a protein with an SPX domain in the N-terminus and a putative E3 ligase domain at its C-terminus (Lin et al., 2013; Peng et al., 2007). Loss of *NLA* results in hyper-accumulation of Pi in the shoots (Kant et al., 2011) similar to *pho2*. NLA in turn, targets for degradation specific PHT1 proteins (Lin et al., 2013; Park et al., 2014). NLA might also be involved in phosphate sensing, given that proteins with an SPX motif, such as PHO81 and PHO87, have been implicated in Pi sensing in yeast (Duan et al., 2008; Ghillebert et al., 2011; Hurlimann et al., 2009; Swinnen et al., 2005). Unlike phosphate transporters in yeast, Arabidopsis high-affinity phosphate transporters lack an SPX motif and are not involved in sensing (Ayadi et al., 2015). Interestingly, the recently identified vacuolar Pi transporters contain an SPX motif that modulates their transport activity, however it is unclear whether this motif is important for Pi sensing (Liu et al., 2015; Liu et al., 2016). An Arabidopsis SPX motif-containing nuclear protein, SPX1, has been shown to directly interact with PHR1 (Figure 1). This interaction

results in PHR1 inhibition in a Pi-dependent manner, thus linking Pi sensing and signaling (Puga et al., 2014). Overall, the SPX motif is essential for Pi sensing, signaling and transport, therefore it plays a major role in Pi homeostasis.

Pi transport and translocation

Two types of active carriers of Pi are known in plants, high- and low-affinity Pi transporters. High-affinity transporters are involved in acquisition of Pi from soil solution when Pi levels are in the micromolar range (Rausch and Bucher, 2002). Low-affinity transporters are involved in Pi uptake when Pi levels are in the milimolar range, and in Pi distribution and remobilization across the plant including the translocation from root to shoot (Raghothama, 1999). In addition, there are intracellular phosphate transporters that function in redistribution, compartmentalization or storage of Pi.

Pi moves within the root via apoplastic or symplastic transport. Pi levels are estimated at 2 μM or less in the apoplast, but they are thousand fold higher in the cytoplasm. This large concentration gradient across the plasma membrane implies that Pi uptake into the cytosol requires an energy-driven transport mechanism. Once Pi is imported into the cell, it gets compartmentalized in various organelles such as chloroplasts, mitochondria and mainly the vacuole, where Pi concentrations reach roughly 120 mM. The Pi stored in the vacuole corresponds to 85-95% of the total Pi available in a particular cell (Lauer et al., 1989; Mimura et al., 1990). The cytoplasmic Pi levels by and large stay constant, while the vacuolar Pi pools fluctuate depending on the Pi demand. Vacuolar Pi levels can even get exhausted during low Pi conditions, as confirmed by the noninvasive measurements of vacuolar pools by ^{31}P-NMR (Lee et al., 1990; Mimura et al., 1990; Pratt et al., 2009). Additionally, Pi transport across the

tonoplast membrane requires ATP (Massonneau et al., 2000). Phosphate transporters at the tonoplast have been identified in plants only recently (Liu et al., 2015; Liu et al., 2016), though it is unclear whether these proteins transport Pi into the vacuole, out of it or bidirectionally.

There are five PHOSPHATE TRANSPORTER (PHT) families in Arabidopsis: PHT1, PHT2, PHT3, PHT4 and PHT5. All PHT families belong to the Pi:H+ Symporter Family (PHS), which in turn are part of the Major Facilitator Superfamily (MFS). PHT1 family proteins are high-affinity phosphate transporters, mostly constrained to the roots and involved in the active uptake of Pi. The PHT2 family consists of a single copy gene that encodes for a low affinity transporter, restricted to chloroplasts and it plays a role in Pi redistribution in shoots (Versaw and Harrison, 2002). The PHT3 family is composed of 3 mitochondria-localized low affinity transporters which might function as Pi translocators (Poirier and Bucher, 2002). The PHT4 family comprises 6 Pi antiporters, 5 of which are localized to plastids, while PHT4;6 localizes to the Golgi apparatus and transports Pi out of the Golgi (Cubero et al., 2009; Guo et al., 2008). The PHT5 Family has been identified most recently, and it consists of three partially redundant vacuolar transporters that regulate cytoplasmic Pi levels (Liu et al., 2015; Liu et al., 2016). Interestingly, PHT5 proteins contain an SPX domain, a distinctive feature, which is not present in other PHT transporters. Moreover, the SPX motif is very important for the transport activity of PHT5 (Liu et al., 2015; Liu et al., 2016).

A class of Pi exporter proteins, PHOSPHATE1 (PHO1), plays important roles in Pi homeostasis, however these proteins show no homology to PHT proteins (Arpat et al., 2012; Stefanovic et al., 2007; Wang et al., 2004). PHO1 proteins are involved in Pi translocation from root to shoot and more specifically, regulate the efflux of phosphate (Stefanovic et al., 2011). In addition, PHO1 contains a SPX domain, thus it may be involved in Pi sensing. Loss

of PHO1 affects Pi homeostasis, resulting in lower Pi levels in shoots (Poirier et al., 1991) but normal levels in the roots, indicating that the Pi loading of the xylem is impaired. PHO1 localizes to the Golgi and *trans*-Golgi network (TGN) (Arpat et al., 2012) similarly to PHT4;6, implicating these organelles in Pi homeostasis. Altogether, the intra- and inter-cellular Pi movement, and Pi compartmentalization are very important for proper Pi balance.

Transcriptional regulation of *PHT1* genes under low Pi

It has been shown that, under Pi-starvation, plants have developed a plethora of adaptation mechanisms to withstand and thrive, one of the most important being the increased uptake of Pi via PHT1. The PHT1 family of high- affinity transporters has nine members in *Arabidopsis thaliana* and these are homologous to PHO84 in yeast (Muchhal et al., 1996; Raghothama, 2000). *PHT1* are upregulated specifically during Pi-stress, as shown by promoter analyses (Karthikeyan et al., 2002; Muchhal et al., 1996; Muchhal and Raghothama, 1999; Mudge et al., 2002). The gene expression profiles of *PHT1* are predominantly, but not exclusively restricted to roots, and they have a somewhat overlapping pattern in both low and high Pi regimes (Misson et al., 2004; Muchhal and Raghothama, 1999; Mudge et al., 2002).

PHT1;1 is expressed in roots, hydathodes and axillary buds of leaves and in the germinating seeds under both low and high Pi. However, under low Pi there is an induction in *PHT1;1* in the root. Strong *PHT1;1* expression was detected in the epidermis and cortex of the root hair zone, while the trichoblasts had a higher *PHT1;1* expression (Table 1) (Mudge et al., 2002). *PHT1;2* had a similar transcriptional profile to *PHT1;1*, however it lacked expression in the root tip (Table 1). Conversely, *PHT1;3* was expressed predominantly in the stele of the primary root and weakly in the vascular tissue of young leaves (Mudge et al., 2002). *PHT1;4*

expression is induced by Pi starvation predominantly in the roots and root caps, more specifically, in the epidermis and cortex and stele of the secondary roots (Table 1). *PHT1;4* may play a role in Pi uptake and remobilization given that the distribution of Pi is affected in the *pht1;4* mutants (Misson et al., 2004). In addition, *PHT1;4* promoter activity was detected in the aerial parts of the plant, in the apical meristems, and the trichomes and hydathodes of leaves, which is consistent with the in-situ hybridization data (Misson et al., 2004; Mudge et al., 2002). In contrast to *PHT1;1/2/3/4*, *PHT1;5* is expressed mainly in the shoots (Table 1). In young seedlings *PHT1;5* is detected in all tissues, while in older plants it is limited to the vasculature (Mudge et al., 2002). *PHT1;6* and *PHT1;7* are expressed in mature pollen, and flowers at stages 9, 10 and 11, additionally, *PHT1;6* was also detected in vascular tissue in the cotyledons during flowering (Table 1). *PHT1;8* and *PHT1;9* promoter activity was undetected, however these genes are expressed exclusively in the roots primarily upon Pi stress (Table 1) (Mudge et al., 2002). The transcriptional profiles of *PHT1;1/2/3/4* are overlapping to a great extent which also suggests there is a great degree of functional redundancy (Ayadi et al., 2015). Overall, the expression profile of *PHT1* genes suggests that there is tight regulation at the transcriptional level dependent upon Pi availability and that *PHT1* genes might have additional roles other than Pi uptake but also Pi redistribution and Pi mobilization (Misson et al., 2004; Mudge et al., 2002). Despite the *PHT1* redundancy there is significant evidence that individual *PHT1* genes undergo distinct posttranscriptional and posttranslational control (Aung et al., 2006; Bayle et al., 2011). For instance, only *PHT1;8* and *PHT1;9* are induced in the phosphate over-accumulator mutant *pho2* (Aung et al., 2006). In addition, the regulation of *PHT1* might differ, given that only *PHT1;8*, *PHT1;4* and *PHT1;5* are systemically regulated by Pi-

deficiency at the transcriptional level (Thibaud et al., 2010). The specific contribution of individual *PHT1* genes needs to be further investigated.

PHT1 protein trafficking

The abundance of proteins at the plasma membrane (PM) is controlled by the anterograde and retrograde intracellular vesicular trafficking, exocytosis, and endocytosis. PM proteins are predicted to be targeted to their final destination via the endomembrane system (ES). The ES includes the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network (TGN), the pre-vacuolar compartments (PVCs, or multivesicular bodies, MVB), the recycling endosomes, and the plasma membrane (PM) (Park and Jürgens, 2011; Richter et al., 2009). As PM proteins travel through the endomembrane system, at each step there is a possibility of anterograde and retrograde trafficking. In plants, the TGN corresponds to the early endosome (EE) (Dettmer et al., 2006; Viotti et al., 2010), and is a sorting hub for endocytic and secretory pathways (Viotti et al., 2010). The pre-vacuolar compartment (PVC) corresponds to the multivesicular bodies (MVB), functions similarly as the late endosome of animal cells, and may eventually fuse with the vacuole (Viotti et al., 2010). Trafficking of the PHT1 proteins is expected to involve the ER, Golgi, TGN and PM, given that these proteins contain twelve transmembrane domains and are predicted to be PM localized.

As all membrane proteins, PHT1;1 is synthesized at the ER, however its exit from the ER is conditioned by PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1), which is related to the Sec12 protein in yeast and facilitates efficient PHT1 targeting to the PM (Chen et al., 2011; González et al., 2005). However, Pi uptake is not completely abolished in the loss of function *phf1* mutant (Nussaume et al., 2011). When Pi is abundant PHT1;1 is

retained at the ER, thus not delivered to PM, by phosphorylation at conserved serine residues on the C-terminus (Bayle et al., 2011; Hem et al., 2007; Nuhse et al., 2004). The kinase that phosphorylates the rice PHT1 homologue, OsPT8, has been identified as the β3 subunit of the CK2 hetero-tetramer kinase (CK2β3) (Chen et al., 2015). The phosphorylated OsPT8 was unable to interact with PHF1 and was retained at the ER. Additionally, phosphorus starvation promoted CK2β3 degradation, relieving the negative regulation of OsPT8 during Pi-stress conditions (Chen et al., 2015). Thus, the posttranslational control of PHT1 is in tight coordination with the Pi status of plants.

Most PM proteins cycle between the plasma membrane and the early endosome/TGN compartments, as exemplified by auxin transport proteins (PINs, AUX) and hormone receptors (BRI1), for the purpose of regulating protein abundance at the PM (Abas et al., 2006; Geldner et al., 2003; Geldner et al., 2001; Kleine-Vehn et al., 2006; Kleine-Vehn et al., 2008; Russinova et al., 2004). After endocytosis, these proteins may be recycled back to the plasma membrane or they may be trafficked to the vacuole for degradation. In fact, when Pi levels are low these proteins are recycled back to the PM and exit from the ER of the newly synthesized transporters is promoted (Bayle et al., 2011).The fungal macrocyclic lactone Brefeldin A (BFA) is used as an efficient trafficking inhibitor of the endosomal recycling mediated by the ARF GEF, GNOM in Arabidopsis (Geldner et al., 2003). The high affinity phosphate transporter PHT1;1 and the auxin transporters PIN1, PIN2, AUX1 have been shown to accumulate in BFA endosomes (Geldner et al., 2001; Kleine-Vehn et al., 2006; Kleine-Vehn et al., 2008; Bayle et al., 2011). When Pi levels are high, PHT1;1 undergoes endocytosis and is trafficked to the vacuole for degradation. The targeting for degradation by ubiquitination of PHT1;1 and/or PHT1;4 is achieved by cooperative NLA and PHO2 action (Lin et al., 2013; Park et al., 2014).

The ubiquitinated PHT1;1 is packaged into MVBs with the contribution of ALIX1, which associates with ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT III (ESCRT-III) (Cardona-Lopez et al., 2015). Therefore, the abundance of the PHT1 proteins is tightly regulated and it is by and large Pi-dependent.

Although, all nine PHT1 proteins in Arabidopsis have a high degree of similarity at the amino acid level, their C-termini are rather distinct. This is very interesting given that the substrate recognition in the high affinity phosphate transporter in *Piriformospora indica* (PiPT- a root colonizing fungus) happens mostly at the C domain of this protein (Pedersen et al., 2013). Additionally, PHT1;6/8/9 proteins differ from the other PHT1 family members in that they contain a predicted Proline, glutamic acid (E), Serine, Threonine (PEST) motif at their C-termini. Interestingly, a PEST motif is also present in PHO84, the dual-affinity phosphate transporter and transceptor in yeast (Estrella et al., 2008; Lagerstedt et al., 2004). Proteins with known PEST sequences are short-lived and subject to quick degradation (Rechsteiner and Rogers, 1996). Additionally, these motifs can be located anywhere on the protein, but most commonly at the C-terminus, and are often conditionally activated rather than being constitutive cleavage signals (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Less than 10% of all mammalian proteins contain a PEST motif, though it is unknown how many plant proteins possess this domain. The truncation of the PEST motif is thought to result in metabolically more stable proteins (Rechsteiner and Rogers, 1996). Conversely, adding a PEST motif to a stable protein results in a quickly-degradable protein (Salama et al., 1994). The presence of the PEST domain in PHT1;6/8/9 could mean that these proteins are targeted for a rapid degradation and may be indicative of distinct posttranslational regulation and

trafficking. The importance of the PEST motif in the PHT1;6, PHT1;8 and PHT1;9 needs to be investigated in the future.

Structural and functional analysis of PHT1 transporters

A protein structure is important for its function and may provide clues about its mode of action. The structure of PHT1 transporters is typical for active carriers with twelve transmembrane domains (M1-M12) and cytosolic loops alternating between the extracellular and cytosolic space (Muchhal et al., 1996). Both, the amino- and carboxyl- termini are predicted to face the intracellular space and this topology was confirmed by the crystal structure of PiPT (Pedersen et al., 2013). According to the crystal structure analysis, inorganic phosphate binds to PiPT at specific and highly conserved residues on M4, M5, M7 and M10, while the affinity for Pi is provided by the lysine 459 on M11. The cytosolic tunnel, formed in the middle of the M domains allows proton translocation into the cytosol. Additionally, the crystal structure of the PiPT suggests a role in phosphate import (Pedersen et al., 2013). Based on the PiPT high similarity to PHO84 and thus to PHT1 proteins, the sites important for PHT1 function can be inferred. In fact a recent study used targeted mutagenesis of *At*PHT1;1 on the tyrosine312 (Y312) located on the M7 to show that this residue is crucial for the formation of PHT1;1 homodimers and PHT1;1-PHT1;4 heterodimers (Fontenot et al., 2015). Rigorous testing is required in order to confirm which of the residues are critical for the proper function of individual PHT1 transporters, given that there is a fair amount of dissimilarities at the amino acid level. In conclusion, modeling and targeted mutagenesis based on the PiPT crystal structure as a template will be instrumental for uncovering the role of PHT1 transporters and

whether formation of homo- and hetero-dimers is required for all PHT1 proteins for proper function.

All *At*PHT1 proteins are presumed to be functionally redundant and to have the same affinity for phosphate, however, this has not always been the case. Shin et al. (2004) corroborated that the major role in Pi uptake was provided by PHT1;1 and PHT1;4, since loss of both of the transporters results in a drop of Pi uptake by ~43% during Pi deprivation and ~70% during sufficient Pi. In fact, *pht1;1 pht1;4* double mutants exhibit significant biomass loss and upregulation of phosphate starvation markers (Shin et al., 2004). PHT1;4 alone is responsible for 40-48% Pi uptake given that this is how much Pi absorption capacity is lost in the *pht1;4* mutant under low Pi conditions (Misson et al., 2004). Otherwise, no growth defects or visible phenotypes of *pht1;4* were observed even under fluctuating Pi (Misson et al., 2004). In addition, overexpression of *At*PHT1;1 in tobacco cells induced a doubling in Pi uptake and enhanced growth under Pi-limiting conditions (Mitsukawa et al., 1997), suggesting that the function of high affinity phosphate transporters is conserved throughout plant species.

PHT1;5 has been shown to play a role in translocation of Pi from source to sink organs using a reverse genetics approach (Nagarajan et al., 2011). In WT Arabidopsis *PHT1;5* transcripts were induced by low P in both roots and shoots, consistent with a role in both roots and shoots. The role of PHT1;5 was elegantly tested in the *pht1;5* mutant alleles exposed to ^{33}P. *pht1;5* displayed 35-40% lower ^{33}P distribution compared to WT, while the decrease for *pht1;1-2* was about 20% (Nagarajan et al., 2011). These results indicated that PHT1 proteins are involved in Pi translocation in addition to Pi uptake. Nonetheless, the subcellular localization of *At*PHT1;5 has not been reported so far. Clearly, all PHT1 transporters need to be tested for their implication in Pi uptake and translocation, by using ^{33}P, before assuming

they are involved in Pi uptake exclusively. Convincing evidence, such as the one presented above, is missing**.**

To date, *AtPHT1;6* has been poorly characterized. It is known that its promoter is active in anthers and mature pollen (Mudge et al., 2002). Similarly, *AtPHT1;7*has not been well characterized, so far it has been shown that its promoter is active in mature pollen (Mudge et al., 2002). No promoter activity was detected in the case of *PHT1;8* and *PHT1;9* in promoter:GUS fusions indicating that these genes are of low abundance (Mudge et al., 2002), however the possibility that the promoter fragments chosen were too short and/or lacking important regulatory elements cannot be dismissed.

The characterization of PHT1;8 and PHT1;9 is starting to emerge, but there are plenty of gaps that need to be filled. The most recent report argues that PHT1;8 and PHT1;9 play a role in root-to-shoot translocation, since the loss of these transporters results in normal Pi content in the roots and lower Pi content in the shoots (Lapis-Gaza et al., 2014). Nevertheless, these data contradict a previously published paper by Remy et al. in 2012, which specified that PHT1;8 and 1;9 mediate Pi acquisition. *PHT1;8* and *1;9* are least similar to *PHT1;1-1;4*, and their transcriptional profiles are rather distinct because they are induced exclusively by low Pi. Moreover, *PHT1;8* and *1;9* transcripts increase upon degradation of PHO2 (Aung et al., 2006). Plants with overexpressed miR399 or lacking PHO2, over-accumulate phosphate due to increased Pi uptake and remobilization. Therefore, it was postulated that PHT1;8 and PHT1;9 are downstream of PHO2 even though these are not its direct targets (Huang et al., 2013).

PHT1;9 localizes to the PM (Remy et al., 2012), while PHT1;8 localization has yet to be reported. In addition, PHT1;8 and PHT1;9 share a high degree of amino acid identity while being less similar to the remaining PHT1 members. It is obvious that the high degree of PHT1

regulation at various levels confirms the important role these transporters play in Pi acquisition and homeostasis, however, there might be additional layers of control specific to a subgroup of PHT1 that require testing. For example, PHT1;6, 1;8 and 1;9 are predicted to have PEST motifs that would target these proteins for rapid turnover (Rechsteiner and Rogers, 1996). Further investigation is necessary to uncover the intricate regulation and functions of the PHT1 transporters that play such a vital role in Pi acquisition and homeostasis.

Understanding the exact role of each individual PHT1 member in Pi homeostasis is still lacking. To this end, knowing the intracellular localization and distribution of these proteins would aid in deciphering PHT1 function. Thus far, only PHT1;1 and PHT1;4 have been shown to form homo- and heterodimers (Fontenot et al., 2015) and this needs to be further investigated for the fellow PHT1 transporters. Furthermore, the interacting partners of PHT1 and how these interactions affect their structure/ function have not been reported. Lastly, the impact of Pi itself in regulating PHT1 proteins remains to be further investigated. Reverse genetics, forward genetics, chemical genetics and a rich array of pharmaceutical agents can be employed to dissect the specific role that each of these transporters play in Pi homeostasis.

CONCLUSION AND FUTURE DIRECTIONS

Plants require abundant phosphorus for proper growth and high yields, since P is a fundamental macronutrient. Despite its high abundance in soils, its bioavailability is limited due to the inability of plants to uptake phosphorus other than the inorganic form. Plants have evolved a whole suite of responses targeted to improve phosphorus use efficiency, phosphate availability in the rhizosphere and its subsequent uptake. Phosphate transporters are critical for processes such as Pi uptake, allocation and remobilization within plants and thus have a great potential to be used as targets for crop improvement. The regulation of PHT1 transporters and its impact on the overall Pi homeostasis have been thoroughly researched and key players and mechanisms have been uncovered in the past few years (Bayle et al., 2011; Cardona-Lopez et al., 2015; Chen et al., 2011; Fontenot et al., 2015; González et al., 2005; Huang et al., 2013; Jost et al., 2015; Kant et al., 2011; Lapis-Gaza et al., 2014; Pedersen et al., 2013; Remy et al., 2012; Thibaud et al., 2010). However, virtually nothing was known about the role, subcellular localization and trafficking of PHT1;8 and PHT1;9 when this project started in 2010.

This dissertation is focused on the contribution of the high affinity phosphate transporters, PHT1;8 and PHT1;9 to Pi homeostasis, the genetic interaction amongst *PHT1* genes, the impact of the overexpressing or downregulating these genes on the Pi homeostasis, as well as, the localization and trafficking of these transporters in *Arabidopsis thaliana* (Chapter 2). We show that PHT1;8 and PHT1;9, unlike other PHT1 transporters, localize predominantly at the ER (Chapter 2). Further investigations are needed to test the functionality of PHT1;8 and PHT1;9 at the ER, however, it is plausible that the ER might be a temporary storage site for these transporters, ready to be quickly delivered to the PM when needed for Pi transport. Given that PHT1;1 forms heterodimers with PHT1;4 (Fontenot et al., 2015), it is

possible that the ER-localized PHT1;8 and PHT1;9 might form heterodimers or even higher order oligomers with a PM-localized PHT1, thus facilitating their delivery to the PM. We also show that PHT1;6 has a polar PM localization, and taken together with its expression in mature pollen and flowers, might indicate that PHT1;6 is needed for directional Pi movement to flowers and pollen (Chapter 2). Future studies should incorporate live imaging of the inorganic phosphate in plants with cellular and subcellular resolution, using sensitive Förster Resonance Energy Transfer (FRET)-based Pi sensors (Mukherjee et al., 2015) to monitor the Pi transport and to evaluate the effect of overexpression or downregulation of *PHT1 in planta* in real time.

In addition, we uncovered that PHO2 does exert transcriptional control over *PHT1;8*, as previously reported (Aung et al., 2006; Bari et al., 2006), however, it does not regulate PHT1;8 and PHT1;9 localization or abundance in Arabidopsis (Chapter 3). This raises a pertinent question as to which protein targets PHT1;8 and PHT1;9 for degradation. One possible candidate involved in PHT1;8 and PHT1;9 degradation might be NLA, given that it regulates PHT1;1 and PHT1;4 abundance (Lin et al., 2013; Park et al., 2014), however the PM localization of NLA suggests that it could interact and target for degradation only PHT1;8 and PHT1;9 that reached the PM. Identification of the proteins that regulate degradation of PHT1;8 and PHT1;9 at the ER, given their abundant ER localization in most tissues, needs to be subject of further studies. A forward genetic screen might help in identification of protein(s) involved in regulating PHT1;8 and 1;9 abundance and/or trafficking in Arabidopsis, given the extensive genetic tools available.

Lastly, we provide more evidence for the extensive crosstalk that exists between Fe and Pi, highlighting the antagonistic nature of this interaction (Chapter 4). We uncovered that the loss of *PHT1;8* and *PHT1;9* results in upregulation of Fe uptake genes at the molecular

level, while Fe deprivation caused downregulation of Pi starvation markers. To ultimately demonstrate the effect of *PHT1;8* and *PHT1;9* loss on the Fe content, measuring Fe levels in these mutants will be necessary. We also found that BRUTUS (BTS) and POPEYE (PYE), which are important for Fe deficiency response (Long et al., 2010; Selote et al., 2015), might differentially regulate *PHT1* gene expression. Future experiments are needed to test whether these changes are due to Pi starvation *per se* or due to indirect Fe excess generated during Pi-deplete conditions. In the future, it will be suitable to analyze mutants with abolished or severe deficiency in Fe (*irt1*) and Pi (*pho1*) and their effect on Pi and Fe homeostasis, accordingly. It will also be worthwhile to evaluate the Fe status of mutants with excess Pi (*pho2*) and with disrupted Pi transport in chloroplasts (*pht2;1*). Ideally, the experiments will have to be conducted with combined Fe and Pi shortages and in hydroponic conditions, to avoid artefacts due to trace amounts of these nutrients from agar.

A better understanding of the molecular basis of Pi deficiency responses in plants could lead to a reduced dependence on phosphate fertilizers and ultimately improve crop yields by targeted manipulation of specific genes and/or multiple sets of genes, although translating the findings from model plants to crop species will be a prerequisite.

REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wišniewska, J., Moulinier–Anzola, J.C., Sieberer, T., Friml, J., Luschnig, C. (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol, **8**(3), 249-256.

Abel, S. (2011) Phosphate sensing in root development. Curr Opin Plant Biol, **14**(3), 303-309.

Arpat, A.B., Magliano, P., Wege, S., Rouached, H., Stefanovic, A., Poirier, Y. (2012) Functional expression of PHO1 to the Golgi and trans-Golgi network and its role in export of inorganic phosphate. Plant J, **71**(3), 479-491.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., Chiou, T.J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol, **141**(3), 1000-1011.

Ayadi, A., David, P., Arrighi, J.F., Chiarenza, S., Thibaud, M.C., Nussaume, L., Marin, E. (2015) Reducing the genetic redundancy of Arabidopsis *PHOSPHATE TRANSPORTER1* transporters to study phosphate uptake and signaling. Plant Physiol, **167**(4), 1511-1526.

Bari, R., Datt Pant, B., Stitt, M., Scheible, W.R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol, **141**(3), 988-999.

Bariola, P., Howard, C., Taylor, C., Verburg, M., Jaglan, V., Green, P. (1994) The Arabidopsis ribonuclease gene *RNS1* is tightly controlled in response to phosphate limitation. Plant J, **6**(5), 673-685.

Bates, T.R. and Lynch, J.P. (1996) Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. Plant Cell Environ, **19**(5), 529-538.

Bates, T.R. and Lynch, J.P. (2000) The efficiency of *Arabidopsis thaliana* (Brassicaceae) root hairs in phosphorus acquisition. Am J Bot, **87**(7), 964-970.

Bayle, V., Arrighi, J.F., Creff, A., Nespoulous, C., Vialaret, J., Rossignol, M., Gonzalez, E., Paz-Ares, J., Nussaume, L. (2011) *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell, **23**(4), 1523-1535.

Benning, C. and Ohta, H. (2005) Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. J Biol Chem, **280**(4), 2397-2400.

Berkowitz, O., Jost, R., Kollehn, D.O., Fenske, R., Finnegan, P.M., O'Brien, P.A., Hardy, G.E., Lambers, H. (2013) Acclimation responses of *Arabidopsis thaliana* to sustained phosphite treatments. J Exp Bot, **64**(6), 1731-1743.

Bonnot, C., Pinson, B., Clément, M., Bernillon, S., Chiarenza, S., Kanno, S., Kobayashi, N., Delannoy, E., Nakanishi, T.M., Nussaume, L., Desnos, T. (2015) A chemical genetic strategy identify the PHOSTIN, a synthetic molecule that triggers phosphate starvation responses in *Arabidopsis thaliana*. New Phytol, **209**(1), 161–176.

Brenner, W.G., Romanov, G.A., Kollmer, I., Burkle, L., Schmulling, T. (2005) Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J, **44**(2), 314-333.

Briat, J.F., Rouached, H., Tissot, N., Gaymard, F., Dubos, C. (2015) Integration of P, S, Fe, and Zn nutrition signals in *Arabidopsis thaliana:* potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). Front Plant Sci, **6**, 290.

Burleigh, S.H. and Harrison, M.J. (1999) The down-regulation of *Mt4*-Like genes by phosphate fertilization occurs systemically and involves phosphate translocation to the shoots. Plant Physiol, **119**(1), 241-248.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Perez-Perez, J., Solano, R., Leyva, A., Paz-Ares, J. (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS Genet, **6**(9), e1001102.

Cardona-Lopez, X., Cuyas, L., Marin, E., Rajulu, C., Irigoyen, M.L., Gil, E., Puga, M.I., Bligny, R., Nussaume, L., Geldner, N., Paz-Ares, J., Rubio, V. (2015) ESCRT-III-associated protein ALIX mediates high affinity phosphate transporter trafficking to maintain phosphate homeostasis in Arabidopsis. Plant Cell, **27** (9), 2560-2581.

Chen, J., Liu, Y., Ni, J., Wang, Y., Bai, Y., Shi, J., Gan, J., Wu, Z., Wu, P. (2011) OsPHF1 regulates the plasma membrane localization of low- and high-affinity inorganic phosphate transporters and determines inorganic phosphate uptake and translocation in rice. Plant Physiol, **157**(1), 269-278.

Chen, J., Wang, Y., Wang, F., Yang, J., Gao, M., Li, C., Liu, Y., Yamaji, N., Ma, J.F., Paz-Ares, J., Nussaume, L., Zhang, S., Yi, K., Wu, Z., Wu, P. (2015) The rice CK2 kinase regulates trafficking of phosphate transporters in response to phosphate levels. Plant Cell, **27**(3), 711-723.

Childers, D.L., Corman, J., Edwards, M., Elser, J.J. (2011) Sustainability challenges of phosphorus and food: solutions from closing the human phosphorus cycle. BioScience, **61**(2), 117-124.

Chiou, T., Aung, K., Lin, S., Wu, C., Chiang, S., Su, C. (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell, 18 (2), 412-421.

Chiou, T.J. and Lin, S.I. (2011) Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol, **62**, 185-206.

Clouse, S.D., Langford, M., McMorris, T.C. (1996) A Brassinosteroid-Insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol, **111**(3), 671-678.

Clouse, S.D. (2011) Brassinosteroids. The Arabidopsis Book. **9**, e0151. http://doi.org/10.1199/tab.0151

Cordell, D., Drangert, J.-O., White, S. (2009) The story of phosphorus: Global food security and food for thought. Global Environ Change, **19**(2), 292-305.

Cubero, B., Nakagawa, Y., Jiang, X.Y., Miura, K.J., Li, F., Raghothama, K.G., Bressan, R.A., Hasegawa, P.M., Pardo, J.M. (2009) The phosphate transporter PHT4;6 is a determinant of salt tolerance that is localized to the Golgi apparatus of Arabidopsis. Mol Plant, **2**(3), 535-552.

Danova-Alt, R., Dijkema, C., De Waard, P., Kock, M. (2008) Transport and compartmentation of phosphite in higher plant cells – kinetic and 31P nuclear magnetic resonance studies. Plant Cell Environ, **31**(10), 1510-1521.

del Pozo, J.C., Allona, I., Rubio, V., Leyva, A., de la Pena, A., Aragoncillo, C., Paz-Ares, J. (1999) A *type 5 acid phosphatase* gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J, **19**(5), 579-589.

Dettmer, J., Hong-Hermesdorf, A., Stierhof, Y.D., Schumacher, K. (2006) Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. Plant Cell, **18**(3), 715-730.

Doerner, P. (2008) Phosphate starvation signaling: a threesome controls systemic P(i) homeostasis. Curr Opin Plant Biol, **11**(5), 536-540.

Duan, K., Yi, K., Dang, L., Huang, H., Wu, W., Wu, P. (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J, **54**(6), 965-975.

Edixhoven, J.D., Gupta, J., Savenije, H.H.G. (2013) Recent revisions of phosphate rock reserves and resources: reassuring or misleading? An in-depth literature review of global estimates of phosphate rock reserves and resources. Earth Syst Dynam Discuss, **4**(2), 1005-1034.

Essigmann, B., Güler, S., Narang, R.A., Linke, D., Benning, C. (1998) Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A, **95**(4), 1950-1955.

Fontenot, E., Ditusa, S., Kato, N., Olivier, D., Dale, R., Lin, W., Chiou, T., Macnaughtan, M., AP, S. (2015) Increased phosphate transport of *Arabidopsis thaliana* Pht1;1 by site-

directed mutagenesis of tyrosine 312 may be attributed to the disruption of homomeric interactions. Plant Cell Environ, **38**(10), 2012–2022.

Franco-Zorrilla, J., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet, **39**(8), 1033-1037.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K., Zhu, J.K. (2005) A miRNA involved in phosphate-starvation response in Arabidopsis. Curr Biol, **15**(22), 2038-2043.

Gaude, N., Nakamura, Y., Scheible, W., Ohta, H., Dormann, P. (2008) Phospholipase C5 (NPC5) is involved in galactolipid accumulation during phosphate limitation in leaves of Arabidopsis. Plant J, **56**(1), 28-39.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., Jürgens, G. (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell, **112**(2), 219-230.

Geldner, N., Friml, J., Stierhof, Y., Jurgens, G., Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature, **413**(6854), 425-428.

Ghillebert, R., Swinnen, E., De Snijder, P., Smets, B., Winderickx, J. (2011) Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in *Saccharomyces cerevisiae.* Biochem J, **434**(2), 243-251.

Gilbert, N. (2009) Environment: The disappearing nutrient. Nature, **461**, 716-718.

González, E., Solano, R., Rubio, V., Leyva, A., Paz-Ares, J. (2005) PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in Arabidopsis. Plant Cell, **17**(12), 3500-3512.

Guo, B., Jin, Y., Wussler, C., Blancaflor, E.B., Motes, C.M., Versaw, W.K. (2008) Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters. New Phytol, **177**(4), 889-898.

Harrison, M.J. The arbuscular mycorrhizal symbiosis: an underground association. Trends Plant Sci, **2**(2), 54-60.

Harrison, M.J. (1997) The arbuscular mycorrhizal symbiosis: an underground association. Trends Plant Sci, **2**(2), 54-60.

Harrison, M.J. (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol, **59**, 19-42.

Harrison, M.J., Dewbre, G.R., Liu, J. (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell, **14**(10), 2413-2429.

Hem, S., Rofidal, V., Sommerer, N., Rossignol, M. (2007) Novel subsets of the Arabidopsis plasmalemma phosphoproteome identify phosphorylation sites in secondary active transporters. Biochem Biophys Res Commun, **363**(2), 375-380.

Hermans, C., Hammond, J., White, P., Verbruggen, N. (2006) How do plants respond to nutrient shortage by biomass allocation? Trends in Plant Science, **11**(12), 610-617.

Holford, I. (1997) Soil phosphorus: its measurement, and its uptake by plants. Soil Research, **35**(2), 227-240.

Huang, T.K., Han, C.L., Lin, S.I., Chen, Y.J., Tsai, Y.C., Chen, Y.R., Chen, J.W., Lin, W.Y., Chen, P.M., Liu, T.Y., Chen, Y.S., Sun, C.M., Chiou, T.J. (2013) Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. Plant Cell, **10**, 4044-4060.

Hurlimann, H.C., Pinson, B., Stadler-Waibel, M., Zeeman, S.C., Freimoser, F.M. (2009) The SPX domain of the yeast low-affinity phosphate transporter Pho90 regulates transport activity. EMBO Rep, **10**(9), 1003-1008.

Jost, R., Pharmawati, M., Lapis-Gaza, H., Rossig, C., Berkowitz, O., Lambers, H., Finnegan, P. (2015) Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in *Arabidopsis thaliana* through the application of phosphite. J Exp Bot **66**(9), 2501-2514.

Kant, S., Peng, M., Rothstein, S.J. (2011) Genetic regulation by *NLA* and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in Arabidopsis. PLoS Genet, **7**(3), e1002021.

Karthikeyan, A.S., Varadarajan, D.K., Jain, A., Held, M.A., Carpita, N.C., Raghothama, K.G. (2007) Phosphate starvation responses are mediated by sugar signaling in Arabidopsis. Planta, **225**(4), 907-918.

Karthikeyan, A.S., Varadarajan, D.K., Mukatira, U.T., D'Urzo, M.P., Damsz, B., Raghothama, K.G. (2002) Regulated expression of Arabidopsis phosphate transporters. Plant Physiol, **130**(1), 221-233.

Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., Friml, J. (2006) Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell, **18**(11), 3171-3181.

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., Friml, J. (2008) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. Proc Natl Acad Sci U S A, **105**(46), 17812-17817.

Kobayashi, K., Awai, K., Nakamura, M., Nagatani, A., Masuda, T., Ohta, H. (2009) Type-B monogalactosyldiacylglycerol synthases are involved in phosphate starvation-induced lipid remodeling, and are crucial for low-phosphate adaptation. Plant J, **57**(2), 322-331.

Lapis-Gaza, H.R., Jost, R., Finnegan, P.M. (2014) Arabidopsis PHOSPHATE TRANSPORTER1 genes *PHT1;8* and *PHT1;9* are involved in root-to-shoot translocation of orthophosphate. BMC Plant Biol, **14**, 334-352.

Lauer, M., Blevins, D., Sierzputowska-Gracz, H. (1989) 31P-Nuclear Magnetic Resonance determination of phosphate compartmentation in leaves of reproductive soybeans (*Glycine max L.*) as affected by phosphate nutrition. Plant Physiol, **89**(4), 1331-1336.

Lee, G. and Jones-Lee, A. (2004) Assesing the water quality impacts of phosphorus in runoff from agricultural lands. In Hall,W., and Robarge, W. Environmental impact of fertilizer on soil and water, ACS Sympsium Series 872, American Chemical Society, Washington DC: 207-219.

Lee, R.B., Ratcliffe, R.G., Southon, T.E. (1990) 31P NMR measurements of the cytoplasmic and vacuolar Pi content of mature maize roots: Relationships with phosphorus status and phosphate fluxes. J Exp Bot, **41**(9), 1063-1078.

Li, D., Zhu, H., Liu, K., Liu, X., Leggewie, G., Udvardi, M., Wang, D. (2002) Purple acid phosphatases of *Arabidopsis thaliana*. Comparative analysis and differential regulation by phosphate deprivation. J Biol Chem, **277**(31), 27772-27781.

Lin, S.I., Chiang, S.F., Lin, W.Y., Chen, J.W., Tseng, C.Y., Wu, P.C., Chiou, T.J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiol, **147**(2), 732-746.

Lin, W., Huang, T., Chiou, T. (2013) Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. Plant Cell, **25**(10), 4061-4074.

Liu, C., Muchhal, U.S., Raghothama, K.G. (1997) Differential expression of *TPS11*, a phosphate starvation-induced gene in tomato. Plant Mol Biol, **33**(5), 867-874.

Liu, C., Muchhal, U.S., Uthappa, M., Kononowicz, A.K., Raghothama, K.G. (1998) Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. Plant Physiol, **116**(1), 91-99.

Liu, J., Yang, L., Luan, M., Wang, Y., Zhang, C., Zhang, B., Shi, J., Zhao, F.-G., Lan, W., Luan, S. (2015) A vacuolar phosphate transporter essential for phosphate homeostasis in Arabidopsis. Proc Natl Acad Sci U S A, **112**(47), 6571-6578.

Liu, T.Y., Huang, T.K., Yang, S.Y., Hong, Y.T., Huang, S.M., Wang, F.N., Chiang, S.F., Tsai, S.Y., Lu, W.C., Chiou, T.J. (2016) Identification of plant vacuolar transporters mediating phosphate storage. Nat Commun, **7**:11095

Long, T.A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., Benfey, P.N. (2010) The bHLH transcription factor POPEYE regulates response to iron deficiency in Arabidopsis roots. Plant Cell, 22(7), 2219-2236.

Lopez-Bucio, J., Hernandez-Abreu, E., Sanchez-Calderon, L., Nieto-Jacobo, M.F., Simpson, J., Herrera-Estrella, L. (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. Plant Physiol, **129**(1), 244-256.

Lynch, J. (1995) Root Architecture and Plant Productivity. Plant Physiol, **109**(1), 7-13.

Ma, Z., Baskin, T.I., Brown, K.M., Lynch, J.P. (2003) Regulation of root elongation under phosphorus stress involves changes in *Ethylene Responsiveness1*. Plant Physiol, **131**(3) 1381-1390.

Marschner, H. (1995m) 13 - Nutrient availability in soils. In Mineral Nutrition of Higher Plants (Second Edition) Academic Press, London: 483-507.

Marschner, H. (1995n) 14 - Effect of internal and external factors on root growth and development. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 508-536.

Marschner, H. (1995o) 15 - The soil-root interface (rhizosphere) in relation to mineral nutrition. In Mineral Nutrition of Higher Plants (Second Edition) Academic Press, London: 537-595.

Marschner, H. (1995p) 16 - Adaptation of plants to adverse chemical soil conditions. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 596-680.

Martin, A.C., del Pozo, J.C., Iglesias, J., Rubio, V., Solano, R., de La Pena, A., Leyva, A., Paz-Ares, J. (2000) Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. Plant J, **24**(5), 559-567.

Massonneau, A., Martinoia, E., Dietz, K.J., Mimura, T. (2000) Phosphate uptake across the tonoplast of intact vacuoles isolated from suspension-cultured cells of *Catharanthus roseus* (L.) G. Don. Planta, **211**(3), 390-395.

Masucci, J.D. and Schiefelbein, J.W. (1994) The *rhd6* Mutation of *Arabidopsis thaliana* alters root-hair initiation through an Auxin- and Ethylene-associated process. Plant Physiol, **106**(4), 1335-1346.

Mimura, T., Dietz, K.J., Kaiser, W., Schramm, M.J., Kaiser, G., Heber, U. (1990) Phosphate transport across biomembranes and cytosolic phosphate homeostasis in barley leaves. Planta, **180**(2), 139-146.

Misson, J., Thibaud, M.C., Bechtold, N., Raghothama, K., Nussaume, L. (2004) Transcriptional regulation and functional properties of Arabidopsis *Pht1;4*, a high

affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. Plant Mol Biol, **55**(5), 727-741.

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S., Shibata, D. (1997) Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. Proc Natl Acad Sci USA, **94**(13), 7098-7102.

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A., Raghothama, K., Baek, D., Koo, Y., Jin, J., Bressan, R., Yun, D., Hasegawa, P. (2005) The Arabidopsis SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proc Natl Acad Sci U S A, **102**(21), 7760-7765.

Muchhal, U., Pardo, J., Raghothama, K. (1996) Phosphate transporters from the higher plant *Arabidopsis thaliana.* Proc Natl Acad Sci U S A, **93** (19), 10519-10523.

Muchhal, U.S. and Raghothama, K.G. (1999) Transcriptional regulation of plant phosphate transporters. Proc Natl Acad Sci U S A, **96**(10), 5868-5872.

Mudge, S., Rae, A., Diatloff, E., Smith, F. (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in *Arabidopsis*. Plant J, **31**(3), 341-353.

Mukherjee, P., Banerjee, S., Wheeler, A., Ratliff, L.A., Irigoyen, S., Garcia, L.R., Lockless, S.W., Versaw, W.K. (2015) Live imaging of inorganic phosphate in plants with cellular and subcellular resolution. Plant Physiol, **167**(3), 628-638.

Nacry, P., Canivenc, G., Muller, B., Azmi, A., Van Onckelen, H., Rossigno, M., Doumas, P. (2005) A role for Auxin redistribution in the responses of the root system architecture to phosphate starvation in Arabidopsis. Plant Physiol, **138**, 2061-2074.

Nagarajan, V., Jain, A., Poling, M., Lewis, A., Raghothama, K., Smith, A. (2011) Arabidopsis Pht1;5 mobilizes phosphate between source and sink organs and influences the interaction between phosphate homeostasis and ethylene signaling. Plant Physiol, **156**(3), 1149-1163.

Narang, R., Bruene, A., Altmann, T. (2000) Analysis of phosphate acquisition efficiency in different Arabidopsis accessions. Plant Physiol, **124**(4), 1786-1799.

Nilsson, L., Muller, R., Nielsen, T.H. (2007) Increased expression of the MYB-related transcription factor, PHR1, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. Plant Cell Environ, **30**(12), 1499-1512.

Nuhse, T., Stensballe, A., Jensen, O., Peck, S. (2004) Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell, **16**(9), 2394-2405.

Nussaume, L., Kanno, S., Javot, H., Marin, E., Pochon, N., Ayadi, A., Nakanishi, T.M., Thibaud, M.C. (2011) Phosphate import in plants: Focus on the PHT1 transporters. Front Plant Sci, **2**, 83-94.

Pant, B.D., Buhtz, A., Kehr, J., Scheible, W.R. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J, **53**(5), 731-738.

Park, B.S., Seo, J.S., Chua, N.H. (2014) NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. Plant Cell, **26**(1), 454-464.

Park, M. and Jürgens, G. (2011) Membrane traffic and fusion at post-Golgi compartments. Front Plant Sci, **2**, 111-125.

Pedersen, B.P., Kumar, H., Waight, A.B., Risenmay, A.J., Roe-Zurz, Z., Chau, B.H., Schlessinger, A., Bonomi, M., Harries, W., Sali, A., Johri, A.K., Stroud, R.M. (2013) Crystal structure of a eukaryotic phosphate transporter. Nature, **496**(7446), 533-536.

Peng, M., Hannam, C., Gu, H., Bi, Y.M., Rothstein, S.J. (2007) A mutation in *NLA*, which encodes a RING-type ubiquitin ligase, disrupts the adaptability of Arabidopsis to nitrogen limitation. Plant J, **50**(2), 320-337.

Poirier, Y. and Bucher, M. (2002) Phosphate transport and homeostasis in Arabidopsis. Arabidopsis Book, **1**, e0024.

Poirier, Y., Thoma, S., Somerville, C., Schiefelbein, J. (1991) Mutant of Arabidopsis deficient in xylem loading of phosphate. Plant Physiol, **97**(3), 1087-1093.

Pratt, J., Boisson, A.M., Gout, E., Bligny, R., Douce, R., Aubert, S. (2009) Phosphate (Pi) starvation effect on the cytosolic Pi concentration and Pi exchanges across the tonoplast in plant cells: an in vivo 31P-nuclear magnetic resonance study using methylphosphonate as a Pi analog. Plant Physiol, **151**(3), 1646-1657.

Puga, M.I., Mateos, I., Charukesi, R., Wang, Z., Franco-Zorrilla, J.M., de Lorenzo, L., Irigoyen, M.L., Masiero, S., Bustos, R., Rodriguez, J., Leyva, A., Rubio, V., Sommer, H., Paz-Ares, J. (2014) SPX1 is a phosphate-dependent inhibitor of Phosphate Starvation Response 1 in Arabidopsis. Proc Natl Acad Sci U S A, **111**(41), 14947-14952.

Raghothama, K.G. (1999) Phosphate acquisition. Annu Rev Plant Physiol Plant Mol Biol, **50**, 665-693.

Raghothama, K.G. (2000) Phosphate transport and signaling. Curr Opin Plant Biol, **3**(3), 182-187.

Rausch, C. and Bucher, M. (2002) Molecular mechanisms of phosphate transport in plants. Planta, **216**(1), 23-37.

Rechsteiner, M. and Rogers, S.W. (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci, **21**(7), 267-271.

Remy, E., Cabrito, T., Batista, R., Teixeira, M., Sa-Correia, I., Duque, P. (2012) The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. New Phytol, **195**(2), 356-371.

Richter, S., Voss, U., Jurgens, G. (2009) Post-Golgi traffic in plants. Traffic, **10**(7), 819-828.

Rouached, H., Secco, D., Arpat, B., Poirier, Y. (2011) The transcription factor PHR1 plays a key role in the regulation of sulfate shoot-to-root flux upon phosphate starvation in Arabidopsis. BMC Plant Biol, **11**, 19.

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A., Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev, **15**(16), 2122-2133.

Russinova, E., Borst, J.-W., Kwaaitaal, M., Caño-Delgado, A., Yin, Y., Chory, J., de Vries, S.C. (2004) Heterodimerization and endocytosis of Arabidopsis Brassinosteroid receptors BRI1 and AtSERK3 (BAK1). Plant Cell, **16**(12), 3216-3229.

Schachtman, D.P., Reid, R.J., Ayling, S.M. (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol, **116**(2), 447-453.

Schiefelbein, J. and Somerville, C. (1990) Genetic control of root hair development in *Arabidopsis thaliana.* Plant Cell, **2**(3), 235-243.

Schmidt, W. and Schikora, A. (2001) Different pathways are involved in phosphate and iron stress-induced alterations of root epidermal cell development. Plant Physiol, **125**(4), 2078-2084.

Selote, D., Samira, R., Matthiadis, A., Gillikin, J., Long, T. (2015) Iron-binding E3 Ligase mediates iron response in plants by targeting basic helix-loop-helix transcription factors. Plant Physiol, 167(1), 273-286.

Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., Zhang, W., Zhang, F. (2011) Phosphorus dynamics: from soil to plant. Plant Physiol, 156(3), 997-1005.

Shin, H., Shin, H.S., Chen, R., Harrison, M.J. (2006) Loss of *At4* function impacts phosphate distribution between the roots and the shoots during phosphate starvation. Plant J, **45**(5), 712-726.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J, **39**(4), 629-642.

Singh, A.P., Fridman, Y., Friedlander-Shani, L., Tarkowska, D., Strnad, M., Savaldi-Goldstein, S. (2014) Activity of the brassinosteroid transcription factors

BRASSINAZOLE RESISTANT1 and BRASSINOSTEROID INSENSITIVE1-ETHYL METHANESULFONATE-SUPPRESSOR1/BRASSINAZOLE RESISTANT2 blocks developmental reprogramming in response to low phosphate availability. Plant Physiol, **166**(2), 678-688.

Stefanovic, A., Arpat, A.B., Bligny, R., Gout, E., Vidoudez, C., Bensimon, M., Poirier, Y. (2011) Over-expression of *PHO1* in Arabidopsis leaves reveals its role in mediating phosphate efflux. Plant J, **66**(4), 689-699.

Stefanovic, A., Ribot, C., Rouached, H., Wang, Y., Chong, J., Belbahri, L., Delessert, S., Poirier, Y. (2007) Members of the *PHO1* gene family show limited functional redundancy in phosphate transfer to the shoot, and are regulated by phosphate deficiency via distinct pathways. Plant J, **50**(6), 982-994.

Sun, Y., Fan, X.Y., Cao, D.M., He, K., Tang, W., Zhu, J.Y., He, J.X., Bai, M.Y., Zhu, S., Oh, E., Patil, S., Kim, T.W., Ji, H., Wong, W.H., Rhee, S.Y., Wang, Z.Y. (2010) Integration of Brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev Cell, **19**(5), 765-777.

Swinnen, E., Rosseels, J., Winderickx, J. (2005) The minimum domain of Pho81 is not sufficient to control the Pho85-Rim15 effector branch involved in phosphate starvation-induced stress responses. Curr Genet, **48**(1), 18-33.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Poirier, Y., Nussaume, L. (2010) Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. Plant J, **64**(5), 775-789.

Ticconi, C., Delatorre, C., Abel, S. (2001) Attenuation of phosphate starvation responses by Phosphite in Arabidopsis. Plant Physiol, **127**(3), 963-972.

Ticconi, C.A. and Abel, S. (2004) Short on phosphate: plant surveillance and countermeasures. Trends Plant Sci, **9**(11), 548-555.

Tran, H.T., Hurley, B.A., Plaxton, W.C. (2010) Feeding hungry plants: The role of purple acid phosphatases in phosphate nutrition. Plant Sci, **179**(1–2), 14-27.

Vance, C.P. (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol, **127**(2), 390-397.

Varadarajan, D.K., Karthikeyan, A.S., Matilda, P.D., Raghothama, K.G. (2002) Phosphite, an analog of phosphate, suppresses the coordinated expression of genes under phosphate starvation. Plant Physiol, **129**(3), 1232-1240.

Veljanovski, V., Vanderbeld, B., Knowles, V.L., Snedden, W.A., Plaxton, W.C. (2006) Biochemical and molecular characterization of *AtPAP26*, a vacuolar Purple Acid Phosphatase up-regulated in phosphate-deprived Arabidopsis suspension cells and seedlings. Plant Physiol, **142** (3),1282-1293.

Versaw, W. and Harrison, M. (2002) A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. Plant Cell, **14**(8), 1751-1766.

Viotti, C., Bubeck, J., Stierhof, Y.D., Krebs, M., Langhans, M., van den Berg, W., van Dongen, W., Richter, S., Geldner, N., Takano, J., Jurgens, G., de Vries, S.C., Robinson, D.G., Schumacher, K. (2010) Endocytic and secretory traffic in Arabidopsis merge in the trans-Golgi network/early endosome, an independent and highly dynamic organelle. Plant Cell, **22**(4), 1344-1357.

Wang, X., Yi, K., Tao, Y., Wang, F., Wu, Z., Jiang, D., Chen, X., Zhu, L., Wu, P. (2006) Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. Plant Cell Environ, **29**(10), 1924-1935.

Wang, Y., Ribot, C., Rezzonico, E., Poirier, Y. (2004) Structure and expression profile of the Arabidopsis *PHO1* gene family indicates a broad role in inorganic phosphate homeostasis. Plant Physiol, **135**(1), 400-411.

Ward, J.T., Lahner, B., Yakubova, E., Salt, D.E., Raghothama, K.G. (2008) The effect of iron on the primary root elongation of Arabidopsis during phosphate deficiency. Plant Physiol, **147**(3), 1181-1191.

Wei, Z. and Li, J. (2016) Brassinosteroids regulate root growth, development, and symbiosis. Mol Plant, **9**(1), 86-100.

Yamaryo, Y., Dubots, E., Albrieux, C., Baldan, B., Block, M.A. (2008) Phosphate availability affects the tonoplast localization of PLDzeta2, an *Arabidopsis thaliana* phospholipase D. FEBS Lett, **582**(5), 685-690.

Zhang, Y., Lynch, J., Brown, K. (2003) Ethylene and phosphorus availability have interacting yet distinct effects on root hair development. J Exp Bot, **54**(391), 2351-2361.

Zhang, Z., Liao, H., Lucas, W.J. (2014) Molecular mechanisms underlying phosphate sensing, signaling, and adaptation in plants. J Integr Plant Biol, **56**(3), 192-220.

Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., Zhong, W., Wu, P. (2008) OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. Plant Physiol, **146**(4), 1673-1686.

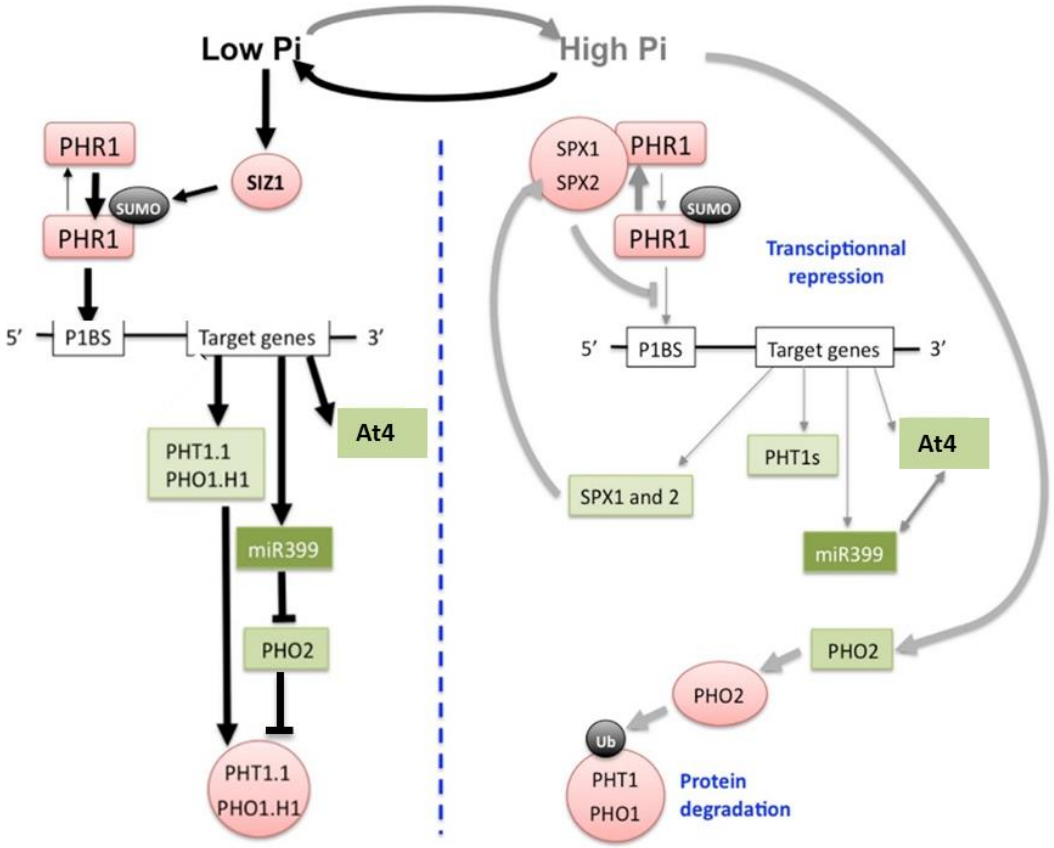
TABLES AND FIGURES

Table 1. Tissue specific expression of *PHT1* genes in *Arabidopsis thaliana.*

Gene name	Gene ID	Tissue expression	Promoter activity
PHT1;1	At5g43350	Shoots, roots, cotyledons, buds, seeds	Epidermis and cortex of root hair zone, hydathodes and axillary buds of leaves
PHT1;2	At5g43370	Roots, cotyledons, leaves	
PHT1;3	At5g43360	Roots, cotyledons, leaves	Stele of primary root, vascular tissue of leaves
PHT1;4	At2g38940	Flowers, siliques, roots	Epidermis and cortex of roots, apical meristem, trichomes and hydathodes of leaves
PHT1;5	At2g32830	Shoots, flowers	Vasculature
PHT1;6	At5g43340	Flowers, pollen	Dry pollen, flowers at stages 9,10, 11, and vascular tissue of cotyledons
PHT1;7	At3g54700	Flowers, roots	Dry pollen
PHT1;8	At1g20860	Roots	None detected
PHT1;9	At1g76430	Roots	None detected

Figure 1. Schematic illustration of the regulatory circuit in response to Pi fluctuations.

When Pi is limiting (left) *PHT1*, *PHO1*, miR399, *At4* genes are transcriptionally activated by PHR1, while PHR1 is activated via sumoylation by SIZ1. MiR399 negatively regulates PHO2. When Pi is sufficient, PHR1 targets are transcriptionally repressed as SPX proteins interact with PHR1 and prevent its binding to P1BS elements. PHO2 expression is activated and it results in Pi transporters degradation via ubiquitination. Color coding as follows: green indicate transcripts, red indicate proteins, black indicate post-translational modifications (Figure adapted from Briat et al., 2015).



CHAPTER 2

Arabidopsis thaliana High Affinity Phosphate Transporters PHT1;8 and PHT1;9 are Important for Adaptation to Pi Stress

ABSTRACT

In *Arabidopsis thaliana* one of the adaptations to inorganic phosphate (Pi) shortage is mediated by the up-regulation of high-affinity phosphate transporters of the PHOSPHATE TRANSPORTER1 (PHT1) Family. *PHT1;8* and *PHT1;9* encode proteins that are 79% identical, thus might have similar functions. Recently, PHT1;8 and PHT1;9 have been implicated in Pi translocation from root to shoot (Lapis-Gaza et al., 2014), however their exact role in acclimation to Pi stress and the cellular mechanisms that regulate their accumulation in the cell remain unclear. A reverse genetic approach was used to characterize the function of *PHT1;8* and *PHT1;9*. Loss of *PHT1;8*, *PHT1;9* or both resulted in reduced basal phosphate levels and longer root hairs in *pht1;8* and *pht1;8 1;9* double mutant. Upon exposure to Pi stress, *pht1;8* and the *pht1;8pht1;9* double, showed reduced tolerance to low Pi, increased rhizosphere acidification and increased anthocyanin accumulation in shoots. Genetic interactions amongst *PHT1* genes is highly probable, as the *pht1;8* and *pht1;9* mutants have increased *PHT1;1/2/3/4* expression, and *PHT1;8* and *1;9* transcripts accumulate at higher levels in the *pht1;1/4* mutant. Overexpression of *PHT1;8* or *PHT1;9* results in a higher basal Pi, suggestive of enhanced Pi uptake and possibly translocation. In addition, fluorescent-protein translational fusions to PHT1;8 and PHT1;9 driven by a constitutive promoter were used to identify their subcellular localization. Transient expression studies in *Nicotiana benthamiana* illustrate that both proteins localize to endoplasmic reticulum (ER). However, in stably-transformed *Arabidopsis thaliana*, PHT1;8 and PHT1;9 localize predominantly to ER and to the plasma membrane

(PM), although to a lower extent. Additionally, the PM pool of PHT1;8 and PHT1;9 is sensitive to Brefeldin A (BFA) suggesting the active role of recycling in regulating the abundance of these proteins at the PM. Altogether, our data suggest that PHT1;8 and 1;9 regulate global Pi status and function in proper adaptation to low levels of Pi.

INTRODUCTION

Albeit the phosphorus (P) pools are abundant in most soils, only a small fraction of these pools is made up of inorganic phosphate (Pi), consequently plants encounter extremely low amounts of usable phosphate for plant growth and development. To overcome this limitation plants have evolved a variety of adaptations destined to promote Pi use efficiency, Pi uptake and Pi bioavailability (del Pozo et al., 1999; Li et al., 2002; Misson et al., 2005; Mudge et al., 2002; Poirier and Bucher, 2002; Thibaud et al., 2010). Root architecture changes enhance Pi foraging given that Pi is a highly immobile macronutrient (Abel, 2011; Bates and Lynch, 1996; Bates and Lynch, 2000). When Pi is low, primary root growth is inhibited, while lateral roots and root hair growth and extension is promoted. These changes result in increases in root surface area and better exploitation of the topsoil (Lynch, 1995). Furthermore, low Pi results in a 5-fold increase in root hair length and density, together with an increase in root diameter as a result of the increase in the number of the trichoblasts in the cortical cells (Bates and Lynch, 1996; Bates and Lynch, 2000). These results highlight the important role of root hairs for Pi acquisition under low Pi.

Upon Pi stress, plants acclimate by increasing phosphate acquisition, either by upregulating *PHT1* genes or by promoting symbiotic associations with specialized fungi. Most vascular flowering plants are capable of symbiotic fungi associations, such as arbuscular mycorrhizae (AM), for the purpose of Pi uptake (Harrison, 1997; Harrison, 2005; Harrison et al., 2002; Vance, 2001). AM fungi are obligate biotrophs that participate in symbiotic relationships with plants to facilitate phosphate uptake, while the plants provide the necessary carbon supply to the fungi. Given that Arabidopsis does not form AM relationships but rather

relies exclusively on the activity of high affinity phosphate transporters, it is an excellent model system to learn about the contributions of *PHT1*.

PHT1 genes encode high affinity phosphate transporters and are responsible for the uptake of Pi from soil. Moreover, *PHT1* genes are upregulated specifically during Pi-stress, as shown by promoter analyses (Karthikeyan et al., 2002; Misson et al., 2004; Muchhal et al., 1996; Muchhal and Raghothama, 1999; Mudge et al., 2002). The gene expression profiles of *PHT1* genes are predominantly, but not exclusively restricted to roots, and they have a somewhat overlapping pattern in both low and high Pi regimes (Misson et al., 2004; Muchhal and Raghothama, 1999; Mudge et al., 2002). *PHT1;1* through *PHT1;4* are highly similar to each other and form a clade distinct from *PHT1;8-1;9.* Of all *PHT1* transcripts, *PHT1;8* and *PHT1;9*, are the least expressed during sufficient Pi, however are highly and specifically induced upon Pi starvation (Misson et al., 2005; Morcuende et al., 2007). PHT1;8 and PHT1;9 are highly similar to each other and share 79% similarity, while they share only 49% similarity with PHT1;1/2/3/5 and 47% with PHT1;4 (Figure S1). PHT1;8 and PHT1;9 proteins are 34% similar to PHO84, the high affinity phosphate transporter in *Saccharomyces cerevisiae* (Figure S1). Moreover, *AtPHT1;9* was able to complement the yeast *pho84* mutation (Remy et al., 2012), just like *AtPHT1;1* and *AtPHT1;4* (Muchhal et al., 1996). This indicates that PHT1;9 is involved in the uptake of Pi. In addition, PHT1;8 and PHT1;9 have been shown to be involved in the translocation of Pi from roots to shoots (Lapis-Gaza et al., 2014).

Taking aside the redundancy, it becomes apparent that there is also a significant genetic interaction amongst *PHT1* genes (Ayadi et al., 2015; Lapis-Gaza et al., 2014; Misson et al., 2004). Furthermore, there is significant evidence that individual *PHT1* genes undergo distinct post-transcriptional and post-translational control (Aung et al., 2006; Bayle et al., 2011). For

instance, in the phosphate over-accumulator mutant *PHOSPHATE2* (*pho2*) only *PHT1;8* expression was upregulated in Pi sufficient conditions, while no difference in the transcript levels of other *PHT1* was observed under Pi deprivation (Aung et al., 2006). This might indicate that PHO2 is upstream of *PHT1;8* to repress its expression, specifically, under Pi replete conditions and this is consistent with the very low abundance of *PHT1;8* transcript in the roots during Pi sufficiency. In addition, the regulation of *PHT1* genes might differ, given that only *PHT1;8*, *PHT1;4* and *PHT1;5* are regulated systemically upon Pi starvation conditions (Thibaud et al., 2010). Moreover, only PHT1;8 and PHT1;9 have a lower affinity for phosphite (Phi), a less oxidized form of phosphorus (P) than phosphate, as suggested by the lower Phi content in the shoots of *pht1;8* and *pht1;9* mutants (Jost et al., 2015). Overall, it is evident that PHT1 transporters have distinct contributions to Pi homeostasis.

As all membrane proteins, PHT1 are synthesized at the ER. The exit of PHT1;1/2 from the ER is conditioned by PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1), which facilitates efficient PHT1 targeting to the PM (Bayle et al., 2011; Chen et al., 2011; González et al., 2005). PHF1 is related to SEC12, a guanine nucleotide-exchange factor (GEF) that participates in budding of transport vesicles from ER in *Saccharomyces cerevisiae* (González et al., 2005). However, Pi uptake is not completely abolished in the *phf1* mutant, suggesting that PM trafficking of some phosphate transporters might be PHF1-independent (González et al., 2005; Nussaume et al., 2011). When Pi is abundant, newly synthesized PHT1;1 transporters accumulate at the ER, and thus are not delivered to the PM, due to phosphorylation at conserved serine residues on the C-terminus of PHT1;1 (Bayle et al., 2011; Hem et al., 2007; Nuhse et al., 2004). The kinase that phosphorylates the rice PHT1 homologue, OsPT8, has been identified as the β3 subunit of the CK2 hetero-tetramer kinase

(CK2β3) (Chen et al., 2015). The phosphorylated form of OsPT8 does not interact with OsPHF1, the latter promoted OsPT8 exit from the ER and thus OsPT8 was retained at the ER. Additionally, phosphorus starvation promoted CK2β3 degradation, relieving the negative regulation of OsPT8 during Pi-stress conditions (Chen et al., 2015).

PHT1 abundance at the PM is regulated by endocytosis, as these proteins may be recycled back to the plasma membrane during Pi-limiting conditions or they may be trafficked to the vacuole for degradation when Pi is plentiful (Bayle et al., 2011). In addition, PHT1;1 employs BFA-sensitive endosomes for active recycling, as indicated by PHT1;1 accumulation in BFA compartments upon BFA treatment under Pi starvation (Bayle et al., 2011). Ubiquitination of PHT1;1 and/or PHT1;4 is achieved by cooperative action of NITROGEN LIMITATION ADAPTATION (NLA) and PHO2, and it represents the first step for endocytosis and vacuolar targeting (Lin et al., 2013; Park et al., 2014). The PHT1;1 packaging into multi vesicular bodies (MVB) requires ALIX1, a protein that associates with ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT III (ESCRTIII) (Cardona-Lopez et al., 2015). This implicates the ESCRT machinery in the targeting of the PHT1;1 to the lytic vacuole for degradation.

Hereon we characterize the role of *Arabidopsis thaliana* high affinity transporter PHT1;8 and its closest homologue PHT1;9 by analyzing loss of function and overexpression mutants. We present evidence for a genetic interaction amongst *PHT1* genes, necessary to maintain proper Pi status. We report that, unlike other PHT1 proteins, PHT1;8 and PHT1;9 localize predominantly to ER in cotyledons, hypocotyls and mature roots, while at the root tips PHT1;8 and PHT1;9 are found mostly at the PM and somewhat at the ER; this might be an indication that the subcellular localization of PHT1;8 and PHT1;9 is developmentally

controlled. We also report for the first time that PHT1;6 transporters localize to the PM in all tissues and in roots these transporters localize preferentially to the apical and basal domains of the PM, suggesting that they may be involved in the directional transport of Pi in flower buds and mature pollen, where this gene is expressed. Furthermore, we show that GFP-PHT1;6 is targeted for degradation at the lytic vacuole.

RESULTS

PHT1;8 and *PHT1;9* genetically interact

A reverse genetics approach was used to determine the contribution to Pi homeostasis of *PHT1;8* and *PHT1;9*. In *pht1;8* (SAIL_116_G06) the T-DNA is inserted in the second exon of *PHT1;8*, while in the *pht1;9* (SALK_073614C) a T-DNA is inserted in the intron of *PHT1;9* (Figure 1A). Partial transcripts could be potentially made from both *pht1;8* and *pht1;9* alleles given the position of the T-DNA insertions (Figure 1A), however these are likely to result in degradation via the nonsense mediated decay (Conti and Izaurralde, 2005). Homozygous plants for the T-DNA insertion in *PHT1;8* and *PHT1;9* were identified by PCR-genotyping. Furthermore, in order to investigate the joint contribution of PHT1;8 and PHT1;9 transporters, we generated the double mutant *pht1;8 1;9* by crossing *pht1;8* to *pht1;9* and by PCR-genotyping in the F_2 population. In order to determine the presence of the full-length *PHT1;8* and *PHT1;9* transcript in the T-DNA insertion lines, RT-PCR was performed. To this end, RNA was extracted from roots of seedlings grown for 7 days at regular conditions and then transferred to media with only 2 µM phosphate for 7 days (low Pi, Figure 1B). No RT-PCR analysis was done in Pi sufficient conditions given that *PHT1;8* and *PHT1;9* transcripts are only detected during Pi starvation (Mudge et al., 2002). RT-PCR analysis revealed that, unlike in the wild type, the full length transcript for *PHT1;8* is undetected in the *pht1;8* mutant and in the *pht1;8 1;9* double mutant under Pi-deficient conditions and therefore, *pht1;8* is a good mutant allele to characterize the function of *PHT1;8* (Figure 1B). Compared to the wild type, lower levels of the full length *PHT1;9* transcript are detected in the *pht1;9* mutant and in the *pht1;8 1;9* double mutant as indicated by the faint band corresponding to *PHT1;9* (Figure 1B). It was also observed that the *PHT1;8* transcript in the *pht1;9* mutant had an increased

abundance relative to WT (Figure 1B), suggesting that *PHT1;8* might compensate for the reduction in *PHT1;9* transcript.

To better characterize the genetic defects of the *pht1;8* and *pht1;9* mutants, transcript abundance of *PHT1;8* and *PHT1;9* was measured by quantitative real-time PCR (qRT-PCR). The *PHT1;8* transcript is reduced to only 5% of the WT level in the *pht1;8* mutant, while *PHT1;9* expression in the *pht1;9* mutant is reduced to 30% of the WT level (Figure 1C). These results indicate that *pht1;8* and *pht1;9* are knockdown mutants rather than null mutants. Remarkably, we also observed that *PHT1;8* is upregulated in the *pht1;9* mutant and vice versa (Figure 1C) consistent with our previous observation (Figure 1B), which suggests that *PHT1;8* and *PHT1;9* genetically interact. In fact, dissecting the function of specific PHT1 transporters is challenging given their high levels of redundancy (Ayadi et al., 2015) and the fact that *PHT1* gene expression is often upregulated when other members of the clade are missing (Ayadi et al., 2015; Lapis-Gaza et al., 2014; Misson et al., 2004; Remy et al., 2012). We therefore examined the expression of *PHT1* genes in *pht1;8* and *pht1;9* mutants by RT-PCR (Figure 1D). All *PHT1* transcripts are increased in the phosphate starved roots of *pht1;8*, *pht1;9*, and *pht1;8 1;9* double mutants when compared to the WT control*;* however, the most drastic increase can be observed in *PHT1;5* expression (Figure 1D). *PHT1;6* and *PHT1;7* have been omitted from this analysis, given their low abundance. We then asked whether *PHT1;8* and *PHT1;9* were also up-regulated in other *pht1* mutants. Indeed, loss of *PHT1;1* and *PHT1;4* also resulted in an increase in *PHT1;8* and *PHT1;9* expression because both transcripts accumulated at –Pi levels in *pht1;1 1;4* mutants exposed to Pi-sufficient conditions (Figure 1E). These data are consistent with the previous observations that *PHT1;8* and *PHT1;9* were induced even in the *pht1;4* mutant alone (Misson et al., 2004) and in the *pht1;1/2/3/4* and

pht1;1/2/3/4 phf1-2 mutants (Ayadi et al., 2015). Additionally, we investigated the impact of *PHT1;8* and *PHT1;9* loss on Pi homeostasis by assessing the expression of a set of *PHOSPHATE STARVATION INDUCIBLE* (*PSI*) genes such as *ACID PHOSPHATASE 5* (*ACP5*) and *At4*, which are highly induced in response to low Pi, and *UBC24/PHO2*, which is repressed in the low Pi conditions. No significant differences in *PSI* gene expression were observed in Pi-starved *pht1;8, pht1;9* and *pht1;8 1;9* seedlings relative to WT (Figure 1D). This could be due to the upregulated *PHT1* genes in the *pht1;8, pht1;9* and *pht1;8 1;9* mutants. Altogether our data indicates that lack of *PHT1;8* and *PHT1;9* cause an upregulation of *PHT1* genes, particularly their closest homologues.

Disrupting *PHT1;8* and *PHT1;9* negatively impacts Pi content

Disruption of *PHT1;8, PHT1;9* or both did not result in severe morphological defects relative to WT, regardless of the Pi availability (Figure S2). This result was expected since there is high degree of redundancy within the *PHT1* family (Ayadi et al., 2015). Given that PHT1;8 and PHT1;9 have been previously implicated in Pi uptake (Remy et al., 2012) and translocation (Lapis-Gaza et al., 2014), we wanted to investigate whether the disruption *PHT1;8* and *PHT1;9* affected phosphate content. In fact it was recently shown in two other *pht1;8* and *pht1;9* mutant alleles, that disruption of *PHT1;8* and *PHT1;9* resulted in lower basal Pi and organic phosphate (Po) content in shoots, but not in roots (Lapis-Gaza et al., 2014). We then tested the basal Pi concentrations in the shoots of *pht1;8* and *pht1;9* and the double mutant. Loss of *PHT1;8, PHT1;9* or both resulted in lower basal Pi levels in the shoots relative to WT levels (Figure 2A), indicating that Pi uptake and/or translocation is impaired in these plants.

Plants respond to low Pi in the environment by promoting the growth and elongation of lateral roots and root hairs, in order to increase root surface area for more efficient mining of Pi (Bates and Lynch, 1996; Bates and Lynch, 2000; Lopez-Bucio et al., 2002; Marschner, 1995n). Additionally, the internal Pi status also influences root hair development (Ayadi et al., 2015; Bustos et al., 2010). For this reason, we analyzed the root morphology of the *pht1;8*, *pht1;9* and *pht1;8 1;9* mutants. Under Pi sufficient conditions, root hairs were 40% longer in *pht1;8,* 30% longer in *pht1;9* and 70% longer in *pht1;8 1;9* double mutants when compared to the WT (Figure 2B). However, when exposed to Pi stress all the genotypes had similar root hair length (data not shown). These results suggest that *pht1;8, pht1;9* and *pht1;8 1;9* plants display a phosphate starvation phenotype when Pi is plentiful.

Upon Pi starvation, plants accumulate high levels of anthocyanins, possibly for photoprotection (Poirier and Bucher, 2002). Although the link between anthocyanin biosynthesis and Pi starvation is not defined, the transcription factor *PHOSPHATE STARVATION RESPONSE 1 (PHR1)* is involved in mediating this connection (Rubio et al., 2001). We surveyed the effect of Pi stress on anthocyanin content in the shoots of *pht1;8*, *pht1;9* and the double *pht1;8 1;9* mutants. Plants were grown on media with sufficient Pi for seven days and subsequently transferred to 2 µM Pi for seven more days. The anthocyanin levels were doubled in the shoots of *pht1;8* and *pht1;8 1;9* compared to the WT seedlings in response to Pi deprivation, suggesting that loss of *PHT1;8* results in an advanced Pi starvation (Figure 2C). Anthocyanin levels in *pht1;9* did not differ from WT, perhaps due to the fact that 30% of *PHT1;9* transcript is produced in *pht1;9,* and this may be sufficient to avoid Pi starvation.

Low Pi triggers production of various root exudates, such as organic acids and acid phosphatases, which alter the rhizosphere pH in order to increase the phosphate availability in the proximate rhizosphere (Gerke, 2015; Jones, 1998; Lopez-Bucio et al., 2000; Marschner, 1995o; Narang et al., 2000). Phosphate-efficient Arabidopsis accessions have been shown to secrete more organic acids than the less efficient counterparts (Narang et al., 2000). Moreover, transgenic tobacco plants overproducing citrate have been shown to have an enhanced Pi uptake (Lopez-Bucio et al., 2000). We therefore investigated the consequences of *PHT1;8* and/or *PHT1;9* loss on the rhizosphere status of Pi starved seedlings. For this purpose, plants were grown on sufficient Pi for 4 days and transferred to a low Pi (2 µM) medium for 4 additional days, after which the seedlings were transferred onto agar plates supplemented with Bromocresol Purple as a pH indicator. *pht1;8* and the *pht1;8 1;9* double mutant displayed a more acidic rhizosphere than the WT seedlings, indicating that the loss of *PHT1;8* results in exacerbated a Pi starvation phenotype, and no phenotype was detected in *pht1;9* mutant when compared to the WT (Figure 2D). Overall, our data indicates that the disruption of *PHT1;8* or both *PHT1;8* and *PHT1;9* results in compromised Pi homeostasis.

GFP-PHT1;8 and GFP-PHT1;9 proteins localize to the endoplasmic reticulum

In order to better understand the function of proteins of interest it is useful to have a fluorescently labeled variant for *in vivo* studies. Thus far, PHT1;1/2/4 have been shown to localize to the plasma membrane and to sorting endosomes in Arabidopsis (Bayle et al., 2011). In addition, *At*PHT1;9 has been detected at the plasma membrane (Remy et al., 2012), but the localization of PHT1;8 has yet to be reported. For this purpose, we investigated the subcellular localization of PHT1;8 and PHT1;9 by generating N-terminal GFP fusions driven by a strong constitutive promoter, the Cauliflower Mosaic Virus 35S promoter (CaMV35S) (Nakagawa et

al., 2007), and N-terminal YFP fusions driven by the moderate constitutive promoter of *UBIQUITIN10* from Arabidopsis (Grefen et al., 2010). Constitutive promoters were used given that both genes are expressed at very low levels and only upon Pi starvation. In both cases, the strong promoters allowed bypassing of the strong transcriptional regulation by Pi of *PHT1;8* and *PHT1;9* genes. Transient expression of GFP-PHT1;8 and GFP-PHT1;9 in *Nicotiana benthamiana* leaves revealed that both localized predominantly to a reticular network resembling the ER (Figure 3 A and B), and similar results were found for YFP-PHT1;8 and YFP-PHT1;9 (Figure S3). To further investigate the network pattern localization of GFP-PHT1;8 and GFP-PHT1;9, we performed a transient co-expression of GFP-PHT1;8 and GFP-PHT1;9 with the ER marker mCherry-HDEL (Nelson et al., 2007) in *N. benthamiana.* The GFP-PHT1;9 signal fully overlapped with the reticular network arrangement of the mCherry-HDEL marker, including in the perinuclear space (Figure 3 D-F), and the same co-localization was detected for GFP-PHT1;8 (not shown). These results indicate that GFP-PHT1;8 and GFP-PHT1;9 localize to the ER when transiently expressed in *N. benthamiana*.

In order to determine whether the localization of GFP-PHT1;8 and GFP-PHT1;9 to the ER was due to ectopic expression, we generated N-terminal GFP and YFP fusions to PHT1;6, a low abundance phosphate transporter that is expressed exclusively in flowers and pollen (Mudge et al., 2002), under the control of the same constitutive promoters. Transient expression of YFP-PHT1;6 in *N. benthamiana* leaves showed that, similar to other PHT1 proteins, PHT1;6 localized to the plasma membrane (Figure 3C). Therefore, the ER pattern of GFP-PHT1;8 and GFP-PHT1;9 cannot solely be explained by ectopic expression of these transgenes. In order to corroborate that the ER localization of GFP-PHT1;8 and GFP-PHT1;9 was not due to overexpression-induced overload of vesicle trafficking, we co-expressed GFP-

PHT1;8 or GFP-PHT1;9 in the same cells with RFP-PHT1;6. Upon co-infiltration of GFP-PHT1;8 or GFP-PHT1;9 with RFP-PHT1;6 in *N. benthamiana* leaves, we observed that the GFP signal did not co-localize with the RFP fluorescence (Figure 3G-L), indicating that PHT1;8 and PHT1;9 do not co-localize with PHT1;6 and that the ER localization of PHT1;8 and PHT1;9 is not due to the overexpression of these transgenes.

GFP-PHT1;8 and GFP-PHT1;9 localize to ER in mature tissues independent of Pi availability

PHT1;8 and *PHT1;9* are expressed predominantly in the roots upon Pi starvation, and it is possible that the localization of these transporters is responsive to Pi availability. In order to further investigate PHT1;8 and PHT1;9 function, we used stable Arabidopsis lines harboring GFP-PHT1;8 and GFP-PHT1;9 and probed their subcellular localization in various tissues when grown in Pi sufficient conditions. PHT1;8 and PHT1;9 exhibited ER localization in cotyledons (Figure 4A and D), hypocotyls (Figure 4B and E), and in mature roots (Figure 4C and F), as evidenced by the network appearance in cortical regions of cells. It is feasible that the ER localization of PHT1;8 and PHT1;9 in cotyledons and hypocotyls is caused by the ectopic expression of these genes in tissues where *PHT1;8* and *PHT1;9* transcripts are not detected in the WT Arabidopsis. However, the fact that GFP-PHT1;8 and GFP-PHT1;9 are detected at the ER in the roots, where these genes are expressed, indicates that the ER localization might be genuine. Moreover, in Arabidopsis plants harboring GFP-PHT1;6, the fluorescence signal was detected at the plasma membrane in all the tissues tested, as expected based on its high similarity to PHT1;1, in spite of its ectopic expression (Figure 4G-I). Finally, we tested the effect of Pi availability on the localization and abundance of GFP-PHT1;8 and

GFP-PHT1;9, but exposure of GFP-PHT1;8 or GFP-PHT1;9 lines to Pi-deficient conditions did not affect the abundance nor the localization of these fusion proteins (Figure S4 A-D).

GFP-PHT1;8 and GFP-PHT1;9 localize to the PM and are BFA-sensitive in root tips

PHT1;9-GFP localization at the plasma membrane has been previously reported in Arabidopsis root tips (Remy et al., 2012) and is not consistent with the ER localization observed in Nicotiana and Arabidopsis seedlings (Figure 3B and 4D-F). To assess whether GFP-PHT1;8 and GFP-PHT1;9 localize to the PM in root tips, co-staining with FM4-64 was carried out. FM4-64 is a lipophilic dye that labels the PM and endosomes (Bolte et al., 2004), and the overall rate of FM4-64 endocytosis is not affected by Pi-starvation (Bayle et al., 2011; Cardona-Lopez et al., 2015). Subcellular distribution of GFP-PHT1;8 and GFP-PHT1;9 in root tips of Arabidopsis revealed noticeable PM localization in addition to ER (Figure 5A and D). This was corroborated by live-cell imaging of the GFP-PHT1;8 and GFP-PHT1;9 treated with 2 µM FM4-64 for 5 minutes, which show a partial co-localization with GFP-PHT1;8 (Figure 5C) and a more pronounced co-localization with the GFP-PHT1;9 (Figure 5F). The overlap of the FM4-64 with GFP-PHT1;8 and GFP-PHT;9 indicates the occurrence of a subpopulation of GFP-PHT1;8 and GFP-PHT1;9 at the PM in addition to the ER. GFP-PHT1;9 appears to have more PM localization, which is consistent with previous reports (Remy et al., 2012), although there is an abundant GFP-PHT1;8 and GFP-PHT1;9 at the ER as well as at the PM in Arabidopsis root tips. These results indicate that the localization of PHT1;8 and PHT1;9 might be developmentally controlled, as suggested by the differential localization of these transporters in root tips versus mature roots, hypocotyls and cotyledons.

The fungal macrocyclic lactone Brefeldin A (BFA) is an efficient trafficking inhibitor of retrograde movement of proteins from endosomes to the PM and it induces the endocytosed cargo to agglomerate into rounded BFA bodies (Geldner et al., 2003). In Arabidopsis, BFA preferentially blocks the endosomal recycling mediated by the ARF GEF, GNOM, while endocytosis remains functional (Geldner et al., 2003). The auxin transporters PIN1, PIN2 and AUX1 have been shown to accumulate in BFA compartments (Geldner et al., 2001; Kleine-Vehn et al., 2006; Kleine-Vehn et al., 2008). Moreover, PHT1;1 has been shown to cycle between PM and BFA-sensitive endosomes (Bayle et al., 2011). We questioned whether the PM population of GFP-PHT1;8 and GFP-PHT1;9 were recycled through BFA sensitive endosomes. For this purpose, we treated Arabidopsis seedlings harboring GFP-PHT1;8, or GFP-PHT1;9 with BFA for 120 min, and concomitantly stained them with FM4-64 for the purpose of labeling of the PM and the endocytic compartments. We then monitored the localization of each fusion protein in root tips by confocal microscopy. PIN2-GFP was used as a positive control as it accumulates in BFA-sensitive endosomes. In the root tips of BFA-treated Arabidopsis seedlings, both GFP-PHT1;8 and GFP-PHT1;9 accumulated in the BFA bodies; nonetheless strong GFP signal was still highlighting the ER network, indicating that a significant amount of GFP-PHT1;8 and GFP-PHT1;9 is localized to ER (Figure 5G-L). Moreover, the FM4-64 stained BFA aggregates overlapped with the GFP-PHT1;8 and GFP-PHT1;9 trapped in the BFA bodies and therefore correspond to BFA-sensitive endosomes (Figure 5I and L). As expected, PIN2-GFP localization was found in the BFA aggregates and at the PM (Figure 5M), and the PIN2-GFP-labeled BFA bodies also co-localized with FM4-64 (Figure 5N, O). These results indicate that a subpopulation of the GFP-PHT1;8 and GFP-PHT1;9 accumulates at the PM, and it traffics via an endocytic pathway sensitive to BFA.

Moreover, the PM localized GFP-PHT1;8 and GFP-PHT1;9 proteins are subject to constitutive recycling.

Plasma membrane localized PHT1;6 transporters are targeted for degradation at the vacuole

Transient expression in tobacco of YFP-PHT1;6 (Figure 3C) and Arabidopsis stably expressing GFP-PHT1;6 (Figure 4G-I) showed that PHT1;6 has a plasma membrane localization. Moreover, we observed that in roots of Arabidopsis seedlings stably expressing GFP-PHT1;6, PHT1;6 preferentially localized to the apical-basal sides of the PM, as seen in the 3D renderings of confocal z stacks (Figure 6A and B). These results indicate that PHT1;6 might be involved in the directional movement of Pi. Next, we wanted to investigate whether PHT1;6 is targeted for degradation at the lytic vacuole, as previously shown for PHT1;1-GFP (Bayle et al., 2011). To this end, we used concanamycin A (concA), an effective inhibitor of vacuolar type H^+-ATPase, that induces an increase in pH at the vacuole, therefore blocking protein degradation (Pali et al., 2004). Arabidopsis seedlings expressing GFP-PHT1;6 grown either at regular or low Pi were treated with 1 μM concA for 180 min, and live-cell confocal microscopy was used to monitor the GFP-PHT1;6 signal. As expected, the GFP signal for PHT1;6 was only detected at the PM in the mock DMSO treatment (Figure 6C and D). In contrast, the concA-treated seedlings showed accumulation of GFP fluorescence in the lumen of the vacuoles in addition to fluorescence at the PM at both Pi regimes (Figure 6E and F), although it was more obvious during low Pi (Figure 6F). Interestingly, after the concA treatment we also observed an enrichment of the GFP-PHT1;6 signal to the apical and basal domains of the PM in both Pi regimes (Figure 6E and F), which was not apparent in the DMSO control. The polar localization suggests that PHT1;6 may be targeted to specific domains of

the plasma membrane. These results overall, indicate that PHT1;6 is sent for degradation to the vacuole, similar to PHT1;1/2 (Bayle et al., 2011). In addition, the polar PHT1;6 localization suggests that PHT1;6 might be involved in directional Pi transport. The strong ER localization of GFP-PHT1;8 and GFP-PHT1;9 prevented us from carrying out similar experiments to identify whether endocytosis of these transporters is followed by targeting to the lytic vacuole.

Ectopic expression of PHT1;8 and PHT1;9 leads to increased Pi content

Overexpression of *AtPHT1;1* in tobacco cell culture induced a doubling in Pi uptake and enhanced cell growth under Pi-limiting conditions (Mitsukawa et al., 1997). Similarly, overexpression of *AtPHT1;9* enhanced tolerance to Pi starvation in Arabidopsis seedlings (Remy et al., 2012). We asked whether the ectopic expression (OX) of *PHT1;8* and *PHT1;9* would enhance the basal Pi content. For this purpose, we measured the basal Pi level in the shoots of 14 day-old seedlings grown on complete media. Two independent transgenic lines harboring GFP-PHT1;8 (PHT1;8OX) and GFP-PHT1;9 (PHT1;9OX), were tested. Basal Pi levels were increased in both PHT1;8OX and PHT1;9OX (Figure 7) when compared to the wild type Arabidopsis seedlings, even though there was not a phenotypical difference between the transgenic lines relative to the WT. These results indicate that overexpression of PHT1;8 or PHT1;9 enhances Pi uptake and/or translocation; and also confirm that GFP-fused versions of PHT1;8 and PHT1;9 are functional phosphate transporters. Future research is needed to measure the actual uptake rate and to visualize the uptake and /or translocation in the PHT1;8 and PHT1;9 overexpressing lines.

DISCUSSION

This study was aimed at deciphering the individual and joint contributions of PHT1;8 and PHT1;9 to Pi homeostasis and the cellular mechanisms that regulate accumulation of these proteins. Recently, PHT1;9 was reported to play a role in Pi uptake (Remy et al., 2012), yet another report, implicated PHT1;8 and PHT1;9 in the Pi translocation from root to shoot (Lapis-Gaza et al., 2014). Markedly, PHT1;4 plays a role in uptake and translocation of Pi, since *pht1;4* clearly displays deficiencies in both processes (Misson et al., 2004). In this study, reverse genetics was used to characterize the function of PHT1;8 and PHT1;9. Consistent with previous reports (Lapis-Gaza et al., 2014; Remy et al., 2012), loss of *PHT1;8* and *PHT1;9* has negative impacts on the Pi status, given that the shoot basal Pi is lower in these mutants when compared to the WT. This indicates that PHT1;8 and PHT1;9 contribute to Pi uptake and/or translocation. Mutant alleles of *PHT1;8* and *PHT1;9* showed only 5% and 30% of the WT transcript levels, respectively (Figure 1C). We also observed that there is transcriptional compensation between *PHT1;8* and *PHT1;9* in the single mutants. Additionally, *PHT1;8* and *PHT1;9* transcripts are induced in the *pht1;1 1;4* double mutant when seedlings are grown under Pi sufficient conditions, where they accumulate at similar levels to those of the WT under low Pi. These results are intriguing given that *PHT1;1/4* and *PHT1;8/9* are the most dissimilar of the *PHT1* family at the gene and protein level. Moreover, there was a noticeable increase of many of the other *PHT1* family transcripts in *pht1;8*, *pht1;9* and *pht1;8 1;9* double mutants, and the most highly induced transcript was *PHT1;5* (Figure 1D). Given the involvement of PHT1;5, PHT1;8 and PHT1;9 in Pi translocation from root to shoot (Lapis-Gaza et al., 2014; Nagarajan et al., 2011), it is possible that *PHT1;5* upregulation in mutants with impaired *PHT1;8* and *PHT1;9* might partially compensate their absence. However, unlike *PHT1;5*

overexpressing plants which accumulate less Pi in the shoots (Nagarajan et al., 2011; Smith et al., 2011), PHT1;8OX and PHT1;9OX plants accumulate more Pi in shoots than WT (Figure 7). This indicates that PHT1;8 and PHT1;9 are functionally distinct from PHT1;5. Taken together these results indicate that there is a strong genetic interaction amongst *PHT1* genes even though these transporters have distinct functions.

Upon low Pi in the environment, the growth and elongation of lateral roots and root hairs is promoted for a more efficient mining of Pi (Bates and Lynch, 1996; Bates and Lynch, 2000; Lopez-Bucio et al., 2002; Marschner, 1995n). Loss of *PHT1;8* and *PHT1;9* resulted in defects on root morphology in the single and double mutants at variable Pi conditions. Loss of *PHT1;8* had a stronger effect on root hair elongation under normal conditions, however losing both *PHT1;8* and *PHT1;9* resulted in almost doubling of the root hair length compared to WT (Figure 2B). Under Pi starvation all of the genotypes tested had a similar root hair length. These results indicate that *pht1;8*, *pht1;9* and *pht1;8 1;9* mutants are more Pi starved when Pi is plentiful, since increasing root hair length is a known strategy to increase the root surface area to access more Pi and facilitate the Pi uptake. Earlier reports have shown that *pht1;1* and *pht1;4* mutants display longer root hairs in plants grown on high Pi-and transferred to low Pi (Shin et al., 2004). Additionally, *phf1* mutants have increased number and length of root hairs under Pi sufficiency conditions, lower sensitivity to arsenate, reduced Pi content and Pi uptake capacity (González et al., 2005). PHF1 is an ER resident protein that allows PHT1 proteins to exit from the ER (Bayle et al., 2011; González et al., 2005), and the root morphology of the *phf1* mutants is likely due to abnormal accumulation of PHT1 proteins at the PM. The root hair phenotype of *pht1;8* is consistent with the association between impaired Pi uptake and increase in root hair length observed in other mutants (Bayle et al., 2011; González et al., 2005; Shin et al.,

2004). Given that Pi uptake per se was not measured here, it is unclear at this point if Pi uptake is affected by loss of PHT1;8 and PHT1;9. The ultimate way to show that PHT1;8 and PHT1;9 are involved in uptake and translocation or only translocation of Pi, would be to visualize radiolabeled Pi in the *pht1;8* and *pht1;9* (Misson et al., 2004; Nagarajan et al., 2011; Nussaume et al., 2011).

It was previously reported that plants produce higher levels of anthocyanins upon exposure to Pi stress, and loss of *PHT1* genes caused enhanced anthocyanin production (Arnaud et al., 2014; Ayadi et al., 2015; Jost et al., 2015; Miura et al., 2005; Nilsson et al., 2007). *pht1;8* and the *pht1;8pht1;9* double mutant displayed a significant increase of the anthocyanin levels in Pi stressed shoots when compared to WT and *pht1;9* (Figure 2C). These results are indicative of the fact that *pht1;8* mutants are more sensitive to low Pi, having an augmented response to Pi stress. Further investigations need to be carried out to characterize the crosstalk between the anthocyanin biosynthetic pathway and the Pi signaling.

It is well-known that plants produce various root exudates when subjected to limited Pi, such as acid phosphatases and organic acids, which acidify the rhizosphere to solubilize metal-Pi complexes and make it more bioavailable (Gerke, 2015; Marschner, 1995o; Narang et al., 2000; Richardson et al., 2011; Shen et al., 2011). The capacity to acidify the rhizosphere was tested in Pi stressed seedlings of WT, *pht1;8, pht1;9* and *pht1;8 1;9.* All of the seedlings acidified their rhizosphere, however, *pht1;8* and *pht1;8 1;9* had noticeably a more acidic pH around their roots (Figure 2D). Taken together, these results indicate that the loss of *PHT1;8* results in reduced tolerance to low Pi. It is conceivable that *pht1;9* phenotypes are similar to WT due to the fact that 30% of *PHT1;9* transcript is still produced.

Subsequently, we investigated the subcellular localization of PHT1;8 and PHT1;9 by transient and stable transformation. Constitutive promoters were used to drive expression of fluorescent protein fusions given that the native promoter-reporter fusions to PHT1;8 and PHT1;9 produced no fluorescence (Mudge et al., 2002). Fluorescent-protein translational fusions to PHT1;8 and PHT1;9 highlighted network-like structures in tobacco leaves transiently expressing these fusions (Figure 3A and B). Furthermore, GFP-PHT1;8 and GFP-PHT1;9 co-localized with the ER marker mCherry-HDEL, confirming that PHT1;8 and PHT1;9 localize to the ER network. PHT1;1-GFP was also detected at the ER when transiently expressed in tobacco, however when PHF1 was co-infiltrated with PHT1;1, PHT1;1-GFP was detected at the PM. In stably transformed Arabidopsis, PHT1;1-GFP was found at the PM and endosomes, suggesting that the *At*PHF1 promoted PHT1;1 exit from the ER (Bayle et al., 2011). Future experiments need to be conducted to test whether the localization of GFP-PHT1;8 and GFP-PHT1;9 shifts from ER to PM in presence of Arabidopsis PHF1 in Nicotiana, to determine whether PHT1;8 and PHT1;9 localization is PHF1-dependent. However, this ER localization of GFP-PHT1;8 and GFP-PHT1;9 cannot be explained by the lack of PHF1 because the Arabidopsis lines that stably express GFP-PHT1;8 and GFP-PHT1;9 also showed ER accumulation of these proteins. Moreover, a stronger PM localization of GFP-PHT1;8 and GFP-PHT1;9 was detected in root tips indicating that ER exit may be more efficient in these cells or it might also be developmentally controlled. Further investigations are needed to decipher the mechanisms that control PHT1;8 and PHT1;9 localization and functionality at PM and ER.

PHT1;6 is expressed exclusively in flowers and mature pollen (Mudge et al., 2002), and GFP-PHT1;6 localized to the PM when transiently expressed in Nicotiana (Figure 3C) and

in stable Arabidopsis plants (Figure 4G-I). Neither GFP-PHT1;8 nor GFP-PHT1;9 co-localized with GFP-PHT1;6 in *Nicotiana benthamiana* (Figure 3G-L) indicating that the ER localization of GFP-PHT1;8 and GFP-PHT1;9 was not due to the overload of the endomembrane system. Given that GFP-PHT1;6 fluorescence was detected only at the PM in tobacco (Figure 3C) without additional PHF1, PHT1;6 does not require PHF1 for exit from the ER, unlike PHT1;1/2/3/4 (Bayle et al., 2011), however, this needs to be further tested. Furthermore, GFP-PHT1;6 in Arabidopsis, localizes more abundantly to the apical and basal domains of the plasma membrane (Figure 6A and B), suggesting that this transporter might be involved in the directional movement of Pi in the flowers and mature pollen, where this gene is expressed (Mudge et al., 2002).

The Arabidopsis PHT1;1/2/3/4 are predicted to have a conserved putative ER-exit motif (D/E-X-D/E) at the C-terminus (Bayle et al., 2011). Interestingly, this motif is missing in the PHT1;8 and PHT1;9 proteins, which might explain their retention at the ER. In addition, a lysine at the 3rd position or a lysine or an arginine at the 4th or 5th position from the C-terminus of transmembrane proteins may function as ER-retention signals (Shin et al., 1991). PHT1;9 has an arginine at the fourth position which might contribute to its strong ER localization. In contrast, PHT1;8 does not contain either lysine nor arginine at the 3rd, 4th or 5th positions from the C-terminus. It is tempting to speculate that the lack of the ER exit motif in both PHT1;8 and PHT1;9 and the presence of an ER-retention signal in PHT1;9 are responsible for their ER localization in mature cells. The presence of PHT1;8 and PHT1;9 at the PM in the root tips was confirmed by partial co-localization with FM4-64 (Figure 5C and F), suggesting that a subpopulation of GFP-PHT1;8 and GFP-PHT1;9 is delivered to the PM. Delivery to the PM in spite of the absence of putative ER-exit motifs on PHT1;8 or PHT1;9 could be possible with

the assistance of a certain specialized protein that facilitates the export from ER or it could be due to a posttranslational modification that would mimic an ER export motif, however this needs to be investigated.

Another interesting aspect of the PHT1;8 and PHT1;9 localization is that in root tips of *Arabidopsis thaliana* harboring GFP-PHT1;8 and GFP-PHT1;9 there is a more prominent PM localization, although the ER localization was still present in those cells. This might indicate a developmental regulation for the PHT1;8 and PHT1;9 localization. These results prompted us to test the trafficking of the PM pool of PHT1;8 and PHT1;9. GFP-PHT1;8 and GFP-PHT1;9 exhibited BFA-sensitivity and aggregated into BFA bodies (Figure 5G-L), which provided evidence for recycling of these proteins at the PM. As expected, the ER pools of these proteins remain and they do not accumulate in the BFA compartments. This result is similar to PHT1;1, as it was shown to be endocytosed via BFA-sensitive endosomes and recycled back to the PM upon Pi starvation (Bayle et al., 2011).These findings suggest that the pool of PM localized GFP-PHT1;8 and GFP-PHT1;9 employ BFA-sensitive endosomes upon their endocytosis, which may be an important mechanism to regulate PHT1;8 and PHT1;9 abundance at the plasma membrane and ultimately Pi acquisition.

PHT1;1 and PHT1;4 have been shown to be polyubiquitinated by the sequential action of PHO2 and NLA (Lin et al., 2013; Park et al., 2014), packaged into MVBs with the assistance of ALIX1 and the ESCRT-III machinery (Cardona-Lopez et al., 2015), and targeted for degradation at the lytic vacuole (Bayle et al., 2011). We tested whether PHT1;8, PHT1;9 and PHT1;6 were trafficked to the vacuole for degradation by concA-mediated inhibition of the vacuolar H+ATPase. GFP-PHT1;6 was detected at the vacuole in concA-treated plants under both Pi regimes (Figure 6E and F), which indicates that this protein is targeted to the vacuole

for degradation. In the case of GFP-PHT1;8 and GFP-PHT1;9, we failed to detect significant GFP signal at the vacuole, probably due to the strong signal from the ER (data not shown). PHT1;6/8/9 proteins differ from the other PHT1 family members in that they contain a predicted Proline, glutamic acid (E), Serine, Threonine (PEST) motif, also present in PHO84, the high affinity phosphate transporter in yeast (Estrella et al., 2008; Lagerstedt et al., 2004). Proteins with known PEST motifs are short-lived and subject to quick degradation; these motifs can be located anywhere on the protein, but most commonly at the C-terminus, and are often conditionally activated rather than being constitutive cleavage signals (Rechsteiner and Rogers, 1996; Rogers et al., 1986). Less than 10% of all mammalian proteins contain a PEST motif, though it is unknown how many plant proteins possess this domain. The truncation of the PEST motifs is thought to result in metabolically more stable proteins (Rechsteiner and Rogers, 1996). Conversely, adding a PEST motif to a stable protein results in a quickly-degradable protein (Salama et al., 1994). The importance of the PEST motif in the PHT1;6, PHT1;8 and PHT1;9 needs to be investigated. Although, it is obvious that this motif does not impair ER or PM localization given that GFP-PHT1;6 localizes to PM while GFP-PHT1;8 and GFP-PHT1;9 localize to both ER and PM. It would be useful to test whether PEST domain presence causes a bypass from the PHO2/ NLA action, and this could be tested by swapping the PEST domain from PHT1;6/8/9 to PHT1;1 or PHT1;4 and analyzing the localization and the abundance of such chimeric proteins.

Altogether, our data indicates that plants lacking *PHT1;8* and *PHT1;9* are more susceptible to Pi stress. However, additional experiments are needed to decipher the PHT1;8 and PHT1;9 roles in Pi uptake, translocation or both. Given the implication of PHT1;1 and PHT1;4 in both acquisition and allocation of Pi (Misson et al., 2004; Shin et al., 2004) it is

highly likely that PHT1;8 and PHT1;9 might also participate in both processes. Moreover, the subcellular distribution to ER and PM of GFP-PHT1;8 and GFP-PHT1;9 in root tips suggests that the localization of these proteins might be developmentally controlled. Deciphering the functional relevance of PHT1;8 and PHT1;9 dual localization is going to be subject of further investigations.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in all experiments, while Wassilewska (Ws) was used as a control for the *pht1;1pht1;4* mutant. The T-DNA insertion lines (Alonso et al., 2003) *pht1;8-5* (SAIL_116_G06) and *pht1;9-3* (SALK_073614C) were obtained from the Arabidopsis Biological Resource Center (ABRC). *pht1;1pht1;4* (Shin et al., 2004), and the PIN2-GFP (Abas et al., 2006) and mCherry-HDEL (Nelson et al., 2007) marker lines were previously described. Homozygous lines for the T-DNA insertions were identified by PCR genotyping using the LBb1.3 primer for *pht1;9* or LB1 primer for *pht1;8* (Table S1). Signal iSect Toolbox (http://signal.salk.edu/isects.html) was used to design the gene-specific primers (Table S1). The double mutant *pht1;8 1;9* was generated by crossing and PCR genotyping. Seeds were sterilized and plated on half-strength complete (MSP01) or P-free (MSP19) Arabidopsis Growth Media (AGM) (Caisson Labs) supplemented with 1% (w/v) sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES) and 4 g/L GelRite (RPI). For low Pi medium, KH_2PO_4 was added to a final concentration of 2 µM to the Pi-free AGM. Plates were kept at 4°C in the dark for 4 days and transferred to a growth chamber at 22 °C with a 16 h light/8 h dark cycle. Wild-type *Nicotiana benthamiana* plants were grown under standard conditions for 5-8 weeks as previously described (Gopinath and Kao, 2007).

Gene expression analysis by RT-PCR and Quantitative Real-Time PCR

Total RNA was extracted using TRI reagent (Ambion) from roots of 14-day-old seedlings grown on Pi media for 7 days and transferred to +Pi or –Pi media for additional 7 days. The cDNA was synthesized from 0.7 µg of DNase I (New England Biolabs)-treated total RNA using iScript cDNA synthesis kit (Bio-Rad). The synthesized cDNA was used as a

template in RT-PCR or Quantitative Real-Time PCR (qRT-PCR) analysis. *UBIQUITIN 5* was used as an endogenous control and the primer sequences used can be found in the supplemental data (Table S2). A StepOnePlus Real-Time PCR system (Life Technologies) and iTaq Universal SYBR Green Supermix (Bio-Rad) were used. Relative expression was calculated and normalized with respect to *UBIQUITIN5,* using the comparative cycle threshold method.

Soluble Pi and anthocyanin content determinations

For soluble Pi levels and anthocyanin quantification, shoot tissue from ~10 seedlings was harvested, rinsed in distilled water, blot-dried, frozen, and ground to a fine powder in liquid nitrogen and separated into 50-100 mg aliquots. Each sample was homogenized in 1% (w/v) Acetic Acid in a 1:10 ratio (1 mg powder to 10 µl 1% (w/v) Acetic Acetic). The mixture was vortexed, and centrifuged at 4º C to pellet the cellular debris. The supernatant was assayed for soluble Pi using the molybdenum-blue method (Ames, 1966). For Pi quantification the absorbance at 660 nm was read while for the anthocyanin quantification the absorbance at 530 nm was measured. A Synergy H1 Hybrid Plate Reader at the Genomic Sciences Laboratory (GSL) Facility at NCSU was used for all the absorbance readings.

Plant phenotype analysis

Seven-day-old Arabidopsis seedlings were used to measure root hair length. Root hairs were visualized in a Leica M205C stereomicroscope equipped with a Leica DFC425C digital camera. The root hairs growing in the region 3–4 mm above the root tips were measured. Data are based on the measurements of root hairs of at least 20 seedlings and at least 10 root hairs per seedling. All measurements were done using the ImageJ software (NIH**)** and Statistical significant deviations from the wild-type were determined by student's t-test ($p > 0.001$). Rhizosphere acidification assay was done as previously described (Yi and Guerinot, 1996).

Briefly, seedlings were grown on regular (1 mM) Pi MS media for 4 days and transferred to low (2 µM) Pi media for an additional 4 days. Three seedlings were grouped together and transferred to a minimal media for 24 h This plate contained 0.006% (w/v) bromocresol purple and 0.2 mM CaSO4, and the pH was adjusted to 6.5 with 1 M NaOH.

Constructs and transgenic plants

The Gateway system (Life Technologies Corporation) was used to generate transgenic plants overexpressing GFP, YFP or RFP fused to *PHT1;8*, *PHT1;9*, and *PHT1;6.* The *PHT1;8* CDS was obtained from ABRC, stock #DQ446267. *PHT1;9* was amplified from Col-0 cDNA from roots of plants grown on –Pi media using PHT1;9-1F-CACCATGCCGGAGTTAAGTTTA and PHT1;9-1R+Stop-TTAGTATTGTCTCCGAAG. *PHT1;6* was amplified from gDNA using the following primers: PHT1;6-1F-CACCATGGCTAACGAAGAACAA and PHT1;6-1R+Stop-CTAAACAACAATCTTCTCCT. Phusion High-Fidelity DNA Polymerase (NEB) was used for PCR. The obtained amplicons were cloned into pENTR™/D-TOPO vector (Life Technologies Corporation) and sequenced by Sanger sequencing. LR recombination was done with the Gateway destination vector pGWB6 (Nakagawa et al., 2007), pUBN-Dest (Grefen et al., 2010) or pGWB455 (Nakagawa et al., 2009). All constructs were transformed into *Agrobacterium tumefaciens* C58. For stable transformation *Arabidopsis thaliana* was transformed by floral dip method as previously described (Clough and Bent, 1998). For transient transformation *N. benthamiana* leaf infiltration standard protocol was used as previously described (Sparkes et al., 2006).

Confocal microscopy

The images were captured using a Zeiss LSM710 confocal microscope with a 40X water objective, at the Cellular and Molecular Imaging Facility (CMIF) at NCSU. To image the samples, combinations of excitation wavelength/emission were 488 nm/492–570 nm for GFP and YFP, 561 nm/588–696 nm for mCherry and RFP, and 514 nm/588–700 nm for FM4-64.

Chemical treatments

BFA (Sigma-Aldrich), concA (Sigma-Aldrich), and FM4-64 (Invitrogen) stock solutions were prepared in 100% DMSO and used at 50 µM, 1 µM and 2 µM, respectively, in liquid +Pi or –Pi MS medium according to the experiment. For control experiments, a 0.5% (v/v) DMSO solution was used. Various drug effects were assayed on 5-d-old seedlings. For BFA, 5-day old seedlings were transferred to liquid 0.5X Murashige and Skoog (MS) supplemented with 1% (w/v) sucrose containing 75 µM BFA and imaged after 120 min.

REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J., Luschnig, C. (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol, **8**(3), 249-256.

Abel, S. (2011) Phosphate sensing in root development. Curr Opin Plant Biol, **14**(3), 303-309.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., Ecker, J.R. (2003) Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana.* Science, **301**(5633), 653-657.

Ames, B.N. (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Method Enzymol, **8**, 115-118.

Arnaud, C., Clement, M., Thibaud, M.C., Javot, H., Chiarenza, S., Delannoy, E., Revol, J., Soreau, P., Balzergue, S., Block, M.A., Marechal, E., Desnos, T., Nussaume, L. (2014) Identification of Phosphatin, a drug alleviating phosphate starvation responses in Arabidopsis. Plant Physiol, **166**(3), 1479-1491.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., Chiou, T.J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol, **141**(3), 1000-1011.

Ayadi, A., David, P., Arrighi, J.F., Chiarenza, S., Thibaud, M.C., Nussaume, L., Marin, E. (2015) Reducing the genetic redundancy of Arabidopsis *PHOSPHATE TRANSPORTER1* transporters to study phosphate uptake and signaling. Plant Physiol, **167**(4), 1511-1526.

Bates, T.R. and Lynch, J.P. (1996) Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. Plant Cell Environ, **19**(5), 529-538.

Bates, T.R. and Lynch, J.P. (2000) The efficiency of *Arabidopsis thaliana* (Brassicaceae) root hairs in phosphorus acquisition. Am J Bot, **87**(7), 964-970.

Bayle, V., Arrighi, J.F., Creff, A., Nespoulous, C., Vialaret, J., Rossignol, M., Gonzalez, E., Paz-Ares, J., Nussaume, L. (2011) *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell, **23**(4), 1523-1535.

Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N., Satiat-Jeunemaitre, B. (2004) FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. J Microsc, **214**(2), 159-173.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Perez-Perez, J., Solano, R., Leyva, A., Paz-Ares, J. (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS Genet, **6**(9), e1001102.

Cardona-Lopez, X., Cuyas, L., Marin, E., Rajulu, C., Irigoyen, M.L., Gil, E., Puga, M.I., Bligny, R., Nussaume, L., Geldner, N., Paz-Ares, J., Rubio, V. (2015) ESCRT-III-associated protein ALIX mediates high affinity phosphate transporter trafficking to maintain phosphate homeostasis in Arabidopsis. Plant Cell, **27**(9), 2560-2581.

Chen, J., Liu, Y., Ni, J., Wang, Y., Bai, Y., Shi, J., Gan, J., Wu, Z., Wu, P. (2011) OsPHF1 regulates the plasma membrane localization of low- and high-affinity inorganic phosphate transporters and determines inorganic phosphate uptake and translocation in rice. Plant Physiol, **157**(1), 269-278.

Chen, J., Wang, Y., Wang, F., Yang, J., Gao, M., Li, C., Liu, Y., Yamaji, N., Ma, J.F., Paz-Ares, J., Nussaume, L., Zhang, S., Yi, K., Wu, Z., Wu, P. (2015) The rice CK2 kinase regulates trafficking of phosphate transporters in response to phosphate levels. Plant Cell, **27**(3), 711-723.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method forAgrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J, **16**(6), 735-743.

Conti, E. and Izaurralde, E. (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol, **17**(3), 316-325.

del Pozo, J.C., Allona, I., Rubio, V., Leyva, A., de la Pena, A., Aragoncillo, C., Paz-Ares, J. (1999) *A type 5 acid phosphatase* gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J, **19**(5), 579-589.

Estrella, L.A., Krishnamurthy, S., Timme, C.R., Hampsey, M. (2008) The Rsp5 E3 ligase mediates turnover of low affinity phosphate transporters in *Saccharomyces cerevisiae*. J Biol Chem, **283**(9), 5327-5334.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., Jürgens, G. (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell, **112**(2), 219-230.

Geldner, N., Friml, J., Stierhof, Y., Jurgens, G., Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature, **413**(6854), 425-428.

Gerke, J. (2015) The acquisition of phosphate by higher plants: Effect of carboxylate release by the roots. A critical review. J Plant Nutr Soil Sci, **178**(3), 351-364.

González, E., Solano, R., Rubio, V., Leyva, A., Paz-Ares, J. (2005) PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 Is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in Arabidopsis. Plant Cell, **17**(12), 3500-3512.

Gopinath, K. and Kao, C.C. (2007) Replication-independent long-distance trafficking by viral RNAs in *Nicotiana benthamiana*. Plant Cell, **19**(4), 1179-1191.

Grefen, C., Donald, N., Hashimoto, K., Kudla, J., Schumacher, K., Blatt, M.R. (2010) A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. Plant J, **64**(2), 355-365.

Harrison, M.J. (1997) The arbuscular mycorrhizal symbiosis: an underground association. Trends in Plant Science, **2**(2), 54-60.

Harrison, M.J. (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol, **59**, 19-42.

Harrison, M.J., Dewbre, G.R., Liu, J. (2002) A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. Plant Cell, **14**(10), 2413-2429.

Hem, S., Rofidal, V., Sommerer, N., Rossignol, M. (2007) Novel subsets of the Arabidopsis plasmalemma phosphoproteome identify phosphorylation sites in secondary active transporters. Biochem Biophys Res Commun, **363**(2), 375-380.

Jones, D. (1998) Organic acids in the rhizosphere – a critical review. Plant Soil, **205**(1), 25-44.

Jost, R., Pharmawati, M., Lapis-Gaza, H., Rossig, C., Berkowitz, O., Lambers, H., Finnegan, P. (2015) Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in *Arabidopsis thaliana* through the application of phosphite. J Exp Bot, **66**(9), 2501-2514.

Karthikeyan, A.S., Varadarajan, D.K., Mukatira, U.T., D'Urzo, M.P., Damsz, B., Raghothama, K.G. (2002) Regulated expression of Arabidopsis phosphate transporters. Plant Physiol, **130**(1), 221-233.

Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., Friml, J. (2006) Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell, **18**(11), 3171-3181.

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., Friml, J. (2008) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. Proc Natl Acad Sci U S A, **105**(46), 17812-17817.

Lagerstedt, J.O., Voss, J.C., Wieslander, Å., Persson, B.L. (2004) Structural modeling of dual-affinity purified Pho84 phosphate transporter. FEBS Letters, **578**(3), 262-268.

Lapis-Gaza, H.R., Jost, R., Finnegan, P.M. (2014) Arabidopsis PHOSPHATE TRANSPORTER1 genes *PHT1;8* and *PHT1;9* are involved in root-to-shoot translocation of orthophosphate. BMC Plant Biol, **14**, 334-352.

Li, D., Zhu, H., Liu, K., Liu, X., Leggewie, G., Udvardi, M., Wang, D. (2002) Purple acid phosphatases of *Arabidopsis thaliana*. Comparative analysis and differential regulation by phosphate deprivation. J Biol Chem, **277**(31), 27772-27781.

Lin, W., Huang, T., Chiou, T. (2013) Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. Plant Cell, **25**(10), 4061-4074.

Lopez-Bucio, J., de La Vega, O.M., Guevara-Garcia, A., Herrera-Estrella, L. (2000) Enhanced phosphorus uptake in transgenic tobacco plants that overproduce citrate. Nat Biotechnol, **18**(4), 450-453.

Lopez-Bucio, J., Hernandez-Abreu, E., Sanchez-Calderon, L., Nieto-Jacobo, M.F., Simpson, J., Herrera-Estrella, L. (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. Plant Physiol, **129**(1), 244-256.

Lynch, J. (1995) Root architecture and plant productivity. Plant Physiol, **109**(1), 7-13.

Marschner, H. (1995m) 13 - Nutrient availability in soils. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 483-507.

Marschner, H. (1995n) 14 - Effect of internal and external factors on root growth and development. Mineral Nutrition of Higher Plants (Second Edition), Academic Press, London: 508-536.

Marschner, H. (1995o) 15 - The soil-root interface (rhizosphere) in relation to mineral nutrition. Mineral Nutrition of Higher Plants (Second Edition), Academic Press, London: 537-595.

Marschner, H. (1995p) 16 - Adaptation of plants to adverse chemical soil conditions. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 596-680.

Misson, J., Raghothama, K.G., Jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., Thibaud, M.-C. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA, **102**(33), 11934-11939.

Misson, J., Thibaud, M.C., Bechtold, N., Raghothama, K., Nussaume, L. (2004) Transcriptional regulation and functional properties of Arabidopsis *Pht1;4*, a high

affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. Plant Mol Biol, **55**(5), 727-741.

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S., Shibata, D. (1997) Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. Proc Natl Acad Sci U S A, **94**(13), 7098-7102.

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A., Raghothama, K., Baek, D., Koo, Y., Jin, J., Bressan, R., Yun, D., Hasegawa, P. (2005) The Arabidopsis SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proc Natl Acad Sci U S A, **102**(21), 7760-7765.

Morcuende, R., Bari, R., Gibon, Y., Zheng, W., Pant, B.D., Sing, O., Usadel, B., Czechowski, T., Udvardi, M.K., Stitt, M., Scheible, W.-R. (2007) Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. Plant Cell Environ, **30**(1), 85-112.

Muchhal, U., Pardo, J., Raghothama, K. (1996) Phosphate transporters from the higher plant *Arabidopsis thaliana.* Proc Natl Acad Sci U S A, **93**(19), 10519-10523.

Muchhal, U.S. and Raghothama, K.G. (1999) Transcriptional regulation of plant phosphate transporters. Proc Natl Acad Sci U S A, **96**(10), 5868-5872.

Mudge, S., Rae, A., Diatloff, E., Smith, F. (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in Arabidopsis. Plant J, **31**(3), 341-353.

Nagarajan, V., Jain, A., Poling, M., Lewis, A., Raghothama, K., Smith, A. (2011) Arabidopsis Pht1;5 mobilizes phosphate between source and sink organs and influences the interaction between phosphate homeostasis and ethylene signaling. Plant Physiol, **156**(3), 1149-1163.

Nakagawa, T., Ishiguro, S., Kimura, T. (2009) Gateway vectors for plant transformation. Plant Biotechnol, **26**(3), 275-284.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng, **104**(1), 34-41.

Narang, R., Bruene, A., Altmann, T. (2000) Analysis of phosphate acquisition efficiency in different Arabidopsis accessions. Plant Physiol, **124**(4), 1786-1799.

Nelson, B.K., Cai, X., Nebenführ, A. (2007) A multicolored set of *in vivo* organelle markers for co-localization studies in Arabidopsis and other plants. Plant J, **51**(6), 1126-1136.

Nilsson, L., Muller, R., Nielsen, T.H. (2007) Increased expression of the MYB-related transcription factor, *PHR1*, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. Plant Cell Environ, **30**(12), 1499-1512.

Nuhse, T., Stensballe, A., Jensen, O., Peck, S. (2004) Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell, **16**(9), 2394-2405.

Nussaume, L., Kanno, S., Javot, H., Marin, E., Pochon, N., Ayadi, A., Nakanishi, T.M., Thibaud, M.C. (2011) Phosphate Import in Plants: Focus on the PHT1 Transporters. Front Plant Sci, **2**, 83-92.

Pali, T., Dixon, N., Kee, T.P., Marsh, D. (2004) Incorporation of the V-ATPase inhibitors concanamycin and indole pentadiene in lipid membranes. Spin-label EPR studies. Biochim Biophys Acta, **1663**(1-2), 14-18.

Park, B.S., Seo, J.S., Chua, N.H. (2014) NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. Plant Cell, **26**(1), 454-464.

Poirier, Y. and Bucher, M. (2002) Phosphate transport and homeostasis in Arabidopsis. Arabidopsis Book, **1**, e0024.

Rechsteiner, M. and Rogers, S.W. (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci, **21**(7), 267-271.

Remy, E., Cabrito, T., Batista, R., Teixeira, M., Sa-Correia, I., Duque, P. (2012) The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. New Phytol, **195**(2), 356-371.

Richardson, A., Lynch, J., Ryan, P., Delhaize, E., Smith, F., Smith, S., Harvey, P., Ryan, M., Veneklaas, E., Lambers, H., Oberson, A., Culvenor, R., Simpson, R. (2011) Plant and microbial strategies to improve the phosphorus efficiency of agriculture. Plant Soil, **349**(2), 121-156.

Rogers, S., Wells, R., Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science, **234**(4774), 364-368.

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A., Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev, **15**(16), 2122-2133.

Salama, S.R., Hendricks, K.B., Thorner, J. (1994) G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover. Mol Cell Biol, **14**(12), 7953-7966.

Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., Zhang, W., Zhang, F. (2011) Phosphorus dynamics: from soil to plant. Plant Physiol, **156**(3), 997-1005.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J, **39**(4), 629-642.

Shin, J., Dunbrack, R.L., Jr., Lee, S., Strominger, J.L. (1991) Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants. Proc Natl Acad Sci U S A, **88**(5), 1918-1922.

Smith, A.P., Nagarajan, V.K., Raghothama, K.G. (2011) Arabidopsis Pht1;5 plays an integral role in phosphate homeostasis. Plant Signal Behav, **6**(11), 1676-1678.

Sparkes, I.A., Runions, J., Kearns, A., Hawes, C. (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat Protoc, **1**(4), 2019-2025.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Poirier, Y., Nussaume, L. (2010) Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. Plant J, **64**(5), 775-789.

Vance, C.P. (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol, **127**(2), 390-397.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., Provart, N.J. (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE, **2**(8), e718.

Yi, Y. and Guerinot, M.L. (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant J, **10**(5), 835-844.

TABLES AND FIGURES

Figure 1. Identification and characterization of *pht1;8* and *pht1;9* mutants.

(A) Schematic representation of the *AtPHT1;8* (top) and *AtPHT1;9* (bottom) genes. The black boxes represent exons, the line between the black boxes indicates the intron, the white boxes represent the untranslated regions (UTRs) and the triangles illustrate the sites of the T-DNA insertions. The red arrows indicate the position of the primers used for full length amplification of the CDS. Bar = 200 bps.

(B) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *PHT1;8* and *PHT1;9* expression in roots of 14-day-old WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* mutants. The seedlings were grown on Pi-sufficient conditions for 7 days and transferred to Pi deficient conditions for additional 7 days. *At4* expression was used as a low Pi treatment control and *UBIQUITIN5* was used as a loading control. Note that there is a faint *PHT1;9* band present in the *pht1;9* mutant indicating that some *PHT1;9* transcript is made.

(C) qRT-PCR analysis of *PHT1;8* and *PHT1;9* expression in roots of 14-day-old WT(Col-0) *pht1;8, pht1;9* and *pht1;8 1;9* double mutant. The seedlings were grown on Pi-sufficient conditions for 7 days and transferred to Pi deficient conditions for additional 7 days. Transcript levels were normalized to those of a housekeeping gene, *UBIQUITIN5* in low Pi conditions. All samples were analyzed with technical and biological triplicates.

(D) RT-PCR analysis of *PHT1* and *PSI* gene expression in 14-day-old WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* mutants. Expression of the *UBIQUITIN5* gene is shown as a loading control.

(E) qRT-PCR analysis of the *PHT1;8* and *PHT1;9* expression in roots of 14–day-old *pht1;1 1;4* and WT (Ws). The seedlings were grown on Pi-sufficient conditions for 7 days and transferred to Pi deficient conditions for additional 7 days. Transcript levels were normalized

to those of the housekeeping gene, *UBIQUITIN5* in Pi sufficient conditions. All samples were analyzed with technical and biological triplicates.

(B-E) Pi sufficient media is 0.5xMS (1 mM) and the low Pi media is 0.5x Pi-free MS supplemented with KH_2PO_4 to a final concentration of 2 µM.

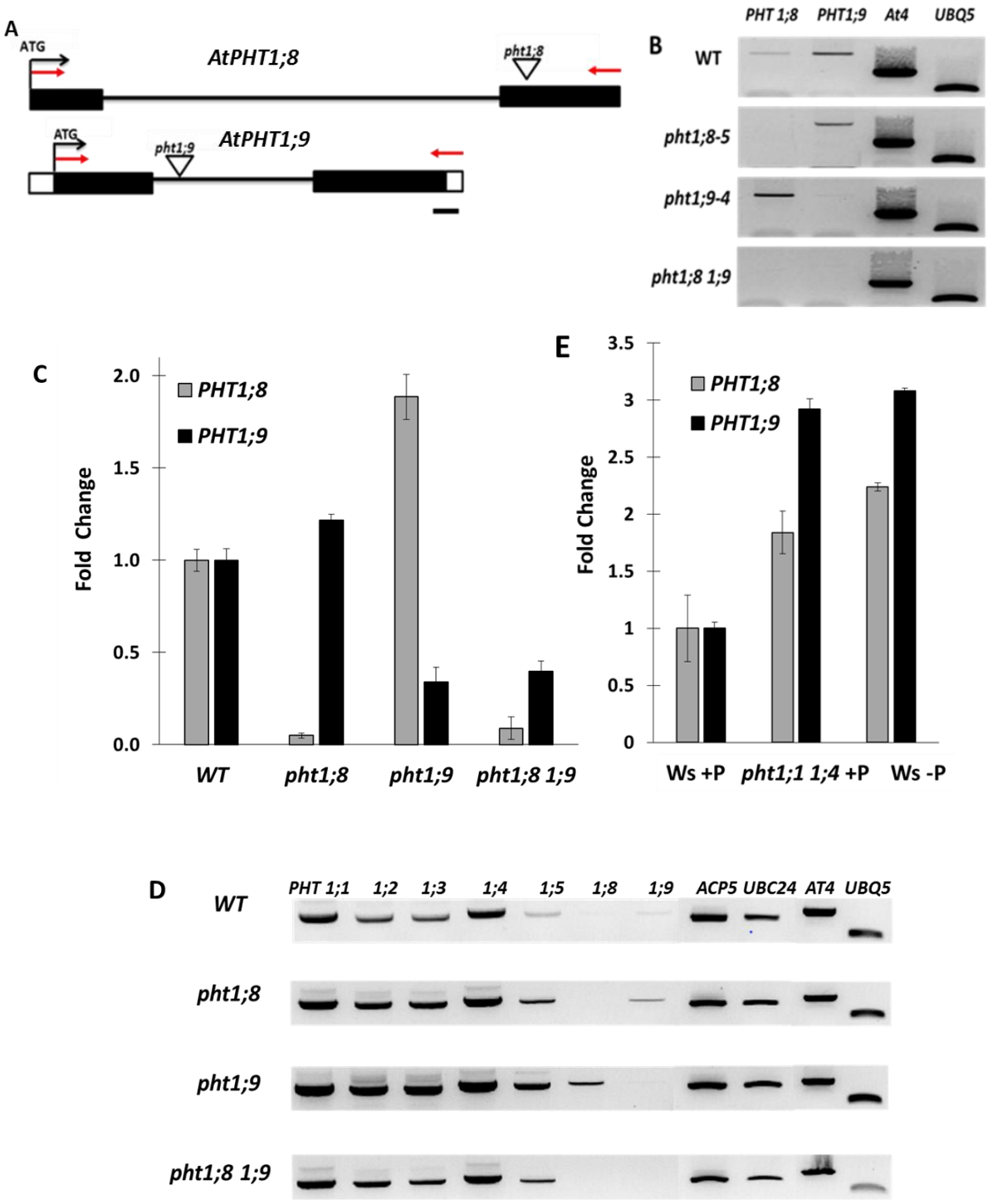

A
ATG
AtPHT1;8
pht1;8
ATG
pht1;9
AtPHT1;9
B
PHT 1;8
PHT1;9
At4
UBQ5
WT
pht1;8-5
pht1;9-4
pht1;8 1;9
C
Fold Change
PHT1;8
PHT1;9
2.0
1.5
1.0
0.5
0.0
WT
pht1;8
pht1;9
pht1;8 1;9
E
Fold Change
PHT1;8
PHT1;9
3.5
3
2.5
2
1.5
1
0.5
0
Ws +P
pht1;1 1;4 +P
Ws -P
D
PHT 1;1
1;2
1;3
1;4
1;5
1;8
1;9
ACP5
UBC24
AT4
UBQ5
WT
pht1;8
pht1;9
pht1;8 1;9

Figure 2. Loss of *PHT1;8* or *PHT1;9* results in exacerbated Pi stress phenotypes.

(A) Effect of *PHT1;8* and/ or *PHT1;9* loss on Pi content. Soluble Pi content was measured in 14-day-old WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* shoots from seedlings grown on Pi-sufficient media. FW= fresh weight. Bars represent means ± SD, n = 5, ***** indicates significant different from Col-0 WT ($p < 0.001$).

(B) Influence of *PHT1;8* and/or *PHT1;9* loss on root hair length. Root hairs of 7-day-old WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* seedlings grown on sufficient Pi media were visualized in a Leica M205C stereomicroscope and the root hair measurements were done using ImageJ software. Bars represent mean ± SD, n = 5, ***** indicates significant difference from Col-0 WT ($p < 0.001$), **#** indicates significant difference from Col-0 WT ($p < 0.05$).

(C) The impact of *PHT1;8* and/or *PHT1;9* loss on anthocyanins levels. Anthocyanin levels were measured in shoots of seedlings grown on Pi-sufficient media for 7 days and transferred to a media with low (2 µM) Pi for 7 more days. Bars represent means ± SD, n = 5, ***** indicates significant difference from Col-0 WT ($p < 0.001$).

(D) Rhizosphere acidification response of WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* to low Pi. Seedlings grown on Pi-sufficient media for 4 days and transferred to a media with low (2 µM) Pi for 4 more days were transferred to minimal media containing Bromocresol Purple for 24 h. Purple color of the media indicates alkaline pH, while yellow color indicates pH below 5.2.

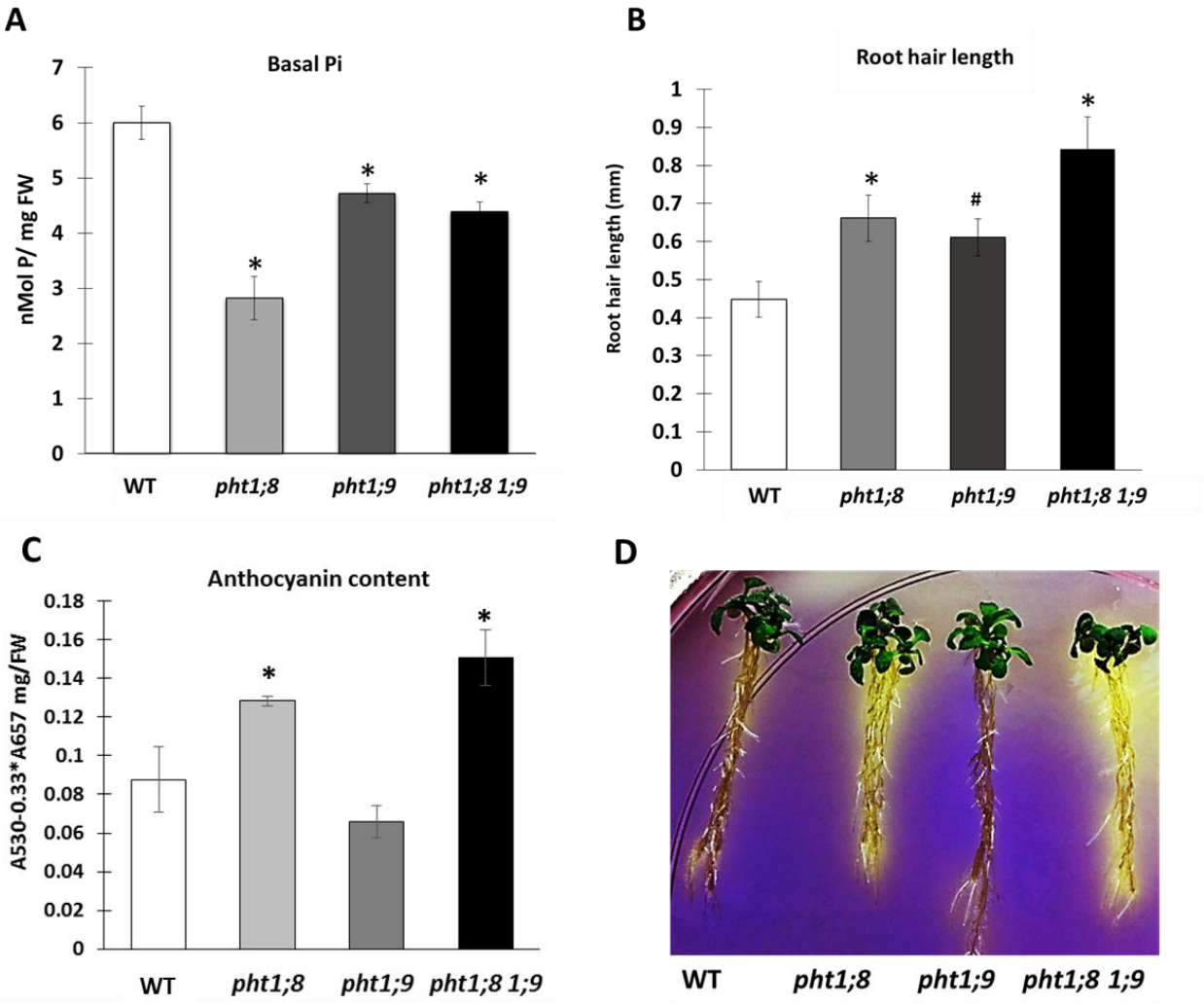

A
Basal Pi
nMol P/ mg FW
7
6
5
4
3
2
1
0
*
*
*
WT
pht1;8
pht1;9
pht1;8 1;9
B
Root hair length
Root hair length (mm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
#
*
WT
pht1;8
pht1;9
pht1;8 1;9
C
Anthocyanin content
A530-0.33*A657 mg/FW
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
WT
pht1;8
pht1;9
pht1;8 1;9
D
WT
pht1;8
pht1;9
pht1;8 1;9

Figure 3. Subcellular localization and distribution of GFP-PHT1;8 and GFP-PHT1;9 in *Nicotiana benthamiana* leaves.

(A-C) 3-D renderings of high-resolution confocal z-stacks acquired from transiently expressed GFP-PHT1;8 (A), GFP-PHT1;9 (B) and YFP-PHT1;6 (C) in *N. benthamiana*. Note the localization of GFP-PHT1;8 and GFP-PHT1;9 to a network structure resembling the Endoplasmic Reticulum (ER), while YFP-PHT1;6 (C) localizes to PM.

(D-F) 3-D renderings of high-resolution confocal z-stacks acquired from transiently co-expressed GFP-PHT1;9 (green, D) with the ER marker mCherry-HDEL (magenta, E) and the merged co-localization in *Nicotiana benthamiana* (F).

(G-L) Confocal laser scanning micrographs of transiently co-expressed GFP-PHT1;8 (G) or GFP-PHT1;9 (J) with RFP-PHT1;6 (H and K) in *Nicotiana benthamiana* leaves.

The GFP fluorescence signal is false colored in green and RFP or mCherry in magenta. The leaf tissue was analyzed 72 h post-infiltration.

All images were acquired by confocal microscopy using a 40X objective.

Scale bar = 20 µm.

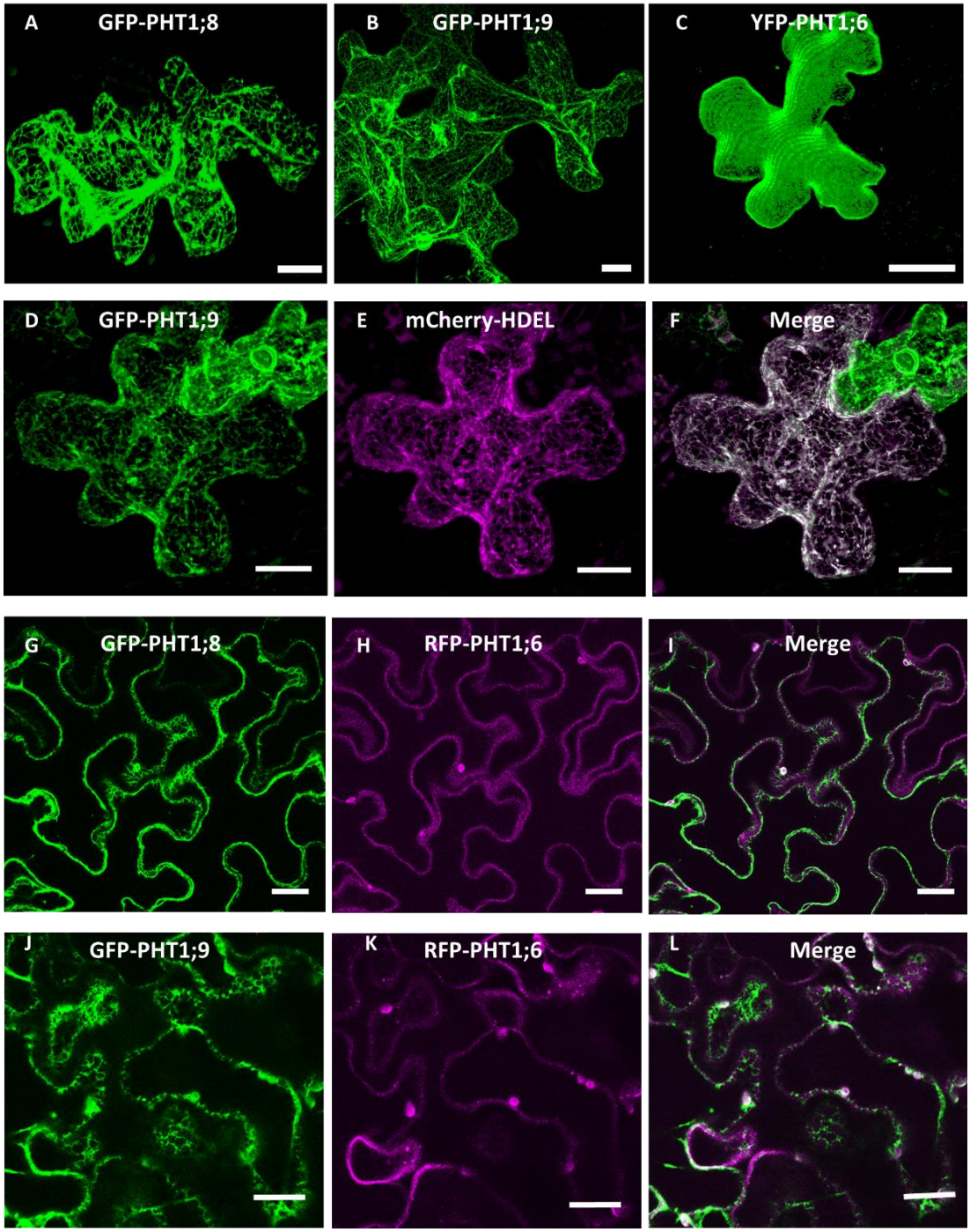

A GFP-PHT1;8
B GFP-PHT1;9
C YFP-PHT1;6
D GFP-PHT1;9
E mCherry-HDEL
F Merge
G GFP-PHT1;8
H RFP-PHT1;6
I Merge
J GFP-PHT1;9
K RFP-PHT1;6
L Merge

Figure 4. GFP-PHT1;8 and GFP-PHT1;9 localize to the ER in cotyledons, hypocotyls and roots of *A. thaliana*.

(A-I) Live-cell fluorescence images of cotyledons (A, D and G) hypocotyls (B, E and H) and roots (C, F and I) of 7-day-old Arabidopsis seedlings stably expressing GFP-PHT1;8 (A-C), GFP-PHT1;9 (D-F) and GFP-PHT1;6 (G-I) grown on Pi-sufficient media and analyzed by confocal laser scanning microscopy using a 40X objective.

Scale bar = 20 µm.

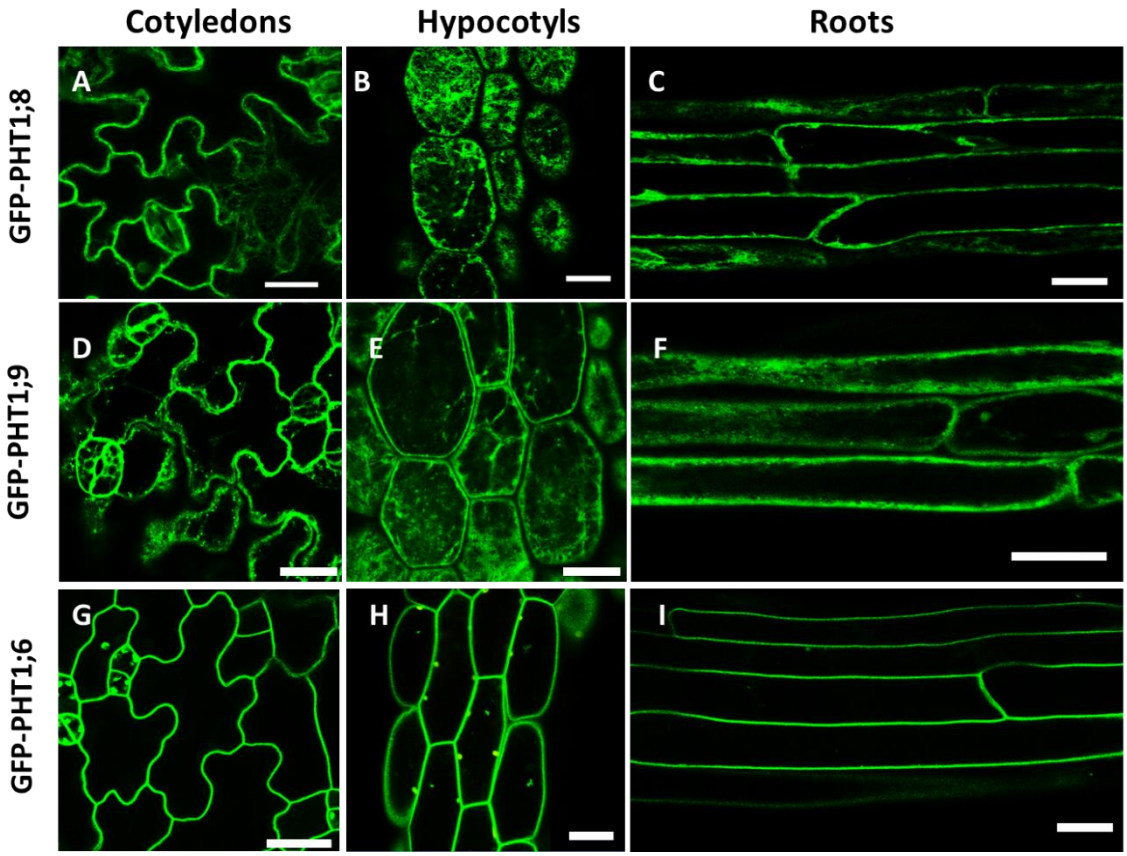


Figure 5. GFP-PHT1;8 and GFP-PHT1;9 localize to ER and PM and are BFA-sensitive in *Arabidopsis* root tips.

(A-F) Root cells of 5-day-old *Arabidopsis thaliana* seedlings stably expressing GFP-PHT1;8 (A) and GFP-PHT1;9 (D) stained with 2 µM FM4-64 (B and E) for 10 min. PHT1;8 and PHT1;9 partially co-localize with FM4-64 as marked by the yellow signal (C and F).

(G-O) Root tips of *Arabidopsis thaliana* 5-day-old seedlings stably expressing GFP-PHT1;8 (G), GFP-PHT1;9 (J) and PIN2-GFP (M). Seedlings were grown on sufficient Pi media and treated with 50 µM Brefeldin A (BFA) and 1 µM FM4-64 (H-N) for 120 min prior to imaging. The BFA-induced compartments were present in the root tips of all genotypes tested (G-O). PIN2-GFP was used as a control for the BFA treatment. The GFP fluorescence signal is false colored in green and the FM4-64 signal in red.

The images were captured using a Zeiss LSM710 confocal microscope with a 40X water objective.

Scale bar = 10 µm.

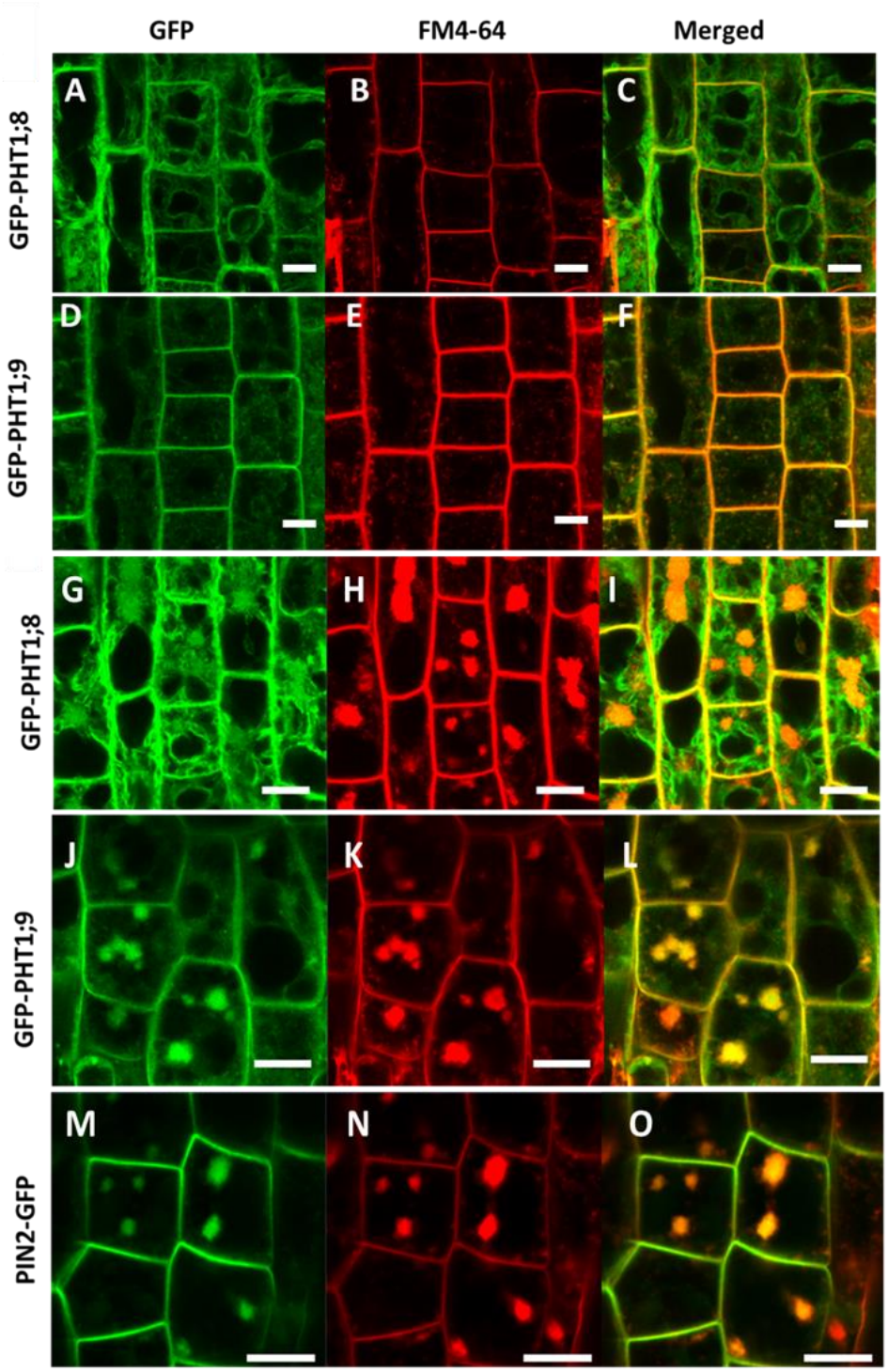

GFP
FM4-64
Merged
GFP-PHT1;8
GFP-PHT1;9
GFP-PHT1;8
GFP-PHT1;9
PIN2-GFP
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 6. GFP-PHT1;6 is targeted to the lytic vacuole for degradation.

(A-F) Live-cell fluorescence images of 7-day-old Arabidopsis roots harboring GFP-PHT1;6. (A) and (B) represent 3-D renderings of high-resolution confocal z-stacks. Arrows in (A) and (B) indicate to the polar localization of GFP-PHT1;6 fluorescence on the apical and basal sides of the PM. (C-F) The seedlings were incubated with DMSO (C and D) or 1 µM concA (E and F) for 2 hours either in Pi- sufficient (C and E) or Pi- deficient (D and F) media.

Scale bar = 20 µm.

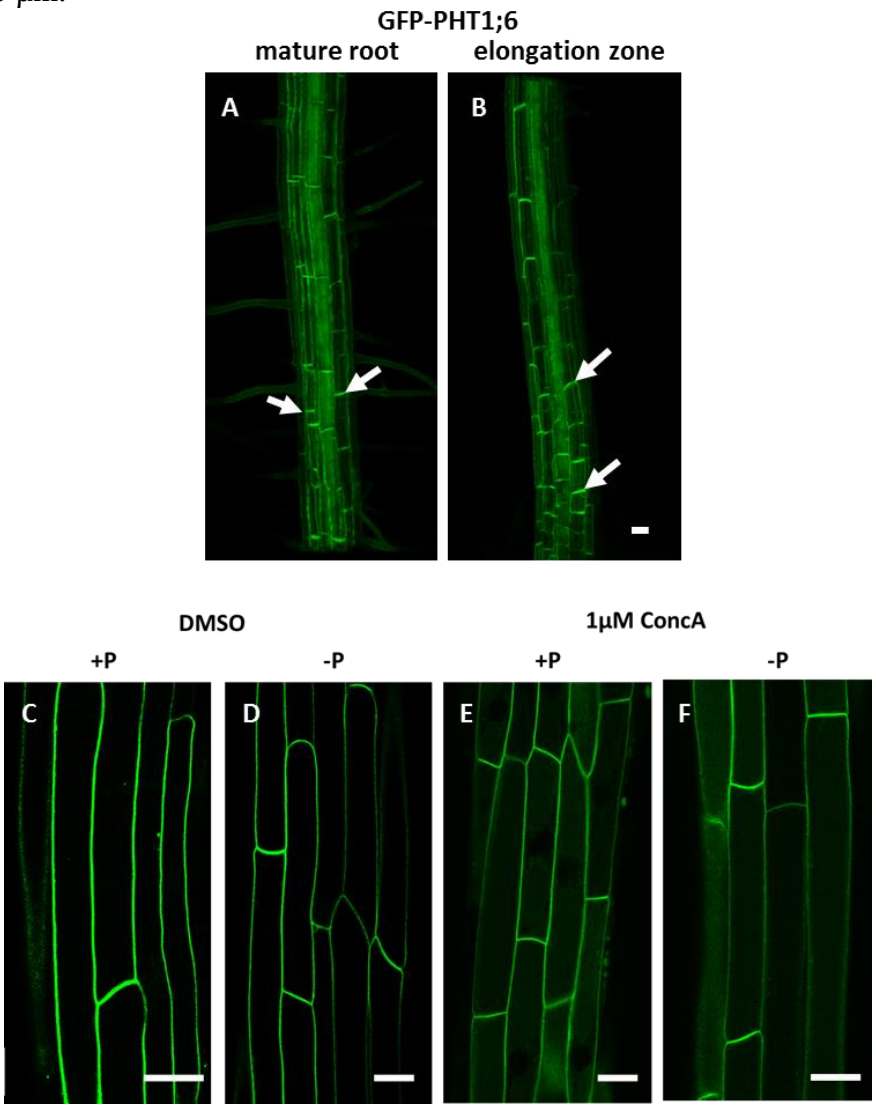
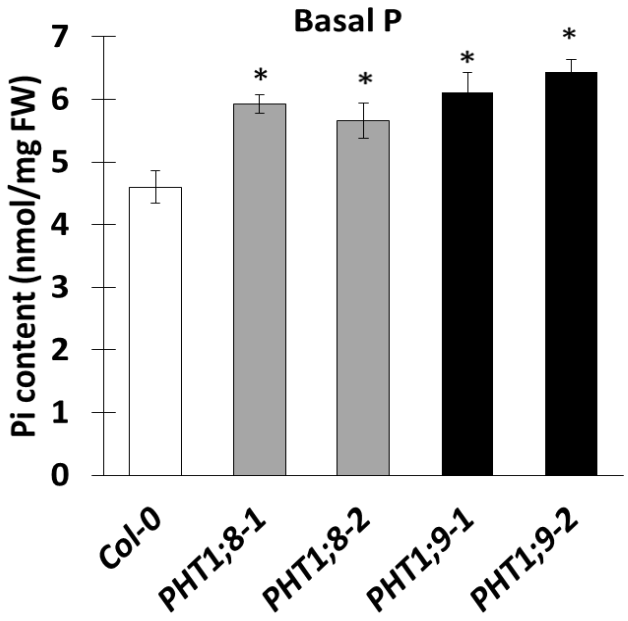


Figure 7. Ectopic expression of PHT1;8 and PHT1;9 leads to increased Pi content.

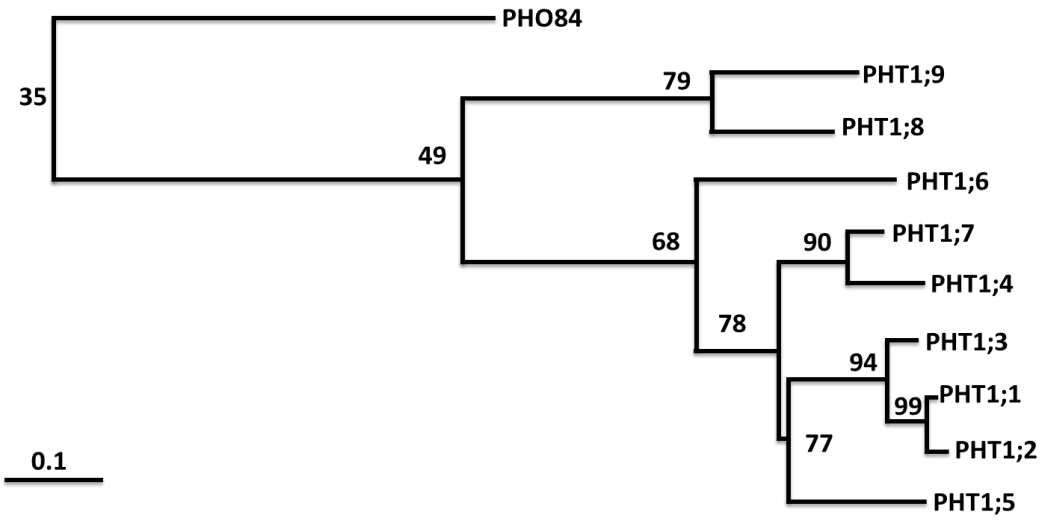
Soluble Pi was measured from shoots of 14-day-old WT (Col-0), 35S::GFP-PHT1;8 and 35S::GFP-PHT1;9 seedlings grown on Pi-sufficient media (1 mM Pi). Two independent transformants were used for each construct. FW= fresh weight. Bars represent means ± SD, n = 5, * indicates significant difference from Col-0 WT ($p < 0.001$) and n represents a pool of 10 plants.



Supplementary Figure 1. Phylogram of *PHT1* Family from Arabidopsis and PHO84 from *Saccharomyces cerevisiae*.

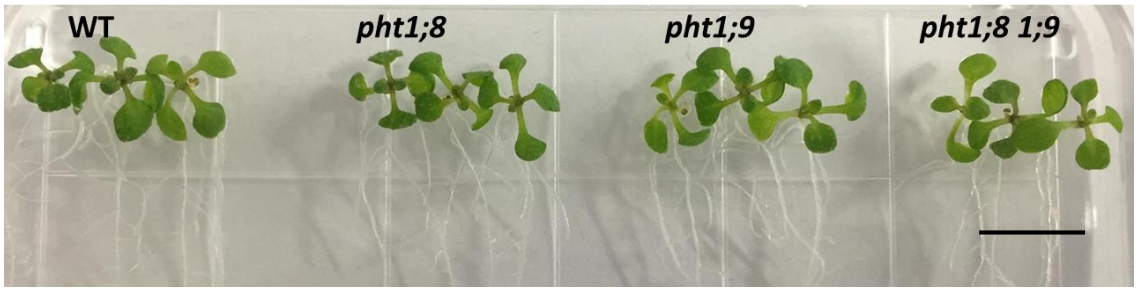
Protein sequence similarity searches with Blastp and pairwise alignments were used to assemble the PHT1 family tree. The closest PHT1 homologue in yeast, PHO84, and the nine PHT1 members were organized to generate a rooted rectangular cladogram using NCBI Blast Tree View. The amino acid similarities are given as percentages by the values presented at each branch point. In the given tree, there are 2 subclades with most closely related members. PHT1;8 and PHT1;9 are divergent to PHT1;1 and PHT1;4.

The scale bar represents the relative distance and 0.1 represents the number of amino acid substitutions per site.



Supplementary Figure 2. *pht1;8, pht1;9* and *pht1;8 1;9* mutants display a WT phenotype under normal conditions.

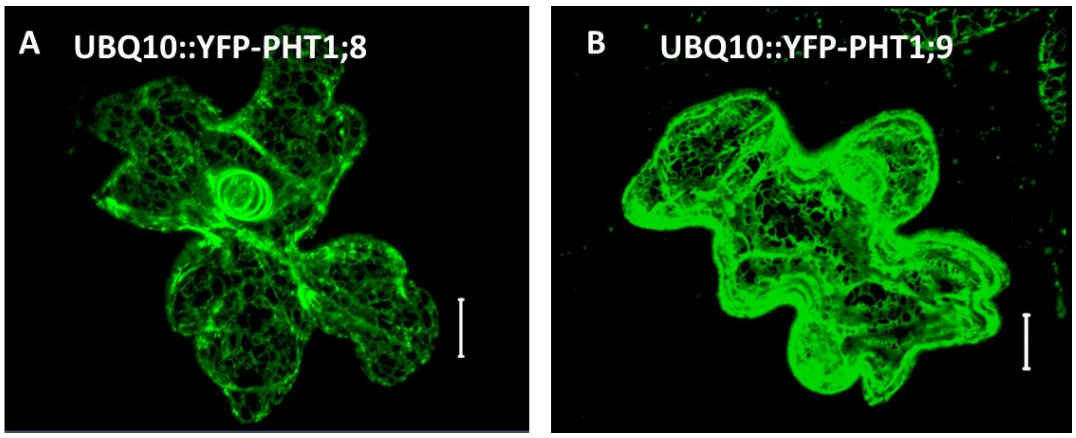
Arabidopsis thaliana 10-day-old seedlings grown under normal conditions on regular 0.5 x MS media. Scale bar = 1 cm.



Supplementary Figure 3. Transiently expressed YFP-PHT1;8 and YFP-PHT1;9 localize to ER in *Nicotiana benthamiana*.

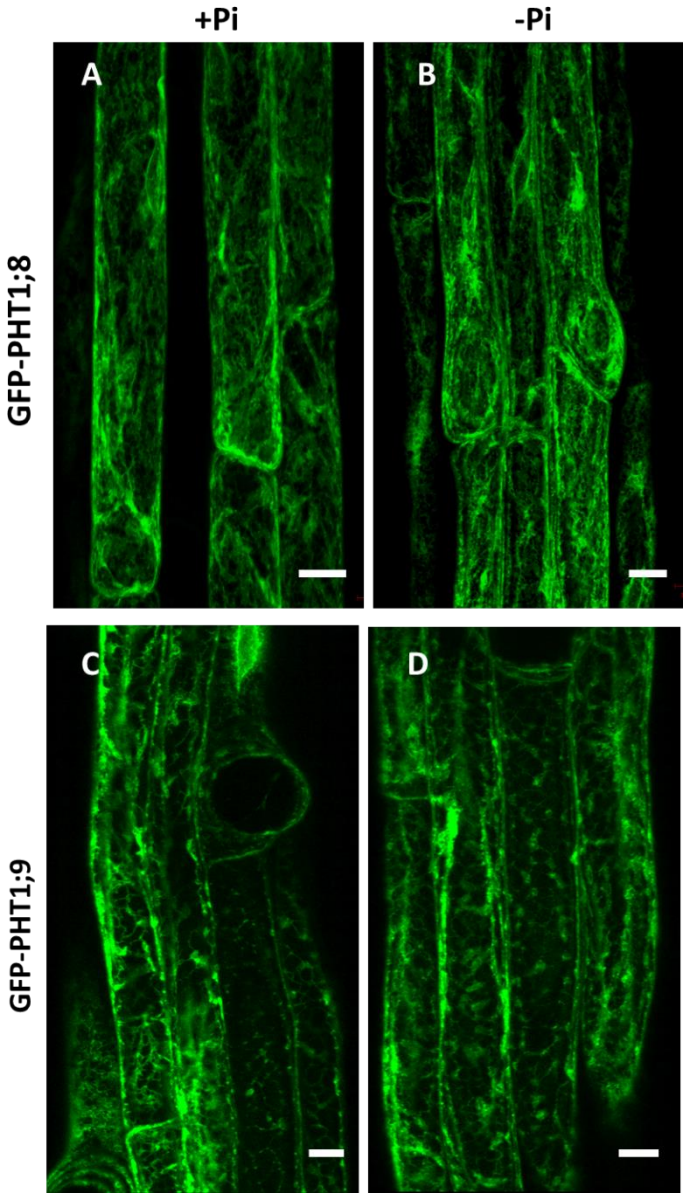
(A-B) 3-D renderings of high-resolution confocal z-stacks acquired from transiently expressed YFP-PHT1;8 (A) and YFP-PHT1;9 (B) in *Nicotiana benthamiana* leaves. The leaf tissue was analyzed 72 h post-infiltration. The YFP fluorescence is false colored to green. All images were acquired by confocal microscopy using a 40X objective.

Scale bar = 10 µm.



Supplementary Figure 4. GFP-PHT1;8 and GFP-PHT1;9 localization and abundance is not affected by Pi levels in *A. thaliana.*

(A-D) 3-D renderings of high-resolution confocal z-stacks acquired from mature roots of 5-day-old *Arabidopsis thaliana* seedlings stably expressing GFP-PHT1;8 (A and B), GFP-PHT1;9 (C and D) grown on Pi-sufficient (A and C) and Pi-deficient (B and D) media. 40X water objective was used. Scale bar = 10 µm.



Supplementary Table 1. List of primers used for genotyping, RT-PCR and qRT-PCR.

Primer Name	Primer Sequence	References
SAIL_116G06 (*pht1;8*)		
LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	
PHT1;8 RP-1F	TCGAGATCATCATCGTGTCTG	
PHT1;8LP-1R	CCTCCTTTCATTTCTCGTTCC	
SALK_073614C (*pht1;9*)		
LBb1.3	ATTTTGCCGATTTCGGAAC	
PHT1;9 RP-1F	CCTCCCAACTCACAAGTCAAC	
PHT1;9 LP-1R	CAACTTCTTACGCAATCGCTC	
RT-PCR and qRT-PCR		
PHT1;8-q1F	GCTCTTCCTGCTGCATTGACG	
PHT1;8-q1R	AGGAGGTGGATCCGTCGTGGCTT	
PHT1;9-q1F	CCGCCAGATACACAGCATTGGT	
PHT1;9-q1R	CGACCGTGGAGACTGAGGAAAC	
PHT1;1-F	GTTCTTAATTTCTCCTGCCAAGCTGATTA	(Shin et al., 2006)
PHT1;1-R	CATCATAACTTAAGGTCAACGAGCCAATA	
PHT1;2-F	GAACAACAACTAGGAGTGCTAAAGGCACT	
PHT1;2-R	CTAACTTCAGCTTCACCAGAGAGTTCTTC	
PHT1;3-F	GATCAACAGCTAGGAGTGCTAAAGGCACT	

PHT1;3-R	TATCAACCTCAGCCTCGCCGGAGAGTTCT	
PHT1;4-F	CTCTCTCAACATTTTCCCCTGAAAATAAG	
PHT1;4-R	AGAACAACTTGAGTTGCTAGAGACAAGGA	
PHT1;5-F	ACGCAGCCAAAACGCAGATGTACCATTTC	
PHT1;5-R	GGACTTTTCTACCGGAATTTGCCACAGTC	
At4-F	ACAAAGAGAGAAGCCATAAAAACCCTAAC	
At4-R	ATAGGAACACACCTGAATGGTGCATCACA	
ACP5-F	GAATTCTGGTCGTCGATCG	(Ward et al., 2008)
ACP5-R	CTACCAACTCTGCATCAACG	
UBQ5-F	GACGCTTCATCTCGTCC	
UBQ5-R	CCACAGGTTGCGTTAG	

CHAPTER 3

PHOSPHATE2 does not Regulate PHT1;8 and PHT1;9 Transporters

ABSTRACT

PHOSPHATE2 (PHO2) encodes the ubiquitin-conjugating E2 enzyme 24 (UBC24/PHO2). Loss of PHO2 function results in excessive Pi accumulation in the shoots, and subsequently, leaf chlorosis and necrosis. The Pi over-accumulation phenotype is due to increased uptake and translocation from roots to shoots and impaired Pi remobilization within the old leaves. PHOSPHATE TRANSPORTER1 (PHT1) proteins are responsible for the uptake of phosphate, however, only *PHT1;8* and *PHT1;9* transcripts were significantly induced in a *pho2* mutant, thus positioning *PHO2* upstream of *PHT1;8* and *PHT1;9.* Given the PHO2 function as an ubiquitin conjugating enzyme, it may mediate the degradation of proteins involved in Pi homeostasis, including, but not limited to PHT1s. In addition, PHO2 localizes to the endoplasmic reticulum (ER) and Golgi apparatus, whereas most PHT1 proteins localize to plasma membrane (Bayle et al., 2011; González et al., 2005), with the exception of PHT1;8 and PHT1;9, which localize predominantly to the ER (Chapter 2). In this work, we queried the posttranslational control of PHO2 over PHT1;8/9. The subcellular localization and PHT1;8/9 abundance are unaffected in the *pho2* mutants, and therefore, their stability appears to be independent of PHO2. However, overexpressing PHT1;8 and PHT1;9 enhances the Pi hyper-accumulation phenotype of the *pho2* mutant.

INTRODUCTION

Phosphate (Pi) levels are tightly regulated in plants given that phosphorus is an important macronutrient for plant growth. Pi homeostasis is accomplished by coordinated regulation of Pi sensing, signaling, uptake, translocation and reutilization (Misson et al., 2005; Muchhal and Raghothama, 1999; Poirier and Bucher, 2002; Shin et al., 2006; Stefanovic et al., 2011; Thibaud et al., 2010). One regulatory module controlling the Pi signaling pathway consists of a family of mobile microRNAs (miR399a-f), their target *PHO2*, and a set of non-coding RNAs, that in turn, regulate the levels of miR399 (Bari et al., 2006; Doerner, 2008; Franco-Zorrilla et al., 2007; Liu et al., 2014; Shin et al., 2006). Upon Pi stress, miR399 is induced and transported via the phloem from shoots to roots (Lin et al., 2008; Pant et al., 2008), where it targets *PHO2* transcripts. Therefore, miR399 is considered to be a systemic signal involved in long distance communication from the shoot to the root (Doerner, 2008; Lin et al., 2008; Pant et al., 2008; Schachtman et al., 1998). The 5'UTR of *PHO2* transcripts contain several miR399 binding sites, and miR399 binding results in the degradation of these transcripts via cleavage in a Pi-dependent manner. Pi-deficiency promotes induction of *At4* and *INDUCED BY PHOSPHATE STARVATION (IPS1)*, two non-coding RNAs somewhat complementary to miR399 (Liu et al., 1997; Shin et al., 2006), thus interfering with the miR399-mediated silencing of *PHO2* by target mimicry (Bari et al., 2006; Doerner, 2008; Franco-Zorrilla et al., 2007; Lin et al., 2008).

Both the *pho2* mutant and *miR399* over-expressing plants show similar phenotypes including increased Pi uptake and translocation together with an increased retention of Pi in the shoots, which overall results in hyper-accumulation of Pi in the shoot (Aung et al., 2006; Chiou et al., 2006). This indicates that miR399 and *PHO2* act in the same Pi signaling pathway.

Furthermore, PHOSPHATE STARVATION RESPONSE 1 (PHR1), a MYB-related transcription factor controlling Pi homeostasis, is required for miR399 induction (Bari et al., 2006). That positions PHR1 upstream of the regulatory module controlling Pi signaling.

In addition to miR399, miR827 and its target gene *NITROGEN LIMITATION ADAPTATION* (*NLA)* have been recently added to the Pi signaling module (Kant et al., 2011; Lin et al., 2013; Park et al., 2014). Similar to miR399, miR827, is induced significantly upon Pi stress and it targets *NLA* for degradation. Loss of *NLA* results in hyper-accumulation of Pi in the shoots (Kant et al., 2011), similar to *pho2*. Moreover, NLA and PHO2 are synergistic given that the *nla pho2* double mutants display a more severe Pi over-accumulation phenotype than the corresponding single mutants (Lin et al., 2013). Interestingly, *NLA* is also a positive regulator for the adaptation to low nitrogen (Peng et al., 2007). This indicates an interaction between N and Pi status (Kant et al., 2011; Lin et al., 2013; Medici et al., 2015; Park et al., 2014; Peng et al., 2007).

Pi is acquired from the soil via specialized high- and low affinity phosphate transporters (PHT). Plants adapt to fluctuating Pi levels in the soil, which are frequently low, by increasing Pi uptake through up-regulation of high-affinity phosphate transporters (Mudge et al., 2002). The increase in Pi uptake in the *pho2* mutant is due to an increased Vmax of PHT1 transporters, which is related to the concentration of the active PHT1 transporters, therefore increasing the total Pi uptake capacity (Aung et al., 2006). However, the affinity of the PHT1 transporters for phosphate is not affected (Aung et al., 2006). The transcript abundance of specific low- and high-affinity phosphate transporters is altered in *pho2* under Pi-sufficient conditions; more specifically, *PHT1;8*, *PHT3;2* and *PHT3;3* levels are increased, while *PHT2;2* level is decreased (Aung et al., 2006). This places *PHO2* upstream of the previously mentioned *PHT1*

and *PHT3* genes, however given the function of *PHO2*, it is probable that this interaction involves some intermediate partners that negatively regulate *PHT1* and *PHT3.*

PHO2 encodes an E2 ubiquitin conjugase, named UBC24 in Arabidopsis (Kraft et al., 2005). *NLA* encodes a protein with an SPX domain in the N-terminus and a putative E3 ligase at its C-terminus (Lin et al., 2013; Peng et al., 2007). Ubiquitination is a known posttranslational modification that results in the targeting of the particular protein for degradation via the 26S proteasome or lytic vacuole targeting. This reaction is catalysed by the consecutive action of three enzymes: E1, E2 and E3. The ubiquitination steps are activation by E1, conjugation by E2 and the ligation achieved by the E3 enzyme. To date, only two E2 and E3 enzymes involved in Pi homeostasis have been identified, PHO2 and NLA, respectively (Aung et al., 2006; Huang et al., 2013; Kant et al., 2011; Park et al., 2014). PHO2 localizes to the endoplasmic reticulum (ER), while the NLA localizes to the nucleus and plasma membrane (PM) (Lin et al., 2013; Liu et al., 2012; Park et al., 2014). The non-overlapping cellular localization of NLA and PHO2 suggests that they function independently; however, they do function sequentially and cooperatively to degrade PHT1;1/4 proteins by polyubiquitination (Lin et al., 2013; Park et al., 2014). It is possible that PHO2 polyubiquitinates the pool of PHT1;1 and PHT1;4 *en route* to the PM while NLA acts on the transporters that are already at the PM.

Most PHT1 proteins have been shown to localize to the PM (Bayle et al., 2011; González et al., 2005; Remy et al., 2012), but PHT1;8 and PHT1;9 also accumulate in the ER (Chapter 2). In this study, we tested the role of PHO2 in the regulation of PHT1;8 and PHT1;9 protein stability, given that the localization of PHT1;8 and 1;9 to the ER coincides that of PHO2. To this end, we used a loss of function mutant *pho2-1* and monitored the localization

and abundance of GFP-PHT1;8 and GFP-PHT1;9 transporters. The overall phenotype of *pho2-1* was slightly enhanced by the overexpression of *PHT1;8* or *PHT1;9*, and the basal Pi level of *pho2-1* was boosted by *PHT1;8* overexpression. PHT1;8 and PHT1;9 localization and abundance were not affected in *pho2* mutants, indicating that their regulation is independent of PHO2.

RESULTS

Ectopic expression of PHT1;8 and PHT1;9 leads to exacerbated Pi toxicity phenotype of the *pho2* mutant

The *pho2* mutant over-accumulates Pi due to enhanced Pi uptake, translocation and reduced remobilization from older tissues (Aung et al., 2006). Excessively high Pi is toxic to plants, therefore the *pho2* plants are smaller and display leaf necrosis at maturity (Aung et al., 2006). We investigated whether *PHT1;8* and *PHT1;9* overexpression would enhance the Pi toxicity of *pho2* mutant given that these overexpressions result in augmented Pi content in the WT Arabidopsis seedlings (Remy et al., 2012); Chapter 2). For this purpose we overexpressed *PHT1;8* and *PHT1;9* under a strong constitutive promoter, the Cauliflower Mosaic Virus 35S (CaMV35S) in *Arabidopsis thaliana*. The shoot phenotype of WT, *pho2* and the *pho2* harboring 35::GFP-PHT1;8 and 35S::GFP-PHT1;9 was analyzed in fifteen-day-old plants. We found that the leaves of the *pho2* mutants are smaller relative to WT due to high Pi toxicity, as previously reported (Aung et al., 2006; Delhaize and Randall, 1995), however, the leaves of the PHT1;8OX *pho2* and PHT1;9OX *pho2* are even smaller than those of *pho2* mutants (Figure 1). This suggests that the overexpression of *PHT1;8* or *PHT1;9* enhances the *pho2* growth phenotype and it is probably due to the augmented Pi uptake and translocation contributed by the PHT1;8 and PHT1;9 overexpression. These results also indicate that PHO2 might be a negative regulator of *PHT1;8* and *PHT1;9,* consistent with the previous reports (Aung et al., 2006; Bari et al., 2006).

Localization and abundance of GFP-PHT1;8 and GFP-PHT1;9 are not affected by the loss of *PHO2*

Earlier reports have shown that most PHT1 proteins localize to the PM (Bayle et al., 2011; González et al., 2005; Remy et al., 2012), but PHT1;8 and PHT1;9 are display a dual localization, at ER and PM, however, the PM localization is detected only in root tips (Chapter 2). In addition, PHT1;8 and PHT1;9 have predicted ubiquitination sites that might implicate the E1/E2/E3 set of enzymes, including PHO2, in their targeting to the vacuole (Huang et al., 2013; Park et al., 2014). We then asked whether PHT1;8 and PHT1;9 are negatively regulated by PHO2 at the posttranslational level. We used stable Arabidopsis lines harboring GFP-PHT1;8 and GFP-PHT1;9 and probed fusion protein localization in roots of WT (Col-0) and *pho2-1* backgrounds. If PHO2 is indeed a negative regulator of PHT1;8 and/or PHT1;9, in the absence of PHO2, we expected a greater fluorescence signal at the PM and/or ER relative to the WT lines. Confocal microscopy analysis revealed that in mature root regions in Col-0 Arabidopsis transgenic seedlings grown on sufficient Pi for 7 days, fluorescence signal corresponding to PHT1;8 and PHT1;9 was detected at the reticular network corresponding to the ER (Figure 2). The localization pattern and intensity of GFP signal from GFP-PHT1;8 and GFP-PHT1;9 in the *pho2-1* mutant is comparable to the localization of these proteins in WT, indicating that the lack of PHO2 does not have an effect on their localization or abundance. This result was unexpected, given that PHO2 does regulate PHT1;1 and PHT1;4 stability (Huang et al., 2013; Park et al., 2014). Therefore, we used the immunoblot analysis to corroborate these results. Membrane and soluble fractions were isolated from Col-0, GFP-PHT1;8 and GFP-PHT1;9 in the WT and *pho2* backgrounds. 30 µg of membrane protein fraction from each sample was loaded onto the SDS gel and probed with an α-GFP antibody.

Prior to protein extractions, GFP fluorescence was confirmed by stereomicroscopic analysis in all the lines expressing GFP fusion proteins, and absence of such a signal in the WT Col-0. Although GFP signal was confirmed by prior microscopic analysis, we were unable to detect the fusion proteins in either WT or *pho2* backgrounds (Figure S1). The expected size for the GFP-PHT1;8 and GFP-PHT1;9 was 90 kDa. When probed with α-GFP we did not detect the expected 90 kDa fragment, but only an unspecific 75 kDa fragment, also present in the Col-0 WT (Figure S1). The 90 kDa fragment corresponding to GFP-PHT1;8 and GFP-PHT1;9 was not detected in the soluble protein fractions either (data not shown). These results suggest that, either GFP-PHT1;8 and GFP-PHT1;9 levels are very low, or we need to further solubilize the membrane proteins. The latter might be unlikely, given that membrane markers for tonoplast (V-ATPase) and ER (Sec12) are detected in the membrane protein fractions in all the samples tested (Figure S1). However, based on the localization and abundance of GFP-PHT1;8 and GFP-PHT1;9 in the *pho2* background, we can conclude that PHO2 is likely not involved in the posttranslational regulation of PHT1;8 and PHT1;9 or it may be that its effect is below the confocal detection capabilities.

Basal Pi content is enhanced upon overexpression of PHT1;8 in the *pho2* background

It was previously reported that loss of PHO2 results in an excessive Pi quantity in the shoots that is achieved by increased efflux of Pi from roots to shoots via PHO1 and an increased Pi uptake via upregulation of *PHT1* transporters (Aung et al., 2006; Delhaize and Randall, 1995; Huang et al., 2013; Liu et al., 2012; Park et al., 2014). We tested whether Pi levels in the *pho2-1* shoots might be further increased by the augmented action of PHT1;8 and PHT1;9. For this, we quantified the level of basal phosphate in the 14-days-old *pho2* and in *pho2* overexpressing GFP-PHT1;8 or GFP-PHT1;9 seedlings. The *pho2* plants harboring GFP-

PHT1;8 had roughly double the amount of soluble Pi relative to the basal Pi levels in the *pho2* mutants, while *PHT1;9* overexpression in *pho2* had a minor effect on the basal Pi content (Figure 4). This result indicates that overexpressing *PHT1;8* is sufficient to enhance the Pi over-accumulation phenotype of the *pho2* mutant, suggesting that PHO2 negatively regulates *PHT1;8* when Pi is sufficient, consistent with earlier reports (Aung et al., 2006).

DISCUSSION

PHO2 plays an important role in Pi homeostasis given that it is a principal component of the Pi-dependent proteolysis machinery (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Delhaize and Randall, 1995; Huang et al., 2013; Lin et al., 2008; Lin et al., 2013; Liu et al., 2012). Previous reports showed that loss of PHO2 results in abnormally high Pi content due to increased Pi uptake and translocation coupled with improper Pi remobilization from source to sink (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Delhaize and Randall, 1995; Huang et al., 2013; Liu et al., 2012). In this report, we investigated the role of PHO2 in regulating PHT1;8 and PHT1;9 proteins and also the contribution of PHT1;8 and PHT1;9 to the *pho2* phenotype.

We found that the overexpression of PHT1;8 and PHT1;9 in the *pho2* background resulted in an enhancement of the Pi over-accumulation phenotype of *pho2* as revealed by the smaller leaves in the 15-day-old seedlings. Monitoring the phenotype of the older plants overexpressing *PHT1;8* or *PHT1;9* in the *pho2* background will be useful to confirm whether the leaf chlorosis and necrosis will be more advanced, since Pi toxicity develops in older plants when the Pi constitutes more that 1% of the plant dry weight (Marschner, 1995l). Furthermore, a detailed quantification of the shoot and root phenotype is pertinent for revealing the effect of *PHT1;8* and *PHT1;9* overexpression on the *pho2* phenotype.

It was previously shown that degradation of the PHT1;1/2/3/4 is PHO2-dependent (Huang et al., 2013; Lin et al., 2013; Park et al., 2014), while Park et al., (2014) reported that PHO2 interacts with PHT1;4 only and no interaction was observed with PHT1;1/2/3/5/6 in a yeast two-hybrid analysis. Our data indicates that GFP-PHT1;8 and GFP-PHT1;9 localization and abundance are not affected by the PHO2, even though their subcellular localization

coincides at the ER. This indicates that PHT1;8 and PHT1;9 are distinct from PHT1;1/2/3/4 and the PHT1;8 and PHT1;9 protein abundance is PHO2-independent, particularly if PHO2 interacts with PHT1;4 only (Park et al., 2014). It is also plausible that the effect of PHO2 is minor or conditional or is exerted at a specific stage of development. It is also, conceivable that PHT1;8 and PHT1;9 degradation mechanisms differ from that of PHT1;1/2/3/4, particularly because PHT1;8 and PHT1;9 are predicted to contain high-confidence putative Proline, glutamic acid (E), Serine, Threonine (PEST) motifs at the C-terminus, which would make these proteins short-lived and subject to quick degradation (Rechsteiner and Rogers, 1996; Rogers et al., 1986). PHT1;1/2/3/4 are not predicted to contain the aforementioned motif. It would be suitable to test whether PEST domain confers a bypass from the PHO2/ NLA action, and this could be tested by swapping the PEST domain from PHT1;8/9 to PHT1;1 or PHT1;4 and analyzing the localization and the abundance of such chimeric proteins.

Our results also show that PHT1;8 enhances the Pi over-accumulation phenotype of the *pho2* as displayed by the heightened basal Pi content in the seedlings overexpressing *PHT1;8* in the *pho2* background, while the *PHT1;9* overexpression had similar basal Pi levels as *pho2* (Figure 3). This is consistent with previous reports where the loss of PHO2 resulted in a greater *PHT1;8* accumulation at Pi sufficient conditions, while the *PHT1;9* increase was only slight (Aung et al., 2006). Our data suggests that PHO2 has a transcriptional control over *PHT1;8*, possibly via an intermediate, to ultimately fine tune the Pi homeostasis, and it has no effect on the localization of PHT1;8 or PHT1;9. Further studies need to be carried out in order to identify proteins involved in the regulation of PHT1;8 and PHT1;9 localization and abundance. One possible candidate involved in PHT1;8 and PHT1;9 degradation might be NLA, given that it regulates PHT1;1 and PHT1;4 degradation (Lin et al., 2013; Park et al., 2014), however the

PM localization of NLA suggests that it could interact and target for degradation only PHT1;8 and PHT1;9 that reached the PM. Additionally, identification of the proteins that regulate degradation of PHT1;8 and PHT1;9 at the ER, given their abundant ER localization in most tissues (Chapter 2), needs to be subject of further studies.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Colombia was used in all experiments. The *pho2-1* was obtained from the Arabidopsis Biological Resource Center (ABRC). The seeds were sterilized and plated on half-strength complete (MSP01) or Pi-free AGM from Caisson Labs (MSP19) supplemented with 1 % (w/v) sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES) and 4 g/L GelRite (RPI). For low Pi medium, KH_2PO_4 was added to a final concentration of 2 µM to the Pi-free AGM. Plates were kept at 4° C in the dark for 4 days and transferred to a growth chamber at 22° C with a 16 h light/8 h dark cycle.

Constructs and transgenic plants

The Gateway system (Life Technologies Corporation) was used to generate transgenic plants harboring GFP or YFP fused to *PHT1;8* and *PHT1;9*. The *PHT1;8* CDS was obtained from ABRC, stock #DQ446267. *PHT1;9* was amplified from Col-0 cDNA from roots of plants grown on low Pi media using PHT1;9-1F: CACCATGCCGGAGTTAAGTTTA and PHT1;9-1R+Stop: TTAGTATTGTCTCCGAAG as primers. Phusion High-Fidelity DNA Polymerase (NEB) was used for amplification of *PHT1;9*. The obtained amplicons were cloned into pENTR™/D-TOPO vector (Life Technologies Corporation) and sequenced by Sanger sequencing. LR recombination was done with the Gateway destination vector pGWB6 (Nakagawa et al., 2007) the constructs were transformed into *Agrobacterium tumefaciens* C58. For stable transformation *Arabidopsis thaliana* Col-0 and *pho2* mutant were used for transformation by floral dip method as previously described (Clough and Bent, 1998).

Confocal microscopy

The images were captured using a Zeiss LSM710 confocal microscope with a 40X water objective, at the Cellular and Molecular Imaging Facility (CMIF) at NCSU. To image the samples combinations of excitation wavelength/emission were 488 nm/492–570 nm for GFP.

Immunoblot analysis

Total protein was extracted from 14 day-old Arabidopsis seedlings grown on Pi sufficient 0.5X MS media using HE-8% buffer (50 mM HEPES-KOH, pH 6.5, 5 mM EDTA, 8 % (w/v) sucrose, 1 mM DTT (w/v) and a protease inhibitor cocktail (Roche). The lysate was centrifuged at 100,000 X g at 4º C for 1 hr to separate the cytosolic proteins from the membrane protein. The obtained pellet was resuspended in 1 ml of HE-8% buffer. Protein concentration was quantified by Bradford Assay (Kruger, 2009) and 30 µg per lane of membrane proteins were run through a 10% SDS Polyacrylamide gel and transferred onto a PVDF membrane by a semidry transfer method using a GE Healthcare Amersham™ TE 70 Semi-Dry Transfer Unit. The blots were incubated with blocking solutions [5 % (w/v) powdered milk in TBS-T [20 mM Tris-HC1, pH 7.5; 150 mM NaCl, and 0.1 % (v/v) Tween 20] for 1 hr. Protein blots were probed with monoclonal anti-GFP antibody (Thermo MA5-15256) 1:1000 dilution as a primary antibody and with Peroxidase-Conjugated Goat Anti-Mouse IgG (Thermo 32430) 1:5000 dilution as a secondary antibody. VATPase and Sec12 were used as controls for the membrane fraction, α-VATPase (Katsiarimpa et al., 2014) and α-Sec12 (Bar-Peled and Raikhel, 1997) at 1:2000 dilution were used as a primary antibody and peroxidase-conjugated Stabilized Goat Anti-Rabbit antibody as secondary antibody 1:5000 (Thermo 32460). The

immunoblots were visualized by chemiluminescence with Clarity Western ECL Substrate (BioRad) and imaged using a G:BOX Chemi XRQ system (Syngene).

Soluble Pi content determinations

For soluble Pi levels and anthocyanin quantification, shoot tissue from 10 seedlings was harvested, rinsed in distilled water, blot-dried, frozen, and ground to a fine powder in liquid nitrogen and separated into 50-100 mg aliquots. Each sample was homogenized in 1% (w/v) Acetic Acid in a 1:10 ratio (1 mg powder to 10 µl 1% (w/v) Acetic Acetic). The mixture was vortexed, and centrifuged at 4ºC to pellet the cellular debris. The supernatant was assayed for soluble Pi using the molybdenum-blue method (Ames, 1966). For Pi quantification the absorbance at 660 nm was read using a Synergy H1 Hybrid Plate Reader at the Genomic Sciences Laboratory (GSL) Facility at NCSU.

REFERENCES

Ames, B.N. (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Method Enzymol, **8**, 115-118.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., Chiou, T.J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol, **141**(3), 1000-1011.

Bar-Peled, M. and Raikhel, N.V. (1997) Characterization of AtSEC12 and AtSAR1 (proteins likely involved in endoplasmic reticulum and golgi transport). Plant Physiol, **114**(1), 315-324.

Bari, R., Datt Pant, B., Stitt, M., Scheible, W.R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol, **141**(3), 988-999.

Bayle, V., Arrighi, J.F., Creff, A., Nespoulous, C., Vialaret, J., Rossignol, M., Gonzalez, E., Paz-Ares, J., Nussaume, L. (2011) *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell, **23**(4), 1523-1535.

Chiou, T., Aung, K., Lin, S., Wu, C., Chiang, S., Su, C. (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. Plant Cell, **18**(2), 412-421.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J, **16**(6), 735-743.

Delhaize, E. and Randall, P.J. (1995) Characterization of a phosphate-accumulator mutant of *Arabidopsis thaliana*. Plant Physiol, **107**(1), 207-213.

Doerner, P. (2008) Phosphate starvation signaling: a threesome controls systemic P(i) homeostasis. Curr Opin Plant Biol, **11**(5), 536-540.

Franco-Zorrilla, J., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet, **39**(8), 1033-1037.

González, E., Solano, R., Rubio, V., Leyva, A., Paz-Ares, J. (2005) PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in Arabidopsis. Plant Cell, **17**(12), 3500-3512.

Huang, T.K., Han, C.L., Lin, S.I., Chen, Y.J., Tsai, Y.C., Chen, Y.R., Chen, J.W., Lin, W.Y., Chen, P.M., Liu, T.Y., Chen, Y.S., Sun, C.M., Chiou, T.J. (2013) Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. Plant Cell, **10**, 4044-4060.

Kant, S., Peng, M., Rothstein, S.J. (2011) Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in Arabidopsis. PLoS Genet, **7**(3), e1002021.

Katsiarimpa, A., Muñoz, A., Kalinowska, K., Uemura, T., Rojo, E., Isono, E. (2014) The ESCRT-III-interacting deubiquitinating enzyme AMSH3 is essential for degradation of ubiquitinated membrane proteins in *Arabidopsis thaliana*. Plant Cell Physiol, **55**(4), 727-736.

Kraft, E., Stone, S.L., Ma, L., Su, N., Gao, Y., Lau, O.S., Deng, X.W., Callis, J. (2005) Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol, **139**(4), 1597-1611.

Kruger, N.J. (2009) The Bradford Method for protein quantitation. In The Protein Protocols Handbook (Third Edition), J.M. Walker (Ed), Humana Press, New York, 17-24.

Lin, S.I., Chiang, S.F., Lin, W.Y., Chen, J.W., Tseng, C.Y., Wu, P.C., Chiou, T.J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiol, **147**(2), 732-746.

Lin, W., Huang, T., Chiou, T. (2013) Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane-localized phosphate transporters to maintain phosphate homeostasis in Arabidopsis. Plant Cell, **25**(10), 4061-4074.

Liu, C., Muchhal, U.S., Raghothama, K.G. (1997) Differential expression of *TPS11*, a phosphate starvation-induced gene in tomato. Plant Mol Biol, **33**(5), 867-874.

Liu, T.Y., Huang, T.K., Tseng, C.Y., Lai, Y.S., Lin, S.I., Lin, W.Y., Chen, J.W., Chiou, T.J. (2012) PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in Arabidopsis. Plant Cell, **24**(5), 2168-2183.

Liu, T.Y., Lin, W.Y., Huang, T.K., Chiou, T.J. (2014) MicroRNA-mediated surveillance of phosphate transporters on the move. Trends Plant Sci, **19**(10), 647-655.

Marschner, H. (1995l) 12 - Diagnosis of deficiency and toxicity of mineral nutrients. In Mineral Nutrition of Higher Plants (Second Edition), Academic Press, London: 461-479.

Medici, A., Marshall-Colon, A., Ronzier, E., Szponarski, W., Wang, R., Gojon, A., Crawford, N.M., Ruffel, S., Coruzzi, G.M., Krouk, G. (2015) AtNIGT1/HRS1 integrates nitrate and phosphate signals at the Arabidopsis root tip. Nat Commun, **6**.

Misson, J., Raghothama, K.G., Jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., Thibaud, M.-C. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Ac Sci U S A,**102**(33), 11934-11939.

Muchhal, U.S. and Raghothama, K.G. (1999) Transcriptional regulation of plant phosphate transporters. Proc Natl Acad Sci U S A, **96**(10), 5868-5872.

Mudge, S., Rae, A., Diatloff, E., Smith, F. (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in Arabidopsis. Plant J, **31**(3), 341-353.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Bioscience and Bioengineering, **104**(1), 34-41.

Pant, B.D., Buhtz, A., Kehr, J., Scheible, W.R. (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J, **53**(5), 731-738.

Park, B.S., Seo, J.S., Chua, N.H. (2014) NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. Plant Cell, **26**(1), 454-464.

Peng, M., Hannam, C., Gu, H., Bi, Y.M., Rothstein, S.J. (2007) A mutation in *NLA*, which encodes a RING-type ubiquitin ligase, disrupts the adaptability of Arabidopsis to nitrogen limitation. Plant J, **50**(2), 320-337.

Poirier, Y. and Bucher, M. (2002) Phosphate transport and homeostasis in Arabidopsis. Arabidopsis Book, **1**, e0024.

Rechsteiner, M. and Rogers, S.W. (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci, **21**(7), 267-271.

Remy, E., Cabrito, T., Batista, R., Teixeira, M., Sa-Correia, I., Duque, P. (2012) The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. New Phytol, **195**(2), 356-371.

Rogers, S., Wells, R., Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science, **234**(4774), 364-368.

Schachtman, D.P., Reid, R.J., Ayling, S.M. (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol, **116**(2), 447-453.

Shin, H., Shin, H.S., Chen, R., Harrison, M.J. (2006) Loss of *At4* function impacts phosphate distribution between the roots and the shoots during phosphate starvation. Plant J, **45**(5), 712-726.

Stefanovic, A., Arpat, A.B., Bligny, R., Gout, E., Vidoudez, C., Bensimon, M., Poirier, Y. (2011) Over-expression of PHO1 in Arabidopsis leaves reveals its role in mediating phosphate efflux. Plant J, **66**(4), 689-699.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Poirier, Y., Nussaume, L. (2010) Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. Plant J, **64**(5), 775-789.

TABLES AND FIGURES

Figure 1. Ectopic expression of *PHT1;8* and *PHT1;9* leads to an exacerbated Pi toxicity phenotype of *pho2* mutant.

The effect of overexpressing *PHT1;8* or *PHT1;9* on the *pho2-1* phenotype. The images show the shoot phenotypes of 15-day-old Arabidopsis seedlings grown on 0.5X MS media containing regular (1 mM) Pi media (Pi sufficient condition).

Scale bar= 1 cm.

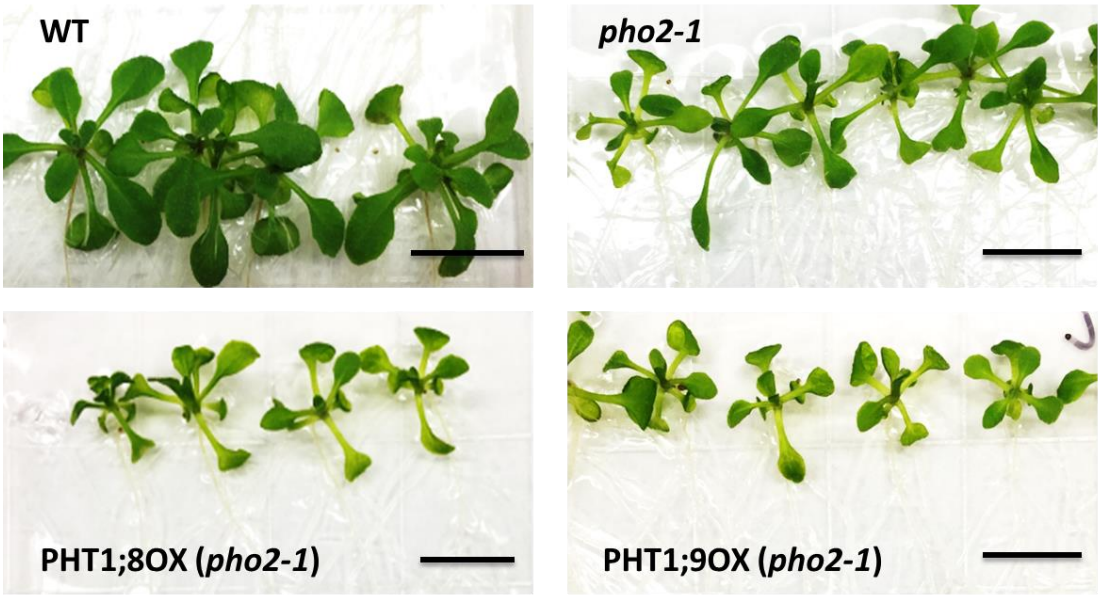


Figure 2. GFP-PHT1;8 and GFP-PHT1;9 subcellular localization and abundance is not altered by the *pho2* mutation.

(A-D) 3D renderings of high-resolution confocal z-stacks acquired from WT and *pho2* *Arabidopsis thaliana* roots of stably expressing GFP-PHT1;8 and GFP-PHT1;9. GFP signal labels the endoplasmic reticulum (ER) network. The seedlings were 7 days old and grown on 0.5X MS media containing 1 mM Pi. GFP fluorescence is false colored in green.

Scale bar = 20 µm.

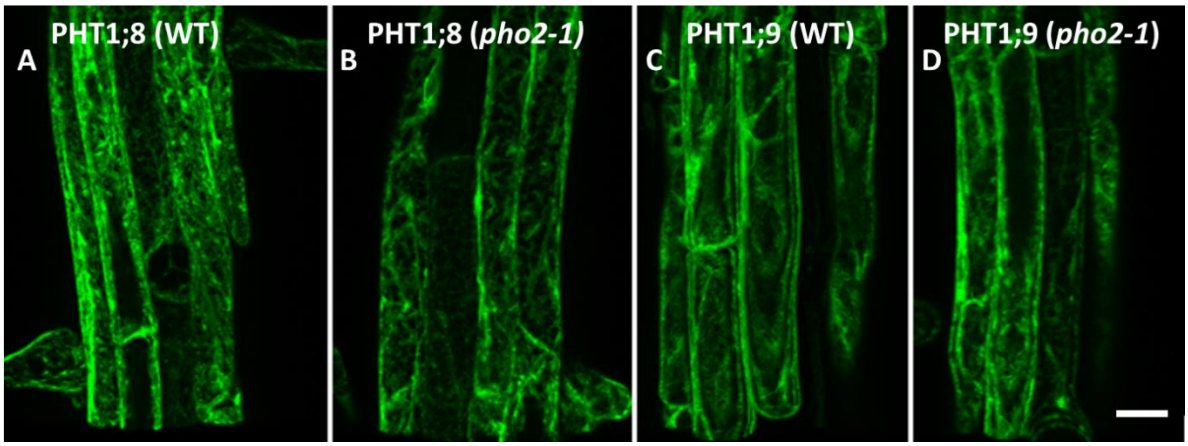
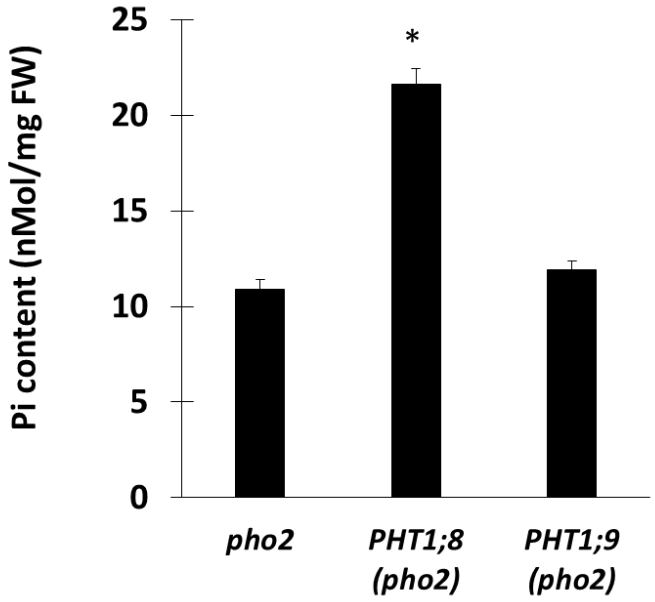


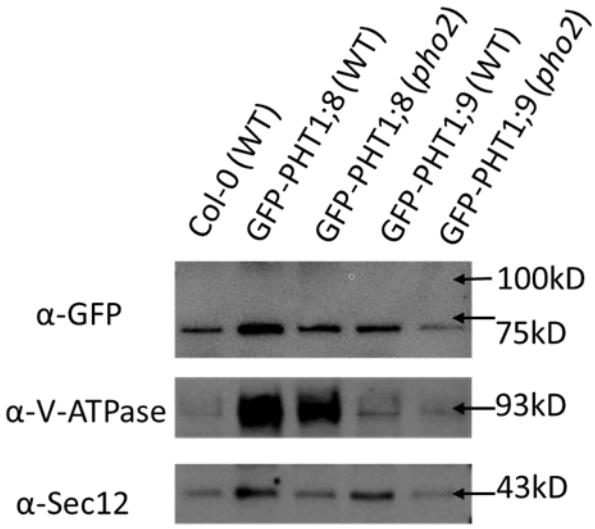
Figure 3. Basal Pi content in PHT1;8 OX and PHT1;9 OX in *pho2* background.

The effect of *PHT1;8* or *PHT1;9* overexpression in the *pho2* background on Pi content. The 14-day-old *pho2*, GFP-PHT1;8 (*pho2)* and GFP-PHT1;9 (*pho2)* seedlings were grown on 0.5X MS media containing regular (1 mM) Pi were used. Soluble Pi was assayed using the Molybdate-blue method as previously described (Ames, 1966). FW= fresh weight. Bars represent means ± SD, n = 5, * indicates $p < 0.001$.



Supplementary Figure 1. Immunoblot analysis of the GFP-PHT1;8 and GFP-PHT1;9 in the wild type and *pho2* mutants.

Immunoblot analysis of GFP-PHT1;8 and GFP-PHT1;9 from membrane fractions of the wild type and *pho2* mutants. 30 µg of membrane protein fraction was loaded and separated through a 10% SDS-Polyacrylamide gel. α-GFP, α-V+ATPase and α-Sec12 antibodies were used. The markers used as controls for the membrane protein fraction were: V+ATPase (93 kDa, tonoplast membrane marker) and Sec12 (43 kDa, an integral ER membrane marker).



CHAPTER 4

The Iron Signaling Proteins BRUTUS and POPEYE Regulate *PHT1* Expression

ABSTRACT

Phosphorus (P) is one of the essential macronutrients indispensable for proper plant growth and development. P is a structural component of nucleic acids and membranes, as well as a fundamental constituent in metabolism and energy production. Most soils are poor in phosphate (Pi), resulting in a significant constraint on plant productivity. Iron (Fe), on the other hand, is an important micronutrient that participates in key processes such as photosynthesis and redox reactions. Insufficient Fe has a negative impact on yield and on the nutritional quality of plants. Pi is a known potent chelator of Fe, and the generated Pi-Fe complexes are unusable for plants resulting in a reduced availability of both elements. Plants developed intricate mechanisms to maintain accurate Fe and Pi homeostasis and there is significant evidence for a crosstalk given that Pi-starved plants accumulate more Fe and the classical inhibition of the primary root during low Pi is due to Fe-toxicity. Recently, PHOSPHATE STARVATION RESPONSE 1 (PHR1), the master regulator of Pi homeostasis has been shown to regulate responses to iron, sulfate and zinc deficiencies, thus PHR1 might be a hub for integrating multiple nutrient stati. We investigated the crosstalk between iron and phosphate homeostasis by analyzing the expression of classical iron and phosphate starvation markers in the high affinity phosphate transporter mutants (*PHT1*), *pht1;8* and *pht1;9* and in the altered iron-deficiency response, *brutus (bts)* and *popeye (pye)* mutants. We observed that loss of *BTS* or *PYE* results in the upregulation of *PHT1;1* and *PHT1;4* transcripts at low and high Pi regimes, in addition to the decrease of these transcripts under Fe-deprivation. Conversely, transcripts for Fe-starvation markers, *Arabidopsis H+-ATPase2* (*AHA2*) and *IRON-REGULATED*

TRANSPORTER1 (*IRT1*) are more abundant in Pi deficient mutants, *pht1;8, pht1;9* and *pht1;8 1;9* independent of the Pi availability. Our results indicate that PHT1;8 and PHT1;9 are involved in Fe homeostasis by controlling the Pi status and that BTS and PYE participate at the crosstalk between phosphate and iron homeostasis by exerting genetic control over *PHT1* and *PSI* genes**.**

INTRODUCTION

Plants are sessile organisms, and thus, maintaining proper balance of macro and micro nutrients is essential for prosperous growth and development (Marschner, 1995f). Pi is often a limiting macronutrient, its availability has an impact on the accumulation of iron, copper, sulfur, zinc, manganese as well as nitrogen, and this points to the existence of a complex nutritional crosstalk in plants (Barben et al., 2011; Briat et al., 2015; Hirsch et al., 2006; Kant et al., 2011; Niu et al., 2015; Perea-Garcia et al., 2013; Rai et al., 2015; Rouached et al., 2010; Rouached et al., 2011; Ward et al., 2008; Kant et al., 2011; Lin et al., 2013; Medici et al., 2015).

Pi is a chelator of Fe in soil/media as well as *in planta*, and the resulting Pi-Fe complexes are unusable for plants. Under normal conditions, Fe mostly accumulates in the vacuole where is it found predominantly in a complex with P, while during low Pi, iron is not detected in the vacuole or cytosol, but in chloroplasts of leaf cells where it is not accompanied by P (Hirsch et al., 2006). Furthermore, Fe is stored in seed vacuoles as Fe-phytate (also known as inositol hexakisphosphate, or IP6) complexes and release of Fe from these complexes is important for seed germination under Fe deprivation (Lanquar et al., 2005). Phosphorus deficiency promotes Fe accumulation and redistribution *in planta* (Hirsch et al., 2006). More specifically, Pi-deficiency results in a more than tenfold increase in Fe content in plants (Hirsch et al., 2006). Root morphology is severely altered during Pi starvation, as the primary root growth is inhibited due to Fe toxicity, which induces cessation of root apical meristem (RAM) activity (Abel, 2011; Rai et al., 2015; Svistoonoff et al., 2007; Thibaud et al., 2010; Ward et al., 2008). In fact, the presence of Fe is required for primary root growth inhibition on low Pi media (Svistoonoff et al., 2007; Ward et al., 2008).

Plants adapt to both Pi and Fe deprivation by acidifying the rhizosphere. Upon Pi deficiency, together with the root system architecture changes, the rhizosphere becomes more acidic as a result of the exudation of acid phosphatases and organic acids, for the purpose of increasing the bioavailability of Pi (del Pozo et al., 1999; Jones, 1998; Li et al., 2002; Lopez-Bucio et al., 2000; Marschner, 1995o; Plaxton and Tran, 2011). The subsequent pH reduction in the rhizosphere results in increased metal solubility (Narang et al., 2000), and as a consequence, it can lead to metal toxicity (Ward et al., 2008). Fe-deficiency conditions also trigger rhizosphere acidification by the action of AHA2, a plasma membrane H+ATPase, and by production of organic acids (Abadía et al., 2002; Santi and Schmidt, 2009). Despite the rhizosphere acidification in response to deprivation in both Fe and Pi, it appears that the acid phosphatase secretion from roots is exclusive to Pi stress.

The Pi-Fe interaction is also apparent at the molecular level. Indeed, split-root experiments have shown that genes involved in metal detoxification and storage are induced, while genes involved in metal absorption and translocation are repressed by Pi starvation, thus pointing to an antagonistic interrelationship between Pi and Fe (Thibaud et al., 2010). *PHOSPHATE STARVATION RESPONSE 1 (PHR1)* and its closest homologue *PHR1-LIKE (PHL1)* are MYB-related transcription factors that regulate gene expression in response to Pi and Fe fluctuations (Bournier et al., 2013; Briat et al., 2015). PHR1 controls the transcript abundance of many *PHOSPHATE STARVATION INDUCIBLE* (*PSI*) genes, such as *INDUCED BY PHOSPHATE STARVATION1 (IPS1*), *At4/IPS2* and *SPX1* in a Pi-dependent manner (Liu et al., 1997; Shin et al., 2006). *IPS1* and *At4/IPS2* are members of the same family of non-coding RNAs that are induced at low Pi and function as riboregulators (Franco-Zorrilla et al., 2007), while *SPX1* encodes a nuclear protein implicated in Pi sensing (Duan et al., 2008;

Puga et al., 2014). PHR1 and PHL1 are involved in the regulation of Fe homeostasis by directly interacting with the promoter of Fe-storage protein *FERRITIN1 (FER1),* as shown by a yeast one-hybrid, to regulate its expression in response to Pi availability (Bournier et al., 2013). Moreover, an alteration in iron homeostasis was observed in the *phr1phl1* mutants, including the misregulation of *FER1* (Bournier et al., 2013). This is consistent with previous reports of upregulation of *FER1* and *FERRIC REDUCTASE DEFECTIVE3 (FRD3),* an effluxer of citrate, in response to low Pi (Thibaud et al., 2010). Clearly, there is robust indication of the cross-communication amongst Fe and Pi responses mediated by PHR1.

Phosphate uptake in Arabidopsis occurs through high affinity phosphate transporters (PHT1) (Misson et al., 2004; Muchhal et al., 1996; Raghothama, 1999; Raghothama, 2000; Raghothama and Karthikeyan, 2005; Shin et al., 2004). Loss of *PHT1* aggravates the Pi starvation phenotypes, while overexpression of these genes results in an increased Pi uptake and Pi content (Ayadi et al., 2015; González et al., 2005; Lapis-Gaza et al., 2014; Misson et al., 2004; Nagarajan et al., 2011; Shin et al., 2004; Smith et al., 2011). Interestingly, loss of *PHT1;1/2/3/4* genes results in an increased Fe content in both leaves and roots (Ayadi et al., 2015). In fact, quantification by Inductively Coupled Plasma (ICP) mass spectrometry shows that Fe content is increased by 30 to 50% in *PHT1* loss of function mutants, depending on how many *PHT1* genes are impaired (Ayadi et al., 2015). This highlights, once more, the connection that exists between Fe and Pi homeostasis.

Phosphate and iron levels regulate the abundance of proteins involved in Fe uptake by transcriptional and/or posttranscriptional mechanisms. IRON-REGULATED TRANSPORTER1 (IRT1) is the main plasma membrane (PM)-localized iron transporter responsible for the iron uptake from soil (Eide et al., 1996; Henriques et al., 2002; Vert et al.,

2002). Upon sufficient Fe, *IRT1* transcript levels are reduced and IRT1 protein is endocytosed from the PM and targeted for degradation at the vacuole (Barberon et al., 2014; Barberon et al., 2011; Vert et al., 2002). Additionally, IRT1 is posttranscriptionally controlled by the helix-loop-helix protein, FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1 (FIT1), which is essential for Fe-deficiency response (Colangelo and Guerinot, 2004). Consistent with a Fe-Pi crosstalk, Pi deficiency results in systemic down-regulation *IRT1* expression, while Pi sufficiency induces accumulation of *IRT1, IRT2* and *FERRIC REDUCTION OXIDASE2* (*FRO2*) (Thibaud et al., 2010), a ferric-chelate reductase enzyme, responsible for the reduction of iron at the root surface (Robinson et al., 1999). Furthermore, FRO2 accumulation is diminished in the loss of function for the ubiquitin-conjugating enzyme UBC24/PHOSPHATE2 (PHO2) mutants, which overaccumulate Pi (Huang et al., 2013). This suggests that the Pi–dependent proteolytic machinery might be involved in the regulation of Fe homeostasis and that Pi homeostasis alterations trigger adjustments of the Fe status.

Under Pi sufficient conditions plants may experience Fe deficiency, given that Pi is a potent chelator of Fe. When Fe levels are low, two key Fe deficiency response regulators *BTS* and *PYE* are upregulated within 72 hours of Fe stress (Long et al., 2010). Although both *bts* and *pye* mutants have elevated Fe content, loss of *BTS* results in increased tolerance to Fe stress, while loss of *PYE* results in reduced tolerance to Fe insufficiency (Long et al., 2010; Selote et al., 2015). Recent studies have shown that BTS binds Fe through its hemerythrin domains (Kobayashi et al., 2013; Selote et al., 2015) and it was proposed to function as an Fe sensor (Kobayashi and Nishizawa, 2014; Selote et al., 2015). BTS also contains a RING domain, which facilities the degradation of ILR3 and bHLH115, two PYL-like proteins that also interact with PYE (Long et al., 2010; Selote et al., 2015). It is thought that by controlling

PYL-like proteins BTS modulates PYE mediated Fe deficiency responses, thus impacting Fe homeostasis.

In the current study, we further elucidate the genetic basis of the Fe and Pi interaction by analyzing mutants with compromised Pi and Fe homeostasis exposed to either stressor. Plants with impaired *PHT1;8* and/or *PHT1;9* expression were used as representative for abnormal Pi homeostasis (Chapter 2). Additionally, plants with loss of *POPEYE (PYE)* or *BRUTUS (BTS)* functions were selected as representative for anomalous Fe homeostasis, as *bts* has an increased tolerance to Fe stress while *pye* mutant has a reduced tolerance to Fe insufficiency and both are critical for regulating Fe homeostasis (Long et al., 2010; Selote et al., 2015). Our experiments showed that loss of *PHT1;8* and *PHT1;9* results in Fe-stress phenotypes at the molecular level, and that BTS and PYE regulate Pi homeostasis by controlling *PHT1* and *PSI*.

RESULTS

Iron homeostasis is affected by the loss of *PHT1;8* or *PHT1;9* at Pi-replete and deplete conditions

In order to further characterize the interaction between Fe and Pi homeostasis, we examined the effect of Pi availability on the expression of iron starvation markers in mutants with compromised Pi. For this purpose we chose mutants with disrupted *PHT1;8* and *PHT1;9,* which are specifically induced by low Pi and their loss results in low tolerance to Pi stress (Chapter 2; Lapis-Gaza et al., 2014; Mudge et al., 2002; Remy et al., 2012). We hypothesized that these mutants might have an altered expression of genes involved in Fe uptake, as a consequence of the Pi- starvation phenotype. Real-time quantitative reverse transcription (qRT)-PCR was carried out to compare the relative expression of genes involved in Fe homeostasis such as *FIT1*, *IRT1* and *AHA2*, in roots of WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* double mutants grown under normal and Pi-deficient conditions. It has been previously shown that all of the above transcripts accumulate during Fe deficiency (Colangelo and Guerinot, 2004; Santi and Schmidt, 2009; Vert et al., 2002). Under sufficient Pi conditions, the expression of *FIT1* is relatively unchanged in all three mutants tested when compared to WT levels (Figure 1), while the *IRT1* transcript levels at are induced 5.4 fold in *pht1;8,* 2.1 fold in *pht1;9* and 3.1 fold in the *pht1;8 1;9* double mutant. Similarly, the *AHA2* relative expression at regular Pi is tripled in *pht1;8,* 5.6 fold in *pht1;9* and sevenfold in *pht1;8 1;9* compared to the *AHA2* levels in the WT (Figure 1). Consistent with previous reports, Pi deprivation caused an overall reduction of the level of *IRT1* (Misson et al., 2005; Thibaud et al., 2010), similarly *FIT1* and *AHA2* transcripts were reduced. Pi depletion led to a drop in *FIT1* expression to 13% in *pht1;8,* 22% in *pht1;9* and 40% relative to WT levels (Figure 1). Likewise, the relative

expression of *IRT1* was reduced in all lines tested to nearly half of the WT levels in response to low Pi (Figure 1). A small change in the *AHA2* transcript abundance was detected at Pi deficient conditions. *AHA2* expression was reduced to 53% of the WT level in *pht1;8,* and almost twofold in the *pht1;8 1;9* double mutant (Figure 1). No change in the *AHA2* expression was detected in the *pht1;9* mutant. The higher expression of *IRT1* and *AHA2* in the *pht1;8*, *pht1;9* and *pht1;8 1;9* under Pi sufficient conditions highlight a perturbation in Fe homeostasis in these mutants. Moreover, the higher levels of *IRT1* and *AHA2* transcripts in all the high-affinity phosphate transporter mutants tested, at abundant Pi, suggest that the iron levels might be higher in these lines. We propose that these perturbations are *FIT1*-independent, given that its expression does not correlate with the *IRT1* and *AHA2* expression in all *PHT1* mutants tested. These results are consistent with previous studies showing that loss of *PHT1;1/2/3/4* resulted in higher Fe content, possibly due to *IRT1* induction (Ayadi et al., 2015).

Pi homeostasis is altered in *bts* and *pye* mutants at insufficient and sufficient Pi

Next, we investigated the impact of Pi availability on plants with compromised Fe homeostasis, and we used the transcript abundance of Pi starvation markers as a readout for Pi status. We chose the *bts* and *pye* mutants for these experiments, because these genes represent two core steps in the regulatory network in response to Fe deprivation (Long et al., 2010; Selote et al., 2015). We tested the relative expression of the two most abundant (*PHT1;1, PHT1;4*) and the two least abundant (*PHT1;8* and *PHT1;9*) members of the *PHT1* genes by qRT-PCR in roots of WT, *bts*, and *pye* mutants. The expression of *PHT1* genes are induced by Pi-deprivation and therefore these are commonly used as Pi starvation markers (Misson et al., 2005; Mudge et al., 2002). Under Pi-replete conditions, the relative expression of *PHT1;1* is comparable to WT in *bts*, while it is nearly doubled in the *pye* mutant (Figure 2). *PHT1;4*, on

the other hand, is induced in both *bts* and *pye*, 2.5 and 2.2 fold, respectively. Interestingly, *PHT1;8* expression is nearly abolished, to 2-3% of the WT level in both *bts* and *pye*, while the *PHT1;9* abundance was not affected in either of the mutants (Figure 2). A similar trend was observed in Pi-starved plants. The relative expression of *PHT1;1* and *PHT1;4*, increased to 1.6 fold in *bts* and 1.4 fold in *pye* when compared to WT. *PHT1;8* expression was reduced to 5% in *bts* and 7% *pye*, and yet again *PHT1;9* expression was unaffected in both *bts* and *pye* mutants (Figure 2). These results indicate that *BTS* and *PYE* might be involved in the regulation of *PHT1* genes independent on the Pi status and that *PHT1* genes appear to be downstream of *BTS* and *PYE*. Furthermore, our results suggest a distinct regulation of *PHT1;1/4, PHT1;8* and *PHT1;9* by PYE and BTS, given their disparate expression pattern in *bts* and *pye* mutants.

In order to further elucidate the Pi status of *bts* and *pye* mutants, we tested the transcript abundance of the genes commonly used as Pi starvation markers. Transcript abundance of *SPX1*, *IPS1* and *At4/IPS2* was analyzed in roots of Arabidopsis WT, *bts*, and *pye* mutants exposed to regular (1mM) or low (2 μM) Pi conditions. At Pi-replete conditions, *bts* mutants showed a ~1.5 fold increase in the relative expression of *SPX1* and *At4/IPS2* compared to the WT (Figure 3). *IPS1* expression was not changed in this mutant. An opposite expression pattern was observed in *pye,* with virtually unaffected *SPX1* and *At4/IPS2* levels, while the relative expression of *IPS1* was reduced to 20% of its levels in the WT (Figure 3). A different response was found in the *bts* mutant exposed to Pi deprivation, with almost doubling in *SPX1* and almost tripling in *IPS1* expression relative to the WT (Figure 3). An analogous increase in *SPX1* and *IPS1* expression was observed in *pye* mutant, where both transcripts more than doubled (Figure 3). In contrast, the *At4/IPS2* transcript was virtually unchanged in both *bts* and *pye* mutants (Figure 3). These data suggest that *bts* is marginally Pi-deficient under sufficient

Pi, while, both *pye* and *bts* mutants display augmented Pi-deficiency in response to low Pi conditions. The differences between *IPS1* and *At4/IPS2* expression in both *bts* and *pye*, suggests that these two genes undergo distinct regulation despite their redundancy.

Altered *PHT1* and *PSI* expression in response to Fe deprivation

Fe deficiency results in major changes of the transcriptome (Dinneny et al., 2008).We inquired whether the expression of known Pi stress markers, such as *PHT1* and *PSI*, would be affected by perturbations in Fe availability. For this purpose, we tested the relative expression of five Pi starvation markers, two *PHT1* (*PHT1;1* and *PHT1;4*) and three *PSI* (*SPX1*, *IPS1* and *At4*) by qRT-PCR, in roots of 14-day-old WT seedlings grown on Fe-sufficient and Fe-deficient conditions. Fe deprivation had a negative impact on all of the Pi starvation markers tested (Figure 4). The relative expression of *PHT1;1* and *PHT1;4* under Fe starvation was reduced to 64% and 34%, respectively, relative to their levels at Fe-sufficient conditions (Figure 4), and this was consistent with a previous microarray data showing a reduction in expression of a highly similar gene, *PHT1;3* (Dinneny et al., 2008). Furthermore, the transcript abundance of Pi starvation markers, *SPX1* and *At4/IPS2*, showed a reduction to 55% and 44% of their levels under Fe-sufficiency. Notably, Fe-deficiency induced the relative levels of *IPS1* to be most attenuated, being reduced to only 2% relative to its levels in normal conditions (Figure 4). Altogether, these results point to the fact that Fe deprived plants exhibit Pi sufficiency at the molecular level. Moreover, the Pi starvation markers are repressed during iron deprivation as highlighted by the lower expression of the Pi-stress markers, pointing to a state of Pi excess. These results are consistent with the antagonistic interaction between the Fe and Pi homeostasis.

Rhizosphere acidification is altered in the *pht1;8* and *pht1;8 1;9* double mutants in response to Fe starvation

It has been previously shown that plants respond to Pi stress by acidifying their rhizosphere for the purpose of increasing the bioavailability of Pi (Gerke, 2015; Jones, 1998; Marschner, 1995o; Narang et al., 2000; Raghothama and Karthikeyan, 2005; Richardson et al., 2011). Moreover, loss of *PHT1;8*, one of the high affinity phosphate transporters, resulted in an enhanced reduction of the pH at the rhizosphere in response to Pi stress (Chapter 2), which indicates that this mutant is more susceptible to Pi shortage. Lowering of the pH at the rhizosphere in response to Fe-deficiency is also an important mechanism to increase Fe availability (Long et al., 2010; Santi and Schmidt, 2009; Yi and Guerinot, 1996). Therefore, we wanted to test the effect of iron starvation on the rhizosphere acidification capacity of the *pht1;8, pht1;9* and *pht1;8 1;9* mutants. For this purpose, seedlings were grown on complete media for 4 days and transferred to Fe-sufficient or Fe- deficient conditions for additional 4 days. Later the seedlings were moved to a minimal media containing Bromocresol Purple, a pH indicator, for additional 24 hours. The purple color of the media indicates a pH of 6.5 or higher, while the yellow color of the media indicates a pH of 5.2 or lower. Under sufficient Fe conditions, the purple color of the media did change slightly, turning lighter in the rhizosphere area of the WT and *pht1;8* mutant, while for the *pht1;9* and *pht1;8 1;9* mutants no major changes of the media proximate to the root were observed (Figure 5A). On the other hand, the rhizosphere of the Fe stressed WT seedlings did get acidic, as indicated by the yellow coloration of the media, as previously reported (Long et al., 2010; Yi and Guerinot, 1996). Interestingly, no acidification was observed in the rhizosphere of the *pht1;8* and *pht1;8 1;9* double mutants in response to low Fe (Figure 5B). These results suggest that these mutants are

more Fe sufficient than the WT even under Fe-deplete conditions, and this is consistent with the fact that *pht1;8* and *pht1;8 1;9* are more Pi starved (Chapter 2), and therefore, they have less Pi to chelate the available Fe. The *pht1;9* mutant acidifies its rhizosphere to a similar extent as the WT control (Figure 5), consistent with its milder Pi starvation phenotype (Chapter 2). Overall, these results suggest that the more Pi stressed *pht1;8* and *pht1;8 1;9* are less Fe-starved.

DISCUSSION

In the current work, we elucidated some of the molecular mechanisms underlying the genetic basis for Fe and Pi interactions in Arabidopsis roots. Although Pi and Fe are both essential for plant growth, they are toxic when present in excess (Abel, 2011; Aung et al., 2006; Delhaize and Randall, 1995; Hirsch et al., 2006; Marschner, 1995h; Marschner, 1995i; Marschner, 1995l). Moreover, Pi and Fe form complexes in soil solutions as well as *in planta*, which results in both nutrients becoming unavailable for plant use (Hirsch et al., 2006; Marschner, 1995d; Ward et al., 2008; Zheng et al., 2009). Low Pi results in Fe toxicity phenotypes in roots and high accumulation and storage of Fe within the plant, thus Pi and Fe are antagonistic (Hirsch et al., 2006; Rai et al., 2015; Thibaud et al., 2010; Ward et al., 2008).This indicates a strict integrated regulation of both Fe and Pi possibly in a PHR1-dependent manner.

We have carried out several experiments to probe Fe homeostasis in response to variable Pi levels and in mutants with defective Pi acquisition. Our study revealed that mutants with increased Pi deficiency, *pht1;8, pht1;9* and *pht1;8 1;9* (Chapter 2) exhibit a reduced expression of the Fe starvation response during Pi deprivation. However, under Pi-sufficient conditions these mutants display enhanced Fe stress phenotypes at the molecular level, as displayed by increased *IRT1* and *AHA2* expression (Figure 1). The expression of *FIT1* was unaffected under sufficient Pi in the *pht1;8* and *pht1;8 1;9* mutants, but it was lower in Pi-deficient conditions (Figure 1), suggesting that these mutants are Fe-sufficient during Pi starvation, possibly, as a result of being more Pi deficient (Chapter 2). During low Pi, the *IRT1* expression is lower, presumably, because the more Pi starved *pht1;8, pht1;9* and *pht1;8 1;9* mutant seedlings have an increased Fe bioavailbaility. This is consistent with the higher Fe

content at regular Pi conditions in the severely Pi-starved *pht1;1/2/3/4phf1* quintuple mutant, previously reported (Ayadi et al., 2015). The increased *AHA2* accumulation in the Pi sufficient conditions further indicates the low Fe status as a consequence of the loss of *PHT1;8* and *PHT1;9.* Additional investigations are needed to confirm effect of *PHT1;8* and *PHT1;9* loss on the Fe levels, which can be tested qualitatively by Perl staining (Selote et al., 2015; Long et al., 2010) and quantitatively by ICP Mass Spec (Ayadi et al., 2015).

Mutants with an increased tolerance to Fe stress (*bts*) and a reduced tolerance to Fe insufficiency (*pye*) showed differential expression of Pi-stress markers in response to variable Pi levels. It has been previously shown that *PHT1* transcripts together with a host of Pi stress markers are highly induced by Pi deprivation (Misson et al., 2004; Mudge et al., 2002; Okumura et al., 1998; Shin et al., 2004). We show that *PHT1;1* and *PHT1;4* are induced in *bts* and *pye* at both low and high Pi (Figure 2). Interestingly, *PHT1;8* expression was almost abolished in both *bts* and *pye*, independent on Pi availability, while *PHT1;9* levels were unaffected at all conditions in both mutants (Figure 2). These results indicates that BTS and PYE might be negative regulators of *PHT1;1/4* and positive regulators of *PHT1;8*, independently of the Pi supply. Additionally, it is tempting to speculate that BTS and PYE might regulate *PHT1;8* in a PHR1-independent manner, since PHR1 controls the expression of all *PHT1* genes. These results also point to a differential regulation of *PHT1;1;/4*, *PHT1;8* and *PHT1;9*, despite their genetic redundancy, given the very different patterns of expression in each of the mutants. Additionally, *bts* appears to be more Pi-starved at Pi-sufficiency, as shown by the increased levels of *SPX1* and *At4/IPS2* at Pi replete conditions. The Pi-starvation phenotype of the *bts* mutant is consistent with its relatively high Fe content (Selote et al., 2015). Interestingly, *pye* might be less Pi-starved than the WT when Pi is plentiful, given the relative

expression of *IPS1* is reduced to 20%. Both *bts* and *pye* are more Pi-starved than the WT under Pi deprivation, given that the Pi starvation markers, *IPS1* and *SPX1* are more than doubled at this condition, consistent with the induction of *PHT1;1* and *PHT1;4* in both mutants. These results highlight the intricate molecular interaction between Pi and Fe, and confirm that compromised Fe homeostasis results in alteration of Pi homeostasis.

Analysis of the abundance of phosphate starvation markers in response to variable Fe availability, has shown that Fe-deficiency has a negative impact on all the Pi starvation induced genes tested, as *PHT1;1, PHT1;4, SPX1, IPS1* and *At4/IPS2* were reduced in response to Fe shortage (Figure 4). Altogether, these results point to the fact that Fe deprived plants exhibit Pi sufficiency at the molecular level. These results further support the antagonistic interaction that exists between Fe and Pi homeostasis. Furthermore, Fe shortage does not trigger the typical rhizosphere acidification in the *pht1;8,* and *pht1;8 1;9* mutants, while the WT and *pht1;9* did acidify their rhizosphere to the same extent. This is suggestive of the fact that *pht1;8* and *pht1;8 1;9* are less Fe-stressed, since they are more Pi starved. Furthermore, it is conceivable that the mechanisms for rhizosphere acidification may differ in response to Pi and Fe deficiency, since *pht1;8* and *pht1;8 1;9* mutants although capable of acidifying their rhizosphere in response to Pi stress, fail to do so in response to Fe deprivation.

This study elucidates some of the molecular aspects that underlie the intricate crosstalk between the Fe and Pi homeostasis. We uncovered that the loss of *PHT1;8* and *PHT1;9* results in Fe-deficiency phenotype at the molecular level when Pi is plentiful, but this was insufficient to trigger acidification of the rhizosphere in response to Fe stress, particularly in the *pht1;8* and the *pht1;8 1;9* mutant. To test whether these changes are due to Pi starvation *per se* or due to indirect Fe excess, future experiments will need to be conducted with combined Fe and Pi

shortages and in hydroponic conditions, to avoid artefacts due to trace amounts of these nutrients from agar. Based on our findings we propose that BTS and PYE have broader roles and might be involved in Pi homeostasis by regulating the expression of *PHT1* and *PSI* genes, regardless of the Pi availability. Future research is needed to uncover the crosstalk at the level of Pi and Fe uptake and the *in planta* distribution of these nutrients in individual and combinatorial Pi-Fe deprivation.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Colombia was used in all experiments. The T-DNA insertion lines (Alonso et al., 2003) used in this experiments were: *pht1;8* (SAIL_116_G06), *pht1;9* (SALK_073614C) (Chapter 2), *bts-1* (SALK_016526) and *pye-1* (SALK_021217) (Long et al., 2010). Seeds were sterilized and plated on half-strength complete (MSP01) or Pi-free (MSP19) AGM (Caisson Labs) supplemented with 1% (w/v) sucrose, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES) and 4 g/L GelRite (Research Products International). For low Pi medium, KH_2PO_4 was added to a final concentration of 2 µM to the P-free AGM. Standard AGM supplemented with 1% (w/v) sucrose, 0.05% (w/v) MES and 4 g/L GelRite, 0.1 mM FeEDTA was added to the Fe-sufficient media and 300 µM ferrozine, an iron chelator, was added to the Fe-free media. Plates were kept at 4 °C in the dark for 4 days and transferred to a growth chamber at 22 °C with a 16 h light/8 h dark cycle.

Gene expression analysis by qRT-PCR

Total RNA was extracted using TRI reagent (Ambion) from roots of 14-day-old seedlings grown on Pi media for 7 days and transferred to +Pi or –Pi media for 7 days. The cDNA was synthesized from 0.7 µg of DNase I (New England Biolabs)-treated total RNA, using iScript cDNA synthesis kit (Bio-Rad). The synthesized cDNA was used as a template in quantitative Real-Time PCR (qRT-PCR) analysis. *UBIQUITIN 5* was used as an endogenous control. The primers sequences for *PSI* and Fe starvation genes used can be found in Table 1. A StepOnePlus Real-Time PCR system (Life Technologies) and iTaq Universal SYBR Green Supermix (Bio-Rad) was used. Relative expression was calculated and normalized with respect to *UBIQUITIN5* using the comparative cycle threshold method.

Rhizosphere acidification

Standard AGM supplemented with 1% (w/v) sucrose, 0.05% (w/v) MES and 4 g/L GelRite, 0.1 mM FeEDTA was added to the Fe-sufficient media and 300 µM ferrozine, an iron chelator, was added to the Fe-free media. Rhizosphere acidification assay was done as previously described (Yi and Guerinot, 1996). Seedlings were grown Fe sufficient media for 4 days and transferred to Fe deficient for additional 4 days. Three seedlings were grouped together and transferred for 24 h to a minimal media containing 0.006% (w/v) bromocresol purple and 0.2 mM CaSO4, the pH was adjusted to 6.5 with 1 M NaOH.

REFERENCES

Abadía, J., López-Millán, A.-F., Rombolà, A., Abadía, A. (2002) Organic acids and Fe deficiency: a review. Plant Soil, **241**(1), 75-86.

Abel, S. (2011) Phosphate sensing in root development. Curr Opin Plant Biol, **14**(3), 303-309.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., Ecker, J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science, **301**(5633), 653-657.

Ayadi, A., David, P., Arrighi, J.F., Chiarenza, S., Thibaud, M.C., Nussaume, L., Marin, E. (2015) Reducing the genetic redundancy of Arabidopsis PHOSPHATE TRANSPORTER1 transporters to study phosphate uptake and signaling. Plant Physiol, **167**(4), 1511-1526.

Barben, S.A., Hopkins, B.G., Jolley, V.D., Webb, B.L., Nichols, B.A., Buxton, E.A. (2011) Zinc, manganese and phosphorus interrelationships and their effects on the iron and copper in chelator-buffered grown Russet Burbank potato. J Plant Nutr, **34**(8), 1144-1163.

Barberon, M., Dubeaux, G., Kolb, C., Isono, E., Zelazny, E., Vert, G. (2014) Polarization of IRON-REGULATED TRANSPORTER 1 (IRT1) to the plant-soil interface plays crucial role in metal homeostasis. Proc Natl Acad Sci U S A, **111**(22), 8293-8298.

Barberon, M., Zelazny, E., Robert, S., Conéjéro, G., Curie, C., Friml, J., Vert, G. (2011) Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants. Proc Natl Acad Sci U S A, **108**(32), 450-458.

Bayle, V., Arrighi, J.F., Creff, A., Nespoulous, C., Vialaret, J., Rossignol, M., Gonzalez, E., Paz-Ares, J., Nussaume, L. (2011) *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell, **23**(4), 1523-1535.

Bournier, M., Tissot, N., Mari, S., Boucherez, J., Lacombe, E., Briat, J.F., Gaymard, F. (2013) Arabidopsis *ferritin 1* (*AtFer1*) gene regulation by the phosphate starvation response 1 (AtPHR1) transcription factor reveals a direct molecular link between iron and phosphate homeostasis. J Biol Chem, **288**(31), 22670-22680.

Briat, J.F., Rouached, H., Tissot, N., Gaymard, F., Dubos, C. (2015) Integration of P, S, Fe, and Zn nutrition signals in *Arabidopsis thaliana*: potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). Front Plant Sci, **6**, 290-305.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Perez-Perez, J., Solano, R., Leyva, A., Paz-Ares, J. (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS Genet, **6**(9), e1001102.

Colangelo, E. and Guerinot, M. (2004) The essential basic helix-loop-helix protein *FIT1* is required for the iron deficiency response. Plant Cell, **16**(12), 3400-3412.

Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., Benfey, P.N. (2008) Cell identity mediates the response of Arabidopsis roots to abiotic stress. Science, **320**(5878), 942-945.

Duan, K., Yi, K., Dang, L., Huang, H., Wu, W., Wu, P. (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. Plant J, **54**(6), 965-975.

Eide, D., Broderius, M., Fett, J., Guerinot, M.L. (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci U S A, **93**(11), 5624-5628.

González, E., Solano, R., Rubio, V., Leyva, A., Paz-Ares, J. (2005) PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in Arabidopsis. Plant Cell, **17**(12), 3500-3512.

Henriques, R., Jásik, J., Klein, M., Martinoia, E., Feller, U., Schell, J., Pais, M., Koncz, C. (2002) Knock-out of Arabidopsis metal transporter gene *IRT1* results in iron deficiency accompanied by cell differentiation defects. Plant Mol Biol, **50**(5), 587-597.

Hirsch, J., Marin, E., Floriani, M., Chiarenza, S., Richaud, P., Nussaume, L., Thibaud, M.C. (2006) Phosphate deficiency promotes modification of iron distribution in Arabidopsis plants. Biochimie, **88**(11), 1767-1771.

Huang, T.K., Han, C.L., Lin, S.I., Chen, Y.J., Tsai, Y.C., Chen, Y. R., Chen, J.W., Lin, W.Y., Chen, P.M., Liu, T.Y., Chen, Y.S., Sun, C.M., Chiou, T.J. (2013) Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. Plant Cell, **10**, 4044-4060.

Jones, D. (1998) Organic acids in the rhizosphere – a critical review. Plant Soil, **205**(1), 25-44.

Jost, R., Pharmawati, M., Lapis-Gaza, H., Rossig, C., Berkowitz, O., Lambers, H., Finnegan, P. (2015) Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in *Arabidopsis thaliana* through the application of phosphite. J Exp Bot, **66**(9), 2501-2514.

Kant, S., Peng, M., Rothstein, S.J. (2011) Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in Arabidopsis. PLoS Genet, **7**(3), e1002021.

Kobayashi, T., Nagasaka, S., Senoura, T., Itai, R.N., Nakanishi, H., Nishizawa, N.K. (2013) Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. Nat Commun 4: 2792

Kobayashi, T. and Nishizawa, N.K. (2014) Iron sensors and signals in response to iron deficiency. Plant Sci, **224**, 36-43.

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., Vansuyt, G., Curie, C., Schroder, A., Kramer, U., Barbier-Brygoo, H., Thomine, S. (2005) Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. Embo J, **24**(23), 4041-4051.

Lapis-Gaza, H.R., Jost, R., Finnegan, P.M. (2014) Arabidopsis PHOSPHATE TRANSPORTER1 genes *PHT1;8* and *PHT1;9* are involved in root-to-shoot translocation of orthophosphate. BMC Plant Biol, **14**, 334.

Lin, S.I., Chiang, S.F., Lin, W.Y., Chen, J.W., Tseng, C.Y., Wu, P.C., Chiou, T.J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiol, **147**(2), 732-746.

Liu, C., Muchhal, U.S., Raghothama, K.G. (1997) Differential expression of *TPS11*, a phosphate starvation-induced gene in tomato. Plant Mol Biol, **33**(5), 867-874.

Liu, T.Y., Aung, K., Tseng, C.Y., Chang, T.Y., Chen, Y.S., and Chiou, T.J. (2011). Vacuolar Ca2+/H+ transport activity is required for systemic phosphate homeostasis involving shoot-to-root signaling in Arabidopsis. Plant Physiol. **156**, 1176–1189.

Long, T.A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., Benfey, P.N. (2010) The bHLH transcription factor POPEYE regulates response to iron deficiency in Arabidopsis roots. Plant Cell, **22**(7), 2219-2236.

Marschner, H. (1995f) 6 - Mineral nutrition and yield response. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 184-200.

Marschner, H. (1995h) 8 - Functions of mineral nutrients: Macronutrients. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 229-312.

Marschner, H. (1995i) 9 - Functions of mineral nutrients: Micronutrients. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 313-404.

Marschner, H. (1995l) 12 - Diagnosis of deficiency and toxicity of mineral nutrients. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 461-479.

Marschner, H. (1995o) 15 - The soil-root interface (Rhizosphere) in relation to mineral nutrition. In Mineral Nutrition of Higher Plants (Second Edition) (H. Marschner, ed, Academic Press, London: 537-595.

Medici, A., Marshall-Colon, A., Ronzier, E., Szponarski, W., Wang, R., Gojon, A., Crawford, N.M., Ruffel, S., Coruzzi, G.M., Krouk, G. (2015) AtNIGT1/HRS1 integrates nitrate and phosphate signals at the Arabidopsis root tip. Nat Commun, **6**: 6274.

Misson, J., Raghothama, K.G., Jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., Doumas, P., Nacry, P., Herrerra-Estrella, L., Nussaume, L., Thibaud, M.-C. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci U S A, **102**(33), 11934-11939.

Misson, J., Thibaud, M.C., Bechtold, N., Raghothama, K., Nussaume, L. (2004) Transcriptional regulation and functional properties of Arabidopsis *Pht1;4*, a high affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. Plant Mol Biol, **55**(5), 727-741.

Muchhal, U., Pardo, J., Raghothama, K. (1996) Phosphate transporters from the higher plant *Arabidopsis thaliana*. Proc Natl Acad Sci U S A, **93** (19), 10519-10523.

Mudge, S., Rae, A., Diatloff, E., Smith, F. (2002) Expression analysis suggests novel roles for members of the *Pht1* family of phosphate transporters in Arabidopsis. Plant J, **31**(3), 341-353.

Narang, R., Bruene, A., Altmann, T. (2000) Analysis of phosphate acquisition efficiency in different Arabidopsis accessions. Plant Physiol, **124**(4), 1786-1799.

Niu, Y., Jin, G., Li, X., Tang, C., Zhang, Y., Liang, Y., Yu, J. (2015) Phosphorus and magnesium interactively modulate the elongation and directional growth of primary roots in *Arabidopsis thaliana* (L.) Heynh. J Exp Bot, **66**(13), 3841-3854.

Perea-Garcia, A., Garcia-Molina, A., Andres-Colas, N., Vera-Sirera, F., Perez-Amador, M.A., Puig, S., Penarrubia, L. (2013) Arabidopsis copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. Plant Physiol, **162**(1), 180-194.

Plaxton, W.C. and Tran, H.T. (2011) Metabolic adaptations of phosphate-starved plants. Plant Physiol, **156**(3), 1006-1015.

Poirier, Y. and Bucher, M. (2002) Phosphate transport and homeostasis in Arabidopsis. Arabidopsis Book, **1**, e0024.

Puga, M.I., Mateos, I., Charukesi, R., Wang, Z., Franco-Zorrilla, J.M., de Lorenzo, L., Irigoyen, M.L., Masiero, S., Bustos, R., Rodriguez, J., Leyva, A., Rubio, V., Sommer, H., Paz-Ares, J. (2014) SPX1 is a phosphate-dependent inhibitor of Phosphate Starvation Response 1 in Arabidopsis. Proc Natl Acad Sci U S A, **111**(41), 14947-14952.

Raghothama, K.G. (1999) Phosphate acquisition. Annu Rev Plant Physiol Plant Mol Biol, **50**, 665-693.

Raghothama, K.G. (2000) Phosphate transport and signaling. Curr Opin Plant Biol, **3**(3), 182-187.

Raghothama, K.G. and Karthikeyan, A.S. (2005) Phosphate acquisition. Plant Soil, **274**(1-2), 37-49.

Rai, V., Sanagala, R., Sinilal, B., Yadav, S., Sarkar, A., Dantu, P., Jain, A. (2015) Iron availability affects phosphate deficiency-mediated responses, and evidence of cross-talk with Auxin and Zinc in Arabidopsis. Plant Cell Physiol, **56**(6), 1107-1123.

Rausch, C. and Bucher, M. (2002) Molecular mechanisms of phosphate transport in plants. Planta, **216**(1), 23-37.

Remy, E., Cabrito, T., Batista, R., Teixeira, M., Sa-Correia, I., Duque, P. (2012) The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. New Phytol, **195**(2), 356-371.

Richardson, A., Lynch, J., Ryan, P., Delhaize, E., Smith, F., Smith, S., Harvey, P., Ryan, M., Veneklaas, E., Lambers, H., Oberson, A., Culvenor, R., Simpson, R. (2011) Plant and microbial strategies to improve the phosphorus efficiency of agriculture. Plant Soil, **349**(2), 121-156.

Robinson, N.J., Procter, C.M., Connolly, E.L., Guerinot, M.L. (1999) A ferric-chelate reductase for iron uptake from soils. Nature, **397**(6721), 694-697.

Rouached, H., Arpat, A.B., Poirier, Y. (2010) Regulation of phosphate starvation responses in plants: signaling players and cross-talks. Mol Plant, **3**(2), 288-299.

Rouached, H., Secco, D., Arpat, B., Poirier, Y. (2011) The transcription factor PHR1 plays a key role in the regulation of sulfate shoot-to-root flux upon phosphate starvation in Arabidopsis. BMC Plant Biol, **11**, 19.

Santi, S. and Schmidt, W. (2009) Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. New Phytol, **183**(4), 1072-1084.

Selote, D., Samira, R., Matthiadis, A., Gillikin, J., Long, T. (2015) Iron-binding E3 ligase mediates iron response in plants by targeting basic helix-loop-helix transcription factors. Plant Physiol, **167**(1), 273-286.

Shin, H., Shin, H.S., Chen, R., Harrison, M.J. (2006) Loss of *At4* function impacts phosphate distribution between the roots and the shoots during phosphate starvation. Plant J, **45**(5), 712-726.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: *Pht1;1* and *Pht1;4* play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J, **39**(4), 629-642.

Svistoonoff, S., Creff, A., Reymond, M., Sigoillot-Claude, C., Ricaud, L., Blanchet, A., Nussaume, L., Desnos, T. (2007) Root tip contact with low-phosphate media reprograms plant root architecture. Nat Genet, **39**(6), 792-796.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Poirier, Y., Nussaume, L. (2010) Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. Plant J, **64**(5), 775-789.

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J.-F., Curie, C. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell, **14**(6), 1223-1233.

Ward, J.T., Lahner, B., Yakubova, E., Salt, D.E., Raghothama, K.G. (2008) The effect of iron on the primary root elongation of Arabidopsis during phosphate deficiency. Plant Physiol, **147**(3), 1181-1191.

Yi, Y. and Guerinot, M.L. (1996) Genetic evidence that induction of root *Fe(III) chelate reductase* activity is necessary for iron uptake under iron deficiency. Plant J, **10**(5), 835-844.

Zheng, L., Huang, F., Narsai, R., Wu, J., Giraud, E., He, F., Cheng, L., Wang, F., Wu, P., Whelan, J., Shou, H. (2009) Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings. Plant Physiol, **151**(1), 262-274.

Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., Zhong, W., Wu, P. (2008) *OsPHR2* is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. Plant Physiol, **146**(4), 1673-1686.

TABLES AND FIGURES

Figure 1. Expression of Fe stress markers in WT *pht1;8*, *pht1;9* and *pht1;8 1;9* at sufficient and low Pi levels.

Quantitative RT-PCR (qRT-PCR) analysis of *FIT1*, *IRT1* and *AHA2* in roots of WT (Col-0), *pht1;8*, *pht1;9* and *pht1;8 1;9* mutants. Total RNA was extracted from roots of 14-day-old seedlings grown for 7 days on Pi sufficient 0.5 x MS media (1 mM, +Pi) and transferred for another 7 days to either +Pi or low Pi media containing 2 µM Pi (-Pi). Relative expression levels were normalized with respect to *UBIQUITIN5* level in the WT at +Pi or –Pi treatment. Data corresponds to the mean of three biological replicates ± SE. Each biological replicate was calculated from three technical replicates.

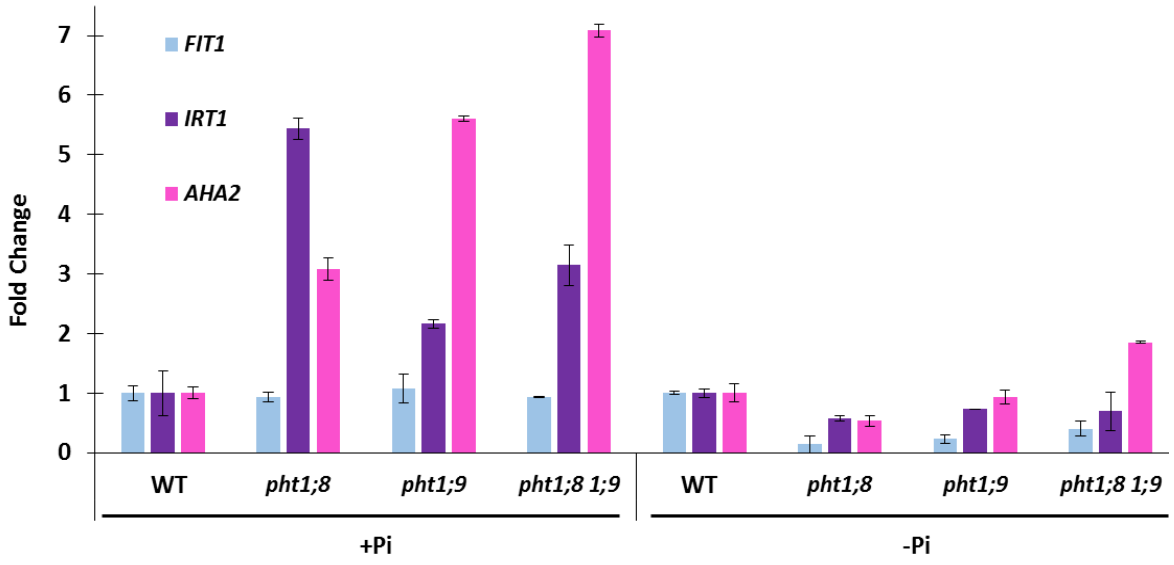


Figure 2. Expression of *PHT1* genes in WT, *bts* and *pye mutants* at regular and low Pi.

qRT-PCR analysis of *PHT1;1*, *PHT1;4*, *PHT1;8* and *PHT1;9* transcripts in roots of WT, *bts*, and *pye* mutants. Total RNA was extracted from roots of 14-day-old seedlings grown for 7 days on Pi sufficient 0.5 x MS media (1 mM, +Pi) and transferred for another 7 days to either +Pi or low Pi media containing 2 µM Pi (-Pi). Relative expression levels were normalized with respect to *UBIQUITIN5* expression in WT at +Pi or -Pi. Data corresponds to the mean of technical triplicates for one biological replicate ± SE.

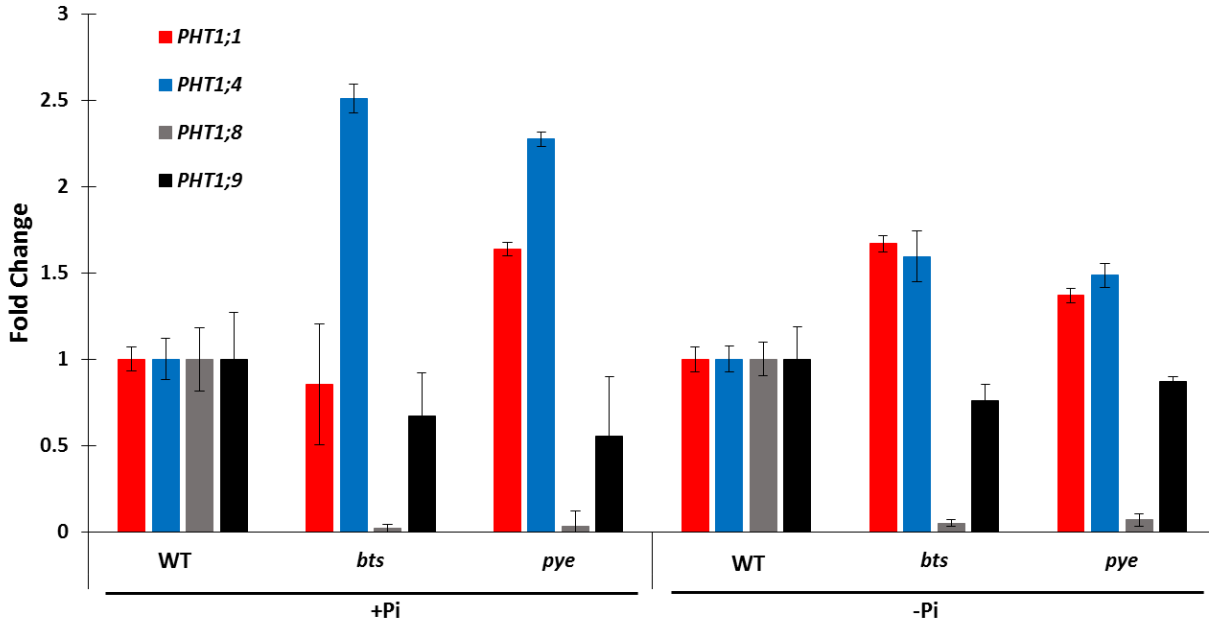


Figure 3. Expression of Pi starvation markers in WT, *bts* and *pye* mutants at regular and low Pi.

qRT-PCR analysis of *SPX1*, *IPS1* and *At4* transcripts in roots of WT, *bts*, and *pye* mutants. Total RNA was extracted from roots of 14 days seedlings grown for 7 days on Pi sufficient 0.5x MS media (1 mM, +Pi) and transferred for another 7 days to either +Pi or low Pi media containing 2 µM Pi. Relative expression levels were normalized with respect to *UBIQUITIN5* expression in WT at +Pi or -Pi. Data corresponds to the mean of technical triplicates for one biological replicate ± SE.

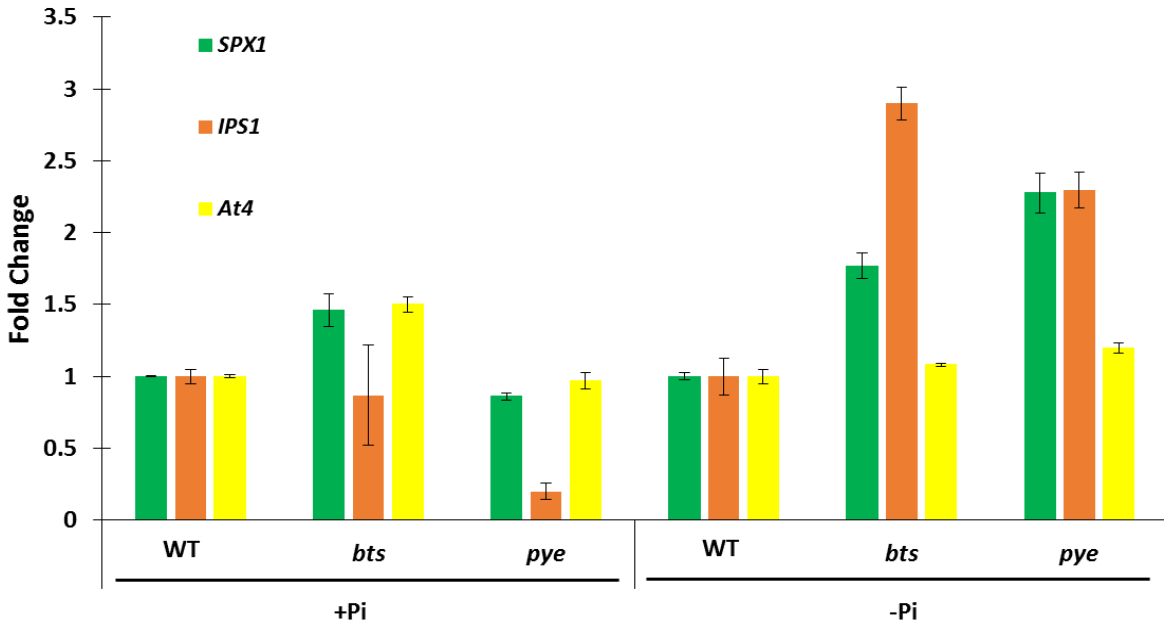


Figure 4. Expression of *PHT1* and *PSI* genes in WT at sufficient and low Fe.

qRT-PCR analysis of *PHT1;1*, *PHT1;4*, *SPX1*, *IPS1* and *At4* transcripts in roots of WT, under Fe sufficient and Fe-deficient conditions. Total RNA was extracted from roots of 14-day-old seedlings grown for 7 days on Fe sufficient media and transferred for additional 7 days on media with (+Fe) or without Fe (-Fe). Relative expression levels were normalized with respect to *UBIQUITIN5* expression in WT at +Fe. Data corresponds to the mean of biological duplicates ± SE. Each biological replicate was calculated from three technical replicates.

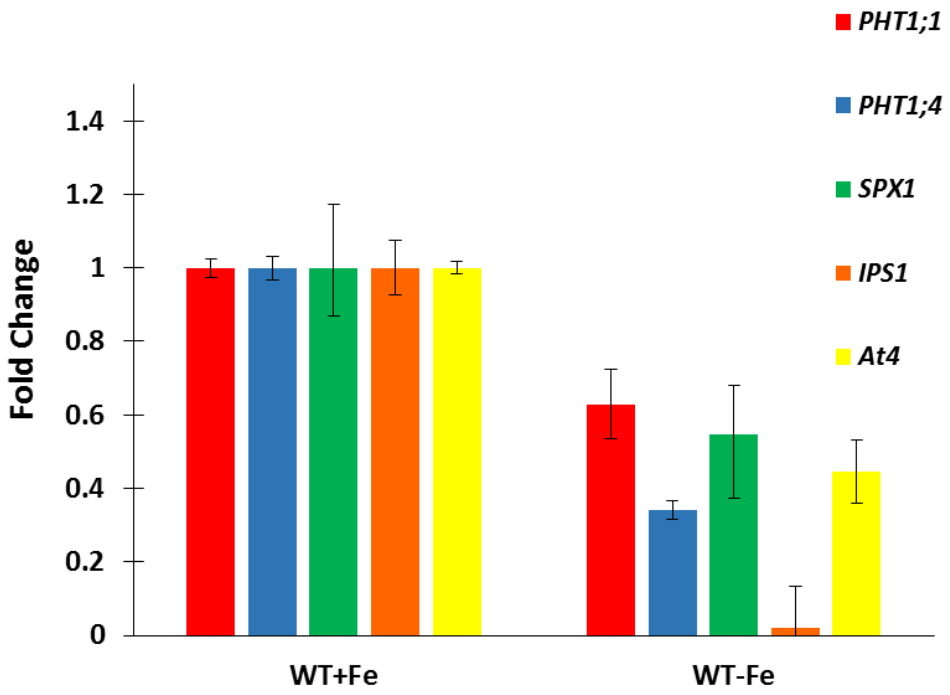


Figure 5. *pht1;8* and *pht1;8 1;9* double mutants have a more alkaline rhizosphere relative to WT under regular and low Fe conditions.

(A and B) Rhizosphere acidification response of WT, *pht1;8*, *pht1;9* and *pht1;8 1;9* to iron stress. Eight-day-old seedlings grown on Fe sufficient media for 4 days were transferred to either Fe-sufficient (A) or Fe-deficient (B) media (with Ferrozine) for 4 days, grouped in 3 and transferred to minimal media containing Bromocresol Purple for 24 h. Purple color of the media indicates alkaline pH, while yellow color indicates pH below 5.2.

Images are representative of two replicated experiments.

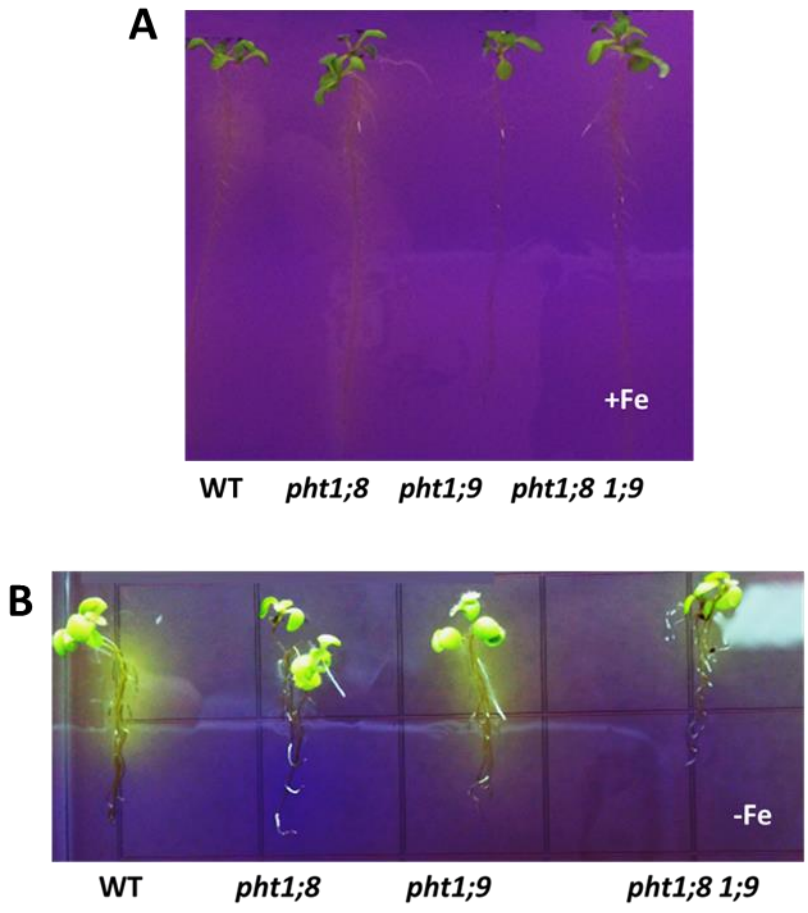


Table 1. List of primers used for qRT-PCR.

Primer Name	Primer Sequence	References
FIT1-q1F	GGAGAAGGTGTTGCTCCATCTC	
FIT1-q1R	TAAGCTCTGTTCGAAGCATGTCC	
IRT1-q1F	GCGATACCGAGTCATTGCCATG	
IRT1-q1R	CCCATGCCTTCGAACATTTGATGG	
AHA2-q1F	GCTAAGAGAAGAGCTGAGATCGCTAG	
AHA2-q1R	CTACACAGTGTAGTGACTGGGAGTTTC	
IPS1-q1F	TAAGGAAAGCGTTTTAAGATATGG	(Thibaud et al., 2010)
IPS1-q1R	TTCACTATAAAGAGAATCGGAAGC	
SPX1-q1F	GATTCCATTGTTGGAGCAAGA	(Liu et al., 2011)
SPX1-q1R	AATCTGTTAGCTTCTTCTATTGTA	
At4-q1F	CCGTGTTTACTTTGTTTCGGT	(Lin et al., 2011)
At4-q1R	AGGAACACACCTGAATGGTGC	
PHT1;8-q1F	GCTCTTCCTGCTGCATTGACG	
PHT1;8-q1R	AGGAGGTGGATCCGTCGTGGCTT	
PHT1;9-q1F	CCGCCAGATACACAGCATTGGT	
PHT1;9 -q1R	CGACCGTGGAGACTGAGGAAAC	
PHT1;1-q1F	CTTGTGCTCTGTCGCTTCT	
PHT1;1-q1R	AGTCACCTCCAATACCAAATCC	
PHT1;4-q1F	GGTTCACAGTTGCGTTCATC	
PHT1;4-q1R	TGGTTGTAAGGAATAGCCAGAG	
UBQ5-F	GACGCTTCATCTCGTCC	
UBQ5-R	CCACAGGTTGCGTTAG	