



REVIEW PAPER

Replace, reuse, recycle: improving the sustainable use of phosphorus by plants

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† Professor Baldwin sadly passed away during the preparation of this review.

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Abstract

The 'phosphorus problem' has recently received strong interest with two distinct strands of importance. The first is that too much phosphorus (P) is entering into waste water, creating a significant economic and ecological problem. Secondly, while agricultural demand for phosphate fertilizer is increasing to maintain crop yields, rock phosphate reserves are rapidly declining. Unravelling the mechanisms by which plants sense, respond to, and acquire phosphate can address both problems, allowing the development of crop plants that are more efficient at acquiring and using limited amounts of phosphate while at the same time improving the potential of plants and other photosynthetic organisms for nutrient recapture and recycling from waste water. In this review, we attempt to synthesize these important but often disparate parts of the debate in a holistic fashion, since solutions to such a complex problem require integrated and multidisciplinary approaches that address both P supply and demand. Rapid progress has been made recently in our understanding of local and systemic signalling mechanisms for phosphate, and of expression and regulation of membrane proteins that take phosphate up from the environment and transport it within the plant. We discuss the current state of understanding of such mechanisms involved in sensing and responding to phosphate stress. We also discuss approaches to improve the P-use efficiency of crop plants and future direction for sustainable use of P, including use of photosynthetic organisms for recapture of P from waste waters.

Key words: Fertilizers, nutrient recycling, membrane transporters, phosphate, phosphate signalling, transcription factors.

Introduction

Phosphate is a non-substitutable plant nutrient, essential for global agriculture. There are two key reasons why the sustainable use of phosphate is of importance: the supply is running out, and paradoxically much of what is produced is wasted and results in environmental damage. Rock phosphate is crucial for the production of inorganic phosphate fertilizers, but

reserves are finite and the supply is expiring rapidly (Cooper and Carliell-Marquet, 2013). In 2010, global extraction was around 176 Mt, and demand is increasing, with global peak phosphorus (P) use expected to be reached by 2030 (Cordell *et al.*, 2009). The best estimates for the longevity of reserves are around 200 years and the worst are 50 years (Rosemarin

Abbreviations: AMF, arbuscular mycorrhizal fungi; ER, endoplasmic reticulum; GFP, green fluorescent protein; miRNA, microRNA; OA, organic acids; P, phosphorus; PAE, amount of P taken up as a function of biomass; Pi, inorganic phosphate; PSI, phosphate starvation inducible; PUE, amount of productivity or yield per unit P; QTL, quantitative trait locus; TF, transcription factor.

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et al., 2011). Moreover, the process of mining rock phosphate and manufacturing fertilizer is expensive and energy intensive (Elser and Bennett, 2011).

In 2013, the UK imported and consumed approximately 140 000 t of phosphate, with 86 000 t used for crop fertilizer and animal feeds (Cooper and Carliell-Marquet, 2013). It is estimated that 2–3 t of phosphate per million people per day enters the UK's watercourses as treated sewage where it is lost to the environment (Kato *et al.*, 2007) and can impact negatively on ecosystems. This equates to 70 000 t or half the country's annual requirement. Prices for diammonium phosphate fertilizer in 2014 were US\$500 t⁻¹ (Argus, 2014), leading to the potential loss of US\$35 million (£22 million) every year.

The majority of phosphate inputs to the environment are from land application as fertilizers (Smil, 2000), animal-generated wastes (Goopy and Murray, 2003) and waste water from human conurbations (organic waste and detergents). These inputs supply waste-water treatment plants with concentrations of dissolved phosphate that is difficult and expensive to remove (Britton *et al.*, 2005), yet provide a potential supply of this resource. P is an essential element in many cellular macromolecules such as nucleic acids, phospholipids, and metabolites such as nucleoside triphosphates and phosphorylated intermediates in many biochemical pathways, and therefore the capacity to replace P (as phosphate) is limited. Consequently, the key to sustainability must be to reuse and recycle P efficiently both within the environment (Elser and Bennett, 2011) and within the plant (Veneklaas *et al.*, 2012). Although several excellent reviews are available on efficient utilization of P nutrition for sustainable crop production (Raghothama, 1999; Rouached *et al.*, 2010; Chiou and Lin, 2011; Nussaume *et al.*, 2011; Richardson *et al.*, 2011; Lopez-Arredondo *et al.*, 2014; Zhang *et al.*, 2014), in this article we present a more holistic view that considers the potential to apply recently developed molecular understanding of plant phosphate responses to reducing crop phosphate requirements and environmental phosphate remediation.

Chemical and biological technologies for capturing phosphate

Methods employed in capturing phosphate from waste outputs depend on available space, cost, and the load applied. In many cases, the addition of metal salts such as Al₂(SO₄)₃, CaCl₂, and FeCl₃ is used to precipitate out the P (de-Bashan and Bashan, 2004). Struvite (NH₄MgPO₄·6H₂O) formation is an alternative method used for nutrient recovery from anaerobic digestates (Britton *et al.*, 2005). These technologies have been used for many years with variable success in achieving low phosphate discharges (<1 mg of P l⁻¹) but carry the burden of cost variations due to fluctuating prices of iron, magnesium, and aluminium (Vidal, 2008; Farchy, 2013). A further issue to tackle when adding these salts is the discharge consent on the salts themselves: high concentrations of iron are not permitted, as it can cause as much harm to the environment as high concentrations of phosphate. In an attempt to tackle the issues surrounding the chemical removal

of phosphates, in recent years much research has been carried out employing biological alternatives.

Phosphate can be removed from waste streams via several different biological methods. These include microbiological, algal, plants (terrestrial and aquatic), and combinations of these. Some are energy-requiring processes and some are not. As well as nutrient removal capacities, biological methods often provide extra benefits such as production of bioenergy crops and animal fodder. Here, we focus on the potential for plant-based remediation.

Microalgae such as *Chlorella* sp. or *Scenedesmus* sp. can be utilized to remove phosphate from wastes (Larsdotter, 2006). Systems include waste-water ponds used for nutrient capture (Chopin *et al.*, 2012) or photobioreactors, which are generally more focused on maximal biomass generation (Michels *et al.*, 2014). The latter tubular systems are energy intensive (artificial lights and temperature control in laboratory settings), while the former makes use of solar energy. While algal and mixed bacterial/algal assemblages have been shown to capture high concentrations of phosphates (Muñoz and Guieysse, 2006), a drawback is the difficulty of harvesting, which can prove uneconomical (Michels *et al.*, 2014).

Terrestrial and aquatic (rooted and free-floating) higher plants (and combinations of these) can be implemented for the capture of several compounds including phosphates (Vermaat and Khalid Hanif, 1998). Waste-water stabilization ponds on farmland and constructed and engineered wetlands as well as constructed tanks for phytoremediation, are all employed globally. Water hyacinth, knotgrass, and cattail can all be grown to capture nutrients in natural or managed wetlands (Fedler and Duan, 2011). Floating macrophytes such as duckweed (E.g. *Lemna* sp. or *Spirodela* sp.) have also shown promise in the uptake of phosphates from waste water, in large-scale batch or variable-flow-rate tank systems (Alaerts *et al.*, 1996; Abuaku *et al.*, 2006; Farrell, 2012). The large quantities of phytobiomass produced by phytoremediative systems (Verma and Suthar, 2014) generally have beneficial by-products that can be used as energy sources such as biogas or biodiesel (Fujita *et al.*, 1999), or as feed for fish or cattle (Goopy and Murray, 2003). The other obvious advantage of using plants in outdoor settings to recapture phosphate is that they are solar powered. However, studies are often descriptive in nature and difficult to compare in terms of efficacy, as very different systems, organisms and conditions have been used, and often control over important variables is lacking, especially in low-cost open systems. Where more controlled studies are performed, results are frequently extrapolated from a small scale to t ha⁻¹ with the associated potential for multiplication of errors. Nevertheless, the drawbacks of chemical removal practices and the energy inputs required by some biological phosphate removal processes highlight the benefits of low-energy phytoremediation. The beneficial by-products from plant nutrient capture systems must also not be overlooked. A clearer understanding of the molecular mechanisms of phosphate uptake in plants would provide great benefits, not least in their manipulation for greater and more reliable phosphate capture from high P waste waters, as well

as the converse goal of maintaining crop plant productivity with reduced P inputs.

Plant responses to low phosphate

Plants operate molecular signalling networks to detect and respond to inorganic phosphate (Pi) starvation. Many recent studies have helped to underpin the molecular signalling networks involved in P homeostasis (reviewed by [Chiou and Lin, 2011](#)). Plants sense and respond to the Pi status both locally and systemically, with separate molecular mechanisms being involved in local and long-distance Pi signalling to maintain homeostasis under Pi starvation ([Thibaud et al., 2010](#); [Lin et al., 2014](#); [Lopez-Arredondo et al., 2014](#)). Typical levels of Pi in soils are low micromolar concentrations, whereas levels in the cytosol of plants under optimal conditions are millimolar, requiring the ability to acquire and buffer cytosolic Pi at concentrations three orders of magnitude above that in the environment. Plants respond to low-P stress in a number of ways ([Fig. 1](#)). These include: release of Pi from vacuolar stores, for example; remodelling of membranes to reduce reliance on phospholipids (reviewed by [Nakamura, 2013](#)), and redistribution of Pi from old(er) source tissues to young, actively growing sink tissues. Remodelling the root system increases the surface area for Pi uptake. Moreover, the secretion of organic acids (OAs) increases Pi solubility, especially in acidic soils, and the secretion of phosphatases releases Pi from soil organic matter. The majority of plant species form mutualistic associations with soil micro-organisms, especially with arbuscular mycorrhizal fungi (AMF), expanding the volume of soil that can be explored and allowing interchange of nutrients in both directions. Membrane proteins are central to many of these adaptations, and examples to be explored in this review are members of the PHT1 family, which are important in both acquisition of Pi from the soil and its recycling within the plant, members of the PHOSPHATE1 (PHO1) family, some of which are involved in export of Pi from roots to shoots, and membrane proteins involved in secretion of OAs. The elaborate machinery that regulates these (and other phosphate-response genes)

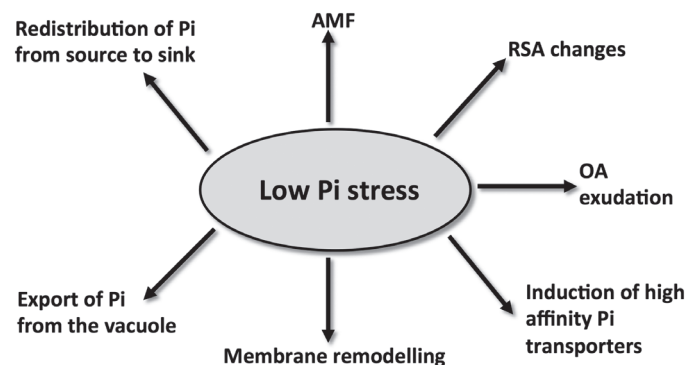


Fig. 1. Schematic representation of plant responses to low Pi stress. Plants respond in multiple ways to low Pi. Some of these responses are local, while others are systemic. Some respond to external Pi concentration, while others respond to internal Pi levels. AMF, arbuscular mycorrhizal fungi; OA, organic acids; RSA, root system architecture.

at multiple levels from transcription through to protein location and stability is also discussed below.

Transcriptional regulation of P responses

PHR1 and its regulatory network

PHOSPHATE STARVATION RESPONSE 1 (PHR1) belongs to the MYB family of DNA-binding proteins and is a major transcription factor (TF) involved in Pi signalling ([Fig. 2](#)). It binds to the phosphate -starvation-related regulatory element [PHR1-binding site (P1BS)] motif (GNATATNC) in the promoter region of Pi-stress-responsive genes ([Rubio et al., 2001](#)). PHR1 is localized in the nucleus, and a SUMO E3 ligase (SIZ1) is known to control Pi homeostasis at the post-translational level through sumoylation of PHR1 ([Miura et al., 2005](#)). PHR1 is involved in the activation of multiple P-starvation-inducible genes including *PHOSPHATE TRANSPORTER1* (*PHT1*), *PHO1*, *At4* and microRNA399 (miRNA399) ([Rubio et al., 2001](#); [Shin et al., 2006](#); [Chen A et al., 2011](#)). miRNA399 has been implicated in Pi-starvation-related signalling in many plants ([Lin et al., 2008](#); [Pant et al., 2008](#); [Liu et al., 2010](#); [Liu and Vance, 2010](#); [Xu et al., 2013](#)) by regulating the levels of *PHO2* mRNA, which produces ubiquitin-conjugating enzyme E2 24 (UBC24; [Pant et al., 2008](#)). Some of these molecules move within the vasculature and therefore function as systemic signals integrating activities in different tissues ([Lin et al., 2014](#)).

PHR1 both regulates and is in turn regulated by SPX domain proteins ([Secco et al., 2012](#)) ([Fig. 2](#)). These proteins are strongly involved in Pi-starvation responses. The transcript abundance of *AtSPX1–AtSPX3* is significantly enhanced while the expression level of *AtSPX4* is reduced to half of that before Pi deprivation ([Duan et al., 2008](#)). Regulation of the *AtSPX* genes was shown to be controlled by PHR1, with *AtSPX1* being proposed as a transcriptional regulator, given its nuclear localization and capacity for upregulating the expression of downstream phosphate-starvation-inducible (PSI) genes when overexpressed ([Duan et al., 2008](#)). However, recent studies have shown that, instead of directly regulating PSI gene expression, *AtSPX1/AtSPX2* are involved in the formation of a protein complex with *AtPHR1* in a Pi-dependent manner ([Puga et al., 2014](#)). Upon Pi starvation, the interaction between *AtSPX1/AtSPX2* and *AtPHR1* is replaced by the binding of *AtPHR1* to the P1BS from PSI genes, thus activating the expression of these genes ([Puga et al., 2014](#)). A similar Pi-dependent interaction between *OsSPX1/OsSPX2* and *OsPHR2* was also detected in rice (*Oryza sativa*) ([Wang et al., 2014](#)). *OsPHR2* is also regulated post transcriptionally by *OsSPX4*, which binds to and prevents its translocation into the nucleus under high-Pi conditions. However, under low-Pi conditions, *OsSPX4* is degraded by the proteasome, allowing *OsPHR2* to traffic to the nucleus and activate gene expression ([Lv et al., 2014](#)). Given the fact that transcription of *PHR1/PHR2* is not greatly influenced by Pi levels, these observations indicate a Pi sensing and signalling function of SPX proteins, although further research is needed to clarify how the Pi level affects the interaction between SPX proteins and *PHR1/*

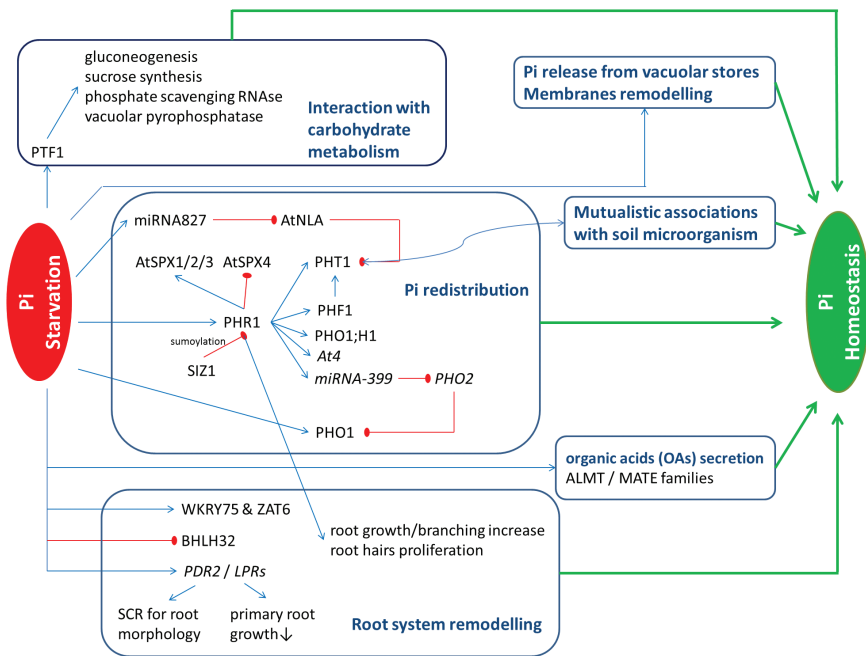


Fig. 2. Regulation and control of key genes in the model plant *Arabidopsis* during phosphate (Pi) starvation responses. Blue arrowheads and red blunt-ended lines show positive and negative regulation, respectively. In the presence of lowered environmental Pi concentrations, root system remodelling is introduced, followed by the upregulation of high-affinity Pi transport systems (PHT1s) to increase Pi uptake from the soil, while specialized transporters (AtPHO1 and AtPHO1;H1) are induced for the movement of Pi within the plant. A rigorous regulation system consisting of the key transcriptional factor PHR1, post-transcriptional regulation by non-coding RNAs, and post-translational regulation by protein trafficking and degradation is also involved in the functional integration of such transporters in the response to Pi starvation.

PHR2. The functional similarities of SPX proteins between monocotyledonous and dicotyledonous plants also suggest that the highly conserved SPX domain could be of great significance in a prevalent Pi sensing and signalling pathway.

Transgenic manipulation of PHR1

Several studies have looked at the impact of overexpressing *AtPHR1* of *Arabidopsis* (Nilsson et al., 2007), *ZmPHR1* of maize (Wang X et al., 2013), *OsPHR2* of rice (Zhou et al., 2008), *BnPHR1* of oil seed rape (Ren et al., 2012), and *TaPHR1-A1* of wheat (Wang J et al., 2013). These studies all observed upregulation at the transcriptional level of several low-phosphate-response genes such as phosphate transporters and the non-coding RNA miRNA399, and corresponding downregulation of *PHO2*, and showed increased levels of Pi in tissues. In several of these studies, the *PHR1*-overexpressing plants showed improved growth under low-Pi conditions (Zhou et al., 2008; Ren et al., 2012; Wang J et al., 2013; Wang X et al., 2013). In some studies, reduced plant growth and performance and Pi toxicity symptoms were observed under high-phosphate growth conditions (Nilsson et al., 2007; Zhou et al., 2008; Ren et al., 2012) but not in others (Wang J et al., 2013; Wang X et al., 2013). This is perhaps not surprising as overexpressing some of the Pi-starvation-responsive genes that are downstream of PHR1 such as *OsmiR399* (Hu et al., 2011) and *OsSPX1* (Wang C et al., 2009) caused Pi toxicity in transgenic plants. In all cases, constitutive strong promoters (35S or maize ubiquitin) were used for overexpression of *PHR1* and the level of overexpression was determined by measuring transcript

abundance. Since active PHR1 is controlled primarily at the post-transcriptional level, this may not be a reliable method of estimating the true level of transcriptionally active PHR1. In studies where growth inhibition at high Pi was not reported, tissue levels of Pi showed only relatively modest increases. The beneficial effects of *PHR1* overexpression included increased root growth/branching (Wang C et al., 2013) and proliferation of root hairs (Zhou et al., 2008).

Other transcription factors

Other TFs involved in P signalling are WKRY75, ZAT6, BHLH32, PTF1, MYB2P-1, and MYB62 (reviewed by Lopez-Arredondo et al., 2014) (Fig. 2). Both WKRY75 and ZAT6 are upregulated during Pi starvation and are found to be involved in regulating the modification of root architecture (Devaiah et al., 2007a, b). In contrast, the *BHLH32* TF is downregulated during Pi starvation and has been found to be associated with the modifications of root architecture and carbon metabolism in response to Pi stress (Chen ZH et al., 2007).

Overexpression of *OsMYB2P-1* conferred Pi-starvation tolerance in rice (Dai et al., 2012). Transgenic plants had shorter roots than wild-type controls on P-sufficient medium and longer roots and more tillers on Pi-deficient medium. The *OsMYB2P-1*-overexpressing plants had retarded growth and lower biomass on high Pi but better growth than the wild type on low Pi (Dai et al., 2012). As with *PHR1* overexpressing plants, the *OsMYB2P-1*-overexpressing transgenics had enhanced expression of Pi-responsive genes including *IPS* and *miRNA399* in both Pi-sufficient and -deficient conditions.

PHO2 was repressed and *OsPT2* was upregulated under Pi-deficient conditions, and the transgenics had increased Pi levels compared with the wild type (Dai *et al.*, 2012).

Transgenic plants overexpressing rice *PHOSPHATE STARVATION-INDUCED TRANSCRIPTION FACTOR 1* (*OsPTFI*) showed improved growth and yield characteristics in hydroponics, pots, and the field. At low Pi, root and shoot biomass and Pi content were higher, as were the number of tillers, reproductive development, and yield (Yi *et al.*, 2005). Overexpression of maize *ZmPTFI* also showed improved tolerance to Pi starvation and resulted in increased partitioning of carbohydrate to the roots, leading to larger root biomass on low Pi (Li *et al.*, 2011). Interestingly, overexpression of *PTFI* upregulated a different set of genes to those under *PHR1* control and included genes involved in gluconeogenesis (phosphoenolpyruvate carboxykinase) and sucrose synthesis (sucrose synthase 2), as well as phosphate-scavenging RNase and vacuolar pyrophosphatase (Yi *et al.*, 2005; Li *et al.*, 2011). These results emphasize the interaction between phosphate levels and carbohydrate metabolism, and indicate the importance of carbohydrate supply to maintain growth under low-Pi stress. Sugars are hence another important group of metabolites involved in Pi-starvation-related signalling that influence the expression of many Pi-stress-related genes in a number of species (Liu *et al.*, 2005; Karthikeyan *et al.*, 2007; Hammond and White, 2008; Hernandez *et al.*, 2009).

Other regulatory genes

It is well established that an important response to Pi stress is through changes in root architecture. Plants produce more lateral roots and root hairs in response to Pi stress, which expands the adsorptive area in the soil (reviewed by Rouached *et al.*, 2010). The phenotypic changes in root architecture are genotype dependent and have been shown to be important for overcoming Pi stress in bean, soybean, maize, and barley (reviewed by Zhang *et al.*, 2014). Key regulatory genes involved in Pi-starvation-associated signalling linked to root system architecture changes are *LOWPHOSPHATE ROOT* (*LPR1*, *LPR2*, and *LPR3*) and the *PHOSPHATE DEFICIENCY RESPONSE 2* (*PDR2*) genes (Fig. 2). Both *LPR* and *PDR2* are involved in root architecture modification in response to Pi starvation. *LPR* genes encode multi-copper oxidases expressed in the meristematic regions of the root tip, including the root cap, and have been demonstrated to reduce the primary root growth capacity under Pi starvation (Svistoonoff *et al.*, 2007). *PDR2* encodes a P5-type ATPase that functions in the endoplasmic reticulum (ER) and is involved in close monitoring of Pi status around the roots (Ticconi *et al.*, 2004). *PDR2* is essential for the expression of *SCARECROW* (*SCR*), a key regulator for root morphology during Pi starvation (Ticconi *et al.*, 2009). In the root tip, both *PDR2* and *LPR1* function to sense the external Pi status and regulate the root architecture through an ER-resident pathway (Rouached *et al.*, 2010). Interactions with auxin and sugar signalling are also of critical importance in the modulation of root architecture in response to phosphate deprivation (reviewed by Rouached *et al.*, 2010).

PHT1 phosphate transporters

P enters into the plant as Pi via plasma membrane transporters of the PHOSPHATE TRANSPORTER1 (PHT1) family, and the process is affected by soil pH, which influences the predominant form of Pi (HPO_4^{2-} or H_2PO_4^- available (Schachtman *et al.*, 1998). Following the first identification and characterization of PHT1 family members in *Arabidopsis* (Muchhal *et al.*, 1996), subsequent PHT1 members have been characterized in many plants including potato, white lupin, tomato, Madagascar periwinkle, barrel medic, barley, tobacco, rice, maize, and wheat (Table 1) (Nussaume *et al.*, 2011).

The PHT1 proteins belong to the major facilitator superfamily (MFS), which is the largest superfamily of active transporters, and these are generally symporters or antiporters driven by proton or sodium gradients. The PHT1 proteins are predicted to contain 12 transmembrane α -helices divided into two domains (N and C) of six transmembrane helices each (Karandashov and Bucher, 2005). The PHT1s are encoded by a family of genes found in each plant species; for example, the *Arabidopsis* genome contains nine genes (Mudge *et al.*, 2002), rice has 13 genes (Paszowski *et al.*, 2002), soybean has 14 genes (Fan *et al.*, 2013), and barley (Rae *et al.*, 2003) and foxtail millet (Ceasar *et al.*, 2014) contain 12 genes each. The first crystal structure of a eukaryotic fungal (*Piriformospora indica*) high-affinity phosphate transporter was recently solved at 2.9 Å in an inward-facing occluded state (Pedersen *et al.*, 2013). Pi is located between the two domains buried in the middle of the membrane at a location similar to the substrate-binding sites in other major facilitators. The same study also proposed a model for the mechanism of Pi import into the cell (Pedersen *et al.*, 2013).

PHT1 proteins transport Pi into the epidermal cortical cells of the root via a proton–Pi co-transport mechanism (Ullrich and Novacky, 1990). Different members of the *PHT1* gene family show different patterns of expression with respect to tissue and phosphate status (reviewed by Nussaume *et al.*, 2011). The *PHT1* genes have been found to be expressed predominantly in roots, especially in epidermal cells and the outer cortex of the root hair (Mudge *et al.*, 2002; Misson *et al.*, 2004; Schunmann *et al.*, 2004; Xiao *et al.*, 2006). For example, eight out of nine PHT1s in *Arabidopsis* have been found to be expressed in roots (Karthikeyan *et al.*, 2002; Mudge *et al.*, 2002). Furthermore, localization studies on these transporters in different plant species confirmed that PHT1 is most specifically targeted to the plasma membrane (Gonzalez *et al.*, 2005; Bayle *et al.*, 2011; Jia *et al.*, 2011; Preuss *et al.*, 2011; Fan *et al.*, 2013). In addition, members of the PHT1 family have been found to be expressed in aerial parts including shoot, leaves, and flowers, suggesting their involvement in both acquisition and remobilization of Pi in the plant. For example, in *Arabidopsis*, AtPHT1;5 is involved in removing Pi from senescing leaves (Nagarajan *et al.*, 2011), and AtPHT1;6 has been found to be expressed in pollen (Karthikeyan *et al.*, 2002; Mudge *et al.*, 2002).

The PHT1s show a range of affinities for Pi and are divided into high- and low-affinity transporters. The affinities of

Table 1. PHT1 genes upregulated by low Pi in different plants and their affinities

Some of the PHT1s reported to be induced by Pi starvation are listed along with the name of the host plant, site of expression, and affinities of the known transporters. The expression patterns of these transporters have been analysed by reverse transcription PCR, quantitative real-time PCR and promoter β -glucuronidase or green fluorescent protein fusion studies.

PHT1 gene	Plant	Affinity	Site of induction by low Pi	Reference
AtPHT1;1	<i>Arabidopsis</i>	High	–	Mitsukawa et al. (1997)
AtPHT1;7, AtPHT 1;8, AtPHT1;9	<i>Arabidopsis</i>	–	Root	Mudge et al. (2002)
CfPHT1;1, CfPHT1;2, CfPHT1;3, CfPHT1;4, CfPHT1;5	Cayenne pepper	–	Only in AMF inoculated roots	Chen A et al. (2007)
GmPHT1;1 to GmPHT1;12	Soybean	–	Root	Fan et al. (2013)
GmPHT1;1, GmPHT1;2, GmPHT1;5, GmPHT1;7, GmPHT1;10	Soybean	High	Root	Fan et al. (2013)
HvPHT1;1	Barley	High	Root	Rae et al. (2003)
HvPHT1;6	Barley	Low	Moderately induced in root and shoot	Rae et al. (2003)
HvPHT1;9	Barley	–	Roots	Huang et al. (2011)
MtPHT1;1	Barrel medic	Low	–	Liu et al. (1998b)
OsPHT1;2, OsPHT1;6	Rice	Low	–	Ai et al. (2009)
OsPHT1;8	Rice	High	Root	Jia et al. (2011)
OsPHT1;8	Rice	High	Shoot	Secco et al. (2013)
PtaPHT1;1, PtaPHT1;2, PtaPHT1;3, PtaPHT1;7	Hardy orange	–	Roots	Shu et al. (2012)
PvPHT1;2	Kidney bean	–	Roots	Tian et al. (2007)
SiPHT1;2	Foxtail millet	–	Leaf	Ceasar et al. (2014)
SiPHT1;4	Foxtail millet	–	Root	Ceasar et al. (2014)
SIPHT1;1, SIPHT1;2	Tomato	–	Roots	Liu et al. (1998a)
SmPHT1;1, SmPHT1;2, SmPHT1;3, SmPHT1;4, SmPHT1;5	Eggplant	–	Leaf and roots	Chen A et al. (2007)
StPHT1;2	Potato	Low	Roots	Leggewie et al. (1997)
ZmPHT1;1, ZmPHT1;2, ZmPHT1;3, ZmPHT1;6,	Maize	–	Root and leaf: ZmPHT1;1, ZmPHT1;2; all parts: ZmPHT1;3; old leaves: ZmPHT1;6	Nagy et al. (2006)

PHT1s have been characterized by expressing them in heterologous systems including the *Saccharomyces cerevisiae* *pho84* mutant, which lacks the equivalent endogenous phosphate transporter (Bun-Ya et al., 1991), and *Xenopus* oocytes. The high-affinity PHT1s are usually expressed at low Pi concentrations and have a K_m ranging from 3 to 10 μ M, whereas the low-affinity ones functional at high Pi availability have a K_m ranging from 50 to 300 μ M (Raghothama and Karthikeyan, 2005; Lopez-Arredondo et al., 2014). These expression patterns and kinetic properties of PHT1s suggest that they play multiple roles for Pi acquisition and remobilization with respect to external Pi status and tissue specificity. Most of the PHT1s are found to be expressed in response to Pi starvation. Examples of PHT1 transporters expressed under Pi starvation and their affinities where known are listed in Table 1.

Post-translational regulation of PHT1 levels

Besides regulation at the transcriptional level in response to phosphate levels, PHT1 transporters undergo regulated trafficking and degradation. These mechanisms have been studied in detail in *Arabidopsis thaliana* and to a lesser extent in rice.

PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1 (PHF1) was identified through a genetic screen as an ER-localized factor required for PHT1;1 targeting to the plasma membrane (Gonzalez et al., 2005). It was subsequently shown to enhance plasma membrane localization of PHT1;2 and PHT1;4 as well (Bayle et al., 2011).

PHF1 shares some sequence similarity to *S. cerevisiae* Sec12p, and its overexpression, like that of Sec12p, inhibited export of COPII-dependent cargo from the ER (Gonzalez et al., 2005); however, PHF1 did not co-localize with other COPII components, suggesting a distinct role (Bayle et al., 2011). PHT1–green fluorescent protein (GFP) fusions are detectable in sorting endosomes regardless of the external Pi concentration, but Pi starvation stabilized the GFP signal at the plasma membrane. In the presence of high Pi and concanavalin A, which inhibits the vacuolar ATPase, GFP fluorescence was detected in vacuole-like structures, consistent with a model where PHT1 is endocytosed and targeted to the vacuole for degradation under high-P conditions (Bayle et al., 2011) (Fig. 3A).

NITROGEN LIMITATION ADAPTATION (NLA) is an E3 ligase that also contains an SPX domain (Table 2) that interacts with PHT1 at the plasma membrane. It targets PHT1;1 and PHT1;4 leading to their ubiquitination and subsequent endocytosis and degradation in the vacuole (Lin et al., 2013) (Fig. 3). It has been shown that *nla* mutants overaccumulate Pi and show toxicity symptoms (Kant et al., 2011). Thus, NLA is an important component of a regulatory system that prevents Pi overaccumulation under conditions of surplus. The ubiquitination pathway requires the sequential action of E1, E2, and E3 enzymes, with UBC8 being the E2 that interacts with NLA (Peng et al., 2007). A further enzyme, PHO2, an ER- and Golgi-localized peripheral membrane protein that may be a chimaeric E2–E3 enzyme

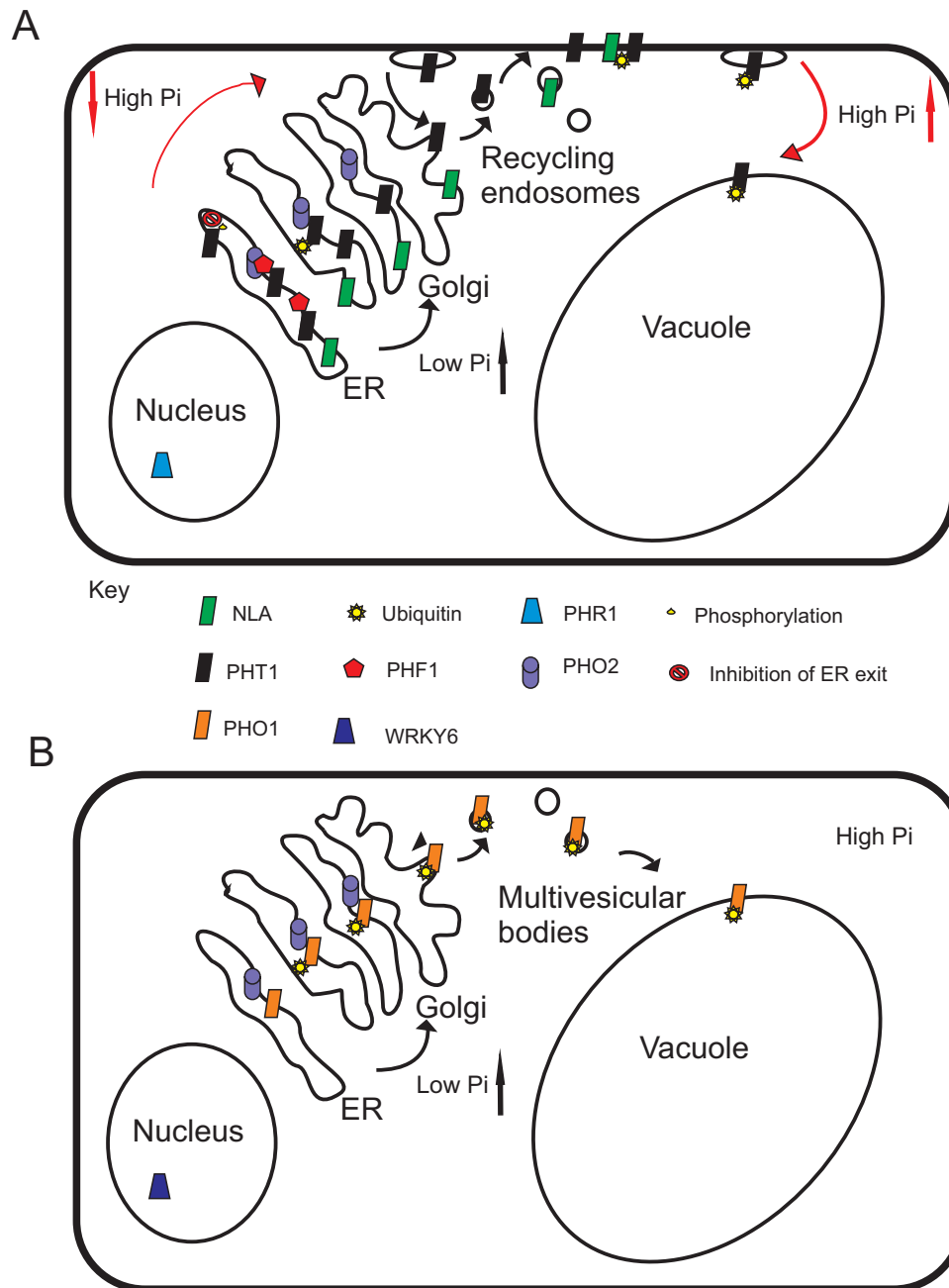


Fig. 3. Regulation of phosphate transporters by post-translational mechanisms. (A) In epidermal cortex and root hair cells, PHT1 transporters under the transcriptional control of PHR1 are translated in the cytosol and targeted to the ER where they pass through the endomembrane system before localization at the plasma membrane. Export from the ER is enhanced by PHF1. PHT1 is present in sorting endosomes, but localization to the plasma membrane is enhanced under low-Pi conditions. Under high-Pi conditions, the level of PHT1 at the plasma membrane is downregulated by multiple mechanisms. NITROGEN LIMITATION ADAPTATION (NLA)-dependent ubiquitination at the plasma membrane results in vacuolar targeting and degradation. Export from the ER is reduced by PHT1 phosphorylation and PHO2 targeting of PHF1. (B) In root cortical cells, PHO2 also targets PHO1 for ubiquitination and degradation in high-Pi conditions. PHT1, PHOSPHATE TRANSPORTER1; PHO1, PHOSPHATE1; PHO2, PHOSPHATE2; PHF1, PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1; PHR1, PHOSPHATE STARVATION RESPONSE1; WRKY6, a TF.

(Liu *et al.*, 2012), is also important in phosphate-deficiency responses and regulates PHT1 transporters [and also other targets such as PHO1 (Liu *et al.*, 2012) and PHF1 (Huang *et al.*, 2013)] via ubiquitination (Fig. 3B). However, double mutants in *nla* and *pho2* showed aggravated phenotypes. They were smaller, accumulated higher levels of Pi in shoots, and had much higher steady-state levels of PHT1;1/2/3, suggesting that they function independently in regulation of

phosphate transporter levels (Lin *et al.*, 2013). When Pi is limiting, AtNLA is downregulated by miRNA827 (Hsieh *et al.*, 2009), relieving this inhibition, while PHO2 is a target of miRNA399 (Aung *et al.*, 2006). One interesting observation is that PHO2 is found predominantly in the vasculature based on studies with promoter reporter fusions; however, PHT1s are expressed predominantly in the epidermal, cortex, and root hair cells. This discrepancy in potential localization has

Table 2. SPX domain-containing proteins in *Arabidopsis* and rice for which location or functional information is known

Profile of SPX domain-containing proteins in *Arabidopsis* and rice (modified from Secco *et al.*, 2012. The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. *New Phytologist* **193**, 842–851, with permission).

Protein	Function/regulation profile	Subcellular localization	Reference
AtPHO1	Pi transfer from root to shoot; Pi loading into the xylem vessel; possible transcriptional signal transport from root to shoot; controlled by PHO2-mediated endomembrane degradation	Largely at Golgi/trans-Golgi network and uncharacterized vesicles; a minor fraction at plasma membrane	Stefanovic <i>et al.</i> (2011); Rouached <i>et al.</i> (2011a); Liu <i>et al.</i> (2012)
AtPHO1;H1	Pi transfer from root to shoot; regulated by PHR1 and influenced by phosphite	–	Stefanovic <i>et al.</i> (2007)
AtPHO1;H4 (AtSHB1)	Control of hypocotyl elongation under blue light; forms a large protein complex through SPX and EXS domain; regulates endosperm development relevant genes	Nucleus	Zhou and Ni (2010)
AtPHO1;H10	Involved in abiotic/biotic stress response (including wounding, dehydration, cold, salt, and pathogen attack)	–	Ribot <i>et al.</i> (2008)
OsPHO1;2	Pi transfer from root to shoot; gene expression regulated by its <i>cis</i> -natural antisense transcripts	–	Secco <i>et al.</i> (2010)
AtSPX1	Positive regulator of plant adaptation to Pi starvation; interacts with PHR1 in a Pi-dependent manner	Nucleus	Duan <i>et al.</i> (2008); Puga <i>et al.</i> (2014)
AtSPX2	Interacts with PHR1 in a Pi-dependent manner	Nucleus	Duan <i>et al.</i> (2008); Puga <i>et al.</i> (2014)
AtSPX3	Negative regulator of some PSI genes	Cytoplasm speckles	Duan <i>et al.</i> (2008)
AtSPX4	–	Plasma membrane	Duan <i>et al.</i> (2008)
OsSPX1	Positive regulator of plant adaptation to Pi starvation; interacts with PHR2 in a Pi-dependent manner	Nucleus	Wang C <i>et al.</i> (2009); Wang <i>et al.</i> (2014)
OsSPX2	Interacts with PHR2 in a Pi-dependent manner	Nucleus	Wang Z <i>et al.</i> (2009); Wang <i>et al.</i> (2014)
OsSPX3	Negative regulator of some PSI genes	Cytoplasm speckles	Wang Z <i>et al.</i> (2009)
OsSPX4	Interacts with PHR2 mainly in cytoplasm and prevents its translocation into the nucleus; controlled by Pi-dependent 26S proteasome pathway	Nucleus/cytoplasm	Wang Z <i>et al.</i> (2009); Lv <i>et al.</i> (2014)
AtSPX-MFS3	–	Tonoplast	Secco <i>et al.</i> (2012)
OsSPX-MFS1	Pi transport and relocation in leaves; gene expression controlled by miR827	–	Lin <i>et al.</i> (2010)
OsSPX-MFS2	Gene expression controlled by miR827	–	Lin <i>et al.</i> (2010)
AtNLA (AtBAH1)	Involved in the nitrogen starvation response; regulates Pi homeostasis by ubiquitination of PHT1 family members; gene expression regulated by a miR827 in a Pi-dependent manner	Endomembrane system	Peng <i>et al.</i> (2007); Kant <i>et al.</i> (2011); Lin <i>et al.</i> (2013)

led to the proposal that *PHO2* mRNA or PHO2 protein may undergo cell-to-cell trafficking (Huang *et al.*, 2013), adding yet another layer of complexity to regulation of phosphate transporters.

Lin *et al.* (2013) showed that NLA regulation of PHT1 levels is also conserved in rice, while in *S. cerevisiae*, Pho84p is internalized and degraded subsequent to phosphorylation and ubiquitination (Lundh *et al.*, 2009). Interestingly PHT1;1 and PHT1;4 also show increased phosphorylation under Pi-replete conditions. A phosphorylation mimicking mutation Ser514 to Asp promoted intracellular localization, probably through inhibiting ER exit. Phosphorylation of Ser320 also increased under Pi-replete conditions, but whether this

affected endocytosis was not tested (Bayle *et al.*, 2011). In rice, OsPHF1 also regulates trafficking of phosphate transporters (Chen J *et al.*, 2011), whereas in *S. cerevisiae*, *pho86* mutants retain Pho84p in the ER (Kota and Ljungdahl, 2005). Thus, an ancient conserved mechanism for regulation of phosphate transporter activity appears to operate across kingdoms, although the molecular components differ.

Manipulation of PHT1 expression levels

Several studies have investigated the effects of upregulating expression of phosphate transporters on the ability of plants to grow on low levels of Pi. *OsPHT1;1* (*OsPHT1*) is widely

expressed in rice plants and is not markedly induced by Pi deficiency (Seo *et al.*, 2008; Sun *et al.*, 2012). In these studies, transgenic plants that expressed *OsPHT1;1* under the control of the cauliflower mosaic virus 35S promoter (Seo *et al.*, 2008) or the ubiquitin promoter (Sun *et al.*, 2012) were characterized. In both cases, plants with increased levels of *OsPHT1;1* transcript were selected, and these plants accumulated higher levels of Pi in shoots under Pi-sufficient conditions. However, under Pi-limiting conditions, no difference in Pi content was seen in 21-d-old plants (Sun *et al.*, 2012). In older plants grown in fertilized soil, Pi levels were almost double the levels in the xylem of transgenic compared with control plants (Sun *et al.*, 2012), and field-grown plants grown on unfertilized soil had a much higher Pi content, as well as 20% more panicles at harvest, although the plants were 30% shorter (Seo *et al.*, 2008). The *OsPHT1;1* overexpresser lines took up more phosphate and also produced more root hairs than control plants, even under Pi-replete conditions (Sun *et al.*, 2012). A similar enhancement of root hair production even under high Pi was seen when *Arabidopsis PHT1;5* was expressed under the control of the *ACTIN2* promoter (Nagarajan *et al.*, 2011). *AtPHT1;5* is expressed in root and leaf and is moderately upregulated under Pi deficiency, and characterization of mutants in this gene point to an important role in the allocation of Pi to shoots under P-limitation conditions and in transfer of Pi from shoots to roots under Pi-sufficient conditions (Nagarajan *et al.*, 2011). *AtPHT1;5* overexpressers showed reduced Pi uptake but increased biomass and leaf area, dry weight, and stalk thickness under both long and short days. However, overexpression lines senesced earlier (Nagarajan *et al.*, 2011). *OsPHT1;8* (*OsPT8*) is also a widely expressed phosphate transporter that is not strongly induced by low Pi (Jia *et al.*, 2011). Overexpression of *OsPHT1;8* under the control of the maize ubiquitin promoter resulted in increased Pi uptake, high-level accumulation of Pi in roots and shoots, and toxicity symptoms under conditions of high-Pi supply. The transgenic plants displayed stunted growth under both high- and low-Pi conditions (Jia *et al.*, 2011). Overexpression of *AtPHT1;9* under the 35S promoter resulted in enhanced tolerance to Pi deficiency in seedlings with plants in soil growing similarly to controls (Remy *et al.*, 2012). However in contrast to the effects of overexpressing *AtPHT1;5* (Nagarajan *et al.*, 2011) and *OsPHT1;1* (Sun *et al.*, 2012), *AtPHT1;9* overexpression resulted in no difference in root-hair density on high Pi and less proliferation of lateral roots under Pi deficiency (Remy *et al.*, 2012), while overexpression of *BnPHT1;4* in *Arabidopsis* resulted in longer primary roots and reduced lateral root density in low-Pi compared with control plants (Ren *et al.*, 2014).

AMF interactions with PHT1 genes

AMF play an important role in mobilization of Pi from new sites in soil to Pi depletion zones that form around the root surface by extending their hyphae far beyond the Pi depletion zone (Becquer *et al.*, 2014). The AMF in turn receive carbon photosynthetically manufactured by the host plant (Smith and Read, 2008). A comprehensive discussion of the

role of AMF in increased P uptake is beyond the scope of this review; however, it should be noted that several *PHT1* genes are mycorrhiza specific and inducible only upon inoculation of AMF. *PHT1* genes that are known to be induced by AMF are given in Table 3. There is a complex and still not well understood interplay between plant and fungus. Barel medic MtPHT1;4 is specifically localized to the plant-derived peri-arbuscular membrane and the specific delivery to this membrane is proposed to arise through a transient reorientation of polarized secretion to this membrane during arbuscle development (Pumplin *et al.*, 2012). MtPHT1;4 is essential for the acquisition of Pi delivered by the AMF and also critical for arbuscular mycorrhiza symbiosis. Loss of MtPHT1;4 function leads to premature death of the arbuscules; the fungus is unable to proliferate within the root and symbiosis is terminated (Javot *et al.*, 2007). Similarly, in rice, both *OsPHT1;11* and *OsPHT1;13* are important for AM symbiosis, although only *OsPHT1;11* is required for Pi transfer to the plants (Yang *et al.*, 2012).

PHO1 and its homologues

Since the concentration of bioavailable Pi in the soil solution is frequently 1000-fold lower than that in the plant intracellular compartments (Bieleski, 1973), an efficient Pi homeostasis system requires not only the acquisition of Pi but also the reallocation of this element. The *Arabidopsis pho1* mutant displays a series of Pi-deficiency symptoms including a prominent decrease in leaf Pi content (Poirier *et al.*, 1991). Intriguingly, it was also found in the same study that mutating *pho1* does not impact the root Pi uptake or shoot Pi movement, thus suggesting that PHO1 is specifically playing a crucial role of exporting Pi from root cortical cells to the xylem before the element is delivered to the shoot (Poirier *et al.*, 1991). This proposed Pi-exporting function of PHO1 was later confirmed by transgenic overexpression of PHO1 in *Arabidopsis* shoot tissues, resulting in enhanced shoot Pi content and intense release of Pi into the extracellular medium (Stefanovic *et al.*, 2011; Arpat *et al.*, 2012). Transient expression of *AtPHO1* in tobacco leaves revealed that the protein was localized predominantly to the Golgi/trans-Golgi network, but a certain proportion of total PHO1 was relocalized to the plasma membrane upon high Pi infiltration (Arpat *et al.*, 2012). PHO1 may be more than a Pi exporter. *Arabidopsis* lines with reduced levels of PHO1 (2–10-fold decrease compared with the wild type) showed a reduction in shoot Pi levels comparable to *pho1* mutants. However, unlike the *pho1* mutant, growth rates similar to those of the wild type were maintained, and gene expression profiles indicative of Pi stress were not observed, showing that it is possible to uncouple Pi levels in the shoot from changes in gene expression (Rouached *et al.*, 2011a). The authors proposed that PHO1 may also be involved in transporting a root-to-shoot signal (other than Pi) that leads to induction of the suite of Pi-deficiency responses in shoot, and that it is this transcriptional response rather than low Pi *per se* that leads to growth inhibition in the *pho1* mutant (Rouached *et al.*, 2011a).

Table 3. *PHT1* genes induced by AMF in various plants

The PHT1s reported to be induced by inoculation with AMF are listed with the name of the plant and the name of the AMF species used. The expression patterns of these transporters were analysed by reverse transcription PCR and quantitative real-time PCR after inoculating the roots with specific AMF.

<i>PHT1</i> gene	Plant species	AMF species used	Reference
<i>AsPHT1;1</i> , <i>AsPHT1;3</i> , <i>AsPHT1;4</i>	Chinese milkvetch	<i>Gigaspora margarita</i> and <i>Glomus intraradices</i>	Xie et al. (2013)
<i>BdPHT1;3</i> , <i>BdPHT1;7</i> , <i>BdPHT1;12</i> , <i>BdPHT1;13</i>	Purple false brome	<i>Glomus candidum</i>	Hong et al. (2012)
<i>CiPHT1;3</i> , <i>CiPHT1;4</i> , <i>CiPHT1;5</i>	Red pepper	<i>Glomus intraradices</i>	Chen A et al. (2007)
<i>GmPHT1;11</i> , <i>GmPHT1;12</i> , <i>GmPHT1;13</i>	Soybean	<i>Glomus intraradices</i>	Tamura et al. (2012)
<i>HvPHT1;8</i> , <i>HvPHT1;11</i>	Barley	<i>Glomus intraradices</i> , <i>Glomus</i> sp. WFVAM23 and <i>Scutellospora calospora</i>	Glassop et al. (2005); Sisaphaithong et al. (2012)
<i>LjPHT1;3</i> , <i>LjPHT1;4</i>	Miyakogusa	<i>Glomus mosseae</i> and <i>Glomus intraradices</i>	Maeda et al. (2006)
<i>MtPHT1;1</i> , <i>MtPHT1;4</i>	Barrel clover	<i>Glomus versiforme</i>	Harrison et al. (2002); Javot et al. (2007)
<i>OsPHT1;11</i> , <i>OsPHT1;13</i>	Rice	<i>Glomus intraradices</i>	Paszowski et al. (2002); Guimil et al. (2005); Glassop et al. (2007)
<i>PhPHT1;3</i> , <i>PhPHT1;4</i> , <i>PhPHT1;5</i>	Petunia	<i>Glomus intraradices</i>	Wegmuller et al. (2008)
<i>PtaPHT1;4</i>	Hardy orange	<i>Glomus etunicatum</i> , <i>Glomus diaphanum</i> and <i>Glomus versiforme</i>	Shu et al. (2012)
<i>PtPHT1;9</i> , <i>PtPHT1;10</i> , <i>PtPHT1;12</i>	Black cottonwood	<i>Glomus intraradices</i> and <i>Gloimus mosseae</i>	Loth-Pereda et al. (2011)
<i>SiPHT1;8</i> , <i>SiPHT1;9</i>	Foxtail millet	<i>Glomus mosseae</i>	Ceasar et al. (2014)
<i>SiPHT1;3</i> , <i>SiPHT1;4</i> , <i>SiPHT1;5</i>	Tomato	<i>Glomus margarita</i> , <i>Glomus caledonium</i> and <i>Glomus intraradices</i>	Nagy et al. (2005); Xu et al. (2007)
<i>SmePHT1;3</i> , <i>SmePHT1;4</i> , <i>SmePHT1;5</i>	Eggplant	<i>Glomus intraradices</i>	Chen A et al. (2007)
<i>StPHT1;3</i> , <i>StPHT1;4</i> , <i>StPHT1;5</i>	Potato	<i>Glomus intraradices</i>	Rausch et al. (2001); Nagy et al. (2005)
<i>TaPHT1;8</i> , <i>TaPHT1;10</i> , <i>TaPHT1;11</i> , <i>TaPHT1;12</i>	Wheat	<i>Glomus</i> sp. WFVAM23, <i>Scutellospora calospora</i> and <i>Glomus intraradices</i>	Glassop et al. (2005); Sisaphaithong et al. (2012)
<i>ZmPHT1;6</i>	Maize	<i>Glomus intraradices</i>	Nagy et al. (2006)

Arabidopsis genomic sequence analysis identified 10 homologues of *PHO1*. These genes encode proteins (PHO1;H1–PHO1;H10) each of which has a well-conserved hydrophilic SPX domain at the N terminus and a hydrophobic EXS domain with six to eight potentially membrane-spanning segments at their C terminus (Hamburger et al., 2002). Among these 10 PHO1 homologues, PHO1;H1 and PHO1;H10 were shown to exhibit the same Pi-stress-inducible expression as PHO1 (Yuan and Liu, 2008), while only PHO1;H1 had a similar Pi exporting function and restored the Pi xylem loading in *pho1* mutant plants (Stefanovic et al., 2007). However, expression of PHO1;H1 and PHO1 has been shown to be either dependent on the regulation of transcription factor PHR1 or controlled by PHO2-mediated endomembrane degradation, respectively (Figs 2 and 3) (Stefanovic et al., 2007; Liu et al., 2012). Such observations suggest that, when facing Pi stress, plants utilize complex signalling pathways at multiple levels of regulation with potentially complex cross-talking among these pathways to maintain the intracellular Pi level. Three similar PHO1 family members, OsPHO1;1–OsPHO1;3, have also been found in rice. So far, OsPHO1;2 is the only member

found to resemble AtPHO1 in Pi transfer from roots to shoots, although all three rice PHO1 members are phylogenetically close to AtPHO1 and AtPHO1;H1 (Secco et al., 2012) and are potentially regulated by their *cis*-natural antisense transcripts under Pi deprivation (Secco et al., 2010). The closest mammalian homologue of PHO1, xenotropic and polytropic retrovirus receptor XPR1, has also been demonstrated recently to exhibit Pi export activity when expressed in metazoan cells (Giovannini et al., 2013) and ectopically expressed in tobacco epidermal cells (Wege and Poirier, 2014).

Despite all *Arabidopsis* PHO1 family members containing some common primary structural features and reverse transcription PCR analysis indicating a broad range of gene expression throughout the plant corpus (Wang et al., 2004), to date only AtPHO1 and AtPHO1;H1 have been shown to play critical roles in Pi signalling and transport. AtPHO1;H4, otherwise known as SHB1 (SHORT HYPOCOTYL UNDER BLUE1) has been demonstrated to control hypocotyl elongation under blue light through the formation of a protein complex (Zhou and Ni, 2010), while homologue AtPHO1;H10 is intensely induced upon various abiotic and biotic stresses

apart from Pi starvation (Ribot *et al.*, 2008). The relatively high level of similarity among PHO1 family members and the conservation of their N-terminal SPX domain throughout homologues from different species indicate an important role of SPX-domain-possessing proteins and the importance of this domain itself in Pi homeostasis maintenance (Table 2).

Secretion of OAs to enhance P availability

Acid soils suffer from Pi deficiency as it is sequestered by positively charged components of the soil (Fig. 4), such as the toxic Al^{3+} ions that become mobilized at a soil pH below 4.5. Importantly, approximately 50% of the world's potentially arable soils are acidic (von Uexküll and Mutert, 1995), and 60% of these are in developing nations, so this is a widespread problem compromising a large portion of potentially arable land (Kochian, 1995). Thus, plants have developed a range of mechanisms to deal with growth on acidic soils, chief among these being OA exudation. By this mechanism, plants release organic anions, such as malate and citrate, into the soil, and these anions overcome the dual problems of soil that is both deficient in phosphate and replete with Al^{3+} ions by protecting the plant from Al^{3+} ion toxicity and helping to mobilize phosphate as shown in Fig. 5. Phosphate can be mobilized by organic anions either by anion exchange, freeing bound Pi, or by chelation of the metal ions that immobilize Pi in the soil (Sas *et al.*, 2001). OA exudation is well established as a major trait in plants with resistance to aluminium toxicity and improved productivity or yield per unit P (PUE) such as wheat (Ryan *et al.*, 2001). The importance of OA exudation can be seen by the fact that up to 20% of a plant's carbon usage can be invested in OA exudation in the roots (Lynch and Ho, 2005), and this loss of carbon may account for some of the loss in yield of P-starved plants.

There are two main families of membrane proteins involved in OA exudation, the channels of the aluminium-activated malate transporter (ALMT) family, and transporters of the

multidrug and toxic compound extrusion (MATE) family, which export malate and citrate, respectively (Ryan *et al.*, 2011). These proteins are α -helical membrane proteins that form pores through the plasma membrane of root epidermal cells in order to release OAs into the soil. The MATE family is large, with many members still uncharacterized; however, a sorghum homologue (*SbMATE*) has been shown to confer Al^{3+} tolerance by facilitating the release of citrate into the rhizosphere in response to Al^{3+} (Magalhaes *et al.*, 2007). In addition, the barley gene *HvAACT1* has been identified as a plasma-membrane-localized MATE transporter expressed at the root tips of barley root epidermal cells responsible for citrate efflux in the presence of Al^{3+} (Furukawa *et al.*, 2007).

The first gene of the ALMT family to be characterized was *ALMT1* in wheat, and it has been shown that TaALMT1 releases malate in an aluminium-activated manner (Zhang *et al.*, 2008). The protein senses free Al^{3+} , which is a signifier of acidic soils, and releases malate through its central pore, down a concentration gradient into the soil. It acts as a channel, passively releasing the malate, rather than a transporter. There is a pressing need for a greater understanding of the structure and mechanism of these channels; although some research has attempted to explore their topology by immunocytochemical or bioinformatics approaches, no settled model has yet been agreed upon (Motoda *et al.*, 2007; Dreyer *et al.*, 2012). Recent work has shown that the first 48 residues and a C-terminal helix of TaALMT1 are vital for its function in oocytes (Sasaki *et al.*, 2014). As yet, several areas remain unresolved including the mechanisms by which these proteins are activated, how they function at a molecular level, and their atomic-level structure. Interestingly, although TaALMT1 has been shown to be expressed constitutively before being activated directly by Al^{3+} , activity of the *Arabidopsis* homologue *AtALMT1* is controlled at the transcriptional level by transcription factors STOP1 (Sawaki *et al.*, 2009) and WRKY1 (Ding *et al.*, 2013) in response to the presence of Al^{3+} .

Manipulation of OA exudation through transgenic modification

Transformation of barley (*Hordeum vulgare* L., which does not have a functional equivalent) with TaALMT1 from wheat resulted in plants that were able to take up more phosphate from the soil and which thrived when grown in acidic, highly P-fixing ferrosol (Delhaize *et al.*, 2009). This boost in yield was seen both in short-term 26 d pot trials and a longer-term experiment to physical maturity after 156 d. The improvement was due to a combination of effects. First, the transgenic plants were able to thrive in acid soil, enabling more root growth and so increasing the area of its rhizosphere. Even in limed conditions, the wild-type barley had a severely restricted rhizosphere, while ALMT1-transformed plants grown in both limed and non-limed conditions produced a larger rhizosphere. Secondly, there was an increase in phosphate uptake per unit root length, indicating that the amount of P taken up as a function of biomass (PAE) was increased by the release of malate into the soil by mobilization of Pi. These experiments show that the creation of a transgenic line with just a single gene addition (that of TaALMT1) was able

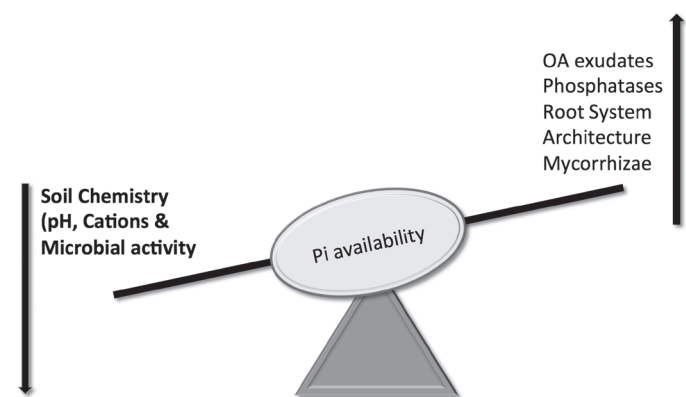


Fig. 4. Factors affecting Pi availability in soil. The bioavailability of Pi in the soil is affected by physicochemical and biological factors such as soil pH, soil type, and the concentrations of cations such as various metals that can complex phosphate, as well as microbial activity. Plants counteract these limitations through different strategies, which may include exudation of phosphatases to liberate phosphate from organic molecules, organic anions to chelate metal cations and increase phosphate solubility, and increasing the volume of soil that can be explored through modifications to the architecture of the root system and interaction with AMF.

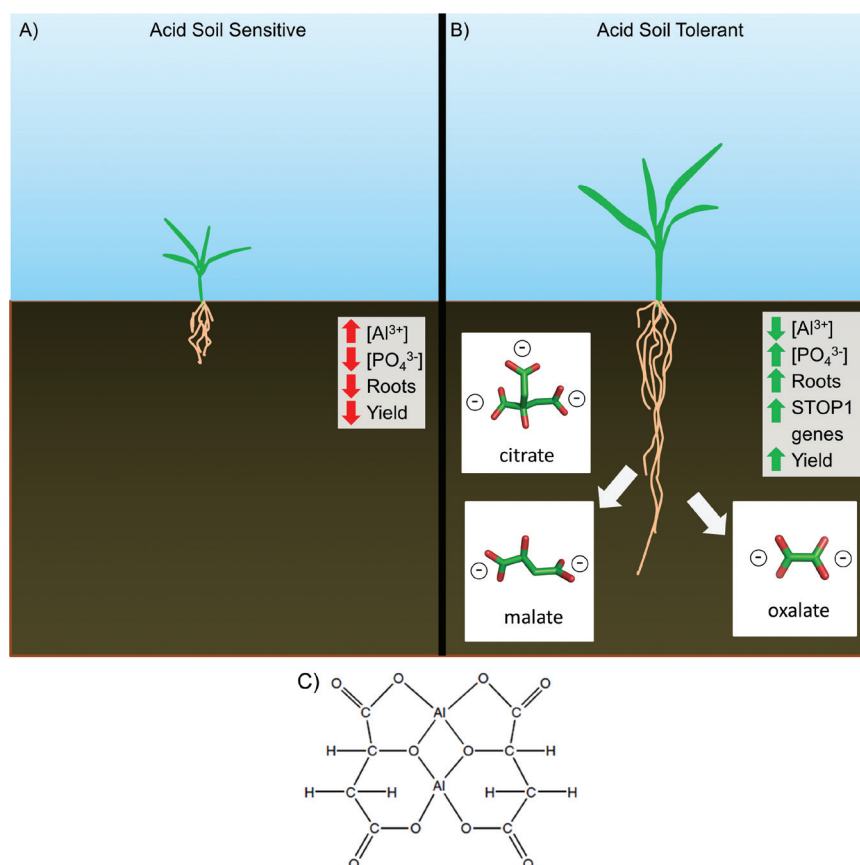


Fig. 5. OA exudation is an important mechanism to improve Pi availability in acid soil. (A) Acid-soil-sensitive plants are compromised on acid soils by toxic Al^{3+} restricting root growth and the low availability of Pi in the soil lowering yields. (B) When acid-soil-tolerant varieties are grown (whether transgenic or not), TFs, such as STOP1 in *Arabidopsis*, upregulate genes involved in protection from Al^{3+} toxicity. Mechanisms differ between different plant species but responses include release of OAs such as malate, citrate, or oxalate by ALMT or MATE genes, depending on the plant species, which leads to lower free Al^{3+} and higher free Pi in the soil and thus higher yields. The upregulation of OA secretion can be by transcriptional or post-transcriptional mechanisms. (C) Structure diagram to show malate chelating aluminium, sequestering it to reduce its toxicity. (This figure is available in colour at JXB online.)

to more than double the grain yield of barley plants grown in acid soil, producing yields close to growth in ideal non-acidic conditions (with no loss of productivity on limed soil). This large effect is very promising for the potential production of transgenic crops with improved PAE and PUE on acid soils.

In connection with the effects on the rhizosphere, it is notable that, even on limed soil and soil with added P, the deeper regions of the soil remain depleted of P. Wild-type barley roots were nearly non-existent below 50 cm, but growth below this depth could be enabled by *TaALMT1*. This restricted root growth impairs yield due to decreased uptake of nutrients such as P but also by restricting access to deep water sources. These transgenic approaches also impact on water usage and drought susceptibility, facilitating integration with other transgenic crop approaches. Although work assessing transgenic barley has been promising, a question remains over the viability of a transgenic strategy to increase yields, as no work has been undertaken at the field scale.

Exploitation of knowledge for crop improvement

The results of manipulation of levels of specific membrane transporters, channels, and TFs suggest that such

an approach could be beneficial to both PAE and PUE. However, it is still unclear exactly how plants sense Pi levels internally and the contribution of levels of phosphate in specific cell types and subcellular compartments to perception and response. As excess accumulation of phosphate results in toxicity, simply driving plants to take up more is not necessarily the solution and runs the risk of further depletion from the soil. It is also difficult to compare the results of different studies when different growth conditions and developmental stages of plants are used. More sophisticated approaches using targeted gene expression in specific tissues, analysis of protein levels (which may not reflect transcript levels because of the extensive post-transcriptional regulation), and whole lifecycle comparisons of control and transgenic plants under conditions more closely replicating those in the field are required. Perturbation of phosphate transporter expression clearly alters these balances in as yet unpredictable ways and provokes changes in the transcription of other genes as reported previously (Jia et al., 2011; Nagarajan et al., 2011; Sun et al., 2012). The uncoupling of transcriptional responses to phosphate starvation from phosphate levels that was seen in *Arabidopsis* lines with reduced PHO1 expression (Rouached et al., 2011a) may present a useful tool for further investigation, as does the recent discovery of a

small molecule ‘phosphatin’ that can attenuate Pi-starvation responses and partially uncouple growth inhibition from Pi levels (Arnaud *et al.*, 2014). Furthermore, as it is becoming apparent that there is significant cross-talk between phosphate and other nutrient pathways such as nitrogen (Kant *et al.*, 2011), sulfur (Moseley *et al.*, 2009; Rouached *et al.*, 2011b), iron (Thibaud *et al.*, 2010; Bournier *et al.*, 2013), and zinc (Khan *et al.*, 2014), a more holistic approach that considers multiple nutrients may be necessary. However, there may also be specific instances where overexpression of a single gene or combination of relatively few genes could make a significant contribution, such as the expression of *TaALMT* in barley (Delhaize *et al.*, 2009).

As an alternative to targeting individual genes, plant breeders have developed crops with improved tolerance to acid soils, which are also improved in P-uptake efficiency (David and Brett, 2003). Screening for quantitative trait loci (QTLs) for low-Pi-tolerant varieties is also a useful method of identification of new components in the P homeostasis pathway and a potential means of marker-assisted breeding. Several studies have been conducted for phenotyping the root traits and marker development in order to produce the low-Pi-tolerant varieties (reviewed by Richardson *et al.*, 2011). In rice, a major QTL, *PHOSPHORUS UPTAKE 1 (PUP1)* was identified from an *aus*-type Pi-starvation-tolerant Indian rice variety Kasalath (Chin *et al.*, 2010), and this has been recommended for marker-assisted breeding. This gene was named for the phosphate starvation tolerance locus (*PSTOLI*) and was missing in the non-tolerant rice genome Nipponbare; expression of *PSTOLI* is also found to be upregulated under Pi starvation (Gamuyao *et al.*, 2012). In barley, an increased level of expression of the low-affinity PHT1 transporters *HvPHT1;6* and *HvPHT1;3* was correlated with genotypes with higher PUE (Huang *et al.*, 2011).

Identification of root trait variations among the genotypes has been another important area of study to identify and develop Pi-stress-tolerant varieties (Lynch, 2007). Variation in root growth angles has been identified as an important trait for Pi-deficiency tolerance in maize (Zhu *et al.*, 2005b), bean (Bonser *et al.*, 1996; Liao *et al.*, 2001), and wheat (Manske *et al.*, 2000). Root-hair variation has also been considered as an important trait for improving Pi-stress tolerance. Several studies have been conducted to assess the genotype variation for root-hair density and root-hair length (reviewed by Richardson *et al.*, 2011), and QTLs associated with root hairs have also been identified in maize (Zhu *et al.*, 2005a) and common bean (Yan *et al.*, 2004). More studies are needed to utilize marker-assisted breeding to release new varieties with increased PAE and PUE.

Concluding statements

The development of integrated and sustainable approaches to agriculture is essential to meet humankind’s future needs. Increased understanding and exploitation of genes, TFs, and proteins involved in uptake, utilization, and signalling of Pi will be useful for efficient utilization of P in the future. Transgenic approaches to modulate the expression levels of

some of these genes holds promise but needs to be decoupled from detrimental knock-on effects on other aspects of plant physiology. Marker-assisted breeding and improvement is a complementary approach for the production of Pi-efficient crops. As well as improved farming methods and improved crop varieties with superior PAE and PUE, it will be crucial to develop more-efficient and environmentally benign methods to recover nutrients including P from waste, and here too plants have a role to play. Thus, P sustainability is a major challenge requiring the efforts of government and industries, engineers, soil scientists, plant scientists, agronomists, plant breeders, and farmers.

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