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REPLACE, REUSE, RECYCLE: IMPROVING THE SUSTAINABLE USE OF PHOSPHORUS BY PLANTS

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Novelty statement.

The phosphate problem is one of matching supply and demand. Understanding molecular mechanisms of phosphate response can help in addressing recapture as well as efficiency of use to increase sustainability.

Abstract

The ‘phosphorus (P) problem’ has recently received strong interest with two distinct strands of importance. The first is too much P is entering into waste water creating a significant economic and ecological problem. Secondly, whilst agricultural demand for phosphate fertiliser 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 whilst 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 synthesise 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 recently been made in our understanding of local and systemic signalling mechanisms for phosphate and expression and regulation of membrane proteins that take phosphate up from the environment and transport it within the plant. We discuss the current status 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.

6 key words in alphabetical order: fertilisers, phosphate, nutrient recycling, membrane transporters, phosphate signalling, transcription factors

Abbreviations:

AMF Arbuscular Mycorrhizal Fungi, MAB marker assisted breeding, miRNA micro RNA, NATS natural antisense transcripts, OA organic acids, PAE, the amount of P taken up as a function of biomass. PUE the amount of productivity or yield per unit P. Pi, inorganic phosphate. SPX, protein domain named for founding members Syg1, Pho81, XPR1. TF 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 fertilisers but reserves are finite and the supply is expiring rapidly (Cooper and Carliell-Marquet, 2013). In 2010 global extraction was *c*. 176Mt and demand is increasing, with global peak phosphorus use expected to be reached by 2030 (Cordell *et al*., 2009). The best estimates for longevity of reserves are around 200 years and the worst are at 50 years (Rosemarin *et al*., 2011). Moreover, the process of mining rock phosphate and manufacturing fertiliser is expensive and energy intensive (Elser and Bennett, 2011).

In 2013 the UK imported and consumed *c*. 140,000 tonnes of phosphate, with 86,000 tonnes used for crop fertiliser and animal feeds (Cooper and Carliell-Marquet, 2013). It is estimated that 2-3 tonnes of phosphate per million people per day enter the UK's watercourses as treated sewage where it is lost to the environment (Kato *et al*., 2007) and can negatively impact on ecosystems. This equates to 70,000 tonnes or half the country's annual requirement. Prices for diammonium phosphate fertiliser in 2014 were \$500 tonne^{-1} (Argus, 2014) leading to the potential loss of \$35M (£22M) every year.

The majority of phosphate inputs to the environment are from land application as fertilisers (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. Phosphorus (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, therefore capacity to replace phosphorus (as phosphate) is limited. Consequently, the key to sustainability must be to reuse and recycle phosphorus 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 (Chiou and Lin, 2011; Lopez-Arredondo *et al*., 2014; Nussaume *et al*., 2011; Raghothama, 1999; Richardson *et al*., 2011; Rouached *et al*., 2010; 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 load applied. In many cases, the addition of metal salts such as $Al_2(SO_4)_3$, $CaCl_2$ or $FeCl_3$ is used to precipitate out the phosphorus (de-Bashan and Bashan, 2004). Struvite ($NH_4MgPO_46H_2O$) 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 (*c*. <1mg P L^{-1}), but carry the burden of cost variations due to fluctuating prices of iron, magnesium and aluminium (Farchy, 2013; Vidal, 2008). 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 utilised 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 all) can be implemented for the capture of several compounds including phosphates (Vermaat and Khalid Hanif, 1998). Waste water stabilisation ponds on farmland, 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 (Abuaku *et al*., 2006; Alaerts *et al*., 1996; Farrel 2012). The large quantities of phyto-biomass produced by phyto-remediative systems (Verma and Suthar, 2014) generally all have beneficial by-products as energy sources such as for biogas, biodiesel (Fujita *et al*., 1999), or 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 small scale to tonnes/ha with the associated potential for multiplication of errors. Nevertheless, the drawbacks of chemical removal practices and the energetic 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 Pi starvation. Many recent studies have helped to underpin the molecular signalling networks involved in P homeostasis (reviewed in Chiou and Lin, 2011). Plants sense and respond to the Pi status both locally and systematically, with separate molecular mechanisms being involved in local and long distance Pi signalling to maintain homeostasis under Pi starvation (Lin *et al*., 2014; Lopez-Arredondo *et al*.,

2014; Thibaud *et al.*, 2010). Typical levels of inorganic phosphate (Pi) in soils are low µM, whereas levels in the cytosol of plants under optimal conditions are mM, requiring the ability to acquire and buffer cytosolic Pi at concentrations 3 orders of magnitude above that in the environment. Plants respond to low P stress in a number of ways (Figure 1). These include: release of Pi from vacuolar stores for example; remodelling of membranes to reduce reliance on phospholipids (reviewed in 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 microorganisms, 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 that are important in both acquisition of Pi from the soil and its recycling within the plant, members of the PHO1 family some of which are involved in export of Pi from roots to shoots, and membrane proteins involved in secretion of organic acids. The elaborate machinery, that regulates these (and other phosphate response genes) at multiple levels from transcription through to protein location and stability, is also discussed in this article.

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 (Figure 2). It binds to the phosphate starvation related regulatory element (P1BS) motif (GNATATNC) in the promoter region of Pi stress responsive genes (Rubio *et al.*, 2001). PHR1 is localized to the nucleus and a SUMO E3 ligase (SIZ1) is known to control Pi homeostasis at the posttranslational 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 micro-RNA399 (*miRNA-399*) (Chen *et al.*, 2011a; Rubio *et al.*, 2001; Shin *et al.*, 2006). The *miRNA-399* has been implicated in Pi starvation related signalling in many plants (Lin *et al.*, 2008; Pant *et al.*, 2008; Liu and Vance, 2010;

Liu et al., 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) (Figure 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). The regulation of the *AtSPX* genes was shown to be controlled by PHR1 with AtSPX1 being proposed to be a transcriptional regulator, given its nuclear localization and capacity of up-regulating the expression of downstream PSI (Phosphate Starvation Inducible) genes when over-expressed (Duan *et al*., 2008). However, recent studies have shown that instead of directly regulating the PSI genes 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 (PHR1 Binding Site) 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 (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 signaling function of SPX proteins, although further research is needed to clarify how Pi level affects the interaction between SPX proteins and PHR1/PHR2. The functional similarities of SPX proteins between monocotyledons and dicotyledons also suggest the highly conserved SPX domain could be of great significance in a prevalent Pi sensing and signaling pathway.

Transgenic manipulation of PHR1

Several studies have looked at the impact of over expressing *PHR1* of *Arabidopsis* (Nilsson *et al.,* 2007*)*, *ZmPHR1* of maize (Wang *et al*., 2013b), *OsPHR2* of rice (Zhou *et al*., 2008) *BnPHR1* of oil seed rape (Ren *et al*., 2012) and *TaPHR1-A1* of wheat (Wang *et al*., 2013a).These studies all observed up regulation at the

transcriptional level of several low phosphate response genes such as phosphate transporters and non-coding RNA *miRNA399*, and corresponding down regulation of *PHO2*, and showed increased levels of Pi in tissues. In several of these studies the *PHR1* over expressing plants showed improved growth under low Pi conditions (Wang *et al* 2013a, b, Ren *et al.,* 2012 Zhou *et al*., 2008). 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 *et al*., 2013a,b). This is perhaps not surprising as over expressing some of the Pi-starvation responsive genes that are downstream of PHR1 such as *OsmiR399* (Hu *et al*., 2011) and *OsSPX1* (Wang *et al*., 2009a) caused Pi toxicity in transgenic plants. In all cases constitutive strong promoters (35S or maize Ubiquitin) were used for over expression of *PHR1* and the level of over expression 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 the studies where growth inhibition at high Pi was not reported, tissue levels of Pi showed only relatively modest increases. The beneficial effects of *PHR1* over expression included increased root growth/branching (Wang et al., 2013a) 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 in (Lopez-Arredondo *et al*., 2014) (Figure 2). Both WKRY75 and ZAT6 are up-regulated during Pi starvation and are found to be involved in regulating the modification of root architecture (Devaiah *et al*., 2007a; Devaiah *et al*., 2007b). In contrast, the *BHLH32* TF is down regulated 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 *et al*., 2007b).

Over expression 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* over expressing plants had retarded growth and lower biomass on high Pi, but better growth than wild type on low Pi (Dai *et al*., 2012). As with PHR1 over expressing plants, the *OsMYB2P-1* over expressing 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 to wild type (Dai *et al*., 2012).

Transgenic plants over expressing *Oryza sativa Phosphate Starvation–Induced Transcription Factor 1* (*OsPTF1*) showed improved growth and yield characteristics in hydroponics, pots and field. At low Pi root and shoot biomass and Pi content was higher, as was the number of tillers, reproductive development and yield (Yi *et al*., 2005). Over expression of maize *ZmPTF1* 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, over expression of *PTF1* up regulated a different set of genes to those under PHR1 control and included genes involved in gluconeogenesis (phosphoenolpyruvate carboxykinase PEPCK) 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 emphasise the interaction between phosphate levels and carbohydrate metabolism and point to 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 which 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 in Rouached *et al*., 2010). The phenotypic changes of root architecture are genotype dependent and have been shown to be important for overcoming Pi stress in bean, soybean, maize and barley (reviewed in 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 (figure 2). Both *LPR* and *PDR2* are involved in root architecture modification in response to Pi starvation. *LPRs* encode multi copper oxidases expressed in the meristematic regions of the root tip, including 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 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*) which is 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 endoplasmic reticulum-resident pathway (Rouached *et al*., 2010). Interactions with auxin and sugar signalling are also of critical importance in modulation of root architecture in response to phosphate deprivation (reviewed in 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 Super family (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 trans-membrane alpha helices divided into two domains (N and C) of 6 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 9 genes (Mudge *et al*., 2002), rice has 13 genes (Paszkowski *et al*., 2002), soybean has 14 genes (Fan *et al*., 2013), 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 in Nussaume *et al.*, 2011). The *PHT1s* have been predominantly found to be expressed in roots, especially in epidermal cells and the outer cortex of the root hair (Misson *et al.*, 2004; Mudge *et al.*, 2002; Schunmann *et al.*, 2004; Xiao *et al.*, 2006). For example 8 out of 9 PHT1s in Arabidopsis have been found to be expressed in roots (Karthikeyan *et al.*, 2002; Mudge *et al.*, 2002). Further, localization studies on these transporters in different plant species confirmed that PHT1 is most specifically targeted to the plasma membrane (Bayle *et al.*, 2011; Fan *et al.*, 2013; Gonzalez *et al.*, 2005; Jia *et al.*, 2011; Preuss *et al.*, 2011). 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 PHT1s have been characterized by expressing in heterologous systems including the *S. 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 µM, whereas the low-affinity ones functional at high Pi availability have a K_m ranging from 50 to 300 µM (Lopez-Arredondo *et al.*, 2014; Raghothama and Karthikeyan, 2005). 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 Table1.

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 localised factor required for PHT1;1 targeting to the plasma membrane (Gonzalez *et al*., 2005). It was subsequently shown to enhance plasma membrane localisation 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-localise with other COPII components suggesting a distinct role (Bayle *et al*., 2011). PHT1-GFP fusions are detectable in sorting endosomes regardless of the external Pi concentration, but Pi starvation stabilised 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) (Figure 3A).

NITROGEN LIMITATION ADAPTATION (NLA) is an E3 ligase which also contains an SPX domain (Table 3) 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) (Figure 3). *nla* mutants over accumulate Pi and show toxicity symptoms (Kant *et al*., 2011). Thus NLA is an important component of a regulatory system that prevents Pi over accumulation under conditions of surplus. The ubiquitination pathway requires 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 localised peripheral membrane protein which may be a chimeric E2-E3 enzyme (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 (Figure 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 they function independently in regulation of phosphate transporter levels (Lin *et al*., 2013). When Pi is limiting AtNLA is down regulated by miRNA827 (Hsieh *et al*., 2009) relieving this inhibition, whilst PHO2 is a target of miRNA399 (Aung *et al*., 2006). One interesting observation is that PHO2 is predominantly in the vasculature based on studies with promoter reporter fusions; however PHT1s are predominantly expressed in the epidermal, cortex and root hair cells. This discrepancy in potential localisation has 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 showed that NLA regulation of PHT1 levels is also conserved in rice (Lin *et al*., 2013), and in *S. cerevisiae* Pho84p is internalised 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 localisation, probably through inhibiting ER exit. Phosphorylation of Ser 320 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 *et al*., 2011b) 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 up regulating expression of phosphate transporters on the ability of plants to grow on low levels of Pi. *OsPHT1;1* (*OsPT1*) is widely expressed in rice plants and not markedly induced by Pi deficiency (Seo *et al*., 2008; Sun *et al*., 2012). In these studies transgenic plants that express *OsPHT1;1* under the control of the CaMV 35S promoter (Seo *et al*., 2008) or the ubiquitin promoter (Sun *et al*., 2012) were characterised. In both cases plants with increased level 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 day old plants (Sun *et al*., 2012). In older plants grown in fertilised soil, Pi levels were almost double the levels in the xylem of transgenic compared to control plants (Sun *et al*., 2012) and field grown plants grown on unfertilised soil had 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 Actin 2 promoter (Nagarajan *et al*., 2011). *AtPHT1;5* is expressed in root and leaf and moderately upregulated under Pi deficiency, and characterisation 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). *At PHT1;5* over expressers showed reduced Pi uptake but increased biomass and leaf area, dry weight and stalk thickness under both long and short days. However, over expression 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). Over expression 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 over expression of *BnPHT1;4* in Arabidopsis resulted in longer primary roots and reduced lateral root density in low Pi compared to 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. *PHT1s* that are known to be induced by AMF are given in Table 2. There is a complex and still not well understood interplay between plant and fungus. Barel medic MtPHT1;4 is specifically localised to the plant-derived periarbuscular membrane and the specific delivery to this membrane is proposed to arise through a transient reorientation of polarised secretion to this membrane during arbuscle development (Pumplin *et al*., 2012). MtPHT1;4 is essential for the acquisition of Pi delivered by the AM fungus and also critical for AM 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 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 (Arpat *et al*., 2012; Stefanovic *et al*., 2011). Transient expression of *AtPHO1* in tobacco leaves, revealed that the protein was predominantly localised to the Golgi/trans-Golgi network, but a certain proportion of total PHO1 was re-localised 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 to wild type), showed reduction of shoot Pi levels comparable to *pho1* mutants. However, unlike the *pho1* mutant, growth rates similar to those of 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 propose 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 it is this transcriptional response rather than low Pi *per se* which leads to growth inhibition in the *pho1* mutant (Rouached *et al*., 2011a).

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-termini and a hydrophobic EXS domain with six to eight potentially membrane-spanning segments at their C-termini (Hamburger *et al*., 2002). Among these 10 PHO1 homologues, PHO1;H1 and

519 PHO1;H10 are shown to exhibit the same Pi stress inducible expression as PHO1
520 (Yuan and Liu, 2008), while only PHO1;H1 has a similar Pi exporting function and
521 restores the Pi xylem-loading in *pho1* mutant plants (Stefanovic *et al*., 2007).
522 However, expression of PHO1;H1 and PHO1 has been shown to be dependent on
523 either the regulation of transcription factor PHR1, or controlled by PHO2 mediated
524 endomembrane degradation, respectively (Figures 2 and 3) (Liu *et al*., 2012;
525 Stefanovic *et al*., 2007). Such observations suggest that when facing Pi stress,
526 plants utilise complex signaling pathways at multiple levels of regulation with
527 potentially complex cross-talking among these pathways to maintain the intracellular
528 Pi level. Three similar PHO1 family members, OsPHO1;1-OsPHO1;3, have also
529 been found in rice. So far, OsPHO1;2 has been the only member found to resemble
530 AtPHO1 in Pi transfer from roots to shoots, although all three rice PHO1 members
531 are phylogenetically close to AtPHO1 and AtPHO1;H1 (Secco *et al*., 2012a) and are
532 potentially regulated by their cis-Natural Antisense Transcripts (NATs) under Pi
533 deprivation (Secco *et al*., 2010). The closest mammalian homolog of PHO1,
534 xenotropic and polytropic retrovirus receptor XPR1, has also recently been
535 demonstrated to exhibit Pi export activity when expressed in metazoan cells
536 (Giovannini *et al*., 2013) and ectopically expressed in tobacco epidermal cells
537 (Wege and Poirier, 2014).
538
539 Despite all *Arabidopsis* PHO1 family members containing some common
540 primary structural features and RT-PCR analysis indicating a broad range of gene
541 expression throughout the plant corpus (Wang *et al*., 2004), to date, only AtPHO1
542 and AtPHO1;H1 have been shown to play critical roles in Pi signaling and transport.
543 AtPHO1;H4, otherwise known as SHB1 (Short Hypocotyl Under Blue1) has been
544 demonstrated to control hypocotyl elongation under blue light through the formation
545 of a protein complex (Zhou and Ni, 2010), while homologue *AtPHO1;H10* is
546 intensely induced upon various abiotic and biotic stresses apart from Pi starvation
547 (Ribot *et al*., 2008). The relatively high level of similarity among PHO1 family
548 members and the conservation of their N-terminal SPX domain throughout
549 homologues from different species indicate an important role of SPX domain-
550 possessing proteins and this domain itself in Pi homeostasis maintenance (Table 3).
551

552 **Secretion of organic acids to enhance P availability**

553 Acid soils suffer from Pi deficiency as it is sequestered by positively charged
554 components of the soil (Figure 4), such as the toxic Al^{3+} ions that become mobilised

555 at soil pH below 4.5. Importantly, approximately 50% of the world's potentially
556 arable soils are acidic (von Uexküll and Mutert, 1995) and 60% of these are in
557 developing nations, so this is a widespread problem compromising a large portion of
558 potentially arable land (Kochian, 1995). Thus, plants have developed a range of
559 mechanisms to deal with growth on acidic soils, chief among these is organic acid
560 (OA) exudation. By this mechanism plants release organic anions, such as malate
561 and citrate, into the soil and these anions overcome the dual problems of soil that is
562 both deficient in phosphate and replete with Al^{3+} ions by protecting the plant from
563 Al^{3+} ion toxicity and helping to mobilise phosphate as shown in Figure 5. Phosphate
564 can be mobilised by organic anions either by anion exchange, freeing bound Pi, or
565 by chelation of the metal ions that immobilise Pi in the soil (Sas *et al.*, 2001). OA
566 exudation is well established as a major trait in plants with resistance to Al-toxicity
567 and improved PUE such as wheat (Ryan *et al.*, 2001). The importance of OA
568 exudation can be seen by the fact that up to 20% of a plant's carbon usage can be
569 invested in OA exudation in the roots (Lynch *et al.*, 2005) and this loss of carbon
570 may account for some of the loss in yield of P-starved plants.
571

572 There are two main families of membrane proteins involved in OA exudation,
573 the channels of the Aluminium-activated Malate Transporter (ALMT) family, and
574 transporters of the Multidrug and Toxic compound Extrusion (MATE) family, which
575 export malate and citrate, respectively (Ryan *et al.*, 2011). These proteins are
576 alpha-helical membrane proteins that form pores through the plasma membrane of
577 root epidermal cells in order to release OAs into the soil. The MATE family is large,
578 with many members still uncharacterised, however a sorghum homolog (*Sb*MATE)
579 has been shown to confer Al^{3+} tolerance by facilitating the release of citrate into the
580 rhizosphere in response to Al^{3+} (Magalhaes *et al.*, 2007). In addition, the barley gene
581 *Hv*AACT1 has been identified as a plasma-membrane-localised MATE transporter
582 expressed at the root tips of barley root epidermal cells responsible for citrate efflux
583 in the presence of Al^{3+} (Furukawa *et al.*, 2007).
584

585 The first gene of the ALMT family to be characterised was *ALMT1* in wheat
586 and it has been shown that *Ta*ALMT1 releases malate in an Al-activated manner
587 (Zhang *et al.*, 2008). The protein senses free Al^{3+}, which is a signifier of acidic soils,
588 and releases malate through its central pore, down a concentration gradient into the
589 soil. It acts as a channel, passively releasing the malate, rather than a transporter.
590 There is a pressing need for a greater understanding of the structure and

mechanism of these channels; although some research has attempted to explore topology via either immunocytochemical or bioinformatics approaches no settled model has yet been agreed upon (Dreyer *et al*., 2012; Motoda *et al*., 2007). Recent work has shown that the first 48 residues and a C-terminal helix of *Ta*ALMT1 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 constitutively expressed before being directly activated by Al^{3+}, activity of the Arabidopsis homologue *At*ALMT1 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 organic acid exudation through transgenic modification

Transformation of barley (*Hordeum vulgare* L.), (which does not have a functional equivalent) with *Ta*ALMT1 from wheat resulted in plants that were able to take up more phosphate from the soil and which thrived when grown in acid, highly-P-fixing ferrosol (Delhaize *et al*., 2009). This boost in yield was seen both in short-term 26-day pot trials and a longer term experiment to physical maturity after 156 days. The improvement is due to a combination of effects. Firstly, the transgenic plants were able to thrive in acid soil, enabling more root growth and so increasing the area of its rhizosheath. Even in limed conditions the wild type barley had a severely restricted rhizosheath, while ALMT1-transfomed plants grown in both limed and non-limed conditions produced a larger rhizosheath. Secondly, there was an increase in phosphate uptake per unit root length indicating that the PAE was increased by the release of malate into the soil by mobilisation of Pi. These experiments show that the creation of a transgenic line with just a single gene addition (that of *Ta*ALMT1) was able 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 rhizosheath 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 near-non-existent below 50 cm, but growth below this depth could be enabled by *Ta*ALMT1. 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 field-scale.

Exploitation of knowledge for crop improvement

The results of manipulation of levels of specific membrane transporters, channels and transcription factors 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 toxcity, 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 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 transcription of other genes as reported (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 attentuate 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 (Bournier *et al*., 2013; Thibaud *et al*., 2010) 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 over expression of a single gene or combination of relatively few genes could make a

significant contribution such as the expression of *Ta*ALMT 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 QTLs for low Pi tolerant varieties is also a useful method of identification of new components in the P homeostasis pathway and 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 in (Richardson *et al*., 2011). In rice, a major QTL, *phosphate uptake1* (*Pup1*) was identified from *aus* type Pi starvation-tolerant Indian rice variety Kasalath (Chin *et al*., 2010), and this has been recommended for MAB. This gene was named for phosphate starvation tolerance locus (*PSTOL1*) and was missing in the non-tolerant rice genome, Nipponbare; expression of *PSTOL1* is also found to be up regulated under Pi starvation (Gamuyao *et al*., 2012). In barley, increased level of expression of a low affinity PHT1 transporter *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 the Pi stress tolerance. Several studies have been conducted to assess the genotype variation for root hair density and root hair length (reviewed in 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 MAB 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, transcription factors and proteins involved in uptake, utilization and signalling of Pi will be useful for efficient utilization of P in future. Transgenic

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

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Tables

Table 1. PHT1 genes up regulated 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 with reference. The expression patterns of these transporters have been analysed by RT-PCR, qRT-PCR and promoter GUS or GFP fusion studies.

Name of the PHT1 gene	Plant	Affinity	Site of induction by low Pi	Reference
AtPHT1;1	Arabidopsis	High affinity	-	Mitsukawa *et al.*, 1997
AtPHT1;7, AtPHT 1;8, AtPHT1;9	Arabidopsis	-	Root	Mudge *et al.*, 2002
CfPHT1;1CfPHT1;2 CfPHT1;3 CfPHT1;4 CfPHT1;5	Cayenne pepper	-	Only in AMF inoculated roots	Chen *et al.*, 2007a
GmPHT1;1 to GmPHT1;12	Soybean	-	Root	Fan *et al.*, 2013
GmPHT1;1, GmPHT1;2, GmPHT1;5, GmPHT1;7, and GmPHT1;10	Soybean	High affinity	Root	Fan *et al.*, 2013
HvPHT1;1	Barley	High affinity	Root	Rae *et al.*, 2003
HvPHT1;6	Barley	Low affinity	Moderately induced in root and shoot	Rae *et al.*, 2003
HvPHT1;9	Barley	-	Roots	Huang *et al.*, 2011
MtPHT1;1	Barrel medic	Low affinity	-	Liu *et al.*, 1998b
OsPHT1;2 OsPHT1;6	Rice	Low affinity	-	Ai *et al.*, 2009
OsPHT1;8	Rice	High affinity	Root	Jia *et al.*, 2011
OsPHT1;8	Rice	High affinity	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

Name of the PHT1 gene	Plant	Affinity	Site of induction by low Pi	Reference
SiPHT1;4	Foxtail millet	-	Root	Ceasar *et al.*, 2014
SlPHT1;1 *SlPHT1;2*	Tomato	-	Roots	Liu *et al.*, 1998a
SmPHT1;1 *SmPHT1;2* *SmPHT1;3* *SmPHT1;4* *SmPHT1;5*	Eggplant	-	Leaf and roots	Chen *et al.*, 2007a
StPHT1;2	Potato	Low affinity	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

Table 2.PHT1 genes induced by AMF in various plants

The PHT1s reported to be induced by inoculation with AMF have been listed with the name of the plant and the name of the AMF species used with references. The expression patterns of these transporters have been analysed by RT-PCR, qRT-PCR after inoculating the roots with specific AMF.

Name of the PHT1 gene	Plant species	AMF species used	Reference
AsPHT1;1 *AsPHT1;3* *AsPHT1;4*	Chinese Milkvetch	*Gigaspora margarita* and *Glomus intraradices*	Xie *et al.*, 2013
BdPHT1;3 *BdPHT1;7* *BdPHT1;12* *BdPHT1;13*	Purplefalsebrome	*Glomus candidum*	Hong *et al.*, 2012
CfPHT1;3 *CfPHT1;4* *CfPHT1;5*	Red pepper	*Glomus intraradices*	Chen *et al.*, 2007a
GmPHT1;11 *GmPHT1;12* *GmPHT1;13*	Soybean	*Glomus intraradices*	Tamura *et al.*, 2012
HvPHT1;8 *HvPHT1;11*	Barley	*Glomus intraradices, Glomus sp*,WFVAM23 and *Scutellospora calospora*	Glassop *et al.*, 2005; Sisaphaithong *et al.*, 2012
LjPHT1;3 *LjPHT1;4*	Miyakogusa	*Glomus mosseae, Glomus intraradices*	Maeda *et al.*, 2006
MtPHT1;1 *MtPHT1;4*	Barrel Clover	*Glomus versiforme*	Harrison *et al.*, 2002; Javot *et al.*, 2007
OsPHT1;11 *OsPHT1;13*	Rice	*Glomus intraradices*	Paszkowski *et al.*, 2002; Guimil *et al.*, 2005
PhPHT1;3 *PhPHT1;4* *PhPHT1;5*	Petunia	*Glomus intraradices*	Wegmuller *et al.*, 2008
PtaPHT1;4	Hardy orange	*Glomus etunicatum, Glomus diaphanum* and *Glomus versiforme*	Shu *et al.*, 2012
PtPHT1;9 *PtPHT1;10* *PtPHT1;12*	Black cottonwood	*Glomus intraradices*and *Glomus mosseae*	Loth-Pereda *et al.*, 2011
SiPHT1;8 *SiPHT1;9*	Foxtail millet	*Glomus mosseae*	Ceasar *et al.*, 2014
SlPHT1;3 *SlPHT1;4* *SlPHT1;5*	Tomato	*Glomus margarita, Glomus caledonium* and *Glomus intraradices*	Nagy *et al.*, 2005

Name of the PHT1 gene	Plant species	AMF species used	Reference
SmePHT1;3 *SmePHT1;4* *SmePHT1;5*	Eggplant	*Glomus intraradices*	Chen *et al.*, 2007a
StPHT1;3 *StPHT1;4* *StPHT1;5*	Potato	*Glomus intraradices*	Rausch *et al.*, 2001; Nagy *et al.*, 2005
TaPHT1;8 *TaPHT1;10* *TaPHT1;11* *TaPHT1;12*	Wheat	*Glomus sp*,WFVAM23, *Scutellospora calospora* and *Glomus intraradices*	Glassop *et al.*, 2005; Sisaphaithong *et al.*, 2012
ZmPHT1;6	Maize	*Glomus intraradices*	Nagy *et al.*, 2006

Table 3.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.*, 2012b)

Protein	Function/Regulation profile	Subcellular localization	Reference
AtPHO1	Pi transfer from root to shoot; Pi loading into the xylem vessel Possible transcriptional signal transporting 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 *et al.*, 2011; Rouached *et al.*, 2011a; Liu *et al.*, 2012
AtPHO1;H1	Pi transfer from root to shoot Regulated by PHR1 and influenced by phosphite	-	Stefanovic *et al.*, 2007
AtPHO1;H4 (AtSHB1)	Control hypocotyl elongation under blue light Form a large protein complex through SPX and EXS domain Regulate endosperm development relevant genes	Nucleus	Zhou & Ni, 2010
AtPHO1;H10	Involved in abiotic/biotic stresses response (including wounding, dehydration, cold, salt and pathogen attack)	-	Ribot *et al.*, 2008
OsPHO1;2	Pi transfer from root to shoot Gene expression regulated by its cis-natural antisense transcripts	-	Secco *et al.*, 2010
AtSPX1	Positive regulator of plant adaptation to Pi starvation Interacts with PHR1 in a Pi dependent manner	Nucleus	Duan *et al.*, 2008; Puga *et al.*, 2014
AtSPX2	Interacts with PHR1 in a Pi dependent manner	Nucleus	Duan *et al.*, 2008; Puga *et al.*, 2014

AtSPX3	Negative regulator of some PSI genes	Cytoplasm speckles	Duan *et al.*, 2008
AtSPX4	-	Plasma membrane	Duan *et al.*, 2008
OsSPX1	Positive regulator of plant adaptation to Pi starvation Interacting with PHR2 in a Pi dependent manner	Nucleus	Wang *et al.*, 2009a; Wang *et al.*, 2014
OsSPX2	Interacting with PHR2 in a Pi dependent manner	Nucleus	Wang *et al.*, 2009b; Wang *et al.*, 2014
OsSPX3	Negative regulator of some PSI genes	Cytoplasm speckles	Wang *et al.*, 2009b
OsSPX4	Interacting with PHR2 mainly in cytoplasm and preventing its translocation into the nucleus Controlled by Pi dependent 26S Proteasome Pathway	Nucleus/Cytoplasm	Wang *et al.*, 2009b; Lv*et al.*, 2014
AtSPX-MFS3	-	Tonoplast	Secco *et al.*, 2012b
OsSPX-MFS1	Pi transport and relocation in leaves Gene expression controlled by miR827	-	Lin *et al.*, 2010
OsSPX-MFS2	Gene expression controlled by miR827	-	Lin *et al.*, 2010
AtNLA (AtBAH1)	Involved in the nitrogen starvation response Regulating Pi homeostasis by ubiquination of PHT1 family members Gene expression regulated by a miR827 in a Pi dependent manner	Endomembrane system	Peng *et al.*, 2007; Kant *et al.*, 2011 Lin *et al.*, 2013

Figure legends

Figure 1. Schematic representation of plant responses to low Pi stress.

Plants respond in multiple ways to low Pi. Some of these responses are local whilst others are systemic. Some respond to external Pi concentration whilst others respond to internal Pi levels. OA, organic acids; AMF, Arbuscular Mycorrhizal Fungi; RSA, Root System Architecture.

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

Figure3. Regulation of phosphate transporters by post translational mechanisms.

A. In epidermal cortex and root hair cells PHT1 transporters under transcriptional control of PHR1 are translated in the cytosol and targeted to the endoplasmic reticulum (ER) where they pass through the endomembrane system before localisation at the plasma membrane. Export from the ER is enhanced by PHF1. PHT1 is present in sorting endosomes but localisation to the plasma membrane is enhanced under low Pi conditions. Under high Pi conditions the level of PHT1 at the plasma membrane is down regulated by multiple mechanisms. NLA dependent ubiquitination at the plasma membrane results in vacuolar targeting and degradation. Export from the ER is reduced by PHT1 phopshorylation and PHO2 targeting of PHF1.

B. In root cortical cells PHO2 also targets PHO1 for ubquitination and degradation in high Pi conditions. NLA nitrogen limitation adaption; PHT1, phosphate transporter1;

PHO1, phosphate1; PHO2, phosphate2; PHF1 phosphate transporter traffic facilitator1; PHR1phosphate starvation response1; WRKY6 a transcription factor.

Figure 4. Factors affecting Pi availability in soil

Bioavailability of Pi in the soil is affected by physicochemical and biological factors such as soil pH, soil type and concentrations of cations such as various metals that can complex phosphate as well as microbial activity. Plants counteract these limitations through different strategies that 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 arbuscular mychorrizal fungi.

Figure 5. Organic acid (OA) exudation is an important mechanism to improve Pi availability on acid soil.

A) Acid soil sensitive plants are compromised on acid soils by toxic Al^{3+} restricting root growth and low availability of Pi in the soil lowering yields. B) When acid soil tolerant varieties are grown (whether transgenic or not) transcription factors, 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 upon 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) A structure to show malate chelating aluminium, sequestering it to reduce its toxicity.

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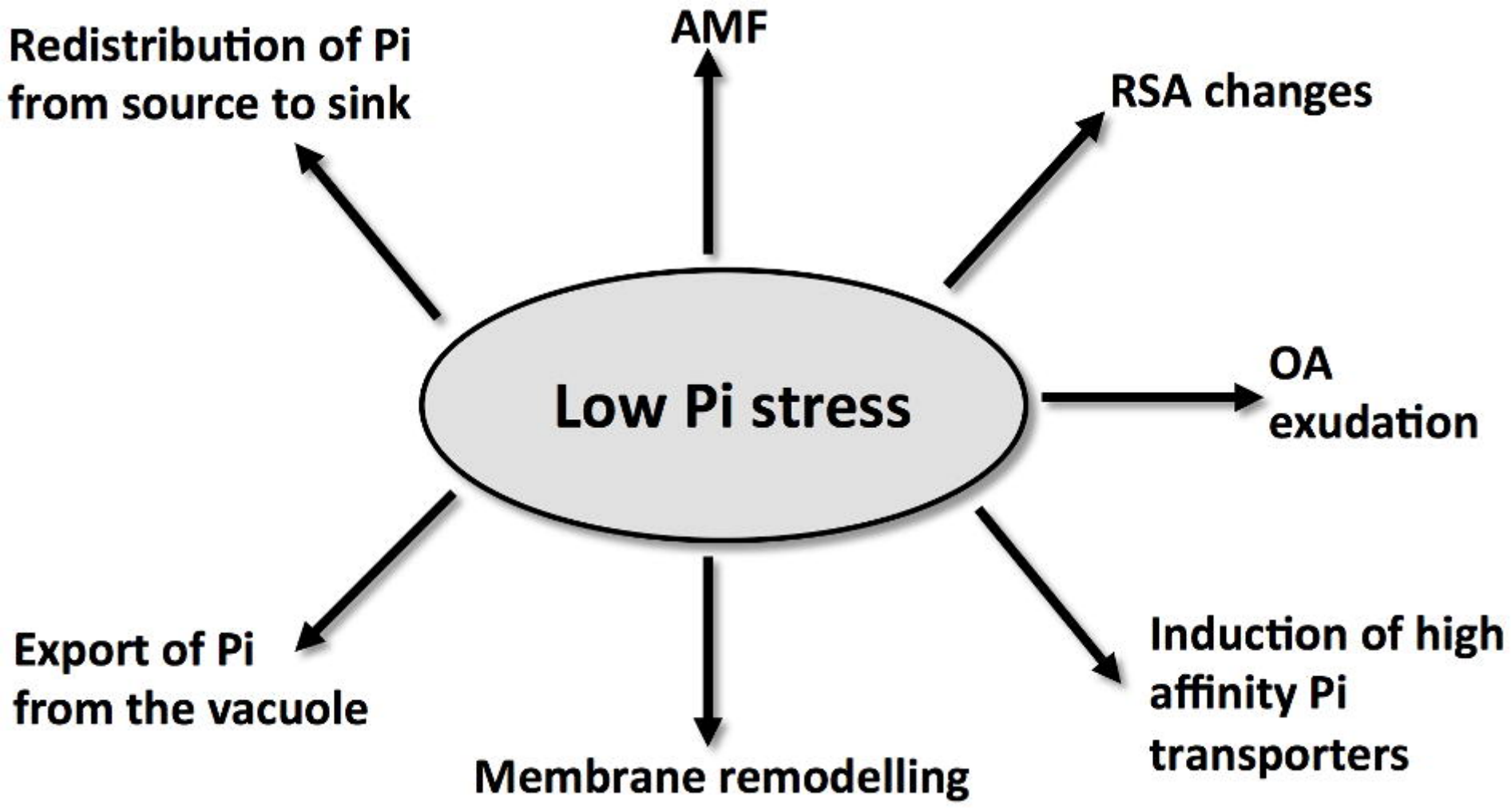
Redistribution of Pi
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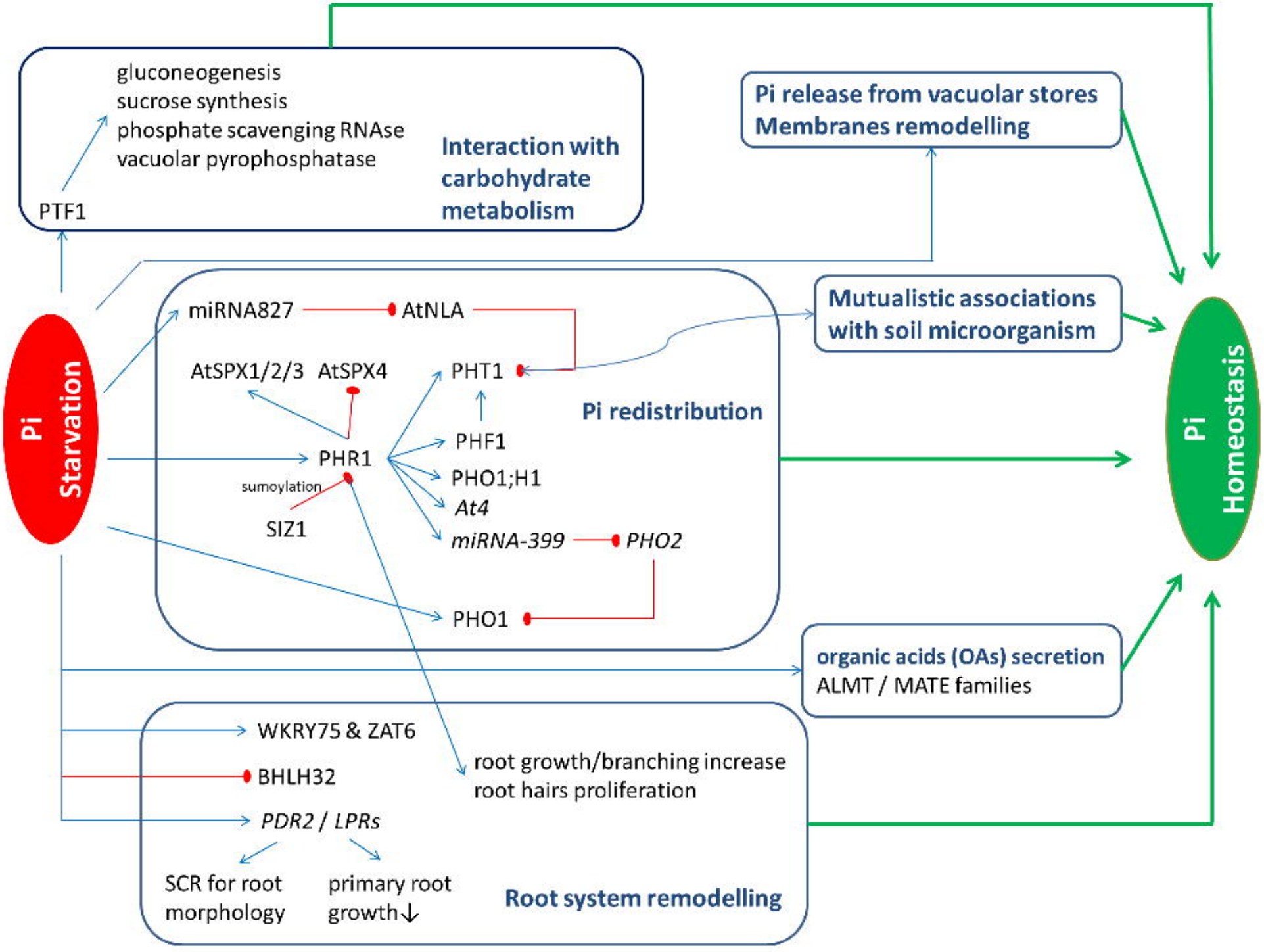
RSA changes
Low Pi stress
OA
exudation
Export of Pi
from the vacuole
Membrane remodelling
Induction of high
affinity Pi
transporters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sucrose synthesis
phosphate scavenging RNAse
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PTF1
Interaction with carbohydrate metabolism
Pi release from vacuolar stores
Membranes remodelling
Pi Starvation
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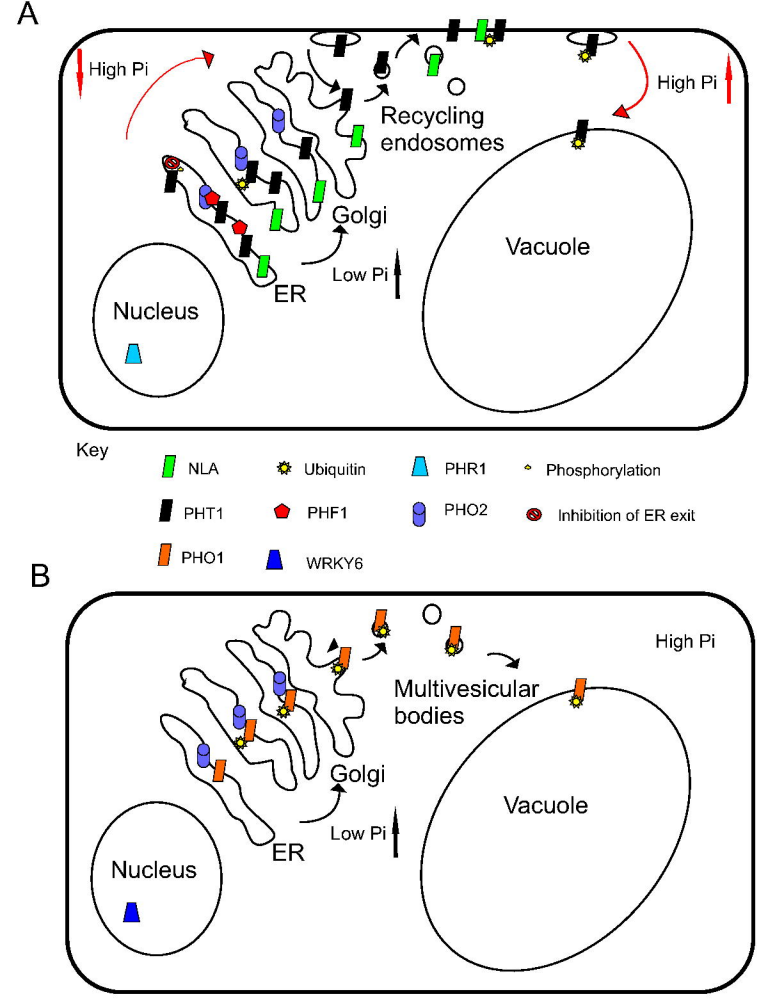
AtNLA
AtSPX1/2/3
AtSPX4
PHT1
Pi redistribution
PHF1
PHR1
sumoylation
SIZ1
PHO1;H1
At4
miRNA-399
PHO2
PHO1
Mutualistic associations with soil microorganism
Pi Homeostasis
organic acids (OAs) secretion
ALMT / MATE families
WKRY75 & ZAT6
BHLH32
PDR2 / LPRs
root growth/branching increase
root hairs proliferation
SCR for root morphology
primary root growth↓
Root system remodelling


A
High Pi
Recycling endosomes
High Pi
Golgi
Vacuole
Nucleus
ER
Low Pi
Key
NLA
Ubiquitin
PHR1
Phosphorylation
PHT1
PHF1
PHO2
Inhibition of ER exit
PHO1
WRKY6
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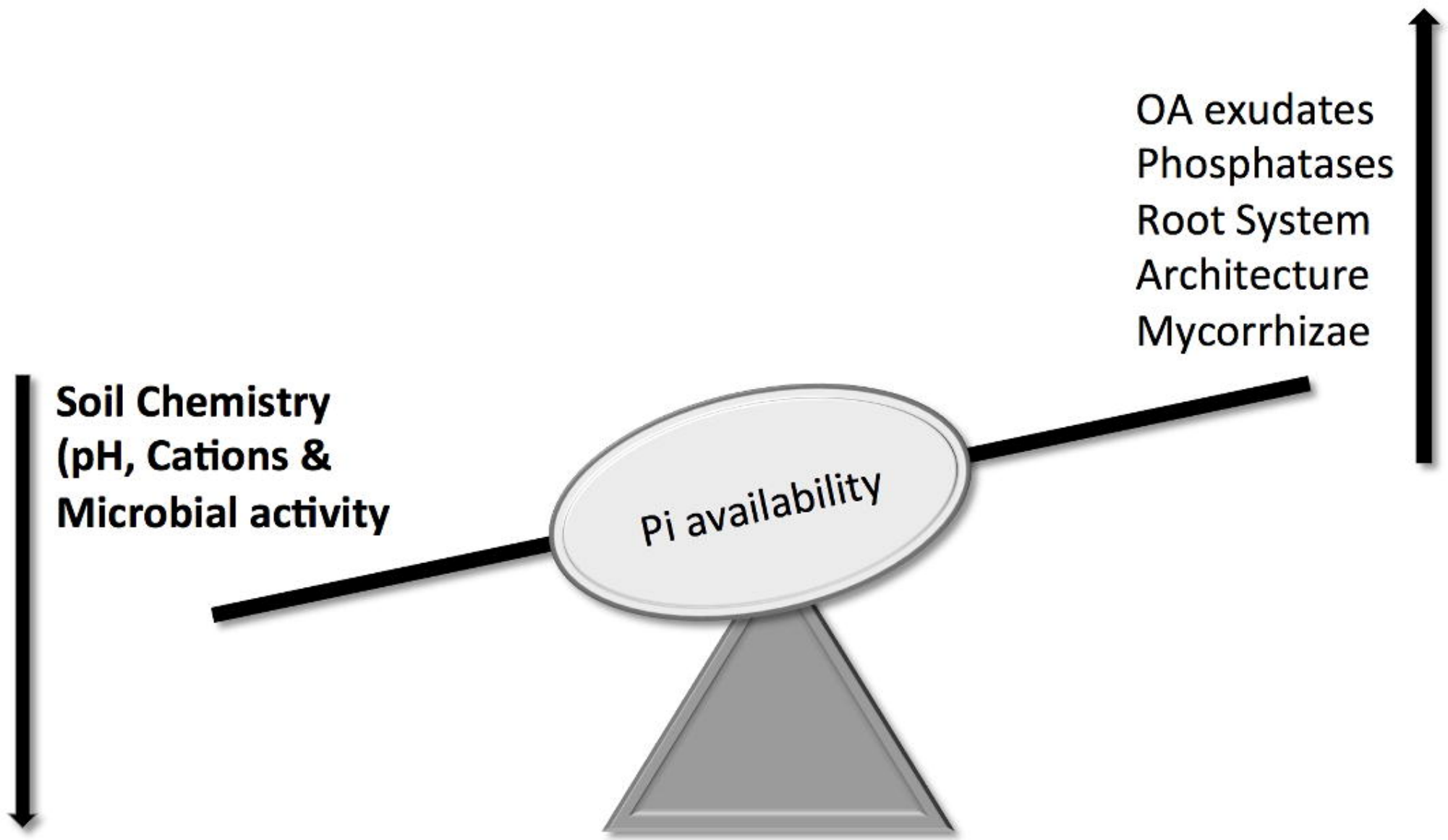
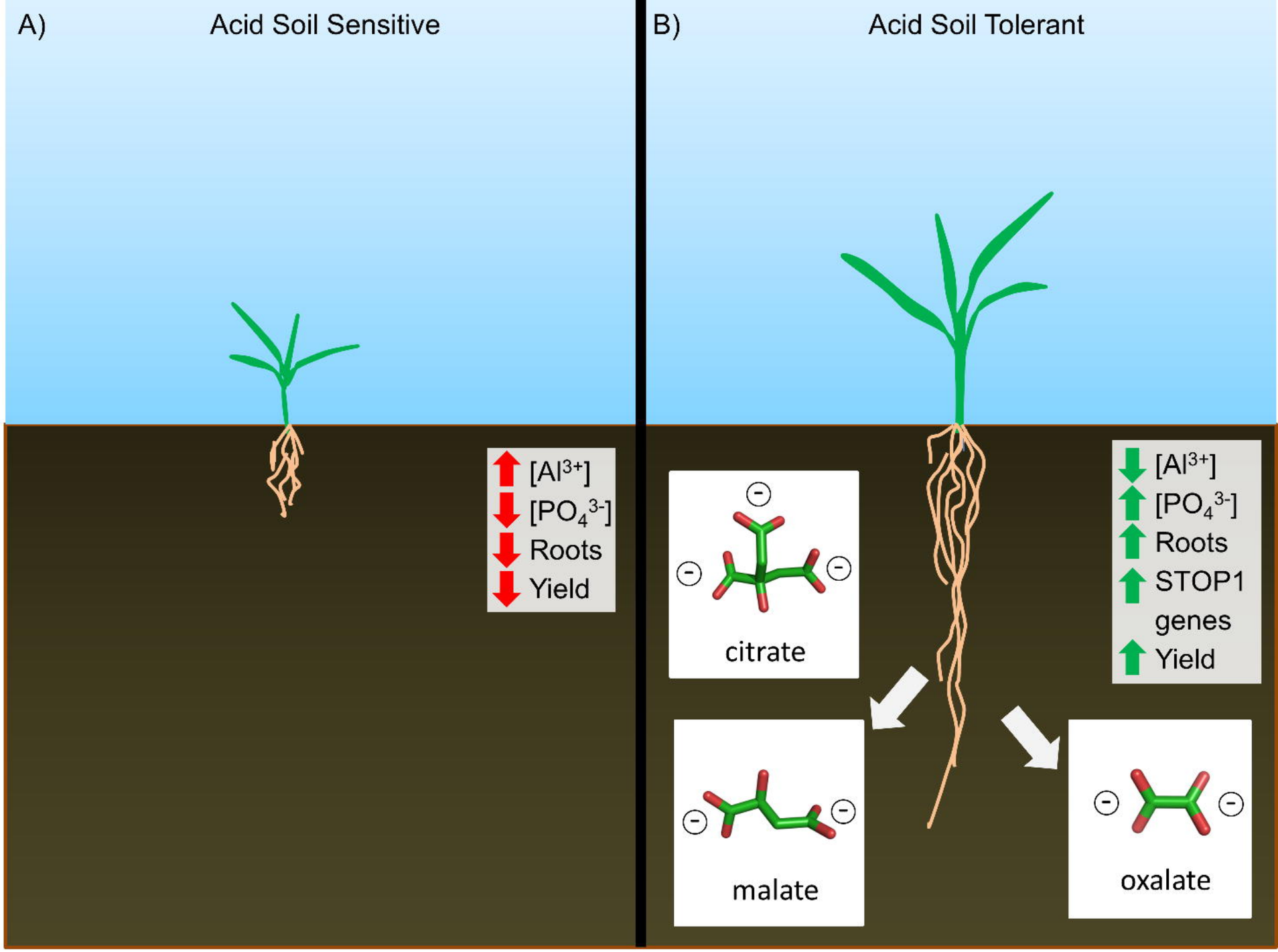
Nucleus
ER


OA exudates
Phosphatases
Root System
Architecture
Mycorrhizae
Soil Chemistry
(pH, Cations &
Microbial activity
Pi availability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Roots
Yield
B)
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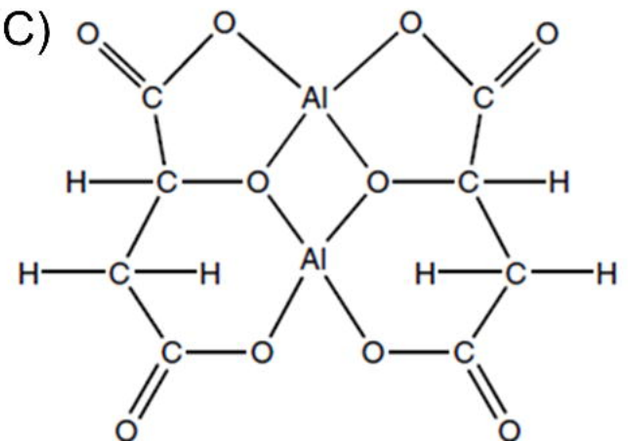
Roots
STOP1 genes
Yield
citrate
malate
oxalate

C)


O
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Al
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