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## Improving phosphorus use efficiency – a complex trait with emerging opportunities

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### **Abstract**

Phosphorus (P) is one of the essential nutrients for plants and indispensable for plant growth and development. P deficiency severely limits crop yield and regular fertilizer applications are required to obtain high yields and prevent soil degradation. To access P from the soil, plants have evolved high and low affinity Pi transporters and the ability to induce root architectural changes to forage P. Also, adjustments of numerous cellular processes are triggered by the P starvation response, a tightly regulated process in plants. With the increasing demand for food due to the growing population, demand for P fertilizer is steadily increasing. Given the high costs of fertilizers and in light of the fact that phosphate rock, the source of P fertilizer, is a finite natural resource, there is a need to enhance P fertilizer use efficiency in agricultural systems and develop plants with enhanced Pi uptake and internal P-use efficiency (PUE).

In this review we will provide an overview of ongoing relevant research and highlight different approaches towards developing crops with enhanced PUE. In this context, we will summarize our current understanding of root responses to low phosphorus conditions and emphasize the importance of combining PUE with tolerance of other stresses, such as aluminum toxicity. Of the many genes associated with Pi deficiency, this review will focus on those that hold promise or are already at an advanced stage of testing (*OsPSTOL1*, *AVP1*, *PHO1*, *OsPHT1;6*). Finally, an update is provided on the progress made exploring alternative technologies, such as phosphite fertilizer.

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## Introduction

For sustainable food production it is an absolute requirement that nutrients removed with the harvest of crops are replaced to prevent nutrient depletion and soil degradation. Of the various nutrients essential for plants, nitrogen (N), phosphorus (P) and potassium (K) are required in the largest quantity and deficiency in either nutrient severely limits crop yield. Given the need to increase food production to keep at pace with the growing population, the global demand for N and P fertilizer is steadily increasing, reaching an estimated 120 Mt of elemental N and 47 Mt of  $P_2O_4$  by 2018 (Figure 1).

N fertilizer is produced from atmospheric nitrogen by the Haber Bosch technology and N supply is, therefore, unlimited and only constrained by the large amount of required energy. In contrast, P fertilizer is produced from phosphate rock and concerns have been expressed that this natural resource will be exhausted in the near future. However, according to the International Fertilizer Development Centre (van Kauwenbergh 2010) easily accessible phosphate rock reserves are estimated to last for another 300–400 years and investments are being made to discover new reserves and develop alternative technologies for isolation of P from marine sediments or human and animal wastes (for a review see Rahman et al 2014). Most of the currently known phosphate rock reserves are located in Morocco, followed by the USA and China (van Kauwenbergh 2010). This unequal global distribution is a potential problem and will require policies to regulate access to P reserves in the future.

Globally, large imbalances in the rate of P fertilizer application exist with adequate or excess application in the Western world and some Asian countries (e.g., China, Japan and Korea) and an increasing P deficit in many Asian, African, and South American countries (Bot et al 2000; MacDonald et al 2011). On a global scale, it is estimated that about 50% of agricultural soils are deficient in P (Lynch 2011), either due to insufficient P replacement into agricultural systems or due to P-fixing soil properties rendering P unavailable to plants (see below). The former is most prevalent in developing

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countries and due to limited financial resources to purchase fertilizer or lack of access to agricultural inputs.

High and low input agricultural systems require different approaches to improve P use efficiency (PUE). For low-input conditions, crops are needed with very efficient, high-affinity  $P_i$  uptake capacity and, at the same time, high internal PUE (Rose and Wissuwa 2012). However, a combination of both, uptake capacity and internal PUE, is equally desirable for high-input systems since it would facilitate reduction of fertilizer doses without yield penalty. In rice, P fertilizer-use efficiency is only about 25% (Dobermann and Fairhurst 2000), providing considerable scope for improvement. Enhanced fertilizer use efficiency would furthermore reduce the risk of eutrophication of water ways due to field runoff, as well as greenhouse gas emissions, which is a major concern about nitrogen fertilizers.

For areas with P-fixing soils, high fertilizer application is currently necessary in order to provide sufficient plant-available P. P fixing soils are generally soils with low or high pH which cause complexation of P by aluminum (Al) or iron (Fe) and, in alkaline soils, by calcium (see Haefele et al 2014 and references therein). At a global scale, P fixing soils and Al toxicity are most widespread in South and Central America, followed by Sub-Saharan Africa and the Asia Pacific with 39%, 19% and 13%, respectively, of the total land affected by Al toxicity (**Figure 2a**). In rice production systems, severe and mild Al toxicity are main constraints in Asia (27% of rice soils affected), Africa (28%) and America (37%) (**Figure 2b**) (Haefele et al 2014).

The main negative effect of Al is inhibition of root growth and it is for this reason that progress in enhancing PUE can only be achieved if plants are also tolerant of Al toxicity or any other major stress limiting root growth. Addressing this, root development and plasticity in response to P deficiency and approaches towards developing combined tolerance of P deficiency and Al tolerance will be discussed within this review.

Macronutrients, such as  $P_i$  and N, are required in large quantities, with  $P_i$  as an important component of e.g., phospho-lipids and N a component of all cellular proteins and enzymes. Both are equally important for DNA and RNA synthesis and other nucleotide-containing molecules. P, in the

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form of ATP, ADP, P<sub>pi</sub>, NADPH and nucleotide sugars provide plant cells with the energy required for virtually all metabolic and catabolic cellular processes.

It is therefore not surprising that plants have developed highly sophisticated mechanisms and regulatory networks to access N and P, and control nutrient homeostasis. Plants have developed a suite of adaptive mechanisms to respond to P<sub>i</sub> deficiency and exudation of e.g., organic acids (to solubilize P complexes) or phosphatases and phytases (to access organic P) have been described in many studies (for a review see Hammond et al 2004; Lopez-Arredondo et al 2014). Likewise, the importance of arbuscular mycorrhizal symbiosis (AM) for P<sub>i</sub> uptake and as an extension of the root surface area has been studied in great detail (for a review see e.g., Lambers et al 2013; Gutjahr and Parniske 2013). The interested reader might be referred to a recent relevant study showing the effect of AM on the grain proteome in field-grown maize (Bona et al 2016 and references therein).

Studies on metabolic responses to P<sub>i</sub> deficiency have revealed an impressive capacity of plants to “save” P<sub>i</sub>, for instance by replacing P-lipids with sulfo- and galacto-lipids (Pant et al 2015 and references therein) or employing metabolic pathways not relying on ATP, for instance P<sub>pi</sub> dependent phosphofructokinase (Plaxton and Tran 2011). In addition, plants have evolved high and low affinity P<sub>i</sub> transporters for P<sub>i</sub> uptake from the soil and internal distribution, as well as for storage and remobilization of P<sub>i</sub> from the vacuole (Pratt et al 2009).

Plant responses and adaptation to P<sub>i</sub> starvation are being studied in great detail, mainly in model species such as Arabidopsis and rice, revealing a multi-layered network regulating gene expression, protein activity and protein turn over. The transcription factor gene *AtPHR1/OsPTF1* is a central regulator of the P-starvation response (for reviews see Yang and Finnegan 2010; Briat et al 2015; Gu et al 2016). In Arabidopsis and rice, it was shown that PHR1 is negatively regulated by SPX-domain containing proteins in a P<sub>i</sub>-dependent manner, and thus serves as a cellular P-sensor (Puga et al 2014; Wang et al 2014). More recent data by Wild et al (2016) support this and furthermore show that the complex formation between the rice transcription factor OsPHR2 and OsSPX4 depends on the presence of pyrophosphate inositol polyphosphates (PP-InsP), rather than P<sub>i</sub>. Strikingly, the

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affinity of complex formation differed between 5-InsP7 and InsP6 enabling cells to sense pyrophosphate groups. Many other regulatory factors/processes have been described. For example, the microRNA399 (miR399) is induced by Pi deficiency and controls the expression of *PHO2* (an ubiquitin E2 conjugase), which in turn represses other Pi responsive genes (see e.g., Lin et al 2008 and references therein). This discovery shed light on the function of *IPS1*, a gene that had earlier been identified as highly Pi responsive. However, because *IPS1* was not coding for a functional protein its function remained elusive until it was recently shown that its transcript sequesters miR399 under a mismatch (“target mimicry”) thereby preventing miR399 binding to and cleavage of *PHO2* mRNA (Franco-Zorilla et al 2007 and references therein).

In light of the complexity of the molecular basis of nutrient uptake and homeostasis in combination with the diversity of agro-environments, soil properties and co-occurrence of multiple stresses, it seems rather unlikely that individual genes can have any beneficial effect. However, there are a few genes that hold promise and/or are already being tested under field conditions and those genes (*OsPSTOL1*, *PHO1*, *AVP1* and *PHT1;6*) are described in more detail within this review.

Further to this, we will discuss some novel approaches currently being tested in rice aiming at the enhancement of internal PUE and the reduction of P removal from rice fields by reducing grain P. As a final chapter, this review will provide an update on the progress being made in developing plants that can use phosphite (Phi) as an alternative Pi source, thereby combining Pi nutrition with weed control.

### **Roots – the entry point for Pi**

Plant roots are located at the soil-plant interface and are the entry point for water and nutrients into the plant. Since P is generally located in the top soil and, in contrast to N, immobile, the ability of plants to access soil P is greatly dependent on their ability to develop a dense, shallow root system. Indeed, the ability to enhance lateral root growth and root hair length are plant-inherent traits as was studied in detail in the model species *Arabidopsis* (for a review see Niu et al 2013) and is also

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found in rice, barley and *Brachypodium* (Jeong et al 2015; Vejchasarn et al 2016; Huang et al 2011; Ingram et al 2012). The development of impressively dense cluster roots is a well described phenomenon in white lupin and other species (see Cheng et al 2011 and references therein).

Root hairs are of pivotal importance to nutrient uptake, particularly for poorly-mobile nutrients such as Pi, because they significantly increase root surface area and enable plants to explore the soil surrounding a root (the rhizosphere). It is therefore not surprising that root hair elongation in response to Pi deficiency is widely conserved in plants. The importance of root hairs has been demonstrated using root-hairless mutants of barley and *Arabidopsis* which were much less effective than their respective wild-type lines in taking up soil P (Bates and Lynch 2000; Gahoonia and Nielsen 2003). Where variation for root hair length within a species is under genetic control, this can be exploited by breeders to develop cultivars with improved Pi uptake efficiency. One of the earliest examples of breeding to increase root hair length is described by Caradus (1981) who developed white clover lines with long root hairs which conferred improved Pi uptake on soils that were steam-sterilised to be non-mycorrhizal. More recently, Miguel et al (2015) showed in field trials that shallow and hairy root traits are synergistic in their effects on Pi uptake by bean. Genes encoding a subset of the basic helix loop helix (bHLH) family of transcription factors have been identified that control root hair length and, importantly, overexpression of specific endogenous *bHLH* genes increased root hair length in *Arabidopsis*, rice and wheat (Han et al 2015; Ding et al 2009; Yi et al 2010).

Despite the fact that plants in general have the capacity to express root plasticity traits (increased lateral roots and root hair length), genetic diversity for the extent of this response has been reported in the above-mentioned studies suggesting that screening for quantitative differences, rather than qualitative screens, is the approach that needs to be applied. However, an inherent problem of working with roots is that they are inaccessible because they are naturally located below ground and it is therefore difficult to non-destructively extract intact root systems. Studies on root architectural changes in response to nutrients and nutrient flux studies are therefore mainly conducted in artificial

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media (hydroponics, agarose) or sand and other substrates that facilitate easy access to roots but might not be representative for soil-grown plants. This is indicated by e.g., a recent study in wheat which showed that genotypic differences in N uptake observed in soil-grown plants was not observed when plants were grown in hydroponics or sand (Melino et al 2015). In rice, dramatic differences in root architecture in different growth substrates (gel, beads, sand) have indeed now been documented using 3D X-ray computed tomography (CT scan) (Rogers et al 2016). With this new exciting technology it is also possible to image roots in pots and columns filled with soil and even with plants grown in competition (e.g., Metzner et al 2015; Mairhofer et al 2012 and 2015; Topp et al 2016 and references therein). One of the remaining limitations is the relatively small pot size which, at some stage, restricts root growth of developing plants. However, if genotypes are being used for those analyses that have shown differences under field conditions (e.g., McDonald et al 2015; Wissuwa and Ae 2001) or field-near screens with unrestricted root growth, chances are very high that we can identify relevant root traits and the underlying QTL and genes for PUE, and many other traits. An even more field-near, alternatively approach was reported by Pfeifer et al (2015) who extracted soil cores for CT scans from around plants grown undisturbed under field conditions.

### **Root development is under complex genetic and hormonal control**

Root development at the molecular level has been studied extensively and, as is the case for the P starvation response, a highly complex multi-faceted picture emerges (for review see Atkinson et al 2014; Bouain et al 2016).

In *Arabidopsis* seedlings, Pi deficiency reduces primary root growth and increases the growth of secondary roots as well as root hair length and density (Desnos, 2008; Svistoonoff et al 2007). A recent study further showed that root tips also play an important role and, despite their small size, accounted for approximately 20% of the total seedling Pi uptake (Kanno et al 2016).

In *Arabidopsis*, mutants have been identified that maintain primary root growth (PRG) even under low P conditions, such as the ferroxidase mutant *lpr1* (*low phosphate root 1*) or show enhanced

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inhibition of PRG (hypersensitivity), such as the P5-type ATPase mutant *pdr2* (*phosphate deficiency response 2*) (Ticconi et al 2004). More recently, it has been shown that Pi deficiency-induced reduction of PRG is influenced by iron (Fe) since the reduction of Fe concentration led to the recovery of primary root elongation under low P conditions (Svistoonoff et al 2007; Ward et al 2008). Furthermore, it has since been shown in *Arabidopsis* that Fe deposition in the root tip meristem leads to an accumulation of reactive oxygen species (ROS) and subsequent callose deposition, likely triggered by LPR1-dependent redox signaling (Mueller et al 2015). This might prevent cell-to-cell symplastic communication, which is required for maintenance of the stem cell niche and causing the inhibition of PRG (Mueller et al 2015).

In addition to Pi and Fe, an interdependence of Pi and Zn homeostasis has also been established as it was shown that, in the absence of Pi, increasing Zn concentration in the medium leads to a better developed root system (Rai et al 2015). These observations suggest that an increase in the internal Zn content could provoke a physiological Fe deficiency, thus alleviating the Pi deficiency responses in roots. Taken together, these data point toward the existence of an interaction between Pi and micronutrients (Zn and Fe) signaling pathways to control root growth.

In addition to interacting with micronutrients, Pi also interacts with macronutrients, such as nitrate and potassium (K). This has recently been studied in detail by Kellermeier et al (2014) revealing a complex interplay of these nutrients and their differential effects on root architectural changes and micronutrient content in *Arabidopsis*. From this work, the protein kinase CIPK23 (Calcineurin B-Like (CBL)-Interacting Protein Kinase 23) emerges as a central integrator of N and K interaction, in support of previous findings showing that CIPK23 also regulates the nitrate transporter NRT1.1 (Ho et al 2009). By combining transcriptome and genetic studies, Medici et al (2015) showed that integration of nitrate and Pi signals in *Arabidopsis* roots involves the transcription factor AtNIGT1/HRS1. HRS1 appeared as an integrator of nitrate and Pi signaling pathways repressing primary root development specifically when Pi is absent and N is present in the media (Medici et al 2015). Strikingly, depending on the N supply, NRT1.1. transports either nitrate or auxin (Krouk et al

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2010) providing a direct link to the hormonal control of nutrient uptake and homeostasis. Likewise, root architectural changes in response to Pi deficiency are regulated by a number of phytohormones including auxin (Nacry et al 2005), gibberellins (Fu and Harberd 2003), strigolactones (SLs) (Kapulnik et al 2011) and ethylene (Neumann 2016).

In light of this complex interplay of nutrients and transcriptional and hormonal regulatory networks modifying root growth in response to nutrient deficiency, it is a challenge to identify key regulators that are sufficiently upstream and robust to be suitable for developing plants with optimized root systems for nutrient uptake. However, genes that integrate the different pathway, such as CIPK23, hold promise and provide first entry points for optimizing root growth in crops.

#### **Aluminum Tolerance – a prerequisite for root growth and Pi uptake on acid soils**

It is critically important to realize that Pi deficiency is most often a companion stress, with Al toxicity by far the most widespread amongst the soil-inherent problems (**Figure 2a, b**) and it is therefore discussed here in more detail.

In acid soils, normally innocuous, Al is solubilised into the toxic Al<sup>3+</sup> ion and for many crop species the primary effect of Al<sup>3+</sup> toxicity is to inhibit root growth (Delhaize et al 2012a). A consequence of reduced root growth is poor exploration of soil and this is particularly important for the uptake of an immobile nutrient such as Pi. *TaALMT1* is the major gene for Al<sup>3+</sup> tolerance in wheat (*Triticum aestivum*) encoding an anion channel located at the plasma membrane (PM) that facilitates the efflux of malate from root apices (Delhaize et al 2012a). The gene was isolated by creating a subtractive cDNA library from root apices of the contrasting near isogenic wheat lines ET8 (Al<sup>3+</sup> tolerant) and ES8 (Al<sup>3+</sup> sensitive) (Sasaki et al 2004). Malate, when exuded by the root apex, binds the Al<sup>3+</sup> into a non-toxic complex. Because TaALMT is activated by Al<sup>3+</sup>, which means that malate is only exuded from root apices when Al<sup>3+</sup> is present, any metabolic cost to the plant is reduced by restricting secretion to root apices and limiting malate efflux to only when it is needed.

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In theory, the secreted malate could itself solubilise complexed soil Pi and consistent with this is the observation that barley lines engineered to express *TaALMT1* are both Al<sup>3+</sup> tolerant and have improved Pi uptake when grown on acid soil (Delhaize et al 2009). However, it is likely that the improved Pi uptake is a consequence of improved root growth, rather than dissolution of poorly-soluble P complexes by the malate, although it is difficult to disentangle primary and secondary effects.

Similarly, a gene (*SbMATE*) for Al<sup>3+</sup> tolerance of root growth in sorghum has been associated with improved Pi uptake efficiency in field trials (Leiser et al 2014; Carvalho et al 2016). *SbMATE* is a member of the Multidrug and Toxic Compound Extrusion (MATE) transporter family and is responsible for citrate efflux from roots. As discussed above for malate in barley, *SbMATE* could result in improved Pi uptake by improving root growth or by enhancing the dissolution of Pi from complexes by citrate. Similar genes have been cloned or identified in barley and maize but their effects on P mobilization have not yet been assessed (Maron et al 2010; Fuji et al 2012).

Although genes can confer Al<sup>3+</sup> tolerance to root growth, the hairs on these roots can still be Al<sup>3+</sup> sensitive. The rhizosheath is the soil that adheres to roots and, in wheat, is a reliable surrogate for root hair length (**Figure 3**). A simple screen for rhizosheath size in young wheat seedlings enabled the development of near isogenic lines (NILs) that vary in root hair length when grown in acid soil (Delhaize et al 2012b). Lines with large rhizosheaths on acid soil (long root hairs) had improved Pi uptake over small rhizosheath lines (short root hairs) in pot experiments using acid soils that contained toxic Al<sup>3+</sup> (James et al 2016). The QTL determining large rhizosheaths on acid soil did not co-locate with QTL for large rhizosheaths on non-acid soil. This indicates that genes involved in protecting root hairs from Al toxicity differ from those that confer long root hairs in the absence of constraints (Delhaize et al 2015).

The above mentioned wheat NILs for root hair length were developed based on a phenotypic screen but molecular markers linked to the identified QTL now provide a much simpler way to screen and

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will greatly improve the efficiency of selection in breeding programs. Future work will need to establish the value of the acid rhizosheath trait to Pi uptake in field trials. It will also be important to establish whether a large rhizosheath in young seedlings is indicative of root hair length in more mature plants grown under field conditions.

### **Phosphate transporters and their posttranslational control**

Pi uptake from the soil is mediated by Pi-H<sup>+</sup> co-transporters located at the PM (*PHT1* gene family) whereas Pi distribution within the plant, i.e., translocation into chloroplasts, mitochondria and Golgi, is mediated by *PHT2*, *PHT3* and the more functionally diverse *PHT4* gene family. The regulation of Pi transporter gene expression, their intracellular localization and function is highly sophisticated and multi-layered and the wealth of information available has been summarized in a recent excellent review by Gu et al (2016).

Under high Pi conditions, plants store excess Pi in the vacuole from where it is remobilized under starvation to maintain cellular homeostasis (Pratt et al 2009). Recently, it was shown that SPX-MFS (SYG1/Pho81/XPR1 (SPX)-Major Facility Superfamily (MFS)) proteins are responsible for Pi transport into the vacuole and were accordingly designated as PHT5 (Liu et al 2016). In contrast, Pi efflux from the vacuole into the cytosol, is mediated by OsSPX-MFS3, a low affinity Pi transporter located in the tonoplast (Wang et al 2015a). Further to these, the SPX-EXS domain protein PHO1 has been implicated with Pi loading into the xylem and long distance transport, and details on this highly interesting gene are provided below.

The *PHT1* gene family has been studied in most detail and comprises nine members in Arabidopsis and 13 genes in rice (Paszkowski et al 2002; Bucher 2007). In rice, *PHT11* and *PHT13* have been shown to be specifically expressed in arbuscular mycorrhiza colonized roots (Paszkowski et al. 2002; Jeong et al 2015). Two other *PHT1* family members, *OsPHT1;2* and *OsPHT1;6* have been functionally

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characterized (Ai et al 2009) and the latter emerges as a promising target as outlined in more detail below.

As was shown in Arabidopsis and rice, the correct location of (at least a subset of) PHT1 transporters in the PM is regulated by PHOSPHATE TRAFFIC FACILITATOR 1 (PHF1) located in the endoplasmic reticulum (ER) (for a review see Gu et al 2016). In elegant experiments Bayle et al (2011) have demonstrated that in the absence of PHF1, the transporter is diffusely distributed within different cell compartments and that only a fraction of the transporter is localized to the PM (as shown by transient expression in onion cells). The authors also showed that, under sufficient P conditions, PHT1 is phosphorylated and thereby retained in the ER and furthermore subjected to more rapid (PHO2-independent) degradation compared with low Pi conditions (Bayle et al 2011). The finding that, upon high transient expression (in onion cells), only a small proportion of PHT1;1 is actually localized to the PM unless co-expressed with PHF1, suggests that overexpression of a transporter gene alone might be a too simplistic approach to enhance Pi uptake.

However, there is some evidence in the literature that overexpression of Pi transporters might be beneficial after all. In rice, two transporters have been functionally characterized in more detail identifying OsPHT1;2 as a low affinity Pi transporter and OsPHT1;6 (*OsPT6*) as a high affinity Pi transporter, with the latter being expressed in the epidermis, cortex and stele of young lateral seedling roots (Ai et al 2009). Despite the complexity of the regulation of Pi uptake, overexpression of *OsPT6* alone revealed promising results in two independent studies. In rice, *OsPT6* was constitutively expressed using the ubiquitin promoter and plants were tested in P deficient soil and under field conditions (Zhang et al 2014). The authors report that the transgenic plants had a higher P content over a range of different P levels and, furthermore, the transgenic plants had a higher grain yield under field conditions, mainly attributed to an increased tiller number (Zhang et al 2014). In soybean constitutive (35S promoter) expression of the *OsPT6* gene enhanced P content and improved growth in low Pi hydroponic culture solution (Yan et al 2014). A comparative study using a set of seven diverse rice genotypes furthermore revealed a relatively higher *OsPT6* gene expression

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in the irrigated variety IR66, which also showed a strong P-starvation induced increase in root growth (Jeong et al 2015). In agreement with that, in two independent field experiments IR66 maintained its grain yield under Pi deficiency, whereas other varieties reduced grain yield by up to 50% (Jeong et al 2015).

Based on the above evidence, it seems worthwhile to explore natural allelic variation within the Pi transporter gene families and their regulatory genes. The availability of an ever increasing amount of genome sequences data now provides us with an unprecedented opportunity to access genetic diversity in rice and other crops to identify natural gene variants for evaluation and breeding applications (for a review see Wissuwa et al 2016).

#### **PHO1 – rendering plants insensitive to P starvation**

Of the many genes that have been implicated with the P starvation response (PSR; for a recent review see Lopez-Arredondo et al 2014), *PHO1* is among the most interesting genes since it was shown that is possible to uncouple Pi deficiency from its major negative effects on plant growth by reducing *PHO1* expression in Arabidopsis (Rouached et al 2011). This raises the question on the role of Pi concentration *per se* and the actual benefit of the PSR.

The genome of Arabidopsis contains 11 members of the *PHOSPHATE1* (*PHO1*) gene family. *PHO1* proteins are composed of three distinct regions (Wang et al 2004). The N-terminal hydrophilic region is composed of a tripartite SPX domain followed by a series of putative transmembrane  $\alpha$ -helices that are partly included in the C-terminal EXS domain (named after the yeast proteins ERD1 and SYG1, and the mammalian XPR1 protein) (Wang et al 2004). So far, in Arabidopsis *PHO1*, *PHO1;H1* and *PHO1;H3* have been shown to be involved in loading Pi into the xylem, which is consistent with their expression in the root vascular cylinder (Hamburger et al 2002; Stefanovic et al 2007; Secco et al 2012; Khan et al 2014). The Arabidopsis *pho1* mutant is impaired in transferring Pi from the root to the shoot, resulting in dwarf plants with normal root but low shoot Pi content (about 3-10% of wild type plants level Pi in the shoot) (Poirier et al 1991).

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The low Pi content in *pho1* shoots was believed to be the cause of the severe reduction in shoot growth, however, this causal link was challenged based on the phenotype of transgenic plants under-expressing *PHO1* (Rouached et al 2011). These low-PHO1 plants, similar to the *pho1* null mutant, were defective in Pi loading into the xylem and Pi accumulation in shoots. However, unlike the *pho1* mutant, low-PHO1 plants did not display the hallmarks of Pi deficiency in the shoot and were indistinguishable from Pi sufficient wild type plants, suggesting that they do not perceive or do not respond to Pi deficiency (**Figure 4**). Indeed, in contrast to *pho1* mutants or Pi deficient wild type plants, low-PHO1 plants showed very limited transcriptional changes despite having a similar low shoot Pi content. These data for the first time demonstrate that it is possible to unlink low shoot Pi content from plant responses thereby enabling plants to retain growth despite Pi deficiency. Importantly, these findings also show that reduced shoot growth is not a direct consequence of Pi deficiency, but is more likely a result of extensive gene expression reprogramming triggered by Pi deficiency.

More recent data furthermore demonstrated that the uncoupling of low shoot Pi and shoot growth can be achieved by expressing only the EXS domain of PHO1 in roots of the *pho1* mutant (Wege et al 2016). Indeed, expression of the EXS domain stimulated shoot growth and the attenuation of the Pi deficiency signalling cascade. Furthermore, a grafting experiment performed by Wege et al (2016) showed that the expression of the EXS domain in *pho1* mutant roots is sufficient to achieve growth improvement of the Pi-deficient shoot. In agreement with the data from Rouached et al (2011), this was associated with reduced expression of a large set of PSR genes. This effect was specific to the EXS domain since the expression of the PHO1 SPX domain had no effect in *pho1* mutants (Wege et al 2016).

Collectively, these data propose a new role for PHO1, and more particularly EXS domains, in transmitting a Pi-deficiency induced root-to-shoot signalling. One attractive hypothesis is that, under non limiting Pi conditions, plant roots transmit an EXS-related signal to the shoot to suppress PSR

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genes. Under Pi-deficient conditions, low Pi roots would no longer produce this repressor signal which would release the repression and trigger the PSR.

Additional examples in the literature suggest that growth retardation following a stress is not necessarily mediated by the damage caused by the stressing agent, but rather by the genetic programs induced by the stress. This was for instance shown for the *executor* mutant of Arabidopsis, which does not respond to oxidative stress induced by light after a dark period and therefore grew better than control plants (Wagner et al 2004). Similarly, a quadruple mutant in DELLA proteins grew better than wild type plants under salt stress, likely due to alteration in the gibberellic acid (GA) signal transduction cascade (Achard et al 2006). DELLA proteins have also been shown to be an important component of the *SUB1A-1* mediated submergence tolerance pathway in rice which suppresses hormone-induced elongation growth in submerged plants (Fukao and Bailey-Serres 2008 and references therein).

The concept that stress tolerance can be achieved by suppressing stress response pathways is striking but more research in this area is needed. In the case of PHO1, it will be important to establish that the data derived from studies using Arabidopsis are transferrable to crops and to confirm its function under relevant field conditions. The finding that the rice PHO1 ortholog can complement the Arabidopsis *pho1* mutant (Rouached et al 2011) suggests that the pathway is conserved in rice. This opens opportunities to explore natural allelic variation or gene editing approaches, such as CRISPR/cas9, for instance, to remove P-responsive PHO1 promoter elements and/or modify PHO1 protein structure.

#### **Exploring genetic diversity in forward genetic screens: The tolerance gene *OsPSTOL1***

For the discussion on the benefit of the PSR and the above-outlined importance of root traits for Pi uptake, the rice gene *PHOSPHORUS STARVATION TOLERANCE 1* (*OsPSTOL1*) provides a good example. *OsPSTOL1* has been identified as the causal gene within a major quantitative trait locus (QTL) enhancing plant growth in highly Pi-deficient soil (Wissuwa and Ae 2001; Gamuyao et al 2012).

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The QTL *Phosphate uptake 1 (Pup1)* was initially identified based on a small diversity study of 30 rice genotypes showing that two rice landraces, Dular and Kasalath, had the highest Pi content (Wissuwa and Ae 2001) (**Figure 5a**). Using a Kasalath x Nipponbare (intolerant japonica type rice) population *Pup1* was subsequently mapped to rice chromosome 12 and a smaller effect QTL was identified on chromosome 6 (**Figure 5b**). (Wissuwa et al 2002). The latter contains a cluster of Pi starvation genes including *OsPTF1* (Heuer et al 2009). In contrast, no obvious candidate genes could be identified within the *Pup1* QTL on chromosome 12 and the region was therefore sequenced and de-novo assembled (Heuer et al 2009). Compared to the Nipponbare reference genome, the Kasalath *Pup1* region is significantly larger due to the presence of a 120 kbp Insertion-Deletion (INDEL) and represents a transposon hot spot. From the 68 predicted gene models, two genes were eventually short listed, namely the dirigent-like gene *OsPupK20-2* and *OsPSTOL1*, which encodes a functional serine/threonine kinase located in the Kasalath-specific INDEL. *OsPSTOL1* is therefore absent from the Nipponbare reference genome (**Figure 5c**) and other intolerant genotypes (Gamuyao et al 2012). When IR64, a rice variety that naturally lacks the *PSTOL1* gene, was transformed with a 35S::*OsPSTOL1* construct, plants showed enhanced growth of nodal roots under both, high and low Pi conditions, in soil and in hydroponics. This was also observed in NILs with the Kasalath *Pup1* introgression. Due to the increase in root surface area, plants were able to take up more P, but also other nutrients, such as N and K (Gamuyao et al 2012). In a pot experiment with P-deficient soil, transgenic plants had up to 60% higher grain yield (Gamuyao et al 2012) (**Figure 5d**).

Although the exact mode of action of *OsPSTOL1* remains to be investigated in detail, two independent microarray studies using *Pup1* NILs and 35S::*PSTOL1* transgenic plants suggest that PSR genes are not primarily involved (Pariasca-Tanaka et al 2009; Gamuyao et al 2012). Rather, in agreement with the observed phenotype, root-growth related genes were identified, such as those encoding xyloglucan endotransglycosylases/hydrolases or *OsHOX1*, amongst other genes including a set of eight transcription factor genes.

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Phenotyping for Pi deficiency tolerance under field conditions is difficult and data are often very variable. As was already mentioned above in the context of the *ALMT* gene, information on the causal gene and genomic QTL regions now enables us to at least partially replace field screenings with marker-assisted selection. However, because of its location in an INDEL, *OsPSTOL1* markers are presence-absence markers (Chin et al 2011) and cannot be assessed by markers based on single nucleotide polymorphism (SNP), which are routinely used in breeding programs today. It is also important to consider that *OsPSTOL1* codes for a protein kinase and is as such member of a large gene family in plants (for a review see Lehti-Shiu and Shiu 2012). Therefore, molecular markers need to be highly specific to the target gene to avoid unspecific amplification when used in breeding programs. In fact, the first set of *OsPSTOL1* specific markers produced unreliable results when tested in accessions of e.g., the African rice *Oryza glaberrima* (STEUD) but this, eventually, led to the identification of a novel *OsPSTOL1* allele in the rice variety CG14, the founder parent of NERICA (New Rice for Africa) upland breeding lines (Pariasca-Tanaka et al 2014). The molecular markers for *OsPSTOL1* and the *Pup1* dirigent-gene (*OsPupK20-2*) have also been tested in an Indian breeding program for acidic lowland conditions (Yumnam et al 2015). However, the authors found that *OsPSTOL1* was conserved in only two out of five tolerant genotypes. This suggests that alternative tolerance genes might be present in these genotypes but also again emphasizes the fact that Pi deficiency is often a companion stress and, in this case, might be caused by inhibition of root growth due to soil acidity and/or the related Al toxicity. Other studies suggest that *PSTOL1* orthologs are associated with enhanced tolerance of Pi deficiency in sorghum (Hufnagel et al 2014; Azevedo et al 2015). *Pup1* rice breeding lines are currently being evaluated in low-input upland rice growing areas, mainly in South East Asia and Africa, to evaluate their performance in different target environments.

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## The H<sup>+</sup>-pyrophosphatase *AVP1* – a gene with multiple benefits

Another interesting gene that has been shown to be beneficial under low Pi conditions is the Arabidopsis vacuolar H<sup>+</sup>-pyrophosphatase AVP1. This gene was originally identified in the context of salt and drought tolerance but was later shown to have other beneficial effects, including enhanced tolerance of Pi starvation (for a review see Gaxiola et al 2016a and b).

The salt tolerant phenotype observed in Arabidopsis was explained by an increased capacity for Na<sup>+</sup> uptake into vacuoles whereas drought resistance was attributed to an enhanced vacuolar osmoregulatory capacity (Gaxiola et al 2001 and 2002). Type I H<sup>+</sup>-PPases were first isolated from vacuoles and were considered to be *bona fide* vacuolar markers (Maeshima, 2000 and references therein) but were later also found in tonoplasts of cells in sink tissues and the PM of companion cells and sieve elements in Arabidopsis and rice (Paez-Valencia et al 2011; Regmi et al 2015; Viotti et al 2013).

In addition to salt and drought tolerance, Arabidopsis, tomato, rice and maize plants engineered with an H<sup>+</sup>-PPase overexpression cassette also showed a more vigorous response to Pi deficiency compared to controls (**Figure 6a**). When grown under Pi deficient conditions they developed significantly more robust root systems (Pei et al 2012; Yang et al 2007) and, in the case of Arabidopsis, 2.5-fold larger and 1.5-fold denser root hairs (Yang et al 2007). In tomato, *AVP1* overexpression increased root and shoot dry weight on average by 13% and 16%, respectively, over control plants under low Pi conditions and fruit dry weight and Pi content per tomato plant were 82% and 30% higher than wild-type, respectively (Yang et al 2007). These tomato plants produced about 25% more marketable ripened fruit per plant under Pi deficiency compared with the controls and also showed increased Pi transport from source leaves to fruits (Yang et al 2014).

It has further been shown that, under Pi deprivation, *AVP1* overexpression in Arabidopsis increased rhizosphere acidification, caused constitutive expression of sugar-induced root ion transporter genes (including the *PHT1.4* transporter) and increased organic acid exudation (Pizzio et al 2015; Yang et al 2007). Increased rhizosphere acidification is a consistent phenotype of transgenic plants expressing

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*H<sup>+</sup>-PPase* genes and is likely contributing to the increased Pi availability and the observed higher Pi uptake (Pei et al 2012; Gaxiola et al 2012; Yang et al 2014). Further support for a role of *H<sup>+</sup>-PPases* under Pi starvation comes from earlier work in Brassica and other species (Palma et al 2000; Ohnishi et al 2007; Kasai et al 1998) and it has also been shown that *H<sup>+</sup>-PPases* are a component of PSR since they were upregulated in *OsPTF1* overexpression lines (Yi et al 2005).

Further to this, it has been shown that, under low N supply, transgenic lettuce (*Lactuca sativa*) had a 2-fold higher shoot and 3-fold higher root biomass, increased rhizosphere acidification and greater total N compared to wild-type plants (Paez-Valencia et al 2013) and transgenic alfalfa (*Medicago sativa*) showed increased solute and water accumulation, greater photosynthetic activity and less damage to cell membranes under drought and salinity stress (Bao et al 2009). Transgenic barley expressing *AVP1* shows a growth advantage under control conditions compared to non-transgenic controls at the seedling stage (**Figure 6b**) as well as under high and low saline field conditions (**Figure 6c**; Schilling et al 2014). Significant increases in grain and fibre yields compared to wild-type plants in a dryland field has furthermore been shown for transgenic cotton expressing *AVP1* (Pasapula et al 2011).

The exact underlying mechanisms conferring the broad benefit of *AVP1* expression in transgenic plants from different species is still being investigated but emerging evidence suggests this is at least partly due to the regulation of photoassimilate partitioning (Khadilkar et al 2016; Pizzio et al 2015; Gaxiola et al 2012; Gaxiola et al 2016b; **Figure 6d**). This is based on the finding that both, constitutive and companion-cell specific overexpression of *AVP1* enhanced shoot and root biomass under control growth conditions. Furthermore, <sup>14</sup>C-labeling experiments showed enhanced photosynthesis, phloem loading and transport, as well as delivery of assimilates to sink organs (Khadilkar et al 2016).

This increased transport of sucrose from source to sink tissues is thought to arise not from the *H<sup>+</sup>-PPase* activity of *AVP1* but rather from its *PP<sub>i</sub>-synthase* activity (Gaxiola et al 2012; Gaxiola et al 2016b). By synthesizing *PP<sub>i</sub>* in the cytosol of phloem companion cells, *AVP1* can facilitate sucrose respiration via UDP-Glucose pyrophosphorylase and *PP<sub>i</sub>-dependent phosphofructokinase*. The latter

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had been already mentioned above as an important alternative, ATP-independent pathway under Pi starvation. Greater sucrose respiration increases the supply of ATP, which in turn increases companion cell PM ATPase activity establishing an electrochemical gradient. This, as the driving force for sucrose/H<sup>+</sup> symporters, would enhance sucrose uptake into companion cells and thus increasing sucrose loading into the phloem (for a review see Gaxiola et al 2016a).

The broad beneficial effect of AVP1 might also be related to the regulation of intracellular PP<sub>i</sub> concentrations. In plant tissues, a large amount of PP<sub>i</sub> is produced as a by-product of various metabolic processes including the synthesis of DNA, RNA, proteins, sucrose and cellulose (Maeshima, 2000; Smyth and Black 1984). Compared to wild-type, an Arabidopsis mutant with a defective AVP1 gene (*fugu5*) had a 2.5-fold higher PP<sub>i</sub> content per seedling, 60% fewer cotyledon cells and lacked the ability to grow heterotrophically (Ferjani et al 2011). A high concentration of cytosolic PP<sub>i</sub> is known to inhibit metabolic processes, including gluconeogenesis, and it was proposed that the hydrolysis of cytosolic PP<sub>i</sub> to P<sub>i</sub> by H<sup>+</sup>-PPases improves heterotrophic growth (Ferjani et al 2011). Indeed, the expression of *IPP1*, a gene encoding a cytosolic soluble inorganic pyrophosphatase from yeast (*S. cerevisiae*), complemented the phenotype of *fugu5* mutants (Ferjani et al 2011). It was further shown that transformation of *fugu5* mutants with uncoupling mutated variants of the Arabidopsis type I H<sup>+</sup>-PPase (which hydrolyse cytosolic PP<sub>i</sub> but do not translocate protons) also complemented the *fugu5* phenotype (Asaoka et al 2016). This suggests that hydrolysis of cytosolic PP<sub>i</sub> by H<sup>+</sup>-PPases is important for plant growth and also likely to be contributing to the improved growth of transgenic plants expressing AVP1 (Asaoka et al 2016).

In summary, AVP1 is one of the most advanced P-related genes currently studied with a proven benefit across different plant species and already tested under field conditions. At least part of the broad function of this gene can be attributed to an increased nutrient uptake via root acidification and its holistic positive effect on Pi homeostasis and the energy status within plants.

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## Internal PUE versus enhanced Pi uptake

Enhancing internal PUE, in contrast to Pi uptake, is an important distinction and requires different aspects to be considered. Although it is highly desirable to enhance Pi uptake from the soil, care has to be taken that nutrients removed from fields are replenished regularly to maintain soil fertility and prevent soil degradation. For low-input agricultural systems, which are most prevalent in developing countries, crops with enhanced Pi uptake might therefore not have a long-lasting benefit but might deplete soil P reserves even faster. This would be especially critical on soils with low total P content and therefore, for those regions crops that require less Pi per unit harvested grain would be the more promising approach.

Internal changes in plants in response to Pi starvation involve changes in many metabolic pathways, remobilizing of P from senescing to younger tissues, the replacement of phospholipids with sulfo- or galactolipids and alternative ATP-independent pathways have been mentioned above and all are associated with adaptive plant responses to Pi deprivation that would increase internal PUE (for a review see Plaxton and Tran 2011). However, there is still little information on whether these genes and phenotypic variations for such traits exist that could be exploited for crop improvement. Indeed, as already mentioned above in relation to *OsPSTOL1*, transcriptional changes in PSR genes were equal or less pronounced than in intolerant controls (Pariasca-Tanaka et al 2009; Gamuyao et al 2012). This emphasizes the necessity of differentiating stress-responses, which can be observed in intolerant genotypes, from true tolerance mechanisms/genes, as was recently reiterated in an RNAseq study comparing four rice cultivars with contrasting tolerance to P-deficiency (Oono et al 2013).

For assessing internal PUE, an inherent complication is that reliable genotypic comparisons require that genotypes have equal tissue P content (Rose et al 2016). Putative QTL for internal PUE, identified in studies concomitantly screening for differences in Pi uptake, tend to be pseudo-QTL because genotypic differences in Pi uptake directly affect tissue P concentrations and, hence, internal PUE estimates. Addressing this, a recent study, conducted at equal plant P content using a

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set of 292 diverse rice genotypes, revealed that internal PUE ranged from 1.4 to 2.8 g biomass per mg Pi provided (Wissuwa et al 2015). Through genome wide association mapping several loci and underlying genes were identified, one of these was significantly upregulated upon Pi-starvation, particularly in old leaves, suggesting a function in Pi remobilization. This gene shows partial homology to a RNA ligase/cyclic nucleotide phosphodiesterase (CPDase), which have been implicated in nuclear tRNA metabolism (Hofmann et al 2000). As RNA constitutes one of the main cellular P pools, the regulation of RNA levels may offer opportunities for P efficiency gains, as recently shown for an Australian native species of the Proteaceae family (for a review see Lambers et al 2013). While studies on candidate genes identified by Wissuwa et al (2015) are ongoing, rice breeding programs in Africa are already using high internal PUE genotypes as donors in their efforts to develop cultivars better adapted to the low-input agriculture practiced in much of the continent.

#### **Reducing P removal from the field**

Another aspect of crop PUE that has not received much attention, is grain P content. Typically, P concentrations in rice seeds harvested from farmer's field range from 2.0 to 2.5 mg g<sup>-1</sup> (Dobermann et al 1996), yet under well-fertilized conditions concentrations in excess of 4 mg g<sup>-1</sup> are observed. This suggests that P translocation to the seed is not tightly regulated but that plants simply tend to load most tissue P into developing grain. The P harvest index (PHI) therefore tends to be higher (PHI= 0.7-0.9) than the grain-biomass harvest index (HI= 0.5-0.6) (Rose et al 2010). As a consequence, a large quantity of P is removed from fields at harvest increasing the need for continued fertilizer application to avoid long-term decrease in soil fertility. As an estimate, a 10-t wheat crop would remove between 25-30 kg P ha<sup>-1</sup> per year whereas a 6-t rice crop would remove about 15 kg P ha<sup>-1</sup> per season. Thus yield level, cropping frequency and grain P concentrations are the dominant factors driving P requirements in agricultural systems, but only grain P concentrations offer opportunities for modification without jeopardizing production (Rose et al 2010; Rose et al 2013). Indeed, there is considerable scope for reducing grain P since it was shown that seedling vigor

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and crop yield can be unimpaired in seeds with P concentrations as low as 1 mg g<sup>-1</sup> (Pariasca-Tanaka et al 2015; Wang et al 2015b). It has been estimated that the P requirement for normal physiological seed function is even below 1 mg P g<sup>-1</sup> and that grain P present above this level serves as storage P, mostly in the form of phytate (Raboy 2009; Wang et al 2015b). Phytate consumed with the grain has very low digestibility and therefore increases P loads in animal and human wastes with negative effects on the environment. Attempts have been made to engineer phytate content in seeds, however, in barley and soybean mutant lines with reduced phytate content the amount of total seed P typically remained unchanged (Raboy 2009). One exception was the case of the barley *lpa1-1* mutant that reduced total seed P by up to 20% as a result of a mutation in a putative sulfate transporter (Ye et al 2011).

Another approach to reduce grain P content would be to manipulate Pi transporters responsible for grain Pi loading. In this context, Wang et al (2015b) analyzed all 26 known rice Pi transporters but found that none of the genes was specifically expressed only in seeds and thus, none of the known Pi transporters can be considered a prime target for mutagenesis. At present, the most promising ongoing approach may be the screening for mutant lines with low seed arsenate content. Arsenate and phosphate are chemical analogs and compete for uptake sites at the root surface (Abedin et al 2002). It is therefore likely that both share the same transport mechanisms in seed loading.

#### **Alternative P fertilizer - Phosphite**

As mentioned above, the problem of low Pi availability in many soils is due to P fixation. Thus, from the total Pi present in the soil only a small fraction is soluble and readily available for plant uptake. To address this problem, different formulations of Pi-fertilizer have been developed in the form of complexes with organic acids that reduce or delay its reactivity, or slow-release particles to render Pi available for longer periods of time. However, because Pi is the only chemical form of P that can be assimilated by plants, no attempts were made in the past to explore the use of other P chemical forms, less prone to be adsorbed onto soil particles or to form insoluble complexes.

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Recently, a novel technology, based on metabolic engineering to allow plants to metabolize phosphite (Phi; a reduced form of P) was developed with the aim of optimizing the effectiveness of P-fertilizers. This technology is based on the expression of a bacterial phosphite oxidoreductase (PTXD) in plants that converts Phi into Pi, enabling engineered plants to produce the Pi required to support plant growth and reproduction (López-Arredondo and Herrera-Estrella, 2012). Phi enters the plant using the same transporter proteins as Pi, however, it is not used by the cellular machinery because plants lack the enzyme necessary to convert it into Pi. Because genetically modified plants expressing PTXD are able to use this non-metabolizable compound as a Pi source, in a low-Pi soil fertilized with Phi these plants can outcompete weeds (unable to use Phi as a P source), thereby reducing the need to apply herbicides for weed control (López-Arredondo and Herrera-Estrella, 2012; Manna et al 2016). To date it has been shown that, at least under greenhouse conditions, several of the most aggressive weeds can be effectively controlled using Phi fertilization (López-Arredondo and Herrera-Estrella, 2012). The effectiveness of the Phi technology as a fertilization and weed control system was initially reported for Arabidopsis and tobacco transgenic plants. More recently, it was reported that PTXD transgenic rice plants can efficiently use Phi as a P source with grain yields comparable to those obtained in Pi fertilized control plots and wild type plants (Manna et al 2016). Additionally, that study reported that Phi is effective as both, pre- and post-emergence control of several weeds that represent serious problems for rice cultivation.

To demonstrate the robustness of the Phi system in different soil types, field trials with tobacco transgenic plants were conducted in two different locations with low levels of available Pi in the soybean-producing region of Argentina. Using a split plot randomized block design with 3 replicates, it was observed that a dose of 80 ppm of Phi was sufficient to promote the growth of the engineered tobacco plants and effective to control weed growth. This was considerably further improved using 180 ppm of Phi (**Figure 7**). An important observation in these field trials is that Phi did not act as a herbicide, but rather inhibited the growth of naturally occurring weeds to an extent that they did not represent competition for PTXD engineered plants (**Figure 7**) Interestingly,

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the Phi technology not only has the potential to significantly reduce the use of agrochemicals with inherent health and ecological benefits, but further to this, the residual weed growth observed in Phi fertilized soils could potentially help prevent soil erosion and water evaporation, which is a long standing demand of agro-ecologists to achieve a more sustainable agriculture.

Based on the concept of using Phi for the control of weeds in agriculture, a novel application of the Phi technology was reported for the control of contaminations with weedy microalgae and other microorganisms during the production of microalgae in closed and open photo-bioreactors (Loera-Quezada et al 2016). It has also been reported that PTXD can be used as a dominant selectable marker for the production of transgenic plants. This was first reported for tobacco and Arabidopsis (López-Arredondo and Herrera-Estrella, 2013) and more recently for the production of transgenic maize (Nahampun et al 2016) eliminating the need of using antibiotic and herbicide resistance genes for the production of genetically modified plants.

Although Phi comes from the same mineral resource as Pi, phosphate rock, this technology will not, at least initially, use the reserves of phosphate rock that are currently used for producing Pi-fertilizer. Rather, Phi is a waste product of the electronic, automotive, pharmaceutical, chemical, construction and other industries that uses sodium hypophosphite as a reducing agent in nickel plating, polishing & reducing agent, among other processes. Since Phi is highly stable as powder, it can be used to design Phi-based fertilizers, which will be stable in the soil solution for two to three months.

### **Summary and Outlook**

The intention of this review was to highlight and summarize a range of diverse ongoing projects and different approaches aiming at the development of crops with enhanced PUE. This will not be a matter of simply enhancing Pi uptake, although this is a highly desirable trait and the importance of root traits and metabolic changes have been described in this context here. However, if not replaced by fertilizers, P removal from agricultural production systems with the harvest ultimately leads to P depletion and soil degradation. Novel approaches, such as reducing grain P content are therefore very valid. Technologies, such as Phi, combining the benefit of P fertilizer with weed control, while

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using industrial waste, serves as an example for a very practical and lateral approach applying detailed knowledge on genes and pathways in novel ways. More of this will be needed in light of the challenges ahead.

In this review, we also attempt to draw attention to the fact that Pi deficiency is often a secondary stress and that, without addressing the primary stress (here exemplified by Al toxicity) there will be no progress in improving PUE. Likewise, there is a need to establish more interdisciplinary collaborations to more efficiently evaluate the many PSR genes identified by the global research community. The evaluation of promising genes, such as those highlighted in this review (*AVP1*, *PSTOL1*, *ALMT*, *PHO1*) require considerable resources and long-term funding for testing under field conditions and in different genetic backgrounds. Because this is beyond the capacity and life time of most research projects, strategic partnerships need to be established to ensure that research findings are being translated into developing crops with the best possible combination of high-value genes, fine-tuned to the diverse agro-ecological systems that secure global food production.

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## Figure legends

### Figure 1. Global macronutrient demand over time.

The demand for fertilizer nutrients is increasing due to increasing demand for food production. Nitrogen fertilizer is required in the largest amount, followed by phosphorus ( $P_2O_4$ ) and potassium ( $K_2O$ ). Data source: FAOSTAT Date: Fri Apr 15 04:22:14 CEST 2016 (2009–2014); IFA Heffer June 2011 (2010–2013); FAO world fertilizer trends and outlook to 2018 (2014–2018)

## Figure 2. Global and rice soils with phosphorus deficiency and aluminum toxicity.

Phosphorus (P) fixation and aluminum (Al) toxicity are major soil constraints in South and Central America, Sub-Saharan Africa and the Asia Pacific (a). Numbers in the bars indicate % of total land area. Soils with Al toxicity and P fixation are also major problems in rice agricultural systems (b). Data source: Bot et al (2000) and Haefele et al (2014).

## Figure 3. Root rhizosphere of wheat seedlings in acid soil.

Seedlings of the wheat cultivars EGA-Burke (a–c) and Fronteira (d–f) were grown on acid soil for 3 days. The rhizosphere is defined as the soil that remains adhered to roots when seedlings are gently removed from soil. Close up views of the rhizosphere and root hairs on EGA-Burke (b, c) and Fronteira (e, f). Fronteira is a Brazilian cultivar that has Al<sup>3+</sup>-tolerant root hairs and EGA-Burke is an Australian cultivar with sensitive root hairs. Both cultivars have an Al<sup>3+</sup>-tolerant allele of *TaALMT1* that allows roots to grow in the presence of Al<sup>3+</sup>. The arrows on panels (a) and (d) indicate the region of the root (apices) from where malate is secreted. This region is well away from where the rhizosphere is largest, indicative of the zone where root hairs elongate (modified with permission from Delhaize et al 2012b).

## Figure 4. The role of PHO1 in Arabidopsis

Sufficient inorganic phosphate (+Pi) is required for shoot growth of wild type (WT) plants and, under this condition, P starvation response (PSR) genes are repressed. Pi-deficiency (–Pi) leads to a strong decrease in shoot growth which is associated with the induction of PSR genes, including Pi transporters in the roots (indicated by black dots). A striking phenotype of PHO1-underexpressing plants (low PHO1) and also of *pho1* mutants complemented with the EXS domain (*pho1* + EXS) (Wege et al 2016; see text for details) is the maintenance of shoot growth comparable to a Pi-sufficient WT plant despite their low Pi content. Low levels of PHO1 in roots (indicated as red dots), while insufficient for mediating adequate Pi transfer from root to shoot

via the xylem (blue arrow), would be sufficient for the transmission of the molecule responsible for the suppression of PSR. While the nature of the molecule is unknown, results of Rouached et al (2011) show that it is unlikely to be Pi itself (see text for details).

**Figure 5. The major rice QTL *Pup1* and *OsPSTOL1***

From a screening of thirty rice genotypes in a highly P deficient field, Kasalath was shown to be more efficient in P uptake than Nipponbare and most other genotypes analyzed (**a**; data were derived from Wissuwa and Ae, 2001). Two QTL were identified in a Kasalath x Nipponbare mapping population: The QTL on chromosome 12 had the largest effect and was named *Pup1* (**b**). The QTL on chromosome 6 was less strong although several P starvation response genes co-localized in the region (Heuer et al 2009). The *Pup1* region was sequenced and compared to the Nipponbare reference genome. Of the 67 gene models within the *Pup1* locus, the protein kinase *OsPSTOL1* is located in a large INDEL which is absent in Nipponbare (**c**). The dirigent-like gene *OsPupK20* is downstream of *OsPSTOL1* (Gamuyao et al 2012). Transgenic plants with constitutive gene expression (*35S::OsPSTOL1*) showed enhanced root and shoot growth, and grain yield in P-deficient soil (**d**; modified from Gamuyao et al (2012)).

**Figure 6. The H<sup>+</sup>-PPase gene *AVP1* confers tolerance to P deficiency and salt stress.**

Shoot and root growth of transgenic tomato plants constitutively expressing the H<sup>+</sup>-pyrophosphatase gene *AVP1D* (*35S:AVP1D*) is enhanced compared to wild type control plants when grown in soil with 100 ppm and 44 ppm NaH<sub>2</sub>PO<sub>4</sub> after 40 days. The same positive gene effect was observed in transgenic *35S:AVP1D* rice grown with 10 μM P for 35 days (modified from Yang et al 2007) (**a**). Enhanced shoot growth was also observed in *35S:AVP1* barley after 27 days grown in a sandy loam with sufficient nutrients (**b**) as well as in field experiments under low and high salinity (12 dS/m) conditions (field site near Kunjin, Western Australia) (**c**). A schematic of an Arabidopsis plant without (wild type) and with constitutive overexpression of

*AVP1* (35S:*AVP1*) indicating the different beneficial effects of *AVP1* overexpression (see text for details) (d).

**Figure 7. Effectiveness of the phosphite-system under field conditions.**

The effect of phosphite (Phi) as a weed control agent and as a P fertilizer was evaluated in field experiments (split plot randomized blocks with three replicates) using a tobacco transgenic line (ptxDNt80) and a non-transgenic sibling wild type (WT) control. An aerial view is shown of three experimental plots (outlined by white dotted lines) cultivated with WT and PTXD-engineered tobacco plants fertilized with 180 mg kg<sup>-1</sup> and 80 mg kg<sup>-1</sup> Phi and a control plot without Phi application (No P). Phi was applied directly to the soil and other nutrients were applied at the locally recommended rate. It can be observed that, in the absence of Phi application, weeds overgrew both the WT and transgenic plants (No P plot), whereas in the plots treated with Phi the transgenic plants outgrew both, the WT and weeds (a). Suppression of natural weeds in plots fertilized with 80 mg kg<sup>-1</sup> Phi (outlined by dotted lines) can be observed at 11 days in comparison to adjacent plots without Phi application (b). In control plots with Pi fertilization no significant difference was observed between transgenic and control plants. The experiment was carried out in Pergamino, Argentina with the corresponding permits to conduct experimental field trials with tobacco. In contrast to wild type plants, which show similarly reduced biomass when grown without Pi and with Phi fertilization, respectively, PTXD transgenic plants are able to metabolize Phi and develop higher biomass (c).

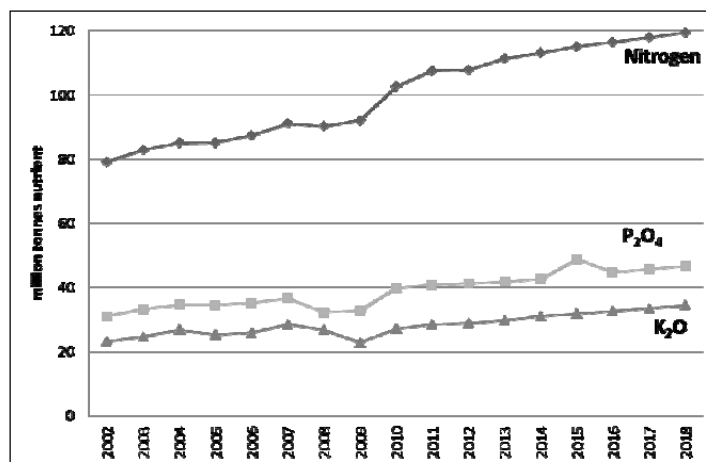


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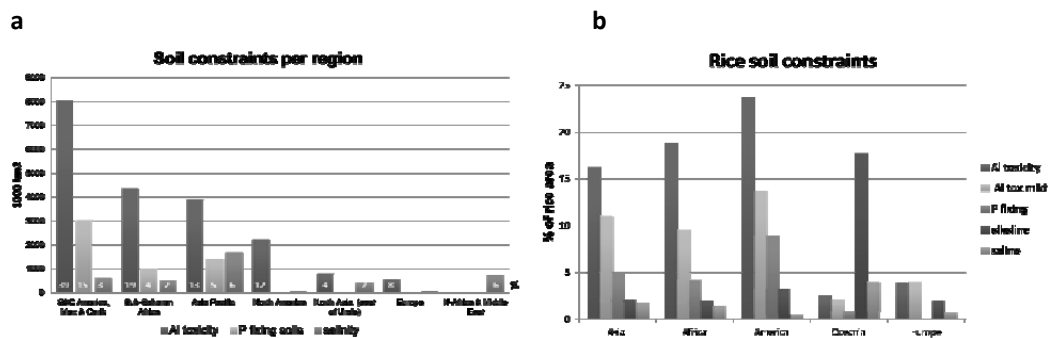


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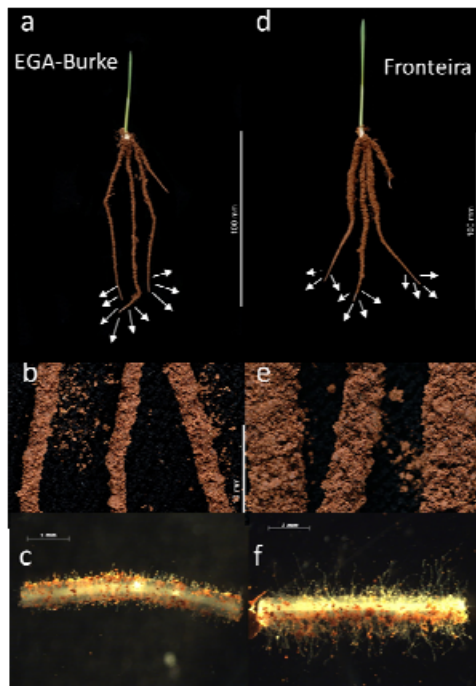


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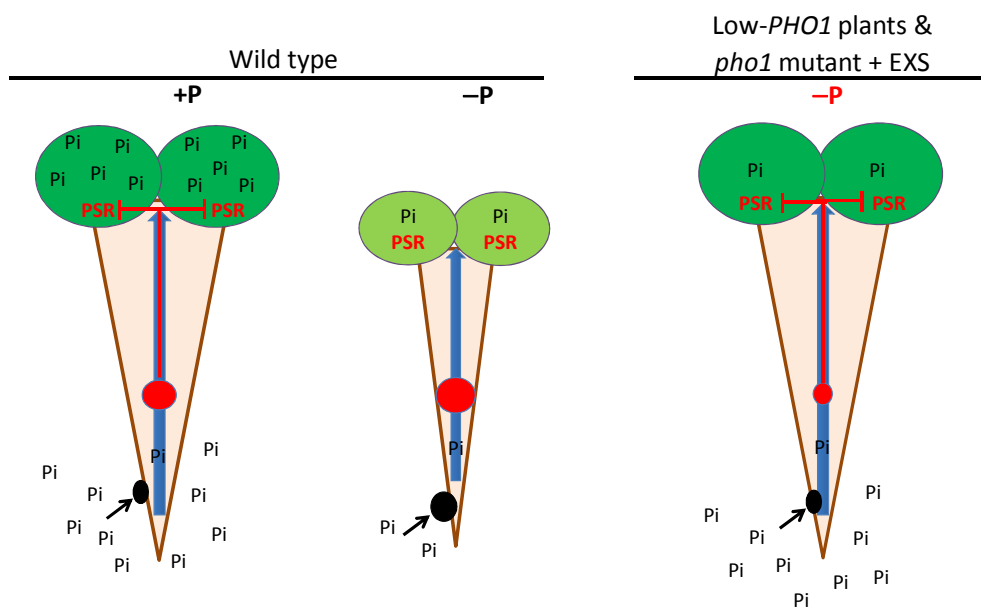


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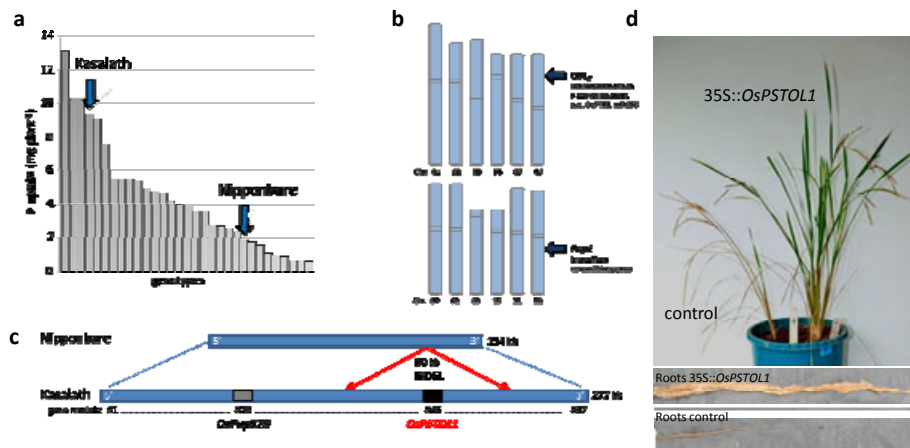


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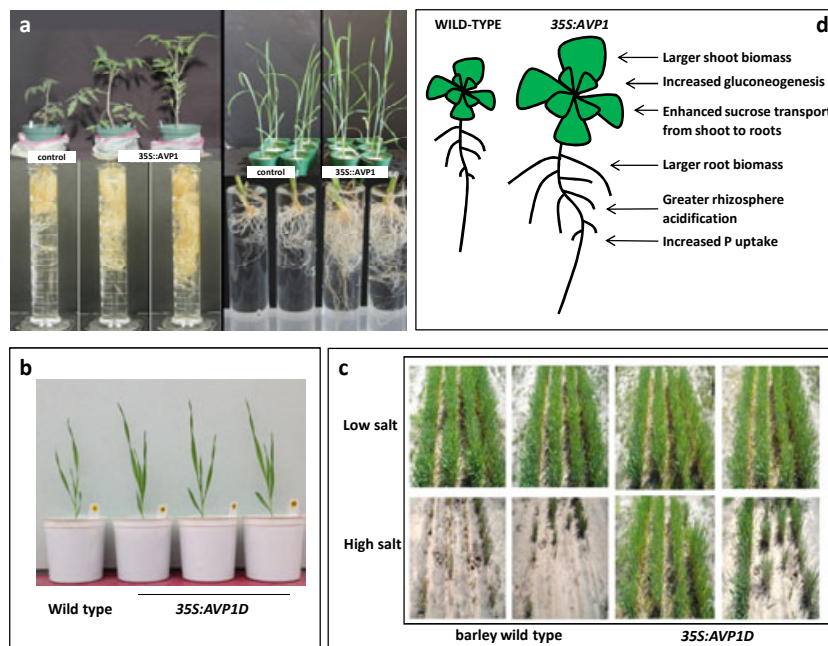


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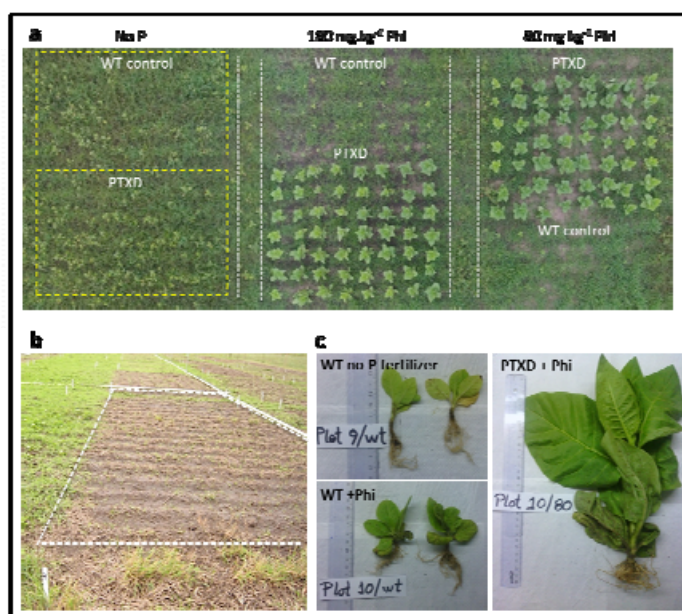


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