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REVIEW

Regulation of phosphate starvation responses in higher plants

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• *Background* Phosphorus (P) is often a limiting mineral nutrient for plant growth. Many soils worldwide are deficient in soluble inorganic phosphate (P_i), the form of P most readily absorbed and utilized by plants. A network of elaborate developmental and biochemical adaptations has evolved in plants to enhance P_i acquisition and avoid starvation.
• *Scope* Controlling the deployment of adaptations used by plants to avoid P_i starvation requires a sophisticated sensing and regulatory system that can integrate external and internal information regarding P_i availability. In this review, the current knowledge of the regulatory mechanisms that control P_i starvation responses and the local and long-distance signals that may trigger P_i starvation responses are discussed. Uncharacterized mutants that have P_i-related phenotypes and their potential to give us additional insights into regulatory pathways and P_i starvation-induced signalling are also highlighted and assessed.
• *Conclusions* An impressive list of factors that regulate P_i starvation responses is now available, as is a good deal of knowledge regarding the local and long-distance signals that allow a plant to sense and respond to P_i availability. However, we are only beginning to understand how these factors and signals are integrated with one another in a regulatory web able to control the range of responses demonstrated by plants grown in low P_i environments. Much more knowledge is needed in this agronomically important area before real gains can be made in improving P_i acquisition in crop plants.

Key words: Phosphate signal, phosphate regulon, transcription factor, non-coding RNAs, phosphate starvation responses.

INTRODUCTION

Phosphorus (P), in the form of phosphate (P_i), is an essential macronutrient for all living organisms, including plants. It is a structural element in nucleic acids and in the phospholipids that make up biomembranes. Many phosphoesters have an indispensible role in metabolic reactions, particularly those that involve energy transfer. P_i, through protein phosphorylation and dephosphorylation, is also a key component of the numerous signal transduction cascades that establish adaptive patterns of gene expression.

A 'phosphorus paradox' in relation to plant nutrition has been pointed out by numerous authors (Bieleski, 1973; Marschner, 1995). Although the total P content of soil is generally high, P availability is frequently a limiting factor for plant growth and productivity. This paradox arises because the concentration of available P_i in the soil solution averages about 1 μM and seldom exceeds 10 μM (Bieleski, 1973). A number of morphological, physiological, biochemical and molecular responses have evolved in plants that allow them to grow and prosper in the presence of such low levels of available P_i. These responses include the development of lateral roots and root hairs, as well as more dramatic root structures such as proteoid and dauciform roots, the secretion from roots of phosphatases and organic acids, and the induction of high-affinity and some low-affinity P_i transporters (Lambers *et al.*, 2006; Ai *et al.*, 2009; Fang *et al.*, 2009). Many plants also establish symbiotic associations with mycorrhizal fungi that aid P_i acquisition (Burleigh *et al.*, 2002).

The application of P fertilizer can compensate for low P_i availability in cropping systems, but high P_i input can cause severe environmental problems such as eutrophication. In addition, the global source of rock P is non-renewable and is being rapidly depleted. It is predicted that easily accessed global P reserves may be depleted in 50–100 years (Steen, 1998; Cordell *et al.*, 2009). A fuller understanding of the strategies used by plants to acquire and utilize P_i efficiently, therefore, is vitally important for the rational breeding and engineering of crop plants with greater capacity to acquire, store and recycle soil P_i. In this review, recent advances in our understanding of the molecular aspects of plant responses to P_i starvation will be discussed. The regulation of P_i transporters, while centrally important, is not covered here, but has been reviewed elsewhere (Raghothama and Karthikeyan, 2005; Bucher, 2007; Lin *et al.*, 2009).

COMPONENTS OF THE P_i REGULON IN PLANTS

A variety of adaptive strategies have evolved in plants that alleviate or help them cope with P_i deficiency. The implementation of these strategies requires changes in the expression profiles of hundreds of genes, as demonstrated by transcriptome analyses in *Arabidopsis thaliana* (arabidopsis), *Oryza sativa* (rice), *Lupinus albus* (white lupin) and *Phaseolus vulgaris* (common bean) (Hammond *et al.*, 2003; Uhde-Stone *et al.*, 2003; Wasaki *et al.*, 2003*a*; Wu *et al.*, 2003; Misson *et al.*, 2005; Hernández *et al.*, 2007). The extent and

complexity of the network of regulatory genes necessary to sense P_i status and regulate the deployment of these adaptive strategies is now being revealed. The network components identified so far include transcription factors, SPX sub-family proteins, non-coding RNAs and protein modifiers, including proteins involved in SUMOylation, phosphorylation, dephosphorylation and protein translocation (Fig. 1).

Transcription factors

Transcription factors bind to specific DNA sequences and regulate gene expression by altering the ability of RNA polymerase to bind to a target promoter sequence. These proteins have vital roles in many plant responses to stress conditions, including P_i deprivation. Transcription factors themselves are quite heavily regulated in response to P_i. In an experiment involving 6172 arabidopsis genes, the transcript abundance of about 30 % of the genes is changed >2-fold within 72 h of P_i deprivation (Wu *et al.*, 2003). Among these were one-third of the 333 transcription factor genes assessed.

At-PHR1 from arabidopsis was the first transcription factor identified to be involved in the control of the P_i starvation response in a vascular plant (Rubio *et al.*, 2001). The gene was identified in a genetic screen set up using a transgenic line harbouring an *At-IPS1::GUS* chimeric reporter gene. The *At-IPS1* promoter is specifically responsive to P_i deprivation. Screening an ethylmethane sulfonate (EMS)-mutagenized M_2 population of the reporter line identified a mutant, subsequently named *phr1*, that had a reduced response of the *At-IPS1::GUS* reporter gene to P_i deprivation. Analysis of the mutant revealed that other well-known P_i starvation responses, such as the accumulation of transcripts from a sub-set of P_i-responsive genes and the accumulation of anthocyanin, were also impaired (Rubio *et al.*, 2001). In addition, the response of 56 of 64 P_i-responsive genes was attenuated in *At-phr1* mutants (Bari *et al.*, 2006). The latter result, together with the knowledge that the P_i responsiveness of

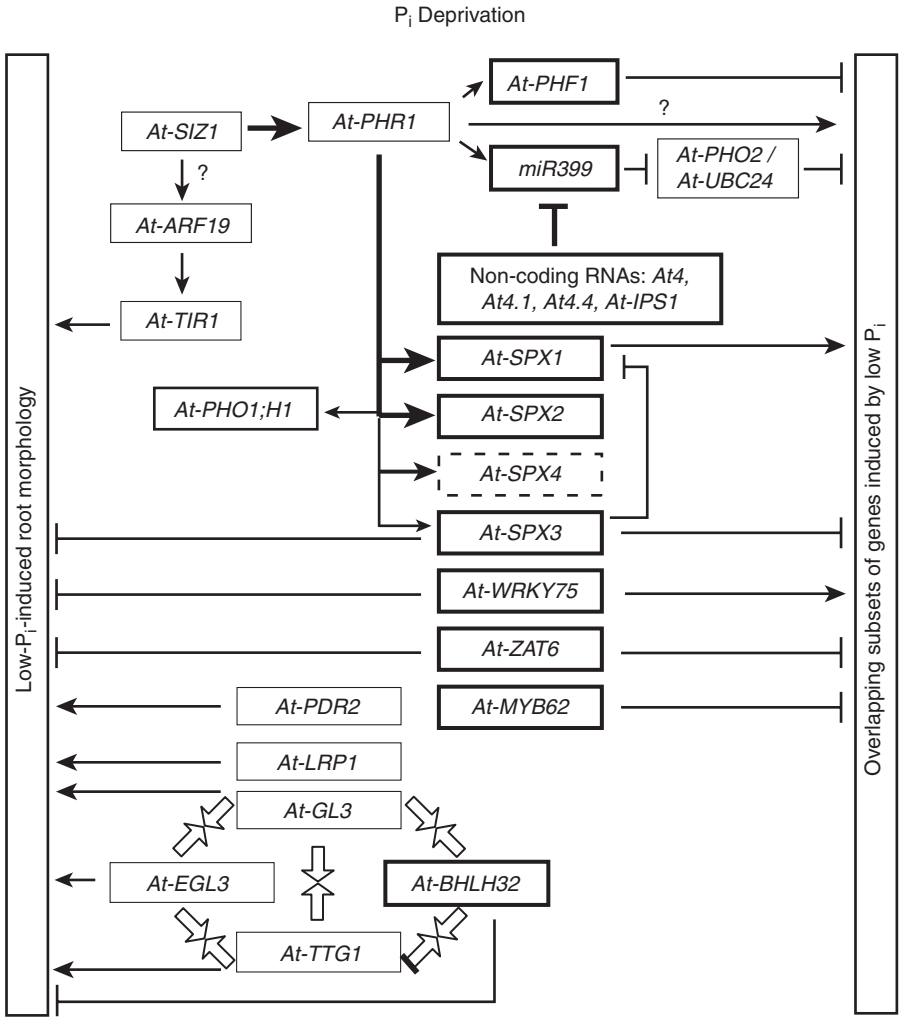

FIG. 1. A model for the plant P_i regulon. For simplicity, the interactions of genes that are induced (heavily outlined boxes), do not respond (lightly outlined boxes) or are repressed (dashed boxes) by P_i deficiency in arabidopsis are shown. The activities of genes from other plants are discussed in the text. The target genes and functions are under either positive (arrowheads) or negative (T-junctions) regulation by the indicated factors. Genes that have been experimentally determined to be influenced by *At-SIZ1* are indicated by heavy arrows. Convergent box arrows show gene products that physically interact with one another as part of a complex. Question marks indicate possible signalling pathways that are not currently supported by experimental evidence.

many of those genes examined was fully reversible within 3 h of P_i resupply, led to the conclusion that *At-PHR1* may be a central positive regulator of most, but not all, P_i starvation-induced genes (Fig. 1). The *At-PHR1* gene was mapped to locus At4g28610 and encodes a member of the MYB transcription factor superfamily. The At-PHR1 protein sequence has high similarity to the sequence of the *PHOSPHORUS STARVATION RESPONSE1* (*PSR1*) gene product from *Chlamydomonas reinhardii* (Wykoff *et al.*, 1999). Both At-PHR1 and Cr-PSR1 have a MYB DNA-binding domain and a coiled-coil domain, indicating the potential for protein–protein interactions. At-PHR1 binds DNA as a dimer to an imperfect palindromic sequence (GNATATNC) present in the promoters of many P_i starvation-responsive genes (Rubio *et al.*, 2001; Franco-Zorrilla *et al.*, 2004). Thus, At-PHR1 acts downstream in the P_i starvation signalling pathway. The *At-PHR1* gene is not itself particularly P_i responsive and the protein is located in the nucleus independently of the P_i status of the plant, indicating that induction of At-PHR1 activity does not require transcription (Rubio *et al.*, 2001).

Two rice genes, *Os-PHR1* and *Os-PHR2*, orthologous to *At-PHR1* and with functions in the P_i stress signalling network similar to *At-PHR1* (Fig. 1), have recently been characterized (Zhou *et al.*, 2008). Transgenic plants with reduced expression levels of either *Os-PHR1* or *Os-PHR2* had lower transcript amounts for several P_i starvation response genes. These same P_i-responsive genes had increased expression in transgenic plants that over-expressed *Os-PHR1* and *Os-PHR2*. Of the two rice *PHR* genes, only *Os-PHR2* over-expression led to an increase of P_i in shoots under P_i-sufficient conditions, phenocopying over-expression of *At-PHR1* in both wild-type and *At-phr1* arabidopsis. Thus, *Os-PHR2* is probably a functional homologue of *At-PHR1*, although it remains possible that *Os-PHR1* and *Os-PHR2* share the regulatory duties encompassed by *At-PHR1*.

At-MYB62 encodes another MYB transcription factor involved in the P_i deprivation response of arabidopsis. Unlike *At-PHR1*, *At-MYB62* expression is induced by P_i starvation (Misson *et al.*, 2005), but only in leaves of young seedlings (Devaiah *et al.*, 2009). The response to low P_i is not mimicked by potassium, iron or nitrogen deficiencies. Expression in roots is apparently always low. At-MYB62 represses at least some P_i-inducible genes when over-expressed. Thus, At-MYB62, itself induced by P_i deprivation, is likely to be a negative regulator of other P_i starvation-inducible genes, and may moderate their activity during P_i starvation. The At-MYB62 protein, like At-PHR1, is localized to the nucleus irrespective of the P_i status of the plant. This observation, coupled with rapid loss of *At-MYB62* transcripts upon P_i resupply and its role in the regulation of genes involved in P_i signalling, high-affinity P_i transport and mobilization, suggests a global role for At-MYB62 during P_i deficiency. Over-expression of *At-MYB62* under P_i-sufficient conditions induced responses reminiscent of P_i starvation, including increased anthocyanin production, reduced primary root length and increased root acid phosphatase activity. Despite increases in P_i uptake and root P_i concentration that probably arose from changes in root architecture, the shoot P_i concentration of over-expressing plants was lower than that of the wild type (Devaiah *et al.*, 2009). The partial rescue of the phenotype by exogenous gibberellic acid and the suppression to varying degrees of the expression of all five genes involved in gibberellic acid biosynthesis suggests that *MYB62* may have a role in regulating biosynthesis of the hormone. Thus, *MYB62* may regulate the P_i starvation responses through changes in gibberellic acid concentration.

The *Os-PTF1* gene encodes a protein with a basic helix–loop–helix (bHLH) motif and was identified by differential screening of rice plants grown under normal and P_i starvation conditions (Yi *et al.*, 2005). Expression of *Os-PTF1* is induced in roots during P_i deprivation, while expression is constitutive in shoots. Like At-PHR1 and At-MYB62, Os-PTF1 is located in the nucleus independently of P_i status. Under P_i-deficient conditions, both in the field and in hydroponics, plants over-expressing *Os-PTF1* have greater tillering, higher root and shoot biomass, and a 25 % higher P content than wild-type plants. Microarray analysis showed that the transcript abundance of 158 genes is altered by >2-fold in plants over-expressing *Os-PTF1* (Fig. 1). The induced genes encode proteins such as nutrient transporters, transcription factors and ATP-binding proteins. The promoters of the induced genes generally have E-box elements (Massari and Murre, 2000), with about 20 % of the genes having at least one copy of the G-box element (Atchley *et al.*, 1999). Further analysis of the responsive genes will enhance our understanding of the function of *Os-PTF1* in the regulation of P_i response pathways.

Microarray analysis revealed that *At-ZAT6* (At5g04340), encoding a cysteine-2/histidine-2 (C2H2) zinc finger transcription factor, is strongly induced by P_i starvation (Hammond *et al.*, 2003). Green fluorescent protein (GFP) fusion experiments showed that At-ZAT6 is located in the nucleus. Suppression of *At-ZAT6* expression through RNA interference (RNAi) is lethal (Devaiah *et al.*, 2007*b*). Over-expression of the gene retards primary root growth independently of the P_i status of the plant and reduces P_i uptake. Over-expression of *At-ZAT6* reduces the expression of a number of P_i starvation-induced genes, confirming its role in regulating P_i homeostasis (Fig. 1). Hence, At-ZAT6 appears to be a repressor of primary root growth, regulating P_i homeostasis through the control of root architecture.

At-WRKY75 is another transcription factor strongly induced by P_i deficiency in arabidopsis (Misson *et al.*, 2005). The At-WRKY75 protein is located in the nucleus and its transcripts increase in abundance, but to various extents, in different parts of the plant during P_i deprivation. RNAi suppression of *At-WRKY75* expression causes anthocyanin accumulation, indicating that the mutant plants are more susceptible to P_i stress than wild-type plants. In *At-WRKY75* RNAi plants deprived of P_i, the expression of several P_i starvation-inducible genes is reduced. In contrast, lateral root length and number, as well as root hair number, significantly increase under both P_i-deficient and P_i-sufficient conditions. Thus, At-WRKY75 has a role in the P_i starvation response as well as in root development (Fig. 1) (Devaiah *et al.*, 2007*a*).

Microarray experiments indicate that the expression of *At-BHLH32* (At3g25710) is induced in both leaves and roots after 48 h of P_i starvation (Wu *et al.*, 2003). In an *At-bhlh32* T-DNA insertion mutant grown under P_i-sufficient conditions,

there is a significant increase in the expression of the P_i starvation-inducible genes *At-PPCK1* and *At-PPCK2* (encoding isoforms of phosphoenolpyruvate carboxykinase), as well as increases in the accumulation of anthocyanins, the formation of root hairs and the total P_i content compared with wild-type plants (Chen *et al.*, 2007). These results indicate that At-BHLH32 acts as a negative regulator in the P_i starvation response (Fig. 1). During P_i deprivation, mutations in *At-TTG1*, *At-GL3* and *At-EGL3*, genes that take part in root hair formation (Bernhardt *et al.*, 2005), lead to the decrease in transcript abundance of *At-PPCK1* and *At-PPCK2* compared with wild-type plants. These results indicate that the three genes are positive regulators in the P_i starvation response pathway (Fig. 1). *Saccharomyces cerevisiae* (yeast) two-hybrid experiments showed that At-BHLH32 can physically interact with At-TTG1 and At-GL3. Thus, At-BHLH32 was inferred to interfere with the function of TTG1-containing complexes (Fig. 1) and consequently influence the biochemical and morphological processes that respond to P_i status (Chen *et al.*, 2007).

SPX domain-containing proteins

The SPX domain appears at the N-terminus of various proteins, especially those involved in signal transduction (Barabote *et al.*, 2006). In yeast, proteins containing the SPX domain are involved in P_i transport and sensing, or the sorting of proteins to endomembranes (Wang *et al.*, 2004). Most SPX-domain proteins with known functions in plants are involved in the regulation of either nutritional homeostasis or the response to environmental cues. The arabidopsis genome contains 20 genes encoding SPX-domain proteins. These proteins have been grouped into four sub-families based on sequence similarity.

Members of three of the four SPX protein sub-families in arabidopsis, a total of 16 proteins, possess an EXS domain in addition to the SPX domain. The *At-PHO1* gene (At3g23430) encodes one such protein that is involved in the regulation of P_i homeostasis (Wang *et al.*, 2004). The N-terminal half of At-PHO1 is mainly hydrophilic, while the C-terminal half has six potential membrane-spanning domains. The *At-pho1* mutant of arabidopsis contains approx. 95 % less P_i and 50–75 % less total P in shoots than wild-type plants (Poirier *et al.*, 1991). The *At-pho1* mutant is deficient in the transfer of P_i from root epidermal and cortical cells to the xylem, but the role of At-PHO1 in P_i loading to the xylem is still unclear. The gene is expressed mainly in roots and is slightly upregulated during P_i deficiency. There are 11 *At-PHO1* homologues in arabidopsis (Hamburger *et al.*, 2002). Among these, only *At-PHO1* and *At-PHO1;H1* (At1g68740) are able to complement the *At-pho1* mutant (Stefanovic *et al.*, 2007). *At-PHO1;H1*, like *At-PHO1*, is involved in the P_i transport pathway; however, its response to P_i status is through a separate signal transduction pathway. The transcript abundance of *At-PHO1;H1* is upregulated by the At-PHR1 transcription factor under P_i starvation conditions (Fig. 1) and suppressed by the P_i analogue phosphite, while *At-PHO1* expression is independent of both At-PHR1 and phosphite (Stefanovic *et al.*, 2007).

The fourth sub-family of SPX-domain proteins does not contain an EXS domain. There are four proteins of this type in arabidopsis, encoded by *At-SPX1* (At5g20150), *At-SPX2* (At2g26660), *At-SPX3* (At2g45130) and *At-SPX4* (At5g15330). In response to P_i starvation, *At-SPX1* and *At-SPX3* are strongly induced, while *At-SPX2* is weakly induced and *At-SPX4* is repressed in both shoots and roots (Duan *et al.*, 2008). Of the six rice homologues in this subfamily, *Os-SPX1–Os-SPX6*, five are induced by P_i starvation in the roots and/or shoots, while no members are repressed (Z. Wang *et al.*, 2009). The SPX1 and SPX2 isoforms from both arabidopsis and rice are targeted to the nucleus, while other the forms are located elsewhere in the cell (Duan *et al.*, 2008; Z. Wang *et al.*, 2009). The induction by P_i, the diversity of cellular locations, coupled with a variety of cell type-dependent transcription patterns suggest that that these SPX proteins may be involved in the P_i signalling networks that regulate the expression of P_i-responsive genes and that each SPX protein has a unique physiological function (Fig. 1).

The strong repression of *At-SPX1* and *At-SPX2* in *At-siz1* (a SUMO E3 ligase gene, see below) and *At-phr1* mutants indicates that both genes are positively regulated by the At-PHR1 regulon (Duan *et al.*, 2008). *At-SPX3* is also strongly repressed in the *At-phr1* mutant but only weakly repressed in the *At-siz1* mutant, suggesting that At-SPX3 acts downstream of At-PHR1. The point of action for *At-SPX4* is unclear, because its expression is repressed about 50 % in both *At-siz1* and *At-phr1* mutants, which may mean that *At-SPX4* is regulated by At-PHR1 in combination with other factors, or independently of At-PHR1.

Over-expression of *At-SPX1* increases the transcript abundance of three P_i starvation-induced genes, more significantly under P_i-sufficient than P_i-deficient conditions, indicating that At-SPX1 is a positive regulator in the P_i signalling pathway (Duan *et al.*, 2008). Partial RNAi repression of *At-SPX3* leads to primary root growth retardation, increased P_i transportation from roots to shoots and enhanced expression of a sub-set of P_i starvation-responsive genes, including *At-SPX1*. These observations suggest that At-SPX3 provides negative feedback in the *At-SPX1* response to P_i starvation (Duan *et al.*, 2008). The functions of *At-SPX2* and *At-SPX4* require further exploration to determine the level of their involvement in the P_i starvation response.

Much more work is needed before strong inferences can be drawn about the functional equivalence, if any, among the SPX isoforms between rice and arabidopsis. Overlapping expression patterns and nuclear localization suggest that *Os-SPX1* might be functionally equivalent to *At-SPX1*. Also, *Os-SPX1* and *At-SPX1* are each positively regulated by *PHR1* orthologues, *Os-PHR2* (C. Wang *et al.*, 2009) and *At-PHR1*, respectively. However, *Os-SPX1* is also functionally dissimilar to *At-SPX1*. *Os-SPX1* and *At-SPX1* are negative and positive regulators, respectively, of different sets of P_i starvation-induced genes. Unfortunately, until overlapping sets of target genes are assessed, it remains possible that the rice and arabidopsis SPX1 proteins act as both positive and negative regulators, targeting similar gene sets in similar ways. Another difference is that *Os-SPX1* regulates the expression of the other five genes in this rice *SPX* sub-family in various ways depending on the tissue and P_i status (Z. Wang

et al., 2009), a function that has not been ascribed to *At-SPX1*. However, *At-SPX3* has been shown to be a negative regulator of *At-SPX1*, highlighting functional similarities between *At-SPX3* and *Os-SPX1*. RNAi suppression of *Os-SPX1* in rice also led to an increase in leaf P_i concentration (C. Wang *et al.*, 2009), as seen in arabidopsis where *At-SPX3* is suppressed by RNAi (Duan *et al.*, 2008). The transcript abundance of certain PHT-type P_i transporters is enhanced in the roots of SPX1-suppressed lines of both rice and arabidopsis, suggesting a mechanism for the enhanced P_i abundance in the leaves. Taken together, these studies show that SPX proteins have complex, and as yet unclear, roles in the response to P_i deficiency.

SUMOylation

Small ubiquitin-related modifier (SUMO) proteins are small polypeptide tags showing greatest primary sequence similarity to ubiquitin. Like ubiquitin, SUMO proteins function through conjugation with other proteins, a post-translational modification that is involved in various cellular processes (Colby *et al.*, 2006). While the ubiquitin system typically tags proteins for proteosome degradation, SUMO conjugation can stabilize the target proteins and alter their sub-cellular localization, as well as indirectly influence ubiquitination and protein degradation (Colby *et al.*, 2006). At-SIZ1 functions as a SUMO E3 ligase in the arabidopsis P_i regulon (Fig. 1), as well as in other cellular processes, including abscisic acid signalling (Miura *et al.*, 2007, 2009). The *At-SIZ1* gene (At5g60410) was originally identified in a genetic screen designed to isolate genes that confer NaCl tolerance (Miura *et al.*, 2005). An *At-siz1* T-DNA insertion mutant showed retarded primary root growth compared with wild-type plants when the nutrient supply was restricted. Wild-type root growth was restored by the addition of P_i, but not other nutrients. The original *At-siz1-1* mutant displayed a similar set of symptoms to wild-type plants in response to P_i deprivation, but to a more severe degree, despite similar intracellular P_i levels. Under P_i-deficient conditions, the abundance of transcripts from several P_i starvation responsive genes is similar in the *At-siz1-1* mutant and wild-type plants, but under P_i-sufficient conditions these transcripts are more abundant in the *At-siz1-1* mutant. Additionally, other genes are induced more slowly by P_i limitation in the *At-siz1-1* mutant. At-SIZ1 was localized to the nucleus and can replace the yeast Sc-Siz2 SUMO E3 ligase in the *in vitro* SUMOylation of an Sc-Cdc3 substrate (Miura *et al.*, 2005), indicating that At-SIZ1 is a SUMO E3 ligase. *In vitro* experiments demonstrated that At-SIZ1 can mediate the SUMOylation of At-PHR1, indicating a role for At-SIZ1 in the P_i deficiency response pathway (Fig. 1) (Miura *et al.*, 2005). In this regard, it is noteworthy that MYB62 contains two functionally untested sites for potential SUMOylation (Devaiah *et al.*, 2009).

At-SIZ1 may work in conjunction with At-PHO2/At-UBC24 in the SUMOylating pathway (Fujii *et al.*, 2005). The *At-UBC24* gene (At2g33770) encodes a putative E2 ubiquitin-conjugase (Aung *et al.*, 2006; Bari *et al.*, 2006). It is responsible for the P_i misallocation phenotype in the *At-pho2* mutant. The *At-pho2* mutant accumulates up to 3-fold more total P in leaves, mostly as P_i, than wild-type plants (Delhaize and Randall, 1995). Like the *At-pho2* mutant, *At-UBC24* T-DNA knockout mutants display increased uptake and translocation of P_i from roots to shoots and reduced P_i remobilization within leaves (Aung *et al.*, 2006). In the *At-pho2* mutant, transcripts from a number of Pi starvation-induced genes remained abundant under P_i-replete conditions, in contrast to wild-type plants where transcripts from these genes are repressed by high P_i supply (Shin *et al.*, 2004; Bari *et al.*, 2006). At-PHO2 and At-PHR1 evidently share a number of downstream targets. A study on the expression of 64 P_i-responsive genes in *At-pho2* and *At-phr1* mutants revealed 21 genes with an altered P_i response in both backgrounds (supplementary data in Bari *et al.*, 2006). That is, the repression of these genes by P_i-replete conditions was impaired in the *At-pho2* mutant, while their induction by P_i-deficient conditions was weakened in the *At-phr1* mutant. This same study found that the abundance of primary transcripts from all five *At-MIRNA399* genes in a *At-pho2* mutant grown under P_i-replete conditions was the same as in wild-type plants, but they were not fully induced in the *At-phr1* background. These results and the location of putative At-PHR1-binding sites 160–270 nucleotides (nt) upstream of several *At-MIRNA399* genes place At-PHO2/At-UBC24 downstream of At-PHR1 in the plant P_i signalling pathway (Fig. 1) (Bari *et al.*, 2006). While the interactions of At-PHO2/At-UBC24 with downstream elements of the P_i signalling pathway remain unclear, the *At-SPX* genes are likely candidates, as *Os-SPX1* functions downstream of *Os-PHO2* (C. Wang *et al.*, 2009).

Protein phosphorylation and dephosphorylation

Protein phosphorylation by kinases and dephosphorylation by phosphatases is a potent binary switch involved in the regulation of most cellular activities and processes (Luan, 2003). Microarray analysis revealed differential regulation of several protein phosphatases at the onset of P_i deprivation in arabidopsis (Wu *et al.*, 2003). However, Le-PS2 from *Lycopersicon esculentum* (tomato) is the only protein phosphatase induced by P_i starvation that has been studied in any detail (Baldwin *et al.*, 2001, 2008). The ability of recombinant Le-PS2 expressed in bacteria to dephosphorylate a phosphopeptide substrate, an activity that is suppressed by okadaic acid, demonstrates that Le-PS2 is a phosphoprotein Ser/Thr phosphatase (Baldwin *et al.*, 2008). However, its target proteins are not known. Given the central importance of reversible protein phosphorylation in other cellular processes and signal transduction pathways, it is surprising that its involvement in the P_i deprivation response has received so little attention.

Protein translocation

One crucial determinant for the activity of P_i transporters is their delivery through the secretory pathway to the plasma membrane. At-PHF1, the first P_i starvation-responsive component of the trafficking pathway to be isolated, enables the high-affinity P_i transporters to exit from the endoplasmic reticulum (ER). The *At-phf1* mutant was isolated (González *et al.*, 2005) using a reporter gene screen similar to that used to isolate the *At-phr1* mutant (Martín *et al.*, 2000; Rubio *et al.*,

2001) and described above. The *At-phf1* mutation causes the retention of the At-PHT1;1 P_i transporter in the ER and leads to a decrease in the concentration of P_i in whole plants. The lesion in the *At-phf1* mutant was positionally cloned to At3g52190, which encodes a SEC12-related protein specific to plants. The *At-PHF1* gene is expressed widely in the plant, but mainly in roots, flowers and senescing leaves. *At-PHF1* has a promoter motif that conforms to the core binding sequence of At-PHR1 (Rubio *et al.*, 2001). The decrease in *At-PHF1* transcript abundance in the *At-phr1* mutant suggests that *At-PHF1* is indeed under direct transcriptional control of At-PHR1 (Fig. 1). The mechanism by which At-PHF1 regulates the intracellular transport of proteins and determines sub-cellular localization is still unclear. The possibility of a separate trafficking apparatus for an individual protein, or a group of proteins involved in a single process, is intriguing, and further investigations may provide new ideas on how plant cells deliver proteins to specific intracellular locations, especially during acclimation to physiological change.

Non-coding RNA

Non-coding RNA genes produce functional RNA molecules rather than proteins. They play key roles in chromosomal silencing, transcriptional regulation, translational repression, developmental control and responses to stress (Axtell *et al.*, 2007). Non-coding RNAs are derived predominantly from introns and intergenic regions, but also from the opposite strand of protein-coding genes (Storz, 2002). MicroRNAs (miRNAs) are non-coding RNAs 20–24 nt in length. They are derived from the stem of hairpin-like precursors of about 75 nt that are in turn derived from longer pri-miRNAs (Zhu, 2008). miRNAs silence genes with complementary or partly complementary sequences by aiding mRNA cleavage or translational repression (Carrington *et al.*, 2003; Bartel, 2004; He and Hannon, 2004). A screen of miRNAs regulated by P_i deprivation led to the identification of miR399 (Sunkar and Zhu, 2004). There are six *MIRNA* loci in arabidopsis that encode miR399 species a–f. In arabidopsis, *At-PHO2*/*At-UBC24* was confirmed to be a target gene for At-miR399 (Fig. 1); it has five miR399 target sites in the 5′-untranslated region (UTR) of its transcripts (Sunkar and Zhu, 2004; Allen *et al.*, 2005). At-miR399 accumulation is induced by P_i deprivation, a condition that suppresses the *At-PHO2*/*At-UBC24* target gene (Fujii *et al.*, 2005). Experiments with transgenic plants that produce artificial *At-PHO2*/*At-UBC24* mRNAs with or without the 5′UTR indicated that miR399 downregulates *At-PHO2*/*At-UBC24* mRNA accumulation by targeting the 5′UTR (Fujii *et al.*, 2005).

The degradation of *At-PHO2*/*At-UBC24* transcripts by miR399 in the P_i regulon is itself regulated by non-coding RNAs from the *Mt4/TPSI1* gene family (Fig. 1). These non-coding RNAs originally came to prominence because of their strong induction in P_i-starved plants (Burleigh and Harrison, 1997, 1998; Liu *et al.*, 1997). Members of the *Mt4/TPSI1* gene family include *Le-TPSI1* in tomato (Liu *et al.*, 1997), *Mt4* in *Medicago truncatula* (Burleigh and Harrison, 1997, 1998), an *Mt4*-like gene in *Glycine max* (soybean) (Burleigh and Harrison, 1999), *At4*, *At4.1*, *At4.2* and *At-IPS1* in arabidopsis (Burleigh and Harrison, 1999; Martín *et al.*, 2000; Shin *et al.*, 2006) and Os-PI1 in rice (Wasaki *et al.*, 2003*b*). The transcripts of *Mt4/TPSI1* genes typically contain multiple short open reading frames that are not conserved among the family members and are unlikely to encode proteins. The lack of conserved open reading frames originally suggested that the transcripts from *Mt4/TPSI1* were the active gene products. The overall sequence identity across the family is quite low, except for a fairly well conserved 23 nt motif in the central region of each transcript.

The *Mt4/TPSI1* genes have a dramatic impact on P_i distribution. For example, an *At4* T-DNA insertion mutant does not redistribute shoot P_i to the roots. Instead, P_i accumulates in the shoots, causing an increase in the shoot to root P_i ratio compared with that in wild-type plants (Shin *et al.*, 2006). The first clue to the mechanism mediating the action of the *Mt4/TPSI1* RNAs came from the observation that the 23 nt sequence in the middle of *At4* that is well conserved among all *Mt4/TPSI1* family members hybridizes to an approx. 22 nt RNA expressed during P_i starvation (Shin *et al.*, 2006). It was also noted that the 23 nt conserved sequence had extensive homology to miR399. However, there are critical mismatches, including a bulge opposite positions 10–11 of miR399, disrupting the base pairing that is required for miR399-guided cleavage of mRNA targets (Jones-Rhoades *et al.*, 2006; Franco-Zorrilla *et al.*, 2007). Indeed, *At-IPS1* RNA is not cleaved in an At-miR399-dependent manner, but instead sequesters At-miR399 (Franco-Zorrilla *et al.*, 2007). Moreover, *At-IPS1* over-expression results in the accumulation of *At-PHO2/At-UBC24* mRNA, an miR399 target, demonstrating that *At-IPS1* represses miR399-dependent transcript cleavage. Therefore, *Mt4/TPSI1* RNAs may function as non-cleavable miR399 substrate competitors, sequestering miR399 in a state where it cannot act upon target gene transcripts (Franco-Zorrilla *et al.*, 2007). Interestingly, At-miR399b and At-miR399c are less efficient in suppressing *At-PHO2/At-UBC24* than At-miR399f. This differential efficiency may be due in part to differences in complementary between the various At-miR399 isoforms and *At4/At-IPS1* transcripts, where higher complementarity may lead to more efficient sequestration by the *At4/At-IPS1* transcript (Doerner *et al.*, 2008). The differential efficiency of its members suggests that the At-miR399 family is part of a fine-tuning mechanism that allows the cell to respond subtly to the dynamics of P_i availability.

Revealing the details of regulatory circuits such as the miR399, *Mt4/TPSI1* and *PHO2/UBC24* system is only the first step in understanding what is likely to be a complex involvement for non-coding RNAs within the P_i starvation regulatory network. Deep sequencing and other global RNA analysis tools are revealing a rapidly growing number of small RNAs whose abundance responds to P_i status (Hsieh *et al.*, 2009; Pant *et al.*, 2009). Several of these small RNAs are miRNAs and are likely to be involved in regulating processes such as P_i uptake and translocation (i.e. At-miR399), anthocyanin biosynthesis, oxidative stress reduction, sulfate translocation and nutrient recycling. While the expression of some of the miRNAs seems to respond only to P_i, others respond not

only to P_i status, but also to the availability of other mineral nutrients, such as N, K, S, Cu and Fe. Among the miRNAs in this latter group, the regulatory response can be either in the same or the opposite direction as the response to P_i status. Another group of P_i-responsive small RNAs are miRNA star strands, such as At-miR399*, indicating that these molecules may have important biological functions. The role of still other P_i-responsive small RNAs in the P_i regulon is entirely unknown. Together, these molecules provide the prospect of many exciting discoveries in the future.

P_i SENSING IN HIGHER PLANTS

Local P_i sensing

The local availability of P_i controls responses such as root hair number and length, and primary root length. Pre-existing root hairs of plants grown under P_i-replete conditions are shorter than new root hairs that grow after plants are transferred to P_i-deficient conditions (Bates and Lynch, 1996). Reciprocally, root hair growth is suppressed in plants transferred from P_i-deficient to P_i-replete conditions. Primary root growth also slows dramatically when the root tip reaches patches of growth medium that contain little P_i (Linkohr *et al.*, 2002). This P_i deficiency-dependent arrest is not a nutritional response but a response to a local P_i signal because primary roots that detached from the growth medium and entered the air phase did not stop growing despite the lack of P_i uptake by the growing tip (Svistoonoff *et al.*, 2007). Moreover, growth of these roots stopped immediately when the tips encountered medium containing low concentrations of P_i (Svistoonoff *et al.*, 2007). Using a reporter gene approach to identify dividing cells at the G_2/M transition, it was shown that the root tips growing through air divided normally, but ceased to divide when the root tips encountered growth medium containing low P_i (Svistoonoff *et al.*, 2007).

Mutant analysis is revealing the mechanism of local P_i sensing. The EMS-induced *At-pdr2* mutant of arabidopsis showed reduced primary root growth compared with wild-type plants when DNA was the only source of P_i (Chen *et al.*, 2000). The mutant had reduced rates of root cell division and elongation when grown in P_i-deficient conditions. The mutant was not affected in the P concentration within the root tip or in P_i uptake rates, excluding a defect in high-affinity P_i acquisition. These results helped lead to the conclusion that the *At-pdr2* phenotype is caused by a defect in the sensing of the local external P_i concentration (Ticconi *et al.*, 2004). *At-PDR2* was recently identified by map-based cloning (Ticconi *et al.*, 2009) and found to encode the single P_5-type ATPase (At5g23630). At-PDR2 was localized to the ER and found to regulate stem cell differentiation and meristem activity through a pair of GRAS family transcription factors, SCR and SHR (Ticconi *et al.*, 2009) that are key regulators of radial root patterning (Di Laurenzio *et al.*, 1996; Gallagher and Benfey, 2009).

At-PDR2 was found to interact genetically with *At-LPR1* in an ER-resident pathway (Ticconi *et al.*, 2009). *At-LPR1*, a quantitative trait locus (QTL) responsible for determining root responses to P_i starvation in arabidopsis, was mapped to the At1g23010 locus, which encodes a multicopper oxidase required for low P_i-dependent growth arrest (Svistoonoff *et al.*, 2007). *At-LPR1*, like *At-PRD2*, is expressed in the root tip, including the meristem and root cap. At-LPR1 may be involved in the switching of primary root growth from indeterminate to determinate, moderating the activity and/or distribution of a hormone-like compound (Svistoonoff *et al.*, 2007). At-LPR1 and At-PDR2 are thought to function together to adjust meristem activity in response to external P_i status (Ticconi *et al.*, 2009). Elucidating the nature of this interaction and uncovering other components of the ER-resident control pathway will provide new insights into the adjustment of root characteristics to local P_i signals.

P_i itself can function as a local signal. Both P_i and phosphite, a supposedly metabolically inert analogue of P_i, could rescue the root meristematic activity of the *At-pdr2* mutant under P_i-limiting conditions (Ticconi *et al.*, 2004). The appearance within 1 h of P_i limitation of transcripts from genes that are P_i starvation inducible and the decrease in abundance of these transcripts within 30 min of P_i resupply, while shoot P_i and carbohydrate status did not change, has also been taken as evidence that local P_i concentration acts as a signal (Wang *et al.*, 2002; Muller *et al.*, 2004).

Long-distance signals

Plants acquire mineral nutrients from the rhizosphere through the roots and transport them to shoots. The balance between shoot demand and root supply for any particular mineral nutrient is determined by long-distance signals that report on nutrient status in the various tissues (Lin *et al.*, 2008). The existence of long-distance signals that report on P_i status in the tissues has been demonstrated in split-root experiments, where the roots of P_i-starved plants were divided, with one part exposed to P_i-replete medium and the other part exposed to P_i-deficient medium. In tomato, genes that were normally induced in response to P_i starvation were systemically repressed in the portion of the root system exposed to P_i-deficient medium when the remaining roots were exposed to P_i-sufficient medium (Liu *et al.*, 1998; Baldwin *et al.*, 2001). Similarly, lateral root elongation is also controlled by shoot P_i status (Linkohr *et al.*, 2002; Shane and Lambers, 2006; Shane *et al.*, 2008). Long-distance signals are also important for the regulation of resource allocation during leaf development, flowering and pathogen defence. These long-distance signals move through the phloem, which contains proteins, sugars, organic acids, amino acids, phytohormones, miRNAs and other types of small RNAs, all of which are potential signal molecules (Lough and Lucas, 2006).

Sugars have been suggested to be a systemic signal for the P_i starvation response in plants, in addition to their more established roles as signals in other plant adaptive responses (Koch *et al.*, 2000; Grigston *et al.*, 2008), substrates for the biosynthesis of complex carbohydrates and in energy metabolism, and in driving phloem transport and delivery of C skeletons to sink tissues for growth and development. Sugars are required for P_i starvation responses, including the stimulation of lateral root growth and the induction of P_i starvation-responsive genes (Karthikeyan *et al.*, 2007). Sucrose induces the accumulation of *At-Pht1;1* and *At4* transcripts in plants grown under P_i-sufficient conditions, and its absence under

P_i-deficient conditions represses transcript accumulation from these genes. Since plants grown under P_i sufficiency contain significantly more P_i than those grown under P_i deficiency, the effect of sucrose on the expression of P_i starvation-responsive genes is not merely the result of sequestration of P_i in sugar–phosphates. Instead, it appears that sucrose acts as a signal in the P_i starvation response pathway (Karthikeyan *et al.*, 2007; Hammond and White, 2008).

The mapping of the *At-pho3* mutation to *At-SUC2*, which encodes a sucrose–proton symporter that is important for phloem loading of sucrose, provides further evidence that sugar acts as a signal during P_i starvation (Lloyd and Zakhleniuk, 2004). The *At-pho3* mutant has reduced root acid phosphatase activity when grown under P_i-deficient conditions (Zakhleniuk *et al.*, 2001). When grown under P_i-sufficient conditions, the total P content is much lower in the shoots and roots of 11-d-old *At-pho3* mutant seedlings than in wild-type seedlings. The *At-pho3* mutant also accumulates much more anthocyanin and starch than wild-type seedlings, clear indicators of a P_i starvation response (Zakhleniuk *et al.*, 2001). While suggesting that sugar acts as a systemic signal in the plant P_i starvation response, these experiments cannot exclude the possibility that sugar is only being used as a substrate. More research needs to be done to understand fully the role of sugars as components of the P_i starvation response pathway.

Small non-coding RNAs are likely to be another important class of long-distance signalling molecules. miR399 has been detected in the phloem sap of *Cucurbita maxima* (Yoo *et al.*, 2004) and *Brassica napus* (Buhtz *et al.*, 2008). The high P_i deficiency-dependent abundance of miR399 in the roots was dwarfed by the abundance of miR399 in phloem sap (Pant *et al.*, 2008). This led to the demonstration through grafting experiments that miR399 is transported from the shoots to the roots through the phloem in both arabidopsis and *Nicotiana benthamiana* (Lin *et al.*, 2008; Pant *et al.*, 2008). When shoots over-expressing miR399 due to the presence of a miR399 transgene were grafted onto the wild-type rootstocks lacking miR399, the roots accumulated mature miR399 to very high levels, while the corresponding primary transcripts were virtually absent. The grafted plants also accumulated 5-fold more P_i in the shoots than the wild-type plants did. Thus, the phloem transport of miR399 from the shoots to the roots apparently systemically controls the maintenance of plant P_i homeostasis (Lin *et al.*, 2008; Pant *et al.*, 2008).

The *At-pho1* mutant may give important insights into how miR399 exerts this systemic control. Under P_i-sufficient conditions, *At-pho1* plants have decreased amounts of P_i in the shoots, despite wild-type amounts of P_i in the roots. The P_i deficiency in the *At-pho1* shoots results in the accumulation in this tissue of both primary and mature miR399 transcripts. The amount of primary miR399 in roots is also increased. It is not yet clear whether the increased abundance of miR399 in roots is the result of increased expression of miR399 in the roots or the translocation of miR399 from the shoots (Lin *et al.*, 2008). Distinguishing between these two possibilities will give us a greater appreciation of the mechanism through which miR399 controls P_i homeostasis.

Phloem sap contains a large variety of RNA molecules, ranging in size from miRNAs to mRNAs (summarized in Zhang *et al.*, 2009), and including miRNA star strand sequences (Buhtz *et al.*, 2008; Pant *et al.*, 2009). Among these RNAs are a group of small RNAs with sequences related to nuclear or organelle genes encoding rRNA and tRNA, as well as nuclear genes for small nucleosomal RNA, processing-related small RNA and signal recognition particle RNA (Zhang *et al.*, 2009). The recent finding that several RNA fragments falling into these classes, as well as several miRNA star sequences, accumulate specifically in P_i-starved plants (Buhtz *et al.*, 2008; Hsieh *et al.*, 2009; Pant *et al.*, 2009) suggests that these classes of RNA may have a biological function. Moreover, their presence in phloem sap (Buhtz *et al.*, 2008; Pant *et al.*, 2009; Zhang *et al.*, 2009) suggests that some may have systemic regulatory functions. This possibility adds a potentially exciting new dimension to the RNA-based regulatory components of the P_i starvation response.

Global integration of P_i signalling

Many of the factors described above are components of a complex regulatory web that dynamically assesses and responds to the availability of P_i at the cellular or whole-plant level. The establishment and maintenance of a mineral nutrient balance that allows optimal growth under the prevailing environmental conditions and within the specific developmental stage of the plant requires the integration of these P_i-specific responses with other signalling networks operating at the whole-plant level. Such signals include information on the availability of other mineral nutrients, such as iron and zinc, and long-distance and local signals embedded within molecules such as phytohormones. A thorough understanding of the integration of the relevant signals and responses is central to obtaining a holistic view of plant nutrient acquisition. Aspects of this integration have been discussed in detail elsewhere (Ward *et al.*, 2008; Rubio *et al.*, 2009; Santner *et al.*, 2009).

The interaction of P with iron is a long-known example where the availability of one nutrient affects the activity of another. The chemical interaction of P and iron in the growth medium, at the root surface or within the plant itself can lead to the formation of complexes that lower the bioavailability of both nutrients. The inhibition of primary root elongation that is commonly observed in arabidopsis upon P_i deprivation is not typical of all plants and was recently linked to iron toxicity (Ward *et al.*, 2008). When plants were grown in media containing decreased iron, the removal of P_i had no effect on primary root elongation. This observation goes some way to explain why a number of iron-responsive genes were found among the genes that respond to P_i deficiency in tomato and arabidopsis (Wang *et al.*, 2002; Misson *et al.*, 2005; Zheng *et al.*, 2009). These genes were likely to be responding to an increase in available iron under low P_i conditions. Also in arabidopsis, increases in plant iron concentration and modifications to iron storage under low P_i conditions are related to enhanced iron availability in the plant and the external medium (Hirsch *et al.*, 2006).

It has also been known for a long time that zinc deficiency can cause high levels of P_i to accumulate in plant tissues, which can lead to toxicity when P_i is readily available

(Cakmak and Marschner, 1986). In barley, the expression of genes encoding the Hv-PT1 and Hv-PT2 high-affinity P_i transporters was induced by zinc deficiency, independently of P_i availability (Huang *et al.*, 2000). The role of zinc in the regulation of the high-affinity P_i transporter genes is specific, and cannot be mimicked by manganese, another divalent cationic micronutrient. From these results, it was suggested that zinc may have a specific role in the signal transduction pathway regulating the high-affinity P_i transporter genes (Huang *et al.*, 2000).

It is becoming clearer that phytohormones are involved in the responses of plants to the availability of mineral nutrients, including P_i (Rubio *et al.*, 2009). However, this role is often secondary to the initial response to low P_i and may only affect specific components or segments of the P_i regulon. Plant hormones are structurally unrelated small molecules that have profound effects as growth regulators (Santner *et al.*, 2009). Generally acting at low concentrations, they mediate plant responses to both biotic and abiotic stresses by acting at a distance from the site of synthesis, and/or by acting locally, at or near the site of synthesis. Hormone signalling generally leads to major changes in transcript profiles. Non-genomic hormonal responses are also likely to occur, but these are not as well characterized. Recent work has made it clear that regulated protein degradation mediated by ubiquitin is a common theme in hormone signalling. Several hormone receptors have now been identified as enzymes in the ubiquitin–protein conjugation pathway, and the abundance of key downstream signalling proteins has also been found to be regulated by ubiquitin-dependent degradation (Santner *et al.*, 2009).

Cytokinins are negative regulators of P_i starvation responses in arabidopsis. The expression of P_i starvation-induced genes, such as *At-PHT1;1*, *At-ACP5* and members of the *Mt4/TPSI1* gene family, was repressed by the application of exogenous cytokinin (Martín *et al.*, 2000; Shin *et al.*, 2006). Mutation of the cytokinin receptor genes *At-CRE1/WOL/AHK4* and *At-AHK3* can attenuate the repression in the presence of sugar (Franco-Zorrilla *et al.*, 2002, 2005). Moreover, P_i deprivation repressed the accumulation of cytokinin and the expression of *At-CRE1*, conditions that would be expected to favour activation of the P_i starvation response (Franco-Zorrilla *et al.*, 2002). Evidence indicating that cytokinins, generally acting through *At-CRE1*, also repress a number of genes that respond to nitrogen, sulfur or iron deficiency (Rubio *et al.*, 2009) is a measure of the complexity of the regulatory network necessary to integrate the nutrient-dependent signals within plants.

Several studies in arabidopsis have examined the role of auxin as a mediator for the reduction in primary root growth and the proliferation of lateral roots induced by P_i starvation. Auxin-resistant mutants show largely wild-type primary root growth responses under both P_i sufficiency and deficiency, suggesting an auxin-independent mechanism for this response (Williamson *et al.*, 2001; López-Bucio *et al.*, 2002; Al-Ghazi *et al.*, 2003). On the other hand, P_i starvation enhances the responsiveness of the root system to the induction of lateral root proliferation caused by the application of exogenous auxin (López-Bucio *et al.*, 2002). The accumulation of auxin at the primary root apex, in pre-initiated lateral root primordia and in young lateral roots suggested that auxin transport or biosynthesis was important (Nacry *et al.*, 2005). However, plants defective either in auxin sensing or in polar transport due to mutation or inhibitors are still able to produce increased lateral roots in response to P_i starvation (Williamson *et al.*, 2001; López-Bucio *et al.*, 2002; Jain *et al.*, 2007; Pérez-Torres *et al.*, 2008). Together, these results point to increased auxin sensitivity, not increased auxin transport or synthesis, as the mediator of proliferative lateral root growth.

The low P_i-induced increase in auxin sensitivity leading to lateral root formation is likely to be mediated by the At-TIR1 auxin receptor (Pérez-Torres *et al.*, 2008). Many auxin-responsive genes are held in a repressed state by the binding of an Aux/IAA protein to an auxin response factor occupying the promoter (Santner *et al.*, 2009). *At-TIR1* is induced in response to low P_i and encodes an F-box protein that binds directly with Aux/IAA, an interaction that is enhanced by auxin. The combination of auxin, At-TIR1 and Aux/IAA induces the ubiquitin-dependent degradation of Aux/IAA by the 26S proteosome, allowing the auxin response factor to proceed with transcription. The arabidopsis transcription factor AUXIN RESPONSE FACTOR 19 (At-ARF19) has been implicated in the increase in lateral root growth in response to low P_i, and is a candidate participant in the TIR1-mediated induction of lateral root formation in response to low P_i (Pérez-Torres *et al.*, 2008).

An involvement of gibberellic acid in the P_i starvation response is beginning to emerge. Gibberellic acid at least partially represses low P_i-induced changes to arabidopsis root and shoot growth and architecture, including the inhibition in primary root growth, the increase in lateral root density and the increase in root-to-shoot ratio (Jiang *et al.*, 2007). Moreover, over-expression of At-MYB62 led to symptoms of gibberellic acid deficiency, which could be partially rescued by the application of exogenous gibberellic acid (Devaiah *et al.*, 2009). The gibberellic acid deficiency arose from a decrease in bioactive hormone brought about by associated changes in the transcript abundance for genes involved in gibberellic acid metabolism.

At least some of the molecular events leading to low P_i-induced changes in plant architecture and anthocyanin accumulation are dependent on the gibberellic acid–DELLA signalling pathway, which involves ubiquitin-dependent protein degradation (Jiang *et al.*, 2007). Gibberellic acid induces plant growth by promoting the destruction of the growth-restraining DELLA proteins (there are five DELLA proteins in arabidopsis). Similarly to the auxin signalling pathway, the binding of gibberellic acid to a specific receptor (GID1a, b or c) promotes binding of the hormone–receptor complex to the DELLA proteins (Santner *et al.*, 2009). DELLA proteins are typically bound to, and inactivate, various transcription factors. The binding of the gibberellic acid–receptor complex to DELLA initiates the ubiquitin-dependent destruction of DELLA by the 26S proteosome, releasing the transcription factors to activate the gibberillin response (Santner *et al.*, 2009).

Other hormones seem to have only a limited role in regulating the P_i starvation response. Mutants with impaired abscisic acid sensitivity (*aba2-1*) or biosynthesis (*aba1*) have a reduced P_i starvation response, including reduced expression of some

P_i-responsive genes and accumulation of anthocyanin (Trull *et al.*, 1997; Ciereszkoa and Kleczkowsk, 2002). However, the mutants were not impaired in the production of P_i starvation-induced phosphatases or in the ability to modulate biomass allocation between roots and shoots. Application of the hormone to the roots of P_i-deprived wild-type plants decreased the transcript abundance in the roots for several members of the *Mt4/TPSI1* gene family (Shin *et al.*, 2006). It is interesting to note in this regard that At-miR399 homologues were identified in a population of arabidopsis plants subjected separately to a number of stresses and abscisic acid before pooling (Sunkar and Zhu, 2004). *At-RAB18*, which is induced by P_i deprivation and sugar (Ciereszkoa and Kleczkowsk, 2002), is also induced by abscisic acid. The induction is through an At-ABI5-dependent signalling pathway, a process attenuated by the At-SIZ1-dependent SUMOylation of At-ABI5, a transcription factor containing a basic leucine-zipper domain (Miura *et al.*, 2009).

Ethylene was found to inhibit root elongation in P_i-sufficient plants, but stimulated it in P_i-deficient plants (Borch *et al.*, 1999; Ma *et al.*, 2003). Conversely, the inhibition of ethylene production or action inhibited root elongation in P_i-deficient plants while stimulating it in P_i-sufficient plants. However, the mechanism mediating the ethylene-dependent response of root elongation to P_i deficiency remains unclear.

It is likely that there is a complex interplay among P_i, hormones and sugar signalling. Mutation of the cytokinin receptor genes *At-CRE1/WOL/AHK4* and *At-AHK3* can attenuate the repression in the presence of sugar (Franco-Zorrilla *et al.*, 2002, 2005). The abscisic acid and Pi starvation-inducible *At-RAB18* gene is also induced by sugar (Ciereszkoa and Kleczkowsk, 2002).

UNIDENTIFIED MUTANT GENES RELEVANT TO P_i NUTRITION IN HIGHER PLANTS

Many of the genes described above that are central to the P_i starvation response network were identified by mutant analysis, clearly reiterating the power of using mutants to determine gene function and dissecting the genetic pathways. There are a number of interesting mutants available that are relevant to P_i nutrition where identification of the mutated gene and functional analysis of the gene product will give insights into the details of the P_i starvation response network.

The At-lpi *mutants*

The reduction in primary root elongation is a conspicuous root developmental change that occurs during P_i starvation (Williamson *et al.*, 2001). The *At-lpi* mutants, representing four different genetic loci, were isolated from an EMS-mutagenized population due to their ability to maintain primary root growth during P_i starvation (Sánchez-Calderón *et al.*, 2006). The abundance of transcripts from a sub-set of P_i starvation-induced genes is reduced during P_i deprivation in the *At-lpi* mutant compared with wild-type plants. The *At-lpi* phenotype is indicative of a function that may be central to the cross-talk between low P_i status and the activation of P_i deficiency-responsive genes that control root development. Isolation of the affected genes from the *At-lpi* mutants may, therefore, identify major control points in the P_i starvation-induced signalling network (Sánchez-Calderón *et al.*, 2006).

The At-psr1 *mutant*

Arabidopsis can grow on a medium containing DNA as the main P_i supply (Chen *et al.*, 2000). The *At-psr1* mutant was isolated from an EMS-mutagenized arabidopsis population due to its inability to use exogenous DNA when P_i is limited. The mutant grows well when P_i is supplied. Biochemical analysis showed that RNase and acid phosphatase activities in the *At-psr1* mutant are generally reduced compared with those in wild-type plants. Genetic analysis indicated that the mutant phenotype is caused by a single recessive allele, implying that *At-PSR1* influences the expression of a sub-set of genes encoding enzymes that degrade exogenous organophosphate substrates, increasing the ability of the plant to scavenge P_i (Chen *et al.*, 2000). Identifying the corresponding gene will give added insight into how plants regulate the scavenging of P_i from the rhizosphere.

The At-pup1 *mutant*

The *At-pup1* mutant was isolated from a T-DNA-mutagenized arabidopsis population due to reduced root staining for phosphatase activity when grown on a P_i-deficient medium (Trull and Deikman, 1998). Analysis of the phosphatases produced by the mutant showed that a 160 kDa acid phosphatase isoform is missing. The response of the *At-pup1* mutant to P_i deprivation, such as the accumulation of anthocyanin and the altered partitioning of P between root and shoot, were the same as in wild-type plants, while the root to shoot ratio was lower in the mutant under P_i-sufficient conditions (Trull and Deikman, 1998). Identifying *At-PUP1* within the 5 cM interval on chromosome 2 to which it has been mapped (Trull and Deikman, 1998) will give us another gene involved in scavenging P_i from the rhizosphere, providing further opportunities to dissect the regulatory pathways used to control the deployment of these strategies.

CONCLUSIONS AND FUTURE PERSPECTIVES

In the past few years, there has been rapid progress made in understanding the ways that plants respond and acclimate to P_i-deficient growth conditions. The PHR1 transcription factors At-PHR1 and Os-PHR2 have emerged as central regulators in the deployment of the adaptive strategies required to cope with P_i deficiency in arabidopsis and rice, respectively. Their constitutive presence in the nucleus suggests they are poised for action, awaiting the signal that P_i concentration is insufficient. But what is the nature of that signal? In future, it will be interesting to see if there are other central regulators, and to determine the conservation of functions across evolutionary distances. For example, why does rice require a second PHR-type transcription factor, Os-PHR1, to adapt to P_i deficiency? Does a transcription factor in arabidopsis have a role analogous to that of Os-PHR1? Are the differences seen between rice and arabidopsis simply an indication of

species diversity, or do they hint at something larger, the divide between monocots and eudicots?

The PHR factors are supported by a host of other regulatory functions, including the transcription factors BHLH32, WRKY75, ZAT6 and MYB62. Some of these factors are positive regulators acting to induce the expression of genes necessary for adaptation, while others are negative regulators. The involvement of both positive and negative effectors illustrates the dynamic process underlying the deployment and adjustment of the P_i starvation response to maintain a balance between nutrient availability and acquisition to satisfy the nutritional demands imposed by the prevailing developmental programme and growth requirements. A fuller understanding of the interplay among factors such as ZAT6 and MYB62 that modulate the intensity of the low P_i response and positive regulators such as PHR1 will reveal the true complexity of plant adaptive responses to limiting P_i.

Details are now rapidly emerging about the interactions of the P_i regulon with the availability of other nutrients and the regulatory networks deployed to adapt to deficiencies in these nutrients. In this regard, hormonal responses and small RNA molecules have central roles that are just being documented. Both hormonal responses and pathways regulated by small RNAs, such as miR399, highlight the importance of post-translational modifications, such as ubiquitination and SUMOylation, in the plant response to P_i availability. Future discoveries on the targets of post-translational modification and the regulation of the modification process in response to P_i availability are areas needing much further work.

The recent finding of numerous small RNA molecules whose abundance is responsive to P_i status (Hsieh *et al.*, 2009; Pant *et al.*, 2009) is both exciting and sobering. The involvement of miRNAs and other small RNAs in regulating plant responses to P_i is clearly very poorly understood, despite the enlightening work done on revealing the regulation of *PHO2/UBC24* by miR399. It will take much effort to reveal the roles of small RNAs in the plant response to P_i. However, the academic and practical rewards for this effort are likely to be large. Especially intriguing is the role of small RNAs, as well as hormones and sugars, as long-distance signals in the P_i starvation response pathway. In the case of RNAs, what is the mechanism of their loading into and unloading from the phloem? What cells are responsible for their synthesis and what cells receive and act upon the signals received?

There are many other intriguing questions yet to be answered in regard to the P_i starvation response in plants. Perhaps top among these is the nature and identity of the sensor that sets the whole P_i starvation response in motion. The finding that local P_i is sensed by an ER-localized pathway involving PDR2 and LPR1 is an excellent step forward, but what is the mechanism of action of these proteins and how does this pathway interlink with the systemic signalling pathways? Might it be something as simple as a protein phosphorylation that is inhibited by the lack of P_i as a substrate or will it be something much more complex? Answering these and many other questions will ultimately reveal the entire P_i starvation regulatory network and provide the understanding needed to develop new molecular genetic strategies for establishing crop plants with improved P_i acquisition and P_i use efficiency.

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