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Control of plant phosphate homeostasis by inositol pyrophosphates and the SPX domain

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Summary

Proteins containing a SPX domain are involved in phosphate (Pi) homeostasis, including Pi transport and adaptation to Pi deficiency. The SPX domain harbors a basic surface binding Pi at low affinity and inositol pyrophosphates (PP-InsPs) at high affinity. Genetic and biochemical studies revealed that PP-InsPs serve as ligands for the SPX domain. Residues in the PHO1 SPX domain involved in PP-InsPs binding are critical for its Pi export activity, and the interaction between SPX proteins and the PHR1 transcription factor, which results in PHR1 inactivation, is promoted by PP-InsPs. Changes in PP-InsPs levels in response to Pi deficiency may thus contribute to the adaptation of plants to stress via the modulation of the activity of SPX-containing proteins and their interactors. Modulating PP-InsP levels or the affinity/specificity of the SPX domain for PP-InsP could potentially be used to engineer crops to maintain high yield under reduced Pi fertilizer input.

Introduction

The availability of phosphorus (P) limits plant growth in natural and agricultural ecosystems. Plants essentially acquire P as water-soluble inorganic orthophosphate (Pi, $H_2PO_4^-$). While P may be quite abundant in most soils, the level of Pi accessible to plants is very low, principally because it readily forms insoluble complexes with calcium as well as with oxides and hydroxides of aluminum and iron. While application of Pi fertilizers to optimize crop yield is an essential practice in modern agriculture, its sustainability has been put into question [1]. P is essentially mined from a limited number of rock phosphate deposits and high quality rock phosphate is a finite resource [1,2]. This fact, combined with the expected increased demand for Pi fertilizers to sustain not only crops but also grassland productivity, raised concerns about its availability for future generations [3]. Furthermore, over-use of fertilizers leads to P run-off in streams and lakes, resulting in eutrophication. In this perspective, one important goal of plant biotechnology is to develop plants that can maintain maximal productivity under reduced fertilizer input. This will require new approaches, as past breeding programs typically focused on improving yield and pest resistance of plants grown under well-fertilized conditions [4].

Plants have evolved complex adaptation mechanisms to grow and survive in Pi-poor soils. Root development is profoundly modified under Pi deficiency: root hair length and density are increased, primary root length is reduced and root branching is enhanced [5]. Pi-deficient roots also secrete more protons, citrate as well as various esterases, and enhance association with mycorrhizae [6]. Collectively, these modifications are aimed at increasing the solubility of soil P, and enhancing the ability of the root system to explore and mine the soil for this precious resource. Plants also adjust their metabolic pathways to optimize internal Pi use, such as shifting phospholipids toward galacto-, glucurono-, and sulfo-lipids [7,8]. At the genetic level, these adaptations are accompanied by the coordinated up-regulation of hundreds of genes (named Phosphate Starvation Induced, PSI) [9,10]. In the last decade, several components have been identified that are involved in sensing and coordinating the complex Pi-deficiency response. One key player is PHR1, a member of the MYB transcription factor family [11]. PHR1 and its closest homologue PHL1 control the expression of the majority of PSI genes and influence numerous metabolic and developmental adaptations to Pi deficiency [10-13]. PHR1 has also recently been shown to integrate the PSI response with the plant immune response and to influence the root microbiota [14]. Since the PHR1 mRNA level is

not modulated by Pi deficiency, questions as to how PHR1 activity is regulated by Pi deficiency remained unanswered. Recently, a role for inositol pyrophosphates and SPX-containing proteins has been uncovered in the PHR1-mediated Pi-deficiency response [15].

SPX-containing proteins in Pi homeostasis

The hydrophilic SPX domain (Pfam PF03105, named after the *Saccharomyces cerevisiae* Syg1 and Pho81proteins, and the mammalian Xpr1) comprises 160-350 amino acids and contains a set of invariant lysine residues forming a conserved sequence fingerprint (Figure 1). SPX-containing proteins are found throughout the eukaryotic tree of life, including fungi, plants, and metazoans. Plant SPX-containing proteins are divided into four sub-families, SPX-EXS (Pfam PF03124, named after the *S. cerevisiae* Erd1, mammalian Xpr1 and *S. cerevisiae* Syg1), SPX-MFS (Pfam Clan CL0015, Major Facilitator Superfamily), SPX-RING (Pfam 13920, Really Interesting New Gene), and SPX, depending on the presence of additional domains, which are always fused to the C-terminus of the SPX domain (Figure 1) [16]. PHO1 is the prototypical member of the SPX-EXS sub-family and encodes a Pi exporter involved in loading Pi into the root vascular cylinder [17]. The EXS domain contains two trans-membrane helices and is implicated in proper localization and activity of PHO1 as a Pi exporter and in participating in the Pi-deficiency responses [18]. The diversity of PHO1-related genes has expanded in dicots compared to monocots. The *Arabidopsis thaliana* genome contains ten PHO1 homologues (PHO1;H1-H10), of which only PHO1;H1 has been directly associated with Pi homeostasis [19-21]. Proteins belonging to the SPX-MFS sub-family contain numerous transmembrane helices and encode the long sought-after tonoplast Pi transporters, named PHT5 or VPT, moving Pi in and out of the vacuole [22-24]. The sub-family SPX-RING includes NLA, an E3 ubiquitin ligase that targets the Pi transporter PHT1 for ubiquitination and degradation [25-27]. The fourth sub-family consists of stand-alone SPX proteins (SPX1 to SPX4), all of which are differentially regulated under Pi deficiency [28]. Modulation of the expression of some members of the SPX sub-family is associated with changes in the expression of PSI genes and influences the adaptation of plants to Pi deficiency [28-30].

The common element of plant SPX-containing proteins is their implication in Pi homeostasis, which also extends to the ten *Saccharomyces cerevisiae* proteins containing a SPX domain, including Pi transporters (Pho87, Pho90, Pho91), a PHO1-homologue (Syg1), a Pi-recycling

enzyme (Gde) and components of the Pi-signaling cascade (Pho81) or subunits of the inorganic polyphosphate polymerase (Vtc2 to Vtc5) [31]. The connection between SPX and Pi homeostasis raised the possibility that this domain may be involved in the direct binding of Pi or some metabolite derived from it. In this context, it is important to know the concentrations of Pi and related metabolites that are available in the plant cytosol for potential interaction with the SPX domain. Measurements of cytoplasmic (e.g. cytosol and organelles except for the vacuole) Pi concentration by in vivo ^{31}P-NMR typically gives estimates of 5-10 mM [32]. However, by using a NMR method allowing the separation of the organellar (primarily mitochondria and plastids) and cytosolic signals, Pi concentrations of 60-80 μM for the cytosol and 4-7 mM for the organelles were obtained [33]. Such a cytosolic Pi concentration would be in the Km range of Pi of several Pi-dependent enzymes but not of others, including SPX proteins ([15] see below). Dynamic changes of cytosolic Pi content in root cells grown on Pi-deficient or Pi-sufficient media have recently been monitored *in vitro* with a Pi-biosensor having a K_d for Pi of 6.4 mM, thus putting the cytosolic Pi concentration potentially in that range [34].

SPX domains are sensors for inositol pyrophosphate signaling molecules

Crystal structures of fungal and human SPX domains revealed a novel protein fold, with a central three-helix bundle preceded by a conserved N-terminal α-hairpin motif (Figure 1) [15]. In different SPX domain structures, the conserved lysine cluster forms a highly basic binding surface at the N-terminus of the protein. This surface can interact with Pi itself, albeit with low millimolar affinity ($K_d \approx$ 5-20 mM) [15]. Furthermore, SPX domains cannot discriminate between Pi and other inorganic anions, such as sulfate (Figure 2A). A combined genetic, biochemical and structural screen identified inositol pyrophosphates (PP-InsPs) as *bona fide* ligands for the SPX domain, which specifically interact with the basic binding surface with nano- to micromolar affintiy ($K_d \approx$ 50 nM- 100 μM) [15,35] (Fig. 2B). PP-InsPs consist of a fully phosphorylated inositol ring that carries additional pyrophosphate groups in one or several positions [36]. In yeast, the concentrations of PP-InsPs change in response to Pi starvation and their biosynthesis route has been well-characterized [15,36,37]. Deletion of the PP-InsP kinase Kcs1 leads to an inhibition of the inorganic polyphosphate polymerase activity of the yeast VTC complex via its SPX domain. In this mutant, Pi is only stored as free inorganic phosphate under Pi sufficient growth conditions [15,38,39]. In plants, homologs of diphosphoinositol pentakisphosphate kinase (PPIP5K/Vip1-like) have been identified [40,41]. However, PP-InsP biosynthesis is generally poorly understood and PP-InsPs have thus far not

been implicated in the response of plants to Pi deficiency [41,42]. This may, in part, be due to the challenges in detecting and quantifying different PP-InsP isomers from plant tissues.

Inositol pyrophosphate binding renders SPX domains competent to interact with other proteins

Previous work has established that SPX proteins can associate with the Arabidopsis PHR1 or its rice homologue PHR2 both in vitro and in vivo [43-45]. Formation of the SPX-PHR1/2 complex in Pi-sufficient plants prevents the binding of PHR1/2 to its target promoters. In contrast, under Pi-deficient conditions, PHR1/2 is free and can act as a transcriptional activator of PSI genes. Initially, Pi itself was proposed as a ligand enabling SPX proteins to interact with PHR1/2 . The dissociation constants for Pi in such a complex range from 10 to 20 mM [43-45]. In contrast, PP-InsPs promote the high-affinity interaction between the rice SPX4 and PHR2, with Kds in the low micromolar range (7-50 μM) [15]. Thus, PP-InsPs may regulate the activity of PHR1/2 via SPX proteins. Consistently, plants unable to synthesize the PP-InsPs precursor inositol hexa*kis*phosphate (InsP6) show constitutive Pi starvation responses, despite having higher cellular Pi levels compared to wild-type [36,46,47]. Couso and colleagues report levels for different PP-InsPs, but it remains an open question which isomer(s) are involved in the PSI response [48]. It is likely that, besides Pi starvation, PP-InsPs control other signaling pathways such as hormone perception, as purification of plant hormone receptors heterologously expressed in insect cells resulted in co-purification of endogenous InsP6 with the auxin receptor complex TIR1-ASK1 and of inositol pentaphosphate (InsP5) with the jasmonate receptor complex COI1-ASK1 [49,50]. In addition, jasmonate specifically induced the production of the PP-InsP InsP8, and *in silico* docking experiments together with site-directed mutagenesis of COI1 hint at InsP8 being essential for COI1-JAZ1 complex formation [41]. Finally, PP-InsPs have also been implicated in COI1-mediated plant wounding responses [51,52]. Interestingly, modulation of TIR1 activity was implicated in the developmental adaptation of roots to Pi deficiency and low Pi induces increased resistance to herbivory via the JA-COI1 pathway [53,54]. It is thus possible that PP-InsPs coordinate multiple aspects of the Pi-deficiency response via their interaction with proteins involved in different signal transduction pathways.

Future perspective

While research on the yeast VTC and the plant PHO1, PHR1 and SPX-containing proteins clearly demonstrated the implication of PP-InsPs in Pi homeostasis and signaling, one may wonder if there is still room for a direct role of Pi as a signal molecule. One of the strongest evidence for this comes from classical studies using phosphite (Phi, $H_2PO_3^-$), a reduced form of Pi. Application of Phi to Pi-deficient plants suppresses a wide range of typical Pi-deficiency responses, including local responses, such as root hair elongation, and systemic responses, such as lipid remodeling and the expression of numerous PSI genes [55,56]. Since plants cannot convert Phi into Pi and Phi itself is non-metabolizable, it can be concluded that Phi likely mimics Pi as a signal molecule. Like Pi, Phi was also shown to promote the interaction between SPX proteins and PHR1/2 [43-45], but it remains to be investigated if and how SPX can sense Phi. Furthermore, ^{31}P-NMR experiments have shown that cytosolic Pi concentration can rapidly fluctuate in response to shifts in external Pi supply, indicating that cytosolic Pi levels are more dynamic than previously thought, a characteristic that would also fulfill an important criterion for a signaling molecule [33]. However, as discussed previously, it is currently uncertain whether the concentration of Pi in the cytosol is sufficient to warrant any significant signaling function via SPX considering the relatively high K_d of Pi for this domain. In this context, it will be important to measure plant cytosolic PP-InsP concentrations under different plant growth conditions. With this information it will be possible to discuss the K_d of PP-InsPs for SPX domains in respect to physiological conditions on the one hand, and to correlate changes in Pi nutrition and cytosolic Pi concentrations with those of PP-InsPs on the other hand.

There is growing evidence for PP-InsPs being master regulators of Pi signaling in plants. Point mutations in the SPX domain of PHO1 in amino acids involved in coordinating with PP-InsPs but not Pi showed that these residues were essential for the complementation of the *pho1* mutant, thus highlighting the role of PP-InsPs versus Pi for PHO1 as a Pi exporter [15]. Similar genetic dissection experiments on different SPX-containing proteins are key experiments enabling to discern the role of Pi and PP-InsPs in their function. It will be also important to determine if the binding of PP-InsPs to the SPX domain of other SPX-containing proteins, such as PHT5/VPT and NLA, regulate their activity through the promotion of interaction with other effector/regulatory proteins, or via distinct mechanisms, such as protein pyrophosphorylation [57].

The high affinity of the SPX domain for PP-InsPs raises the possibility of using this domain as biosensors for the detection of PP-InsPs in vivo [58]. However, distinguishing between different PP-InsPs isomers may be a limiting factor of such a biosensor. In this respect, it is noteworthy that the SPX domain may require protein-protein interaction partners to discriminate PP-InsP isomers [15].

One major goal of future research will be the dissection and characterization of plant PP-InsPs biosynthestic and catabolic pathways as targets for the efficient manipulation of plant Pi homeostasis and the Pi-deficiency signal transduction pathway. Previous studies with PHO1 have shown that it is possible to uncouple shoot phosphate deficiency from its negative effects on growth [59]. Optimization of the PSI response, e.g. enhancement or attenuation, via the engineering of the SPX domain or the modulation of PP-InsPs content may thus be associated with improved growth under low Pi supply. The transfer of such knowledge to agronomically important plant species, such as rice, would contribute to the engineering of crops that have a reduced demand for valuable Pi-fertilizers but maintain maximum yield.

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Figure legends

Figure 1. Structure of the SPX domain and function of SPX domain-containing proteins in plants.

(A) Ribbon diagram of the ScVtc4 SPX domain (http://rcsb.org, PDB-ID 5IIG). The 3-helix bundle, consisting of two long core helices and two smaller C-terminal helices, is shown in orange. The N-terminal α-helical hairpin motif is highlighted in yellow. The position of the conserved lysine residues, which form sequence fingerprints for the SPX domain, are depicted by blue spheres. The C-terminal α6 helix can be connected to extra domains such as RING, MFS, and EXS. (B) In *Arabidopsis thaliana*, there are 20 SPX proteins that are classified into four different sub-families depending on the presence of extra domains: four proteins contain only the SPX domain (SPX1 to SPX4), two proteins combine SPX and a RING domain (NLA and NLA2), three combine SPX and a MFS domain (PHT5 or VPT), and eleven proteins combine SPX and an EXS domain (the PHO1 family). The functions of these proteins are indicated.

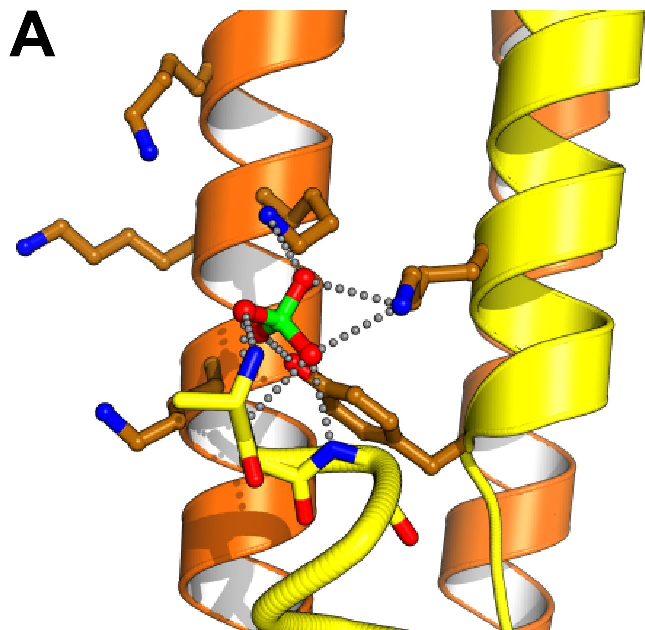
Figure 2. Interaction of the SPX domain with sulfate and InsP6. (A) Structure of the SPX domain of the human XPR1, a PHO1 homologue (http://rcsb.org, PDB-ID 5IJH, colors as in Fig. 1) bound to a sulfate ion (in bonds representation). Note that the SPX domain cannot differentiate between sulfate and Pi and that sulfate is a good structural mimic to Pi. The sulfate ion is in hydrogen bond contact (grey dotted lines) with only few of the lysine residues conserved among SPX domains. (B) Structure of the SPX domain of *Chaetomium thermophilum* Glycerophosphodiester Phosphodiesterase 1 (http://rcsb.org, PDB-ID 5IJJ) in complex with InsP6. The signaling molecule establishes an extensive hydrogen bond network with a tyrosine originating from the N-terminal helical hairpin motif and with the invariant lysine residues.

A

α2 N α1 α5 α4 α3 α6 C

B

Class		*Arabidopsis* gene name (locus identifier)	Function	References
Class 1 (SPX)	SPX	*AtSPX1* (AT5G20150) *AtSPX2* (AT2G26660)	• Negative regulation of Pi starvation responses through the inhibition of PHR1activity.	[44-45]
		AtSPX3 (AT2G45130)		
		AtSPX4 (AT5G15330)	• OsPHR2 translocation from cytoplasm to nucleus in a Pi-dependent manner.	[43]
Class 2 (SPX-RING)	SPX RING	*AtNLA/AtBAH1* (AT1G02860)	• Degradation of Pi transporters for the regulation of Pi homeostasis. • Regulation of nitrate-dependent phosphate homeostasis and source-to-sink remobilization of nitrate.	[25-27]
		AtNLA2 (AT2G38920)		
Class 3 (SPX-MFS)	SPX MFS	*AtSPX-MFS1/PHT5;1* (AT1G63010) *AtSPX-MFS2/PHT5;2* (AT4G11810) *AtSPX-MFS3/PHT5;3* (AT4G22990)	• Vacuolar Pi transporters.	[22-24]
Class 4 (SPX-EXS)	SPX EXS	*AtPHO1* (AT3G23430)	• Pi translocation from root to shoot • ABA-induced stomatal closure.	[17-19]
		AtPHO1;H1 (AT1G68740)	• Pi translocation from root to shoot.	[20]
		AtPHO1;H2 (AT2G03260)		
		AtPHO1;H3 (AT1G14040)	• Pi homeostasis in response to Zn deficiency.	[60]
		AtPHO1;H4/AtSHB1 (AT4G25350)	• Seed development, cryptochrome signaling, photoperiodic and autonomousflowering.	[61-62]
		AtPHO1;H5 (AT2G03240) *AtPHO1;H6* (AT2G03250) *AtPHO1;H7* (AT1G26730) *AtPHO1;H8* (AT1G35350) *AtPHO1;H9* (AT3G29060) *AtPHO1;H10* (AT1G69480)		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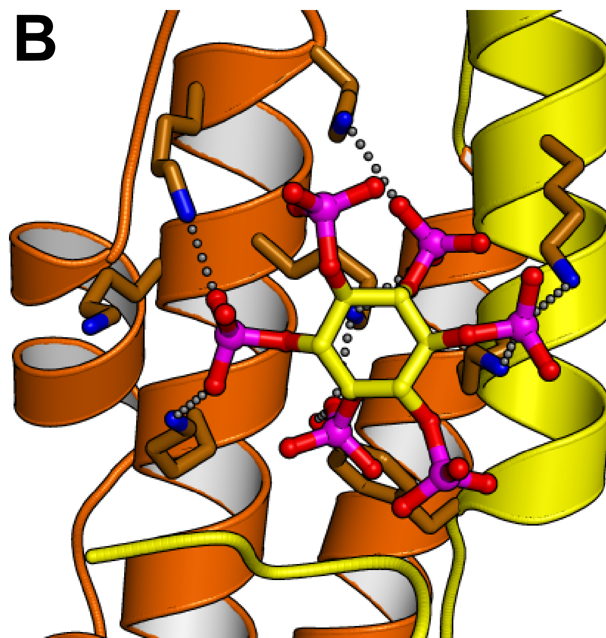
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