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Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis

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Rice is one of the most important cereal crops feeding a large segment of the world's population. Inefficient utilization of phosphate (Pi) fertilizer by the plant in rice production increases cost and pollution. Developing cultivars with improved Pi use efficiency is essential for the sustainability of agriculture. Pi uptake, translocation and remobilization are regulated by complex molecular mechanisms through the functions of Pi transporters (PTs) and other downstream Pi Starvation Induced (PSI) genes. Expressions of these PSI genes are regulated by the Pi Starvation Response Regulator (OsPHR2)-mediated transcriptional control and/or PHO2-mediated ubiquitination. SPX-domain containing proteins and the type I H^+-PPase AVP1 involved in the maintenance and utilization of the internal phosphate. The potential application of posttranscriptional regulation of PT1 through OsPHF1 for Pi efficiency is proposed.

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Introduction

Developing crop cultivars with increased yield and less dependence on the heavy application of fertilizers is essential for the sustainability of agriculture. Phosphorus (P) is an essential macronutrient for plant growth and development. Plants take up P exclusively in the form of inorganic phosphate (Pi). The high chemical fixation rate, slow diffusion and substantial fractions of organically bound P of Pi render it one of the least available nutrients for crop [1]. To obtain maximum crop yield, P fertilizer is often over-applied, which led to accelerating soil degradation and water eutrophication [2]. Rice is one of the most important cereal crops feeding a large segment of the world's population. Developing rice cultivars with higher efficiency in P use is increasingly important for sustainable food production.

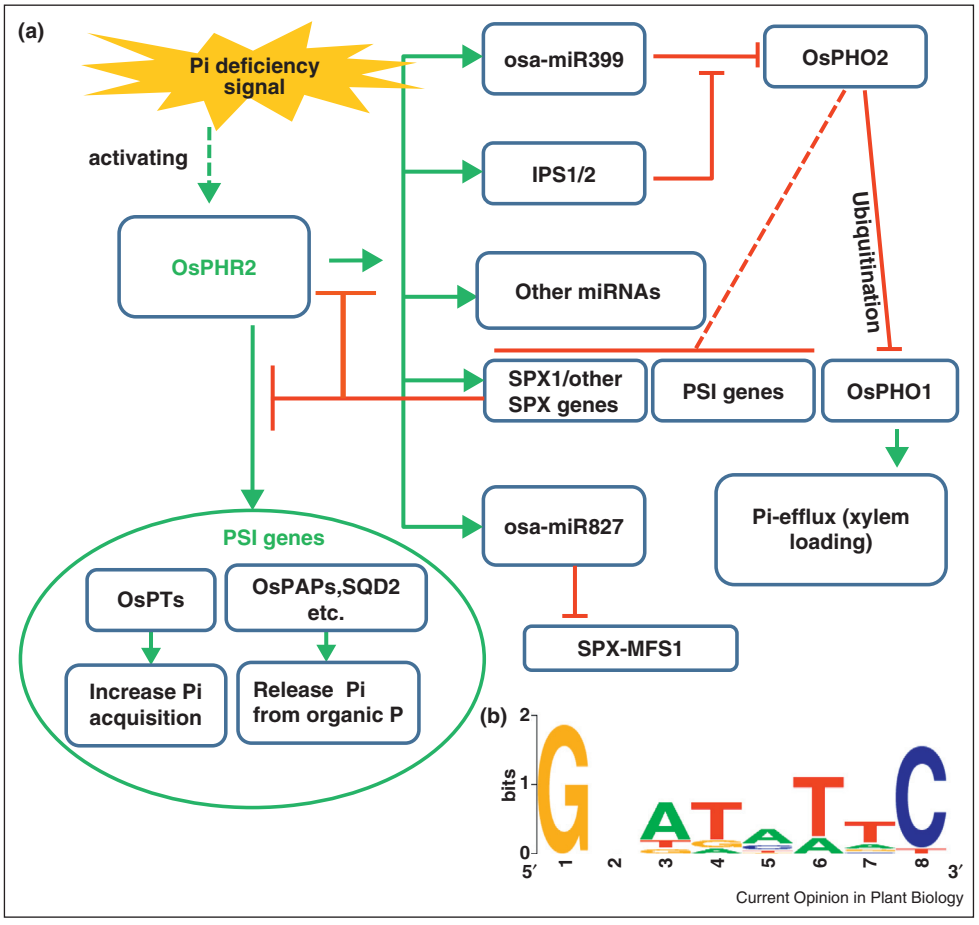
Over the past decades, many scientific studies aimed at elucidating the complex molecular mechanisms and crucial regulators underlying Pi signaling and Pi homeostasis in plants have been performed. In this article, we focus on progress in understanding the molecular regulation of Pi acquisition and homeostasis in the major cereal crop rice. In particular, this review summarizes the recent progress in determining the signal networks of PSI, the functions and regulation of rice PT, and the roles of SPX domain-containing proteins in Pi homeostasis. In addition, we provide new data showing the application of Pi-transporter posttranslational regulation to improve tolerance to low Pi stress in rice.

Phosphate signaling under the control of the central transcription factor OsPHR2

The Arabidopsis Phosphate Starvation Response Regulator 1 (PHR1) is a MYB transcription factor, playing a key role in Pi starvation signaling by binding to a cis-element of GNATATNC, named the PHR1 binding sequences (P1BS) [3]. Genes downstream of PHR1 include genes encoding the signal molecules AtIPS1/At4 [4,5], miRNAs [6], SPXs [7,8••,9,10], biosynthetic genes of sulfolipids and galactolipids [11], PTs [12–14] and purple acid phosphatases (PAPs) [15–17] (Figure 1a). In addition to P1BS, there are other consensus sequences adjacent to P1BS that have proved to be essential to the Pi starvation response [18•,19].

OsPHR1 and OsPHR2 are homologous proteins of PHR1 in rice [20]. Overexpression of *OsPHR2* in rice mimicked the Pi starvation signal. It induced PSI gene expression and resulted in the enhancement of Pi acquisition. The PSI genes that are activated by the overexpression of *OsPHR2* include genes encoding the signaling molecules *OsIPS1/2*, microRNA osa-miR399 and osa-miR827 [20,21,22•,23], and SPX1-3 and SPX5-6 [8••,9,10,22•]; PTs for Pi uptake and translocation; PAPs for releasing Pi from organic P [20,24]; sulfoquinovosyldiacylglycerol 2 (SQD2) for recycling Pi from membrane phospholipids [12–14] (Table 1 and Figure 1a). Analysis of the 2.0 kb sequence upstream of the initiating ATG of the listed PSI genes and a yeast one-hybridization assay showed that at least one additional motif (Figure 1b), named as P1BS-like, adjacent to the PIBS elements at a certain distance is

Figure 1



(a) Regulation of phosphate starvation signal through the transcription factor, OsPHR2. Green arrows represent positive effects, whereas red lines ending with a short bar indicate negative effects. The dotted red line indicates unknown mechanism. **(b)** Weblogo presentation of sequence of P1BS-like motif.

required for OsPHR2 binding. The requirement for the existence of P1BS and P1BS-like sequences in the promoter region of PSI genes is consistent with the fact that OsPHR2 acts as a heterodimer or homodimer.

As was initially shown in Arabidopsis [25–29], the expression of both *osa-miR399* and its antagonists *OsIPS1/2* are induced by *OsPHR2* overexpression, regardless of the status of Pi supply. osa-miRNA399 targets an E2 ubiquitin-conjugase, OsPHO2 [30]. OsIPS1/2 mimics the osa-miR399 target to attenuate the suppressive effect of miR399 on PHO2 mRNA. Overexpression of *OsPHR2* and loss of function of *OsPHO2* lead to excessive accumulation of Pi in the shoot tissue [9,20,30].

A number of rice PTs have been shown to be induced by Pi starvation [8••,13,20,31,32••,33]. While 9 out of the 13 rice PTs contain P1BS sequences in their promoter region, only OsPT2/3/7/10/11 contains the adjacent P1BS-like motif (Table 1). The physical binding of OsPHR2 to the promoter of the *OsPT2* gene, which encodes a low-affinity PT, has been shown [8••]. Whether the Pi starvation-induced PTs, that lack the adjacent P1BS-like element, are regulated by the transcription factor OsPHR2 in conjugation with other protein factors or by other transcription factors needs to be clarified.

The expression levels of 10 out of the 25 identified rice *PAP* genes were upregulated by both phosphate deprivation or overexpression of the transcription factor OsPHR2 [24]. In addition to the 10 PSI PAP genes in the root, the promoters of *OsPAP9a* and *OsPAP15* contained P1BS and the adjacent P1BS-like elements (Table 1). It is not clear why the expression of the two PAP genes does not respond to Pi starvation. In addition, *OsPAP1d* and *OsPAP10a* have only P1BS element(s), not the adjacent P1BS-like element. Whether the induction of these two genes by Pi starvation requires factors other than OsPHR2 needs further analysis.

Table 1

Existence of the P1BS and P1BS-like motifs in promoter regions of rice PSI genes. The presence of P1BS and P1BS-like motifs was analyzed in 2 kb upstream of ATG initiation site of each gene. P1BS sequence: GNATATNC; P1BS-like motif: see supporting data in Figure1

Genes	LOC number	P1BS position	P1BS-(adjacent P1BS-like) position
Signal genes			
OsIPS1	AY568759	−483, −618	−483/(−471)
OsIPS2	AK240849	−154, −224	−154/(−142)
OsmiR827a	MI0010490	−195	−195/(−208)
OsmiR399d	MI0001056	−188, −263	−188/(−263)
OsmiR399j	MI0001062	−156, −194	−156/(−194)
OsSPX1	Os06g40120	−136, −170	−136/(−170)
OsSPX2	Os02g10780	−147, −163	−147/(−163)
OsSPX3	Os10g25310	−208, −954	−208/(−216)
OsSPX4	Os03g61200	−196	None
OsSPX5	Os03g29250	−166, −1636	None
OsSPX6	Os07g42330	−148, −856, −1864	−148/(−138), −856/(−869), −1864/(−1853)
OsPTs			
OsPT2	Os03g05640	−336	−336/(−357)
OsPT3	Os10g30770	−502, −797	−502/(−510)
OsPT5	Os04g10690	−257	None
OsPT8	Os10g30790	−1585, −1754, −2839	None
OsPT9	Os06g21920	−828	None
OsPT10	Os06g21950	879 (within intron)	879/(892)
OsPT11	Os01g46860	−219, −515, −1203	−1203/(−1212)
OsPT12	Os03g05610	−1693	None
OsPT13	Os04g10800	−527	None
OsSQD2	Os01g04920	−54, −936	−54/(−94)
PAPs			
OsPAP1a	Os03g11530	−273, −2496	−273/(−261)
OsPAP1d	Os12g38750	−220	None
OsPAP3b	Os10g02750	−113, −419, −2209	−419/(−406
OsPAP9a	Os07g02090	−96, −2856	−96/(−145)
OsPAP9b	Os01g58640	−366, −985, −1299, −1463	−999/(−985)
OsPAP10a	Os01g56880	−218	None
OsPAP10c	Os12g44020	−139, −516, −1829	−516/(−506), −1829/(−1817)
OsPAP15	Os03g63074	−504, −982, −1134, −1198	−504/(−485)
OsPAP20b	Os12g05540	−573	−573/(−587)
OsPAP21b	Os11g05400	−39	−39/(−51)
OsPAP23	Os08g17784	−107	−107/(−96)
SQDs			
OsSQD2	Os01g04920	−54, −936	−54/(−94)

Phosphate signaling through PHO2-mediated ubiquitination

As outlined in the last section, PHR1/OsPHR2 negatively regulates the transcript level of *PHO2* through miR399-mediated RNA cleavage in Arabidopsis and rice [20,25–29]. It is not surprising that some common genes could be induced by both the activation of *PHR1* and the mutation of *pho2*. However, many of the downstream genes that are antagonistically regulated by *PHR1/OsPHR2* and *PHO2* are PSI genes whose promoter region contains P1BS elements that bind PHR1/OsPHR2. For instance, 21 out of 22 genes deregulated in the *pho2* mutant were regulated by PHR1, including SPX1, Pht1;4, the acid phosphatase 5 (ACP5) and so on [27]. Consistent with the Arabidopsis results, rice *OsSPX1*, *OsPT2*, *OsPT8*, *OsPAP10a* and RNase were induced in OsPHR2 over-expressors and the *pho2* mutant [9,30]. Therefore, the direct binding between the P1BS element (plus other required motifs) and PHR1/OsPHR2 is significant but not sufficient to explain the regulatory mechanism of PSI gene expression (Figure 1). PHO2 negatively regulates a number of PSI genes via an unknown mechanism. The recent discovery of a PHO2-dependent PHO1 degradation pathway sheds new light on our understanding of how PHO2 mediates Pi uptake and translocation [34••]. Further studies are thus required to identify PHR1/OsPHR2 and PHO2 interacting proteins and determine how they are co-regulated.

Roles of OsSPX domain proteins in phosphate homeostasis

In plants, the proteins containing the SPX (named after the yeast Syg1, Pho81, and the human XPR1) domain can be divided into the SPX, SPX-EXS, SPX-MFS and SPX-RING subfamilies on the basis of the presence of additional domains in their protein structure. Proteins

exclusively harboring the SPX domain are referred to as SPX proteins. Except for OsSPX4, the rice SPX genes were highly induced by Pi starvation at the transcription level in roots and/or in shoots [8••,9–10]. The expression of *OsSPX1* can be induced by Pi starvation or the PHR-PHO2 signal pathway, that is, the transcript level of *OsSPX1* is increased in *OsPHR2* overexpressors or *OsPHO2* mutants (*ospho2*) [9]. However, the role of OsSPX1 in Pi accumulation is opposite of that of OsPHR2. While overexpression of *OsPHR2* or knockout of *OsPHO2* resulted in toxicity due to the accumulation of Pi [9,20], knockdown of OsSPX1 increased the expression of some PSI genes, including the Pi transporters OsPT2 and OsPT8 and consequently increased Pi accumulation in the shoots [9]. In contrast, overexpression of *OsSPX1* reduced the shoot Pi concentration of the *OsPHR2* overexpressor to a level similar to that of the wild-type plant [8••,9]. Thus, OsSPX1 is a Pi-starvation-induced negative regulator. OsSPX1-6 were shown to localize in different organelles [10], implying that the six OsSPX proteins have different roles.

The Arabidopsis Pi-deficient mutant *pho1* was the first Pi-deficient mutant identified [35]. In addition to an SPX domain at its N-terminal region, the PHO1 protein contains a C-terminal EXS domain (named after the yeast ERD1, the human XPR1, and the yeast SYG1) [36,37]. PHO1 is involved in the xylem loading of Pi in roots [36]. Interestingly, underexpression of *AtPHO1* resulted in a low Pi concentration in shoots to a low level similar to that of the *pho1* mutant [36], but it had no growth defect [38••]. Expression of the PHO1 rice ortholog, *OsPHO1;2*, in the *pho1* null mutant also results in plants that maintain normal growth and suppression of the Pi-deficiency response, despite the low shoot Pi [38••]. The growth hallmarks of the *pho1* mutant are not a direct consequence of Pi deficiency but are likely to be a result of extensive gene expression reprogramming triggered by Pi deficiency. Thus, the strategy of developing crop cultivars with enhanced efficiency of Pi use may need to consider increasing the Pi uptake ability of plants in lower Pi supply conditions while preventing overreaction to Pi starvation at the gene expression level.

Three members of rice SPX proteins possess an MFS (Major Facilitator Superfamily) domain in their C-terminal region [22•,23]. The mRNA levels of *OsSPX-MFS1* and *OsSPX-MFS3* were suppressed by Pi starvation, whereas that of *OsSPX-MFS2* was induced by Pi deficiency. *OsSPX-MFS1* and *OsSPX-MFS2* are direct targets of a Pi-starvation-induced osa-miR827 [22•,23]. Overexpression of osa-miR827 or mutant of OsSPX-MFS1 increases the Pi concentration in the leaves by reducing Pi remobilization from old to young leaves [22•]. Expression of the *OsSPX-MFS1* gene in the yeast strain PAM2, which lacks the two high-affinity Pi uptake transporters, restored its growth, suggesting that OsSPX-MFS1 is likely function as a Pi transporter.

SPX-domain-containing proteins has a Really Interesting New Gene (RING) domain in the C terminal region was named as SPX-RING protein [39,40]. The Arabidopsis SPX-RING protein NLA (Nitrogen Limitation Adaptation) was shown to be involved in phosphate homeostasis [41•]. Whether rice SPX-RING genes play similar roles in Pi homeostasis should be investigated.

Rice SPX-domain-containing proteins are involved in Pi xylem loading (OsPHO1;2), leaf Pi reallocation (OsSPX1, OsSPX-MFS1), the negative regulation of the expression of downstream PSI genes (*OsSPX1*) and possibly the movement of Pi to and from the vacuole (OsSPX-MFSs). Although the mechanism through which SPX-domain proteins regulate Pi signaling and responses is not yet fully understood, the available evidence from yeast and Arabidopsis suggests that SPX proteins may function by interacting with other proteins via the SPX domain [34••,39,42]. Recently, it was reported that the PHO2 protein, an important mediator of Pi starvation signaling, can specifically interact with the SPX domain of PHO1 and mediate the degradation of PHO1 protein [34••]. Further identification of proteins that physically interact with SPX-domain containing proteins will uncover the molecular networks that are affected by SPX-domain proteins.

Function and regulation of rice Pi transporters

On the basis of amino acid sequence similarity with the yeast PT, complementation test of yeast mutants lacking endogenous high affinity PTs, *Xenopus oocyte* or plant suspension cell expression system, 9 and 13 PTs in the Pht1 family were identified in the Arabidopsis and rice genomes and named as AtPT1-9 and OsPT1-13, respectively [12,13]. Most of them contain P1BS/P1BS-like cis-elements in their promoter regions (Table 1) and were expressed either exclusively or predominately in the roots. The transcript levels of these *PT* genes were strongly induced by a low Pi supply or by inoculation with arbuscular mycorrhizal fungi (AMF) [12,13,31,33], consistent with their role in Pi uptake from soil by roots or AMF. In Arabidopsis, 3 of the 9 PTs have been functionally characterized using T-DNA insertion mutants. AtPT1 and AtPT4 were shown to be responsible for Pi acquisition [43,44]. A recent report showed that AtPT5 may mobilize Pi between the source and sink tissues [45•].

In the rice genome, OsPT1/2/6/8 have been functionally analyzed in detail [46–48]. OsPT2 and OsPT6 were predominately expressed in the roots in response to Pi deprivation, but expression in the leaves also increased upon Pi starvation, especially for OsPT6 [48]. OsPT2 is a low Pi affinity H^+/Pi co-transporter, mediating Pi uptake

in the millimolar (mM) range [48]. OsPT2 was responsible for most of the excessive accumulation of Pi in the shoots of OsPHR2 overexpression lines under Pi sufficient conditions [8••]. Knockdown of *OsPT6* decreased both direct Pi uptake from the culture medium and Pi translocation from roots to shoots [48]. Its diverse expression patterns in roots and shoots and the estimated kinetics, with an apparent mean Km for Pi of 97 μM, suggest that OsPT6 in rice might have similar functions to Arabidopsis AtPT1 and AtPT4, that plays a broad role in Pi uptake, translocation and internal transport throughout the plant to enable adaptation to the changing P status of the soil [43,44]. The root expression of *OsPT8* was upregulated by Pi deprivation, while its shoot expression was not affected by Pi supply status [47]. The apparent mean Km value for Pi transport of OsPT8 was 23 μM in the yeast and 27 μM in the oocyte system. Knock down of *OsPT8* caused large decreases in root and shoot biomass and total Pi uptake at 15 μM Pi [47]. These data support that OsPT8 acts *in planta* as a high affinity PT in rice. In contrast, overexpression of *OsPT8* resulted in excessive Pi in both roots and shoots. Moreover, OsPT8 functions in Pi translocation from vegetative organs to reproductive organs in rice [47]. OsPT1 is expressed abundantly in roots and leaves, irrespective of the Pi supply, suggesting that OsPT1 is a constitutive PT in rice [46]. OsPT1 has a much lower Pi affinity (Km of 177 μM) than its homolog OsPT8 [46]. Overexpression of *OsPT1* could enhance Pi transportation from roots to shoots and P accumulation in young leaves even at a relatively late developmental stage [46].

Maintaining sufficient Pi in the aerial part of the plant depends on not only root Pi acquisition from the external environments but also the transfer of Pi from the roots to the shoots via the xylem and redistribution inside the plant via the phloem. The PHO1 protein, primarily expressed in the root vascular cylinder, is known to mediate Pi efflux to load Pi into the root xylem in Arabidopsis [36,49]. In rice, it has been shown that OsPHO1;2 plays an important role in transferring Pi from roots to shoots [50]. However, it is not clear whether and which Pht1 members are directly responsible for Pi loading, unloading or retrieval in the vascular tissue.

Although there are partial overlaps in the spatial expression patterns of some OsPTs, they have differential involvement in the OsPHR2-regulated or OsPHO2-regulated Pi pathways. Overexpression of *OsPHR2* upregulated the expression of Pi-starvation enhanced OsPTs including *OsPT2*, *OsPT6* and *OsPT8*, but not *OsPT1* [46,47]. In contrast, *OsPT1*, but not *OsPT2* and *OsPT8*, was strongly upregulated in *ospho2* mutant leaves. Their interaction with OsPHR2 or OsPHO2 signaling pathways needs to be further characterized.

Posttranslational regulation of Pi transporters and its application in the improvement of Pi uptake ability in rice

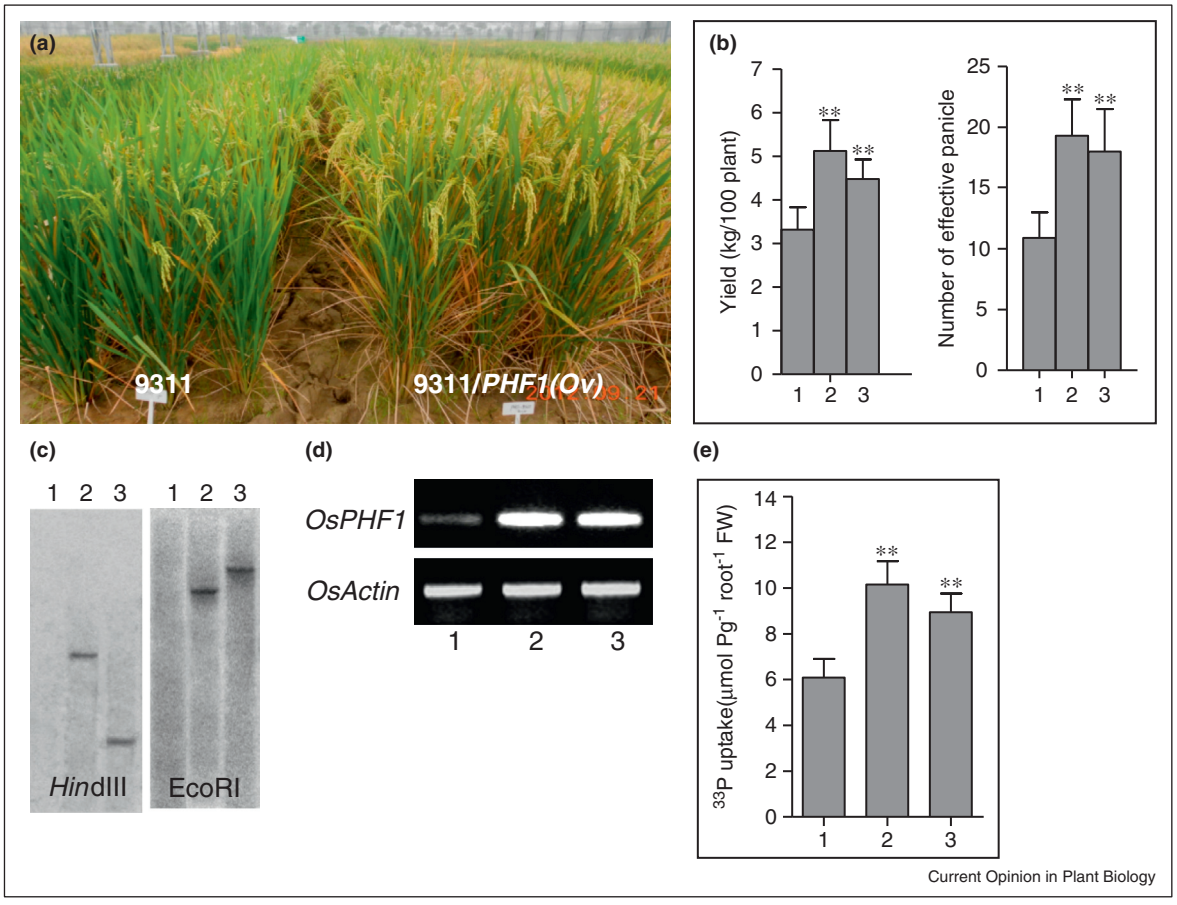
The activity of Pi-transporters can be regulated posttranslationally. The trafficking of the Arabidopsis high-affinity PT, PHT1 from the endoplasmic reticulum (ER) to plasma membrane requires the PT Traffic Facilitator (PHF1) [51]. Recently, it was shown that PHF1 can facilitate the exit of PHT1 and other PHT1 family members from the ER [52•]. It has been hypothesized that most posttranslational regulatory events identified in Arabidopsis are conserved among plant species [52•]. Isolating and performing a functional analysis of OsPHF1, a homolog of PHF1, confirmed this hypothesis [32••].

Because the PHF1-mediated posttranslational regulation of Pi-transporters does not change the cell-specific or tissue-specific expression patterns of Pi-transporter genes, increasing expression of *PHF1* may enhance function of PHT1 to improve Pi uptake ability. In Arabidopsis, overexpression of *AtPHF1* cannot significantly increase the Pi-uptake ability, unless under N-deficient conditions [52•]. In contrast, overexpression of *OsPHF1* in rice increases the Pi-uptake ability and results in enhanced tolerance to low Pi stress [32••]. The different function of PHF1 in the regulation of Pi uptake may be attributed to the different growth ecosystems of Arabidopsis and rice (upland versus flooding) or the different physiological systems of monocot and dicot plants. Overexpression of *OsPHF1* in 9311, an *indica* restorer line of Super Hybrid Rice, led to enhanced tolerance to low Pi stress in transgenic rice in a large-scale field test. Under low Pi soil environment, grain yield per plant of the two transgenic lines 9311/*PHF1*(Ov1) and 9311/*PHF1*(Ov2) were significantly higher than that of the wild type counterpart (Figure 2). The field experiment data demonstrated that posttranscriptional regulation of Pi transporters can be used as a novel strategy to improve Pi uptake ability.

Increase phosphate use efficiency in rice

Generation of plants with enhanced plant Pi use efficiency (PUE) can be achieved by a coordinated increase of Pi acquisition, translocation and internal utilization through either traditional breeding or genetic engineering. Recent studies showed that nutrient acquisition and partitioning depend on the H^+ gradients, which is regulated by the plasma membrane H^+-ATPases [53–55]. Transgenic rice plants that overexpressing the gain-of-function mutant version of the Arabidopsis H^+-PPase (*AVP1D*) maintained shoot growth under Pi-deficient conditions while the WT controls grew poorly. Overexpression of *AVP1D* enhanced P extraction capacity in transgenic rice and other crops under both P-deficient and P-sufficient conditions [53–54]. Rice H^+-PPases was shown to concomitantly be upregulated by OsPTF1, a

Figure 2



Growth performance and yield of *OsPHF1*-overexpressed plants under low phosphate soil. The *indica* line 9311 was used for transformation. **(a)** Field performance. **(b)** Grain yield and numbers of effective panicle per plant: (1) wild-type; (2) *PHF1*(Ov-1); (3) *PHF1*(Ov-2). Data are means ± SD (n = 100). **Significant difference ($P < 0.01$) from the wild type (t-test). **(c)** Southern blot analysis of the two independent transgenic lines overexpressing *OsPHF1* (1, wildtype; 2, *PHF1*(Ov-1); 3, *PHF1*(Ov-2)). **(d)** RT-PCR analysis of the expression levels of two transgenic lines overexpressing *OsPHF1*, using the same primers as in a previous report [32••]. **(e)** Pi-uptake ability analysis of the two transgenic lines using ^{33}P-labled Pi. Two weeks old plants were supplied with 100 mM ^{33}P-labeled Pi for 24 hours. Data are means ± SD (n = 3). Low P soil: Olsen P 3.5 ppm and pH: 7.2. Nitrogen and potassium were applied before planting at usual levels (450 kg urea/ha; 300 kg KCl/ha). No phosphate fertilizer was applied since 2009. The plants were transplanted with 25 cm × 25 cm arranged in randomized plots. The experiment was conducted in the Agricultural Experiment Station of Zhejiang University, Changxing, Zhejiang in 2012.

bHLH transcription factor enhancing rice tolerance to Pi starvation [55]. Whether H^+-PPases is under control OsPTF1 or other transcription factors related with PUE is to be elucidated.

Prospects

While genetic manipulation of key genes in the complicated regulation system involved in a plant's responses to Pi starvation offers solutions to improve rice Pi efficiency, identification and tracking of genetic variation is another effective approach for novel gene and allele discovery. Recently, *PSTOL1* (phosphorus starvation tolerance 1) gene, encoding for a Pup1-specific protein kinase, was found to be responsible for the natural variation in phosphorus starvation tolerance between the intolerant modern varieties and tolerant genotype Kasalath [56]. Overexpression of *PSTOL1* in rice varieties that naturally do not have the gene significantly enhanced grain yield in phosphorus deficient soil by promoting early root growth and Pi acquisition [56]. A single amino substitution of Ser-514 with Ala in Arabidopsis PHT1 can change the status of phosphorylation site regulated by PHF1, and significantly increased the of Pi-transporter activity [52•]. The advances in new platform technologies of genome sequences for rice genotypes together with field screening focusing on the key genes for Pi uptake and utilization may promote the generation of crops with enhanced Pi use efficiency.

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