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Genetic manipulation of a high-affinity PHR1 target *cis*-element to improve phosphorous uptake in *Oryza sativa* L.

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Abstract Phosphorus (P) is an essential macronutrient for crop development and production. Phosphate starvation response 1 (PHR1) acts as the central regulator for Pi-signaling and Pi-homeostasis in plants by binding to the *cis*-element PHR1 binding sequence (P1BS; GNATATNC). However, how phosphate starvation-induced gene expression is regulated remains obscure. In this work, we investigated the DNA binding affinity of the PHR1 ortholog OsPHR2 to its downstream target genes in *Oryza sativa* (rice). We confirmed that a combination of P1BS and P1BS-like motifs are essential for stable binding by OsPHR2. Furthermore, we report that variations in P1BS motif bases affected the binding affinity of OsPHR2 and that the highest affinity motif was GaATATtC (designated the A–T-type P1BS). We also found that a combination of two A–T-type P1BS elements in tandem, namely HA-P1BS, was very efficient for binding of OsPHR2. Using the *cis*-regulator HA-P1BS, we modified the promoters of *Transporter Traffic Facilitator 1* (*PHF1*), a key factor controlling endoplasmic reticulum-exit of phosphate transporters to the plasma membrane, for efficient uptake of phosphorous in an energetically neutral way. Transgenic plants with the modified promoters showed significantly enhanced tolerance to low phosphate stress in both solution and soil conditions, which provides a new strategy for crop improvement to enhance tolerance of nutrient deficiency.

Wenyuan Ruan and Meina Guo have contributed equally to this work.

Accession Numbers Rice Genome Initiative locus identifiers for the genes mentioned in this article are Os07g25710 (*OsPHR2*) and Os07g09000 (*OsPHF1*). The GenBank Accession Numbers: AY568759 (*OsIPS1*), AK240849 (*OsIPS2*).

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Introduction

Phosphorous (P) is an essential plant macronutrient for life cell growth and development. However, the assimilable inorganic phosphate (Pi) form of P for plants is often limited in the soil due to its low mobility in the soil solution and its rapid conversion to organic forms (Marschner 1995). Hence, Pi is often a limiting factor for plant growth in both natural and agricultural ecosystems (Oropeza-Aburto et al. 2012). Although fertilizer application has increased crop productivity by 30–50 %, its manufacture is an energy-intensive process. Moreover, over-application of fertilizers leads to runoff and pollution of waterways, causing a host of environmental problems. One approach to reducing the application of fertilizers in agriculture is to optimize their efficient use by crop plants (Holford 1997; Raghothama 1999; Yang and Finnegan 2010; Secco et al. 2012).

Phosphate starvation response 1 (PHR1), a key transcriptional regulator of Pi starvation responses (Rubio et al. 2001; Bari et al. 2006; Bustos et al. 2010), directly regulates the expression of *IPS1*, a non-coding RNA that mimics the target of *miR399* to block the cleavage of *PHO2* under Pi-deficient conditions (Franco-Zorrilla et al. 2007). PHO2 functions as an ubiquitin-conjugating E2 enzyme (UBC24) and modulates Pi acquisition by regulating the abundance of phosphate transporter 1s (PHT1s) in the secretory pathway destined for plasma membranes (Huang et al. 2013). The RING-type ubiquitin E3 ligase nitrogen limitation adaptation (NLA), as the target of *miR827*, recruits PHO2 for the degradation of Pi transporters in the cellular Pi homeostasis process (Kant et al. 2011; Lin et al. 2013; Park et al. 2014). In the Pi-starvation signaling pathway, PHR1 is sumoylated by an SIZ1-dependent mechanism (Miura et al. 2005). SIZ1 is a plant small ubiquitin-like modifier (SUMO) E3 ligase that is a focal controller of Pi-starvation-dependent responses. In addition, the function of PHR proteins is suppressed by SPX (SYG/PHO81/XPR1)-domain proteins, which are involved in plant Pi homeostasis (Wang et al. 2009a, b, 2014; Liu et al. 2010; Shi et al. 2014; Lv et al. 2014). In Arabidopsis, AtPHR1 also binds to the promoters of *SULTR1;3* and *SULTR3;4*, regulating shoot-to-root flux of sulfate upon phosphate starvation (Rouached et al. 2011).

Pi uptake across the plant is mediated by Pi transporters belonging to the phosphate transporter 1 (PHT1) class (Poirier and Bucher 2002) of plant Pi/H^+ symporters of the major facilitator superfamily (Pao et al. 1998). In Arabidopsis, there are nine PHT1 family members and among them, PHT1;1 and PHT1;4 are reported to be the major players in Pi acquisition under Pi-sufficient conditions (Shin et al. 2004). All nine Arabidopsis *PHT1* genes have the P1BS *cis*-element motif in their promoters, and the mRNA expression of *PHT1;1* is dramatically decreased in the double mutant of *phr1/phl1* (Bustos et al. 2010), consistent with PHR regulation of *PHT1* of expression. In rice, aside from *OsPHT1;1* and *OsPHT1;4*, the other 11 PHT1 family members contain the P1BS motif in the promoter, and *OsPHT1;2* (*OsPT2*) has been confirmed as a direct target of OsPHR2 (Liu et al. 2010). *OsPHT1;11* and *OsPHT1;13* have the P1BS motif but are arbuscular mycorrhiza-specific Pi transporters and are not involved the Pi starvation response (Paszkowski et al. 2002; Gutjahr et al. 2008; Kobae and Hata 2010; Yang et al. 2012). This finding leads to the speculation that the P1BS motif alone might not sufficient for the DNA binding of PHR proteins. In the posttranslational regulation of PHT1 proteins, phosphate transporter traffic facilitator 1 (PHF1), encoded by a downstream gene of PHR1, assists the trafficking of PHT1s to the plasma membrane (PM) (González et al. 2005; Bayle et al. 2011). Both Arabidopsis and rice *phf1* mutants exhibit decreased Pi content and retarded growth (González et al. 2005; Chen et al. 2011).

DNA *cis*-elements are important regulators of gene expression (Segal and Widom 2009) and considerable effort is currently being invested to decipher the rules governing gene expression. The fine structure of the *PHO5* promoter from *Saccharomyces cerevisiae* (yeast) was mapped, revealing that Pho4 binding affinity was ruled by E-box flanking sequences (Rajkumar et al. 2013). In Arabidopsis, PHR1 was found to bind an imperfect palindromic sequence (GNATATNC) that is found in the promoter regions of many phosphate starvation induced (PSI) genes (Franco-Zorrilla et al. 2004; Rubio et al. 2001). Bustos et al. (2010) reported that a B-motif (GAWGATNC) was essential for AtPHR1 to bind an adjacent P1BS site in the *AtIPS1* promoter. In addition, our previous research showed that most PSI genes have a P1BS-like motif, which is similar in structure to P1BS, in the region adjacent to their P1BS motif (Wu et al. 2013).

In this study, we found that OsPHR2 DNA binding affinity is regulated by the P1BS flanking sequences and P1BS variable bases, and that the most efficient P1BS constitution for OsPHR2 binding is 'GaATATtC', herein named the A–T-type P1BS. Further, we used the highest affinity P1BS combination (HA-P1BS) for modification of the *OsPHF1* promoter and generated phosphate-efficient transgenic plants (*OsPHF1-HA-P1BS*) with obviously increased yield under low-phosphate conditions. Our research thus provides a new approach to modify crops without large-scale changes to the original expression pattern of modified genes, which can avoid energy waste and other possible

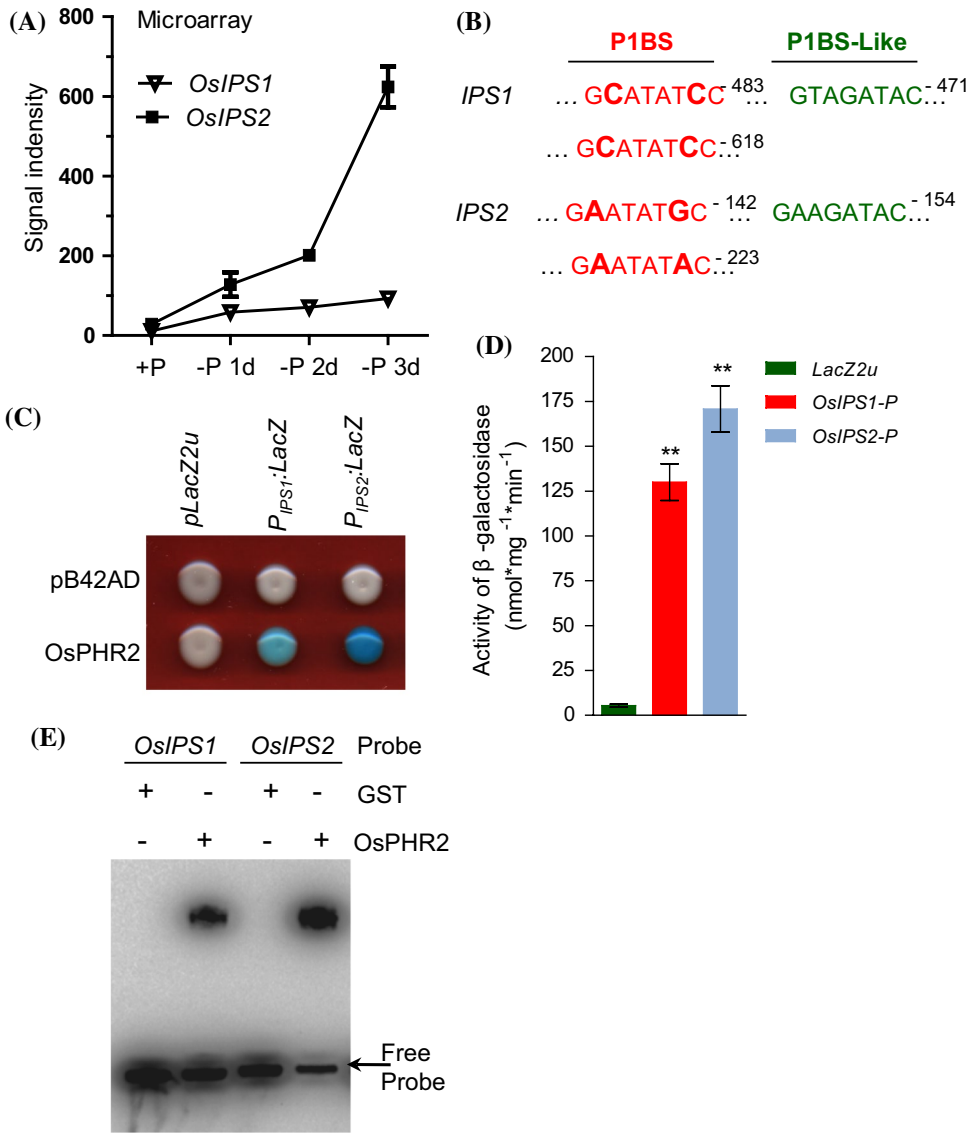

Fig. 1 DNA binding affinity of OsPHR2. **a** The mRNA expression level of *OsIPS1* and *OsIPS2* based on RNA sequencing CHIP data (NCBI Series GEO accession number: GSE35984). **b** The P1BS (*red*) and adjacent P1BS-like (*green*; Wu et al. 2013) motifs in the promoters of *OsIPS1* and *OsIPS2*. The *numbers* indicate the position in the promoter relative to the start codon. **c** Yeast one hybridization assay for OsPHR2 binding to the P1BS motif in the promoters of PSI genes (*OsIPS1* and *OsIPS2*). All four PSI promoters were fused with reporter genes of *LacZ2u* (P_{OsIPS1}-*LacZ2u* and P_{OsIPS2}-*LacZ2u*) for analysis. The empty vectors *pLacZ2u* and *pB42AD* were used as negative control. **d** *β*-Galactosidase activity for OsPHR2 with the different promoters, as described in C. *Values* represent mean ± SD of four replicates. Data significantly different from negative controls are indicated (**$P < 0.01$; Student's *t* test). **e** Electrophoretic mobility shift assay (EMSA) for OsPHR2 with the promoter probes of *OsIPS1* and *OsIPS2*. Recombinant OsPHR2 proteins fused with GST were prepared using the *BL21*(*DE3*) expression system. GST protein was used as negative control and biotin-labeled DNA sequences were used as probes (See Table S2)

deleterious effects compared with ectopic expression driven by the 35S promoter.

Results

Different DNA binding affinities of OsPHR2 for its various downstream target promoters

PHR1 subfamily proteins initiate the transcription of downstream genes by recognizing imperfect palindrome sequences of GnATATnC (Rubio et al. 2001; Bustos et al. 2010). However, different OsPHR2 target genes show different degrees of responsiveness to Pi deficiency; according to the Gene Expression Omnibus (GEO), *OsIPS2* is much more sensitive than *OsIPS1* to phosphate starvation signaling on the early stage, indicating that *OsIPS2* may be an early phosphate starvation response gene (Fig. 1a). To learn whether a difference in OsPHR2 DNA binding affinity leads to such diversity in responsiveness, the promoters of *OsIPS1* and *OsIPS2*, which contain different P1BS-type motifs, were characterized in detail (Fig. 1b). Using yeast one-hybrid (Y1H) analysis, we confirmed that OsPHR2 can physically bind to the tested promoters, as previous reported (Fig. 1c; Rubio et al. 2001; Bustos et al. 2010; Lv et al. 2014). This analysis also showed that the DNA binding affinity of OsPHR2 was significantly greater for the *OsIPS2* promoter than for that of *OsIPS1* (Fig. 1d). In agreement with the Y1H results, electrophoretic mobility shift assays (EMSAs) revealed a greater affinity between OsPHR2 and *OsIPS2* (Fig. 1e). These results are consistent with the idea that greater Pi-starvation responsiveness (e.g., of *OsIPS2*) is related to higher affinity DNA binding of OsPHR2.

P1BS and P1BS-like motifs together determine the binding ability of OsPHR2

We used the *OsIPS2* promoter to further explore the DNA binding ability of OsPHR2. As shown in Fig. 2a, the *OsIPS2* promoter contains two P1BS motifs namely, P1BS1 ('GAATATGC' at −223 nt from transcription start site) and P1BS2 ('GAATATAC' at −153 nt), as well as one P1BS-like motif ('GAAGATAC' at −142 nt). To characterize the contribution of each motif to OsPHR2 binding, we tested different truncated promoter fragments as well as constructs of the entire 1.6-kb promoter lacking only P1BS1 (Pm2), P1BS2 (Pm1) or the P1BS-like motif (Pm3) in Y1H (Fig. 2a). The truncated promoter containing P1BS1 and the P1BS-like motif from −100 to −223 nt (P1), showed no change in OsPHR2 binding ability compared to the full length *OsIPS2* promoter (P0). However, the truncated version from −100 to −149 nt (P2), could not bind to OsPHR2. The 1.6-kb promoter with only the P1BS2 deleted (Pm1) maintained its ability to bind OsPHR2, while the mutated versions lacking P1BS1 (Pm2) or the P1BS-like motif (Pm3) lost their binding ability (Fig. 2a). To quantify the different binding affinities, we monitored the expression of the reporter gene *LacZ2u* in Y1H assays (Fig. 2b). The results consistently showed that removing P1BS2 had little effect on OsPHR2 protein binding ability; however the binding ability almost completely disappeared in the absence of P1BS1 or the P1BS-like motif. These results indicate that the P1BS1 and P1BS-like motifs, but not P1BS2, in the *OsIPS2* promoter are essential for the binding of OsPHR2. We analyzed the promoters of several other PSI genes (*OsIPS1*, *OsIPS2*, *OsmiR399d*, *miR827*, *OsPT2*, *OsPHF1*, *OsSPX1*, *AtIPS1*, *AT4*, *AtmiR399*, *AtmiR827*, *AtPHT1;1*, *AtPHT1;4 AtPHF1 and AtSPX1*) and found a consensus P1BS::P1BS-like structure (Fig. 2c). That the presence of this kind of tandem structure might be of general importance in phosphate-starvation responsiveness.

Fine-tuning of variable P1BS bases to enhance DNA binding affinity of OsPHR2

The promoters of both *OsIPS1* and *OsIPS2* contain the P1BS::P1BS-like combination structure (Fig. 1b); this indicates that the presence or absence of such a structure is not solely responsible for their different affinities for OsPHR2. We tested whether the variable bases of the P1BS motif affected binding, since the two promoters contain different types of P1BS (Fig. 1b). To investigate whether the variable bases of P1BS regulate OsPHR2 affinity, we used the Pm1 version of the promoter, in which the P1BS2 of the *OsIPS2* promoter was deleted. When the first variant base 'A' of P1BS1 was changed to 'T', 'C' or 'G', the OsPHR2

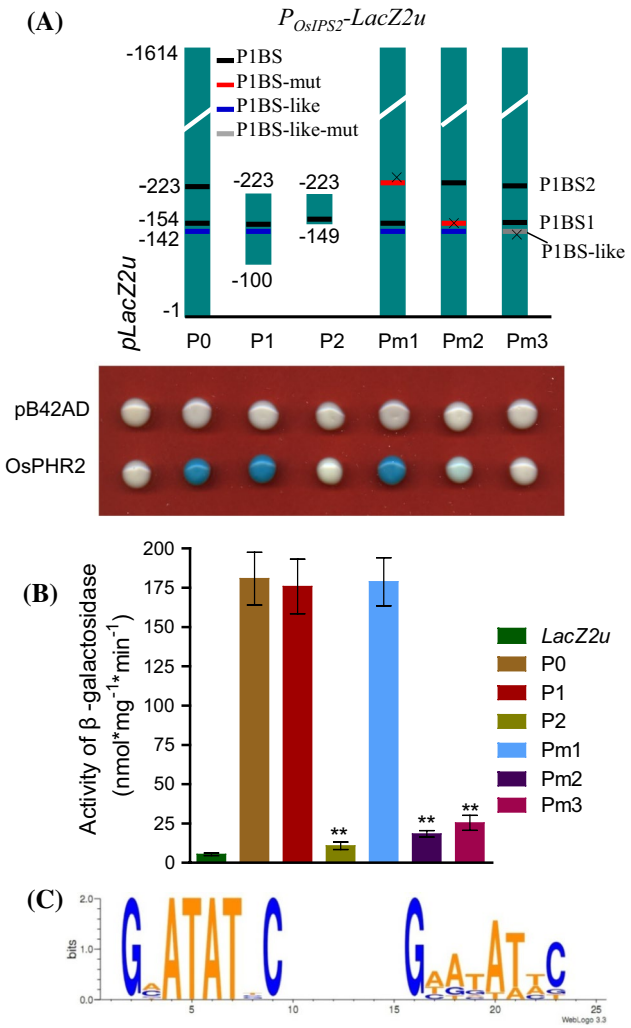

Fig. 2 The combination of P1BS and P1BS-like motifs is essential for the binding of OsPHR2. **a** Yeast one-hybrid assay for OsPHR2 with different truncated and mutated versions of the *OsIPS2* promoter. The *black box* indicates the P1BS motif in the *OsIPS2* promoter, the *red box* indicates the deletion of P1BS, the *blue box* indicates the P1BS-like motif, and the *grey box* indicates the deletion of P1BS-like motif. **b** *β*-Galactosidase activity for OsPHR2 with the constructs described in **a**. *Values* represent mean ± SD of four replicates. Data significantly different from corresponding controls are indicated (P2, Pm2 and Pm3 versus P0, $^{**}P < 0.01$; Student's *t* test). **c** Logo analysis of P1BS::P1BS-like structure in Pi-starvation response genes. The P1BS::P1BS-like motif in *OsIPS1*, *OsIPS2*, *OsmiR399d*, *miR827*, *OsPT2*, *OsPHF1*, *OsSPX1*, *AtIPS1*, *AT4*, *AtmiR399*, *AtmiR827*, *AtPHT1;1*, *AtPHT1;4*, *OsPHF1* and *AtSPX1* were used for Logo analysis (http://weblogo.threeplusone.com)

binding affinity was dramatically decreased, implying that OsPHR2 preferentially binds the 'GAATATnC' P1BS (herein designated the A-type P1BS; Fig. 3a, b). When the second variant base, 'G', was changed to 'C' or 'A', the affinity was not significantly changed compared to the wild-type P1BS. Interestingly, when the first variant base was fixed as 'A' and the second variant base changed to 'T'

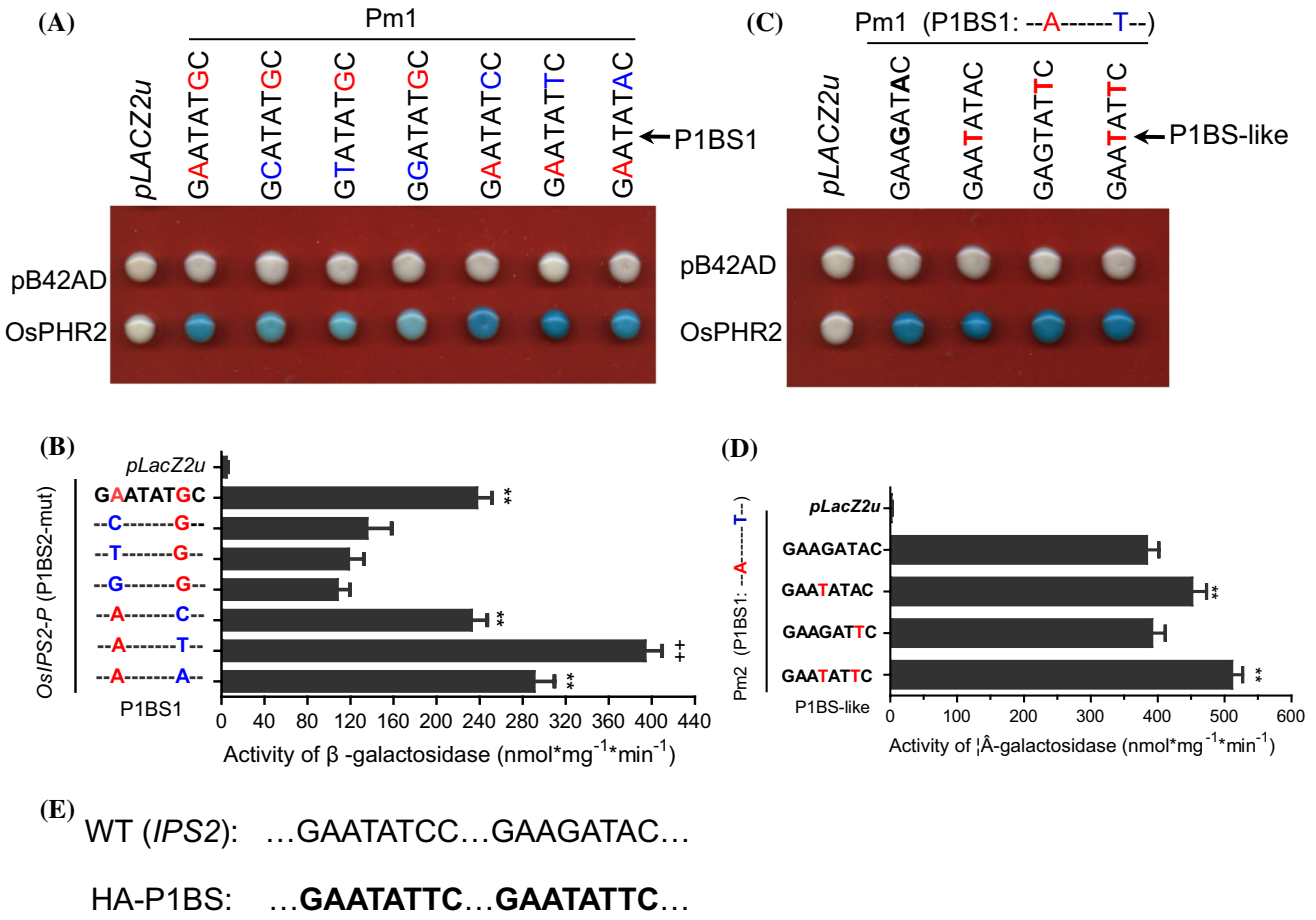

Fig. 3 Fine-tuning the P1BS to increase OsPHR2 affinity. **a** OsPHR2 DNA binding assay in yeast with different versions of P1BS in which the variable bases have been substituted. Pm2 (*OsIPS2-P*-P1BS2-mut: P1BS2 was deleted in *OsIPS2* promoter) was the background for the variable base substitution in this experiment. **c** OsPHR2 DNA binding affinity with different versions of the P1BS-like motif. The P1BS-like motif bases were modified to approach the A–T-type P1BS in the background of the Pm2 promoter in which the P1BS1 was further substituted to the A–T-type-P1BS (GaATATtC). **b**, **d** β-Galactosidase activity for OsPHR2 with the different promoter constructs described in **a** and **c**, respectively. *Values* represent mean ± SD of four replicates. Data significantly different from corresponding controls are indicated (A-type-P1BS versus non-A-type-P1BS, $^{**}P < 0.01$; A-type-P1BS versus other P1BS, $^{++}P < 0.01$; student's *t* test). **e** The sequences of the wild-type *OsIPS2* promoter compared with that of HA-P1BS

(we designated this P1BS as A–T-type), OsPHR2 binding affinity was markedly increased (Fig. 3a, b).

To determine whether modifying the P1BS-like motif, by making it more like the A–T-type-P1BS, could further enhance the affinity for OsPHR2, we tested different versions of the P1BS-like motif in the background of the A–T-type Pm1 (with the P1BS1 substituted by the A–T-type-P1BS; Fig. 3c). When the P1BS-like motif of *OsIPS2* was changed from 'GAAgATaC' to 'GAAgATtC', the affinity was not obviously different, while when the P1BS-like motif was changed to 'GAAtATaC' or 'GAAtATtC', the affinity increased. The substitution 'GAAtATtC' (resembling the A–T-type P1BS) produced the highest affinity for OsPHR2 (Fig. 3b, d). These results indicate that the A–T-type-P1BS::A–T-type-P1BS structure, which we term high affinity P1BS (HA-P1BS), is very efficient for OsPHR2 binding (Fig. 3e).

HA-P1BS can enhance the activity of the OsPHF1 promoter

To further test whether *HA-P1BS* can enhance the DNA binding ability of OsPHR2, we used the *OsPHF1* promoter, because native *OsPHF1* is weakly responsive to Pi starvation (Chen et al. 2011) and its promoter contains the P1BS motif. We generated transgenic plants harboring a *GUS* reporter gene driven by WT or HA-P1BS-modified promoters (Fig. 4a). Compared with the wild-type *OsPHF1* promoter construct (P_{OsFHF1}*:GUS*), the expression driven by the HA-P1BS modified promoter (P_{OsFHF1}*HA:GUS*) was enhanced, based on GUS staining and activity measurement (Fig. 4b, c). It is worth noting that HA-P1BS could efficiently increase the activity of the *OsPHF1* promoter under both Pi-deficient and Pi-sufficient conditions. To

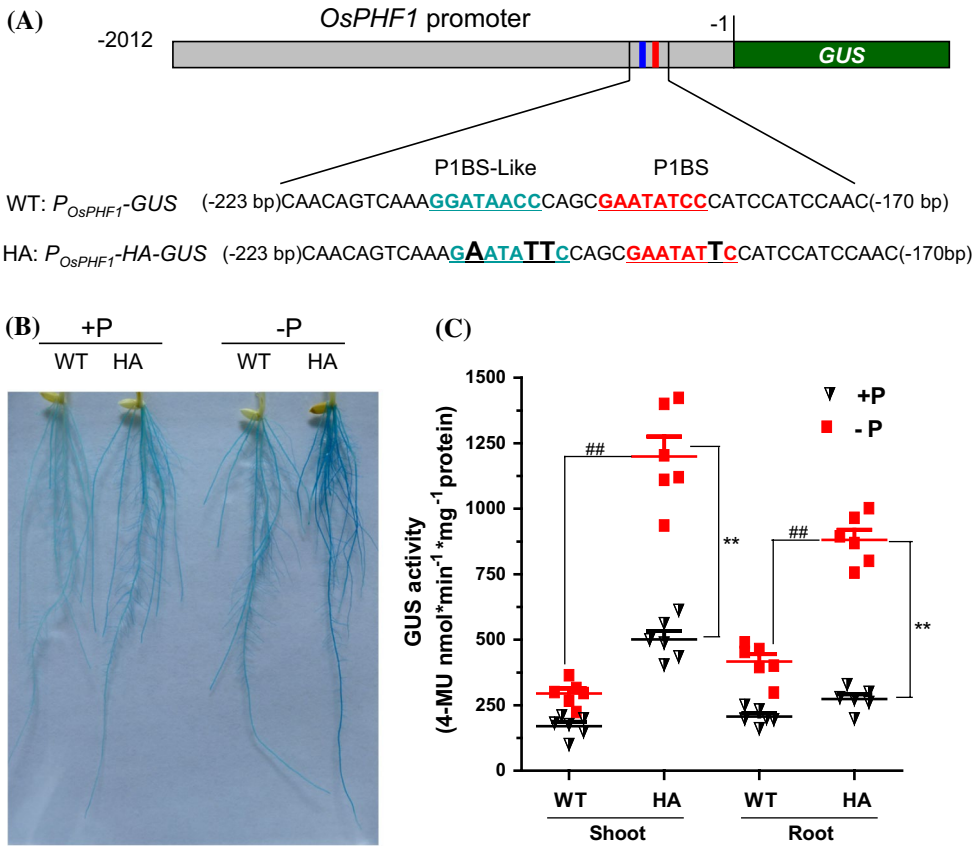

Fig. 4 The HA-P1BS modified *OsPHF1* promoter shows enhanced activity in rice. **a** Diagram of the P_{OsPHF1}-*GUS* reporter gene and modified version. The P1BS and P1BS-like motif in each promoter is highlighted with a *vertical bar* (P1BS, red; P1BS-like, *blue*). In the P_{OsPHF1}-*HA-GUS* promoter, the P1BS and P1BS-like motif were modified to the A–T-type P1BS motif. **b**, **c** GUS activity assays of P_{OsPHF1}-*GUS* and P_{OsPHF1}-*HA-GUS*. All plants were grown for 7 days in +P (200 μM Pi) or −P (0 μM Pi) solution before GUS activity analysis. *Values* represent mean ± SD of three replicates from six different transgenic lines. Data significantly different from corresponding controls are indicated (HA/−P versus WT/−P, $^{\#\#}P < 0.01$; HA/−P versus HA/+P, $^{**}P < 0.01$; Student's *t* test)

examine whether modification of the P1BS and P1BS-like motif of the *OsPHF1* promoter would change the mRNA expression pattern, tissue cross-sections were analyzed. The results showed that the tissue expression pattern of the modified *OsPHF1* promoter was not changed compared with wild type in leaf and root (Fig. S1).

HA-P1BS-modified transgenic plants show tolerance to low phosphate stress

Since Pi starvation triggers the transcriptional activity of *PHR1* and closely related genes (Rubio et al. 2001; Zhou et al. 2008) and our data demonstrated a high affinity P1BS combination (HA-P1BS) for DNA binding by PHR1, we tested the utility of HA-P1BS for enhancement of Pi uptake. PHF1 is reported to be an important factor for the localization of Pi transporters to the plasma membrane in Arabidopsis and rice (González et al. 2005; Chen et al. 2011). Transgenic rice overexpressing *OsPHF1* behind the 35S promoter show excessive accumulation of shoot Pi and Pi-toxicity symptoms (Chen et al. 2011), which might be caused by the non-tissue-specific constitutive overexpression of *OsPHF1*. Here, we generated transgenic plants with the HA-P1BS combination in the promoter of *OsPHF1* (designated as *PHF1-HA-P1BS*; Fig. S2) to test for the improvement of Pi uptake efficiency without ectopic overexpression.

Physiological characterization of two independent lines of *PHF1-HA-P1BS*, for which mRNA expression and copies numbers were determined (Fig. S2), was conducted in solution culture. The *PHF1-HA-P1BS* transgenic plants showed significantly higher Pi concentration in shoots under both Pi-sufficient conditions and Pi-deficient conditions compared with wild-type plants (Fig. 5c–f). Uptake of ^{33}P-labelled Pi was monitored over a 24-h period, and the two *PHF1-HA-P1BS* lines showed much higher Pi uptake than wild type (XS134) under Pi-deficient conditions (Fig. 5b). Additionally, the shoot biomass of *PHF1-HA-P1BS2/5* was significantly higher than wild type (Fig. 5g–j).

T_2 transgenic plants were used for pot experiments with high-phosphate (38.20 mg/kg Pi, HP) or low-phosphate (8.15 mg/kg Pi, LP) fertilizer. The yield, biomass, setting rate and effective tiller numbers of 10 plants from each replicate pot were measured and the P content was determined by ICP analysis (Fig. 6). *PHF1-HA-P1BS* plants under low-Pi stress showed significant increases in yield and effective tiller number compared to wild-type plants. In addition, the total P content was increased by 11 and 20 % under control (high Pi) and low Pi stress conditions, respectively.

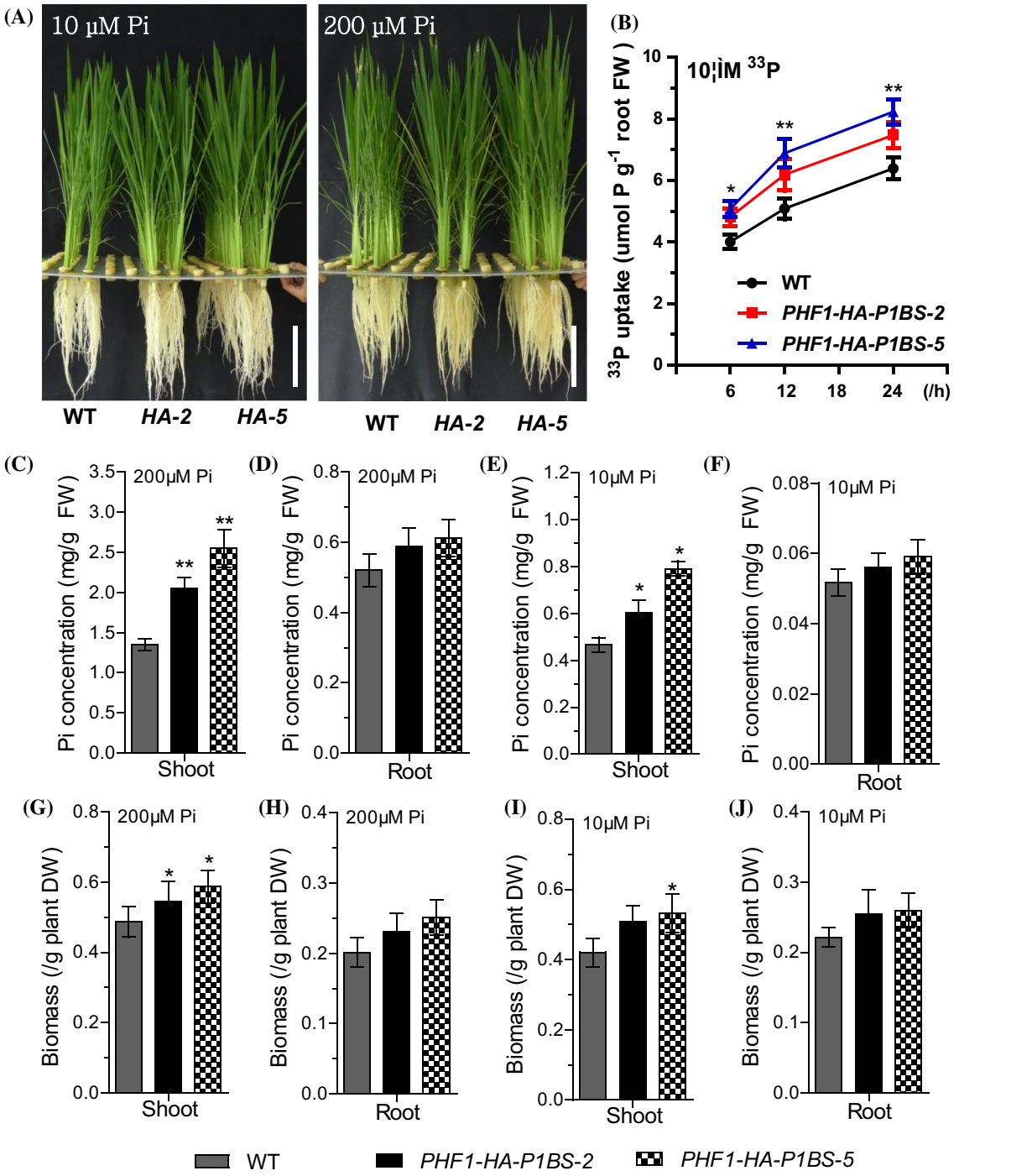

Fig. 5 *PHF1-HA-P1BS* plants take up Pi more efficiently than wild type in solution culture. **a** Phenotype of 30-days-old wild-type plants (Xiushui 134<XS134>, an *japonica* high yield variety) and *PHF1-HA-P1BS* lines (*HA-2* and *HA-5*) under Pi-sufficient (200 μM Pi) and Pi-deficient (10 μM Pi) conditions. The two independent lines of *PHF1-HA-P1BS* were identified by DNA gel blotting analysis (Fig. S2). *Bar* 20 cm. **b** Uptake rate of [^{33}P]-Pi in wild type (XS134) and *PHF1-HA-P1BS* plants. Plants were supplied with ^{33}P-labelled Pi ($H_3^{33}PO_4$) for 6, 12, and 24 h. **c–f** Cellular Pi concentration in the shoots and roots of WT (XS134), PHF1-HA-P1BS-2 and *PHF1-HA-P1BS*-5 plants grown under 200 or 10 μM Pi conditions. *Values* represent mean ± SD of five replicates. Data significantly different from the corresponding wild-type controls are indicated (*$P < 0.05$; **$P < 0.01$; Student's *t* test). **g–j** Shoot and root biomass of WT and *PHF1-HA-P1BS* lines under Pi-sufficient (200 μM Pi) and Pi-deficient (10 μM Pi) conditions. *Values* represent mean ± SD of ten replicates. Data significantly different from the corresponding wild-type controls are indicated (*$P < 0.05$; Student's *t* test)

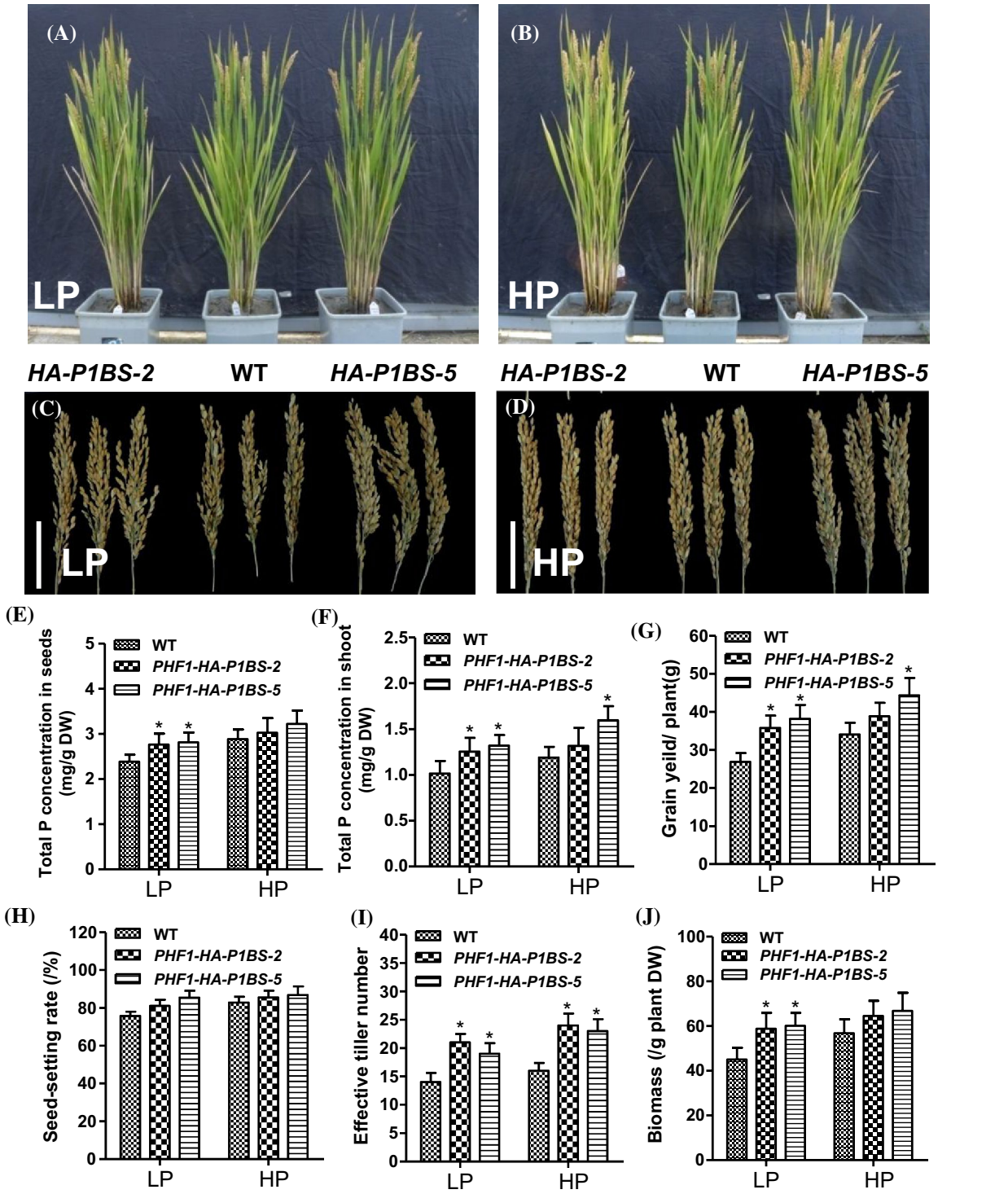

Fig. 6 HA-P1BS-modified *OsPHF1* plants show tolerance to low phosphate stress in soil conditions. **a**, **b** Growth performance and yield of two independent transgenic lines with the HA-P1BS motif combination in the promoter of *OsPHF1* (*PHF1-HA-P1BS-2/5*) under fertilizer (38.20 mg/kg Pi, HP) and low fertilizer (8.15 mg/kg Pi, LP) conditions; **c**, **d** Phenotypes of seed-setting performance in WT and the transgenic *PHF1-HA-P1BS-2/5* plants under HP and LP conditions; **e** total P concentration in shoot; **f** total P concentration in grains; **g** yield of per plant; **h** seed setting rate; **i** effective tiller number; **j** shoot biomass. Total *P values* represent mean ± SD of five replicates. Yield of per plant, seed setting rate, effective tiller number and shoot biomass *values* represent mean ± SD of ten replicates. Data significantly different from the corresponding wild-type controls are indicated (*$P < 0.05$; Student's *t* test)

Whereas there was no obvious difference in the seed setting rate, the shoot biomass of *PHF1-HA-P1BS* plants was also increased under both conditions.

Discussion

Phosphorus is indispensable in agricultural production systems, and much progress has been made to elucidate the phosphate regulation system in Arabidopsis and rice (Chiou and Lin 2011; Wu et al. 2013). In this work, we report fine-tuning of the transcription of an OsPHR2 target gene based on variant bases in the P1BS *cis*-element. We also provide a novel strategy to improve cereal crops under nutrition deficiency stress: modifying this *cis*-element to mildly increase the expression of target genes and thereby avoid non-specific expression and energy waste compared to strategies involving constitutive and high expression promoters.

The P1BS::P1BS-like structure is essential for the DNA binding of PHR protein

The P1BS motif is a Pi-starvation specific response *cis*-regulator in plants (Bustos et al. 2010). A previous study described the essential of P1BS flanking sequences, namely B-motif, for the output of *AtIPS1* promoter (Bustos et al. 2010). Members of the PHR1 subfamily need to form homodimers or heterodimers to bind this motif (Rubio et al. 2001; Bustos et al. 2010). This dimerization may be the reason that the combination of P1BS::P1BS-like structure is essential for stable binding by PHR proteins. As mentioned above, *OsPHT1;11* and *OsPHT1;13* are not involved in the Pi starvation response (Paszkowski et al. 2002; Gutjahr et al. 2008; Kobae and Hata 2010; Yang et al. 2012). Although they both contain a P1BS motif in their promoters, the P1BS::P1BS-like structure could not be found, and it is possible that this explains why *OsPHT1;11* and *OsPHT1;13* do not obviously respond to Pi starvation. Transcriptional and histochemical analyses have shown that all of the *SPX* genes, with the exception of *SPX4*, are responsive to Pi starvation (Duan et al. 2008; Wang et al. 2009b). We found that all of the *SPXs*, with the exception of *SPX4*, contain the P1BS::P1BS-like structure, again suggesting that the P1BS::P1BS-like combination is important, likely for the binding of the PHR1 subfamily. In the Arabidopsis *PHOSPHOLIPASE DZ2* (*PLDZ2*) promoter, a 65-bp Pi-responsive enhancer element (named EZ2) containing two P1BS motifs was characterized, and mutation of either of the two motifs led to the loss of function of EZ2 (Oropeza-Aburto et al. 2012). Based our current work, this loss of function might be due to disruption of a P1BS::P1BS-like structure that was necessary for PHR binding. Although we have revealed the importance of the P1BS::P1BS-like structure for binding of PHRs dimers, the optimal distance between the P1BS and P1BS-like motifs remains unclear. In endogenous PSI promoters, we find the distance between motifs to vary from 1 to ~30 nt, which implies that the distance is flexible to some extent.

The fine-tuning of P1BS variable bases for PHR binding affinity

Recent studies have highlighted the importance of *cis*-element regulatory variants for the transcription factor DNA binding affinity. A typical example is the Pho4 binding site and it flanking sequence, which affect the output of *PHO5* in yeast under different Pi supply conditions and they showed that the promoter output was precisely tunable by subtle changes in binding-site affinity of less than 3 kcal mol^{-1}, which are accessible by modifying 1–2 bases (Rajkumar et al. 2013). In addition, human limb malformations have been shown to be caused by *cis*-regulatory mutations (enhancer mutations; Vander-Meer and Ahituv 2011). In this study, we showed that the P1BS *cis*-regulator can regulate the binding affinity of the OsPHR2 protein. The mutations of first variant base of P1BS1 in the *OsIPS2* promoter can repress the affinity for OsPHR2, whereas binding affinity was strengthened when the second variant base was mutated from 'G' to 'T' (Fig. 3a). These findings allow us to explain why two copies of P1BS4 [EZP1BS4(2X)] had a stronger output ability than two copies of P1BS3 [EZP1BS3(2X)] (Oropeza-Aburto et al. 2012): P1BS4 (GAATATTC) is an A–T-type P1BS, while P1BS3 (GGATATTC) is not. These results further highlight the importance of P1BS variant *cis*-regulators in the affinity of OsPHR2 as it functions in Pi-starvation responses.

HA-P1BS is an efficient *cis*-regulator for cereal crop improvement

Single nucleotide mutations are common during evolution, the variant bases in P1BS are different even for the same *PSI* orthologous genes in different species (such as *PHF1*; Table S1). The evolution of P1BS variant bases may represent a mechanism for adaptation to Pi availability changes in the rhizosphere and at the cellular level. As shown in Fig. 4a, the P1BS::P1BS-like structure of *OsPHF1* is not the most efficient combination for OsPHR2. Therefore, we chose to modify the *OsPHF1* promoter with HA-P1BS. As we expected, the transcription rate of *OsPHF1* was increased (Fig. 5), whereas its expression pattern was not changed (Fig. S1). The mRNA expression level of *OsPHF1* in *OsPHF1-HA-P1BS* was obviously induced under Pi-deficient conditions (Fig. S2), suggesting the utility of HA-P1BS for promoter modification. Importantly, the degree of

induction was mild (about five to tenfold) compared with that of the 35S promoter (Chen et al. 2011). It was previously reported that the mRNA expression of *OsPHF1* is weakly respond to phosphate starvation (Chen et al. 2011), whereas when we modified the *OsPHF1* promoter with the HA-P1BS *cis*-element, mRNA expression was induced. It is possible that the nucleosome structure or the TFs binding threshhold of the *OsPHF1* promoter was changed and the PHR binding sites were exposed by the HA-P1BS modification (Zhou and O'Shea 2011).

Although previous work has shown that the loss of function of *PHO2*, *SPXs*, *CAX1/2* and *NLA1* or the overexpression of *OsPHR2*, *OsPHF1* and *OsmiR827*, which are key members in the plant phosphate regulation system, can lead to Pi-overaccumulation (Aung et al. 2006; Bari et al. 2006; Zhou et al. 2008; Wang et al. 2009a, b, 2012, 2014; Liu et al. 2010; Chen et al. 2011; Shi et al. 2014; Lv et al. 2014), none of those plants grew well under Pi-deficient conditions. Thus, there must have been as-yet unknown side effects in these Pi-accumulating plants. Hence, it is desirable to find an alternative strategy to promote phosphate uptake efficiency without disrupting plant development. In this regard, it is important that the *PHF1-HA-P1BS* transgenic lines did not exhibit retarded growth (Figs. 5a, 6a) and appeared to benefit from the modestly enhanced, but still tissue-specific expression of *OsPHF1*.

The ^{33}P uptake experiment demonstrated the enhanced Pi uptake ability of *OsPHF1-HA-P1BS* (Fig. 5b). Unlike the lines constitutively overexpressing *OsPHF1*, which exhibited Pi accumulation in the shoot and necrosis in the leaves under Pi-sufficient conditions (Chen et al. 2011), the *OsPHF1-HA-P1BS* transgenic lines did not show deleterious effects on growth. Therefore, HA-P1BS, as a Pi-starvation responsive *cis*-regulator, represents a new approach for development of plants tolerant of low nutrient conditions, by modifying a key *cis*-element to modestly increase the expression of genes in situ and avoid the energetic waste or possible harmful effects of ectopic overexpression.

Materials and methods

Plant materials and growth conditions

The hydroponic experiments were performed using rice solution culture (Zhou et al. 2008). Rice plants were grown in Pi-sufficient (+P, 200 mM), low Pi (20 mM) or Pi-deficient (−P, 0 mM) condition under a greenhouse with a 12/12 light/dark cycle (200 mol m^{-2} s^{-1} photon density) at 30/22 °C after germination. Humidity was controlled at approximately 60 %.

Yeast one hybrids assay

To identify and characterize the interaction between OsPHR2 and the promoter regions of *OsIPS1*, *OsIPS2*, *OsmiR827*, *OsPT2* and *OsPHF1* in yeast, the full length cDNA of *OsPHR2* was cloned into pB42AD vector (Clontech), thus generating pB42AD-OsPHR2 (AD:OsPHR2) for yeast one-hybrid. The promoter regions of *OsIPS1* (from −1 to −1,678), *OsIPS2* (from −1 to −1,614), *OsmiR827* (from −1 to −1,827), *OsPT2* (from −1 to −1,680) and *OsPHF1* (from −1 to −600) were cloned into *pLAZ2u* (Clontech). The primers and restriction endonuclease sites used to generate the constructs for these analyses are listed in Table S2. Yeast strain EGY48 was transformed with AD:OsPHR2 and the various reporter gene constructs and grown on synthetic medium lacking urea and tryptophan (Clontech).

The *β*-galactosidase activity was measured according to Calvenzani et al. (2012). Briefly, transformed yeast from an overnight culture was inoculated into fresh −Trp/−Ura culture solution, grown to OD_{600} ≈0.4, and then collected. Total proteins were extracted to measure the *β*-galactosidase activity with reaction buffer (Z-buffer: 60 mM NaH_2PO_4, 40 mM Na_2HPO_4 anhydrous, 10 mM KCl, 1 mM Mg_2SO_4, 50 mM *β*-mercaptoethanol), stopping buffer (1.5 M $CaCO_3$) and substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG: 1.4 mg/mL). Chromogenic reaction plates were used essentially following the MatchMaker One Hybrid System manual (Clontech).

Electrophoretic mobility shift assay (EMSA)

To express GST:OsPHR2 recombinant protein in *E. coli* strain *BL21(DE3)* (Novagen), the full-length CDS of OsPHR2 was amplified with primer pair OsPHR2-GST-F(BamHI)/OsPHR2-GST-R (SalI) and cloned into the pGEX-4T-1 vector (GE Healthcare), producing the GST-PHR2 vector. The fusion protein was extracted and purified according to the manufacturer's instructions. The promoter regions containing PIBS motif was amplified using the primer pairs OsIPS1-probe-F/OsIPS1-probe-R, OsIPS2-probe-F/OsIPS2-probe-R, OsmiR827-probe-F/OsmiR827-probe-R, OsPT2-probe-F/OsPT2-probe-R and were used to obtain biotin-labeled probes. The primer sequences are listed in Table S2. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's instructions.

GUS histochemical analyses

To develop transgenic plants expressing the *GUS* reporter genes P_{OsPHF1}-*GUS* and P_{OsPHF1}-*HA-GUS*, *Nipponbare* genomic DNA fragments containing less than 2,012 bp

promoter (from start codon ATG) sequence were amplified with the primer pair OsPHF1-P-GUS-F (SalI)/OsPHF1-P-GUS-R(KpnI) and cloned into the binary vector GUS-pBI101.3 (Clontech). The primer sequences are listed in Table S2. The GUS constructs were introduced into rice plants (*Nipponbare*) using *Agrobacterium*-mediated transformation. Plant tissues were collected from the transgenic plants containing P_{OsPHF1}-*GUS* and P_{OsPHF1}-*HA-GUS*. The root and leaf sections were vacuum infiltrated and stained in GUS solution according to Jefferson et al. (1987).

GUS activities of six independent transgenic lines (P_{OsPHF1}-*GUS* and P_{OsPHF1}-*HA-GUS*) were determined after 15-days-old plants were grown for 7 days in +Pi (200 μM) or −Pi solution. Plant tissues were ground in GUS extraction buffer, and 2 μg protein was used for the fluorometric assay. Protein extracts were incubated in reaction buffer (2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG), 50 mM Na_2HPO_4 and NaH_2PO_4, pH 7.0, 0.1 % Sarkosyl, 0.1 % Triton X-100, 10 mM β-mercaptoethanol, and 10 mM EDTA) for 30 min, followed by analysis with a fluorometer (Hoefer Scientific Instruments).

RNA isolation, reverse-transcription PCR, and qRT-PCR

Total RNA was isolated from rice root or shoot with the Trizol Reagent kit (Invitrogen), according to the manufacturer's protocol. Reverse-transcription PCR using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) cDNA synthesis kit (Promega) followed the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) was performed as described previously (Liu et al. 2010). The primers for qRT-PCR are listed in Table S2.

DNA gel blot analysis of transgenic plants

DNA gel blotting analysis was performed as previously described (Zhou et al. 2008).

Overlap PCR

To generate different *OsIPS2* and *OsPHF1* promoter mutation constructs, the method of Overlap-PCR (Higuchi et al. 1988) was used. All primers used to make mutations are listed in Table S2.

Measurements of Pi concentration and Pi uptake ability in plants

The concentrations of effective phosphorus and total phosphorus, as well as the plant biomass were measured following the protocols described preciously (Zhou et al. 2008; Wu et al. 2011).

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