SPX1 is a phosphate-dependent inhibitor of PHOSPHATE STARVATION RESPONSE 1 in *Arabidopsis*

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To cope with growth in low-phosphate (Pi) soils, plants have evolved adaptive responses that involve both developmental and metabolic changes. PHOSPHATE STARVATION RESPONSE 1 (PHR1) and related transcription factors play a central role in the control of Pi starvation responses (PSRs). How Pi levels control PHR1 activity, and thus PSRs, remains to be elucidated. Here, we identify a direct Pi-dependent inhibitor of PHR1 in *Arabidopsis*, SPX1, a nuclear protein that shares the SPX domain with yeast Pi sensors and with several Pi starvation signaling proteins from plants. Double mutation of *SPX1* and of a related gene, *SPX2,* resulted in molecular and physiological changes indicative of increased PHR1 activity in plants grown in Pi-sufficient conditions or after Pi refeeding of Pi-starved plants but had only a limited effect on PHR1 activity in Pi-starved plants. These data indicate that SPX1 and SPX2 have a cellular Pi-dependent inhibitory effect on PHR1. Coimmunoprecipitation assays showed that the SPX1/PHR1 interaction *in planta* is highly Pi-dependent. DNA-binding and pull-down assays with bacterially expressed, affinity-purified tagged SPX1 and ΔPHR1 proteins showed that SPX1 is a competitive inhibitor of PHR1 binding to its recognition sequence, and that its efficiency is highly dependent on the presence of Pi or phosphite, a nonmetabolizable Pi analog that can repress PSRs. The relative strength of the SPX1/PHR1 interaction is thus directly influenced by Pi, providing a link between Pi perception and signaling.

phosphate sensor | phosphate starvation signaling

Since the beginning of molecular genetics, phosphate (Pi) starvation rescue systems, especially the Pi starvation rescues systems of bacteria and yeast, have served as emblematic models for studies of regulation of gene activity. In plants, these systems have gained additional interest because of the complexity and multicellular nature of plants (1, 2), and especially due to their potential for improving Pi acquisition and use in crops, a major goal toward sustainable agriculture. Considerable information has been gathered in the past decade on the components of the Pi starvation signaling pathway (reviewed in refs. 3–6). Major findings in plants include (*i*) identification of PHOSPHATE STARVATION RESPONSE 1 (PHR1) and related transcription factors as master regulators of Pi starvation responses (PSRs) (7–11); (*ii*) demonstration of the involvement of ubiquitin system components, including PHO2 and NLA, in Pi signaling (12–16); (*iii*) identification of miRNAs as mobile signals in Pi homeostasis (17, 18); and (*iv*) identification of Pi starvation-induced (PSI) riboregulators of miRNA activity, based on target mimicry (19) and natural antisense RNA that activates translation of PHO1 mRNA (20). In addition, a singular characteristic of nutrient starvation responses in plants is that several of these responses are at long distance, systemically controlled by plant shoot nutrient status, whereas others are controlled by local nutrient concentration. Transcriptomic analyses have clarified details of systemic vs. locally controlled molecular responses to Pi starvation; specifically, systemically controlled responses include Pi recycling and recovery, whereas locally controlled responses affect root development and growth (21). Potential systemic signals that affect Pi starvation have been described, and some components that control local PSRs have been identified genetically (6, 22–27).

Despite this progress in the dissection of Pi starvation signaling in plants, very little is known of how Pi levels are sensed or of the early steps in this signaling pathway. Several Pi homeostasis-related proteins in yeast, including Pi sensors, share an SPX domain (28–32), so-called because it is present in the suppressor of yeast *gpa1* (Syg1), the yeast cyclin-dependent kinase inhibitor (Pho81), and the human xenotropic and polytropic retrovirus receptor 1 (XPR1). Several plant proteins bearing this domain are involved in Pi starvation signaling (33–37); nonetheless, demonstration of a role for plant SPX proteins as Pi sensors is lacking.

Significance

When P levels are low, plants activate an array of adaptive responses to increase efficient acquisition and use of phosphate (Pi), the form in which P is preferentially absorbed, and to protect themselves from Pi starvation stress. Considerable progress has been made recently in dissecting the plant Pi starvation signaling pathway. Nonetheless, little is known as to how Pi levels are perceived by plants. Here, we identify the nuclear protein SPX1 as a Pi-dependent inhibitor of DNA binding by PHOSPHATE STARVATION RESPONSE 1 (PHR1), a master regulator of Pi starvation responses. We show that the Pi dependence of SPX1 inhibition of PHR1 activity can be recreated in vitro using purified proteins, which indicates that the SPX1/PHR1 module links Pi sensing and signaling.

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In contrast to the lack of knowledge on the Pi sensor(s) in plants, there is some information regarding the nature of the signal molecule perceived by the sensing machinery. A role for Pi itself as a signal was inferred from physiological experiments using compounds that sequester this inorganic molecule (38). This conclusion was further substantiated in experiments using phosphite (Phi), a nonmetabolizable analog of Pi that nevertheless inhibits PSRs (39, 40). In yeast, a role for Pi as a signal has also been established (41), although in these microorganisms, additional metabolites, such as *myo*-D-inositol heptakisphosphate, whose synthesis is increased by Pi starvation, act as signals under Pi starvation stress (29).

Here, we identified the *Arabidopsis* SPX1 nuclear protein as a PHR1 interactor. Physiological and transcriptomic analyses of a double mutant, in which *SPX1* and its closely related gene *SPX2* are impaired, indicate that these genes are Pi-dependent inhibitors of PSRs. Coimmunoprecipitation (co-IP) studies showed that the PHR1/SPX1 interaction in vivo is highly Pi-dependent. We also show that the Pi dependence of the SPX1 inhibitory effect on PHR1 can be reconstituted in vitro using purified proteins; these two proteins thus provide a link between Pi starvation perception and signaling.

Results

SPX1 Interacts with PHR1. To identify proteins that act early in Pi sensing and signaling, we sought interacting partners of PHR1, whose gene is only weakly responsive to Pi starvation (7). We examined a functional MYC-tagged form of PHR1 expressed under the control of its own promoter (*PHR1$_{pro}$::PHR1-MYC*), whose activity is Pi-dependent, and found that PHR1-MYC accumulation and its posttranslational modification pattern are relatively unaffected by Pi starvation (Fig. S1 *A* and *B*), suggesting that Pi control of PHR1 activity involves an accessory protein. To search for PHR1-interacting partners, we screened a normalized yeast two-hybrid cDNA library, using as bait a truncated derivative of PHR1 (ΔPHR1, aa 208–362) that lacks transcription activation domains. One candidate PHR1 partner was SPX1 (Fig. S2), described as a nuclear protein involved in Pi signaling (34), which has an SPX domain also present in yeast Pi sensors. We determined that SPX1 interacts with PHR1 *in planta* using a co-IP assay in *Nicotiana benthamiana* plants agroinfiltrated with constructs expressing HA-tagged PHR1 (HA-PHR1) and GFP-tagged SPX1 (GFP-SPX1; Fig. 1*A*). We further confirmed the SPX1/PHR1 interaction in bimolecular fluorescence complementation (BiFC) assays in tobacco leaves, which showed that YFP^{C}-PHR1 interacts with YFP^{N}-SPX1 in the nucleus (Fig. 1*B*). Yeast two-hybrid assays with SPX1 deletion derivatives showed that binding to PHR1 required an intact SPX domain and a flanking region at its C terminus (Fig. S2).

Pi-Dependent Effect of *spx1* and *spx2* Mutations. In *Arabidopsis*, SPX1 is part of a subfamily of three nuclear proteins (SPX1, SPX2, and SPX3) whose genes are highly responsive to Pi starvation (34). We identified single *spx1* and *spx2* transfer DNA (T-DNA) mutants in the Salk collection (42), and used them to generate a double mutant (Fig. S3). We also generated transgenic plants that overexpressed GFP-SPX1, and examined Pi levels in WT, mutants, and two independent transgenic plants grown in four Pi regimens (0, 30, 100, and 2,000 μM). In the 2,000 and 100 μM Pi growth conditions, the *spx1spx2* double mutant showed a significant increase in Pi accumulation relative to WT plants, whereas the opposite was the case for the two *GFP-SPX1*–overexpressing lines (Fig. 2*A*). In 0 and 30 μM Pi growth conditions, however, plants with altered *SPX1* and/or *SPX2* activity showed Pi levels similar to Pi levels of WT plants (Fig. 2*A*). The effect of *SPX1* and *SPX2* on Pi accumulation is therefore Pi-dependent. Single *spx1* and *spx2* mutations had a marginal effect on Pi accumulation, indicating marked functional redundancy between these SPX proteins (Fig. 2*A*).

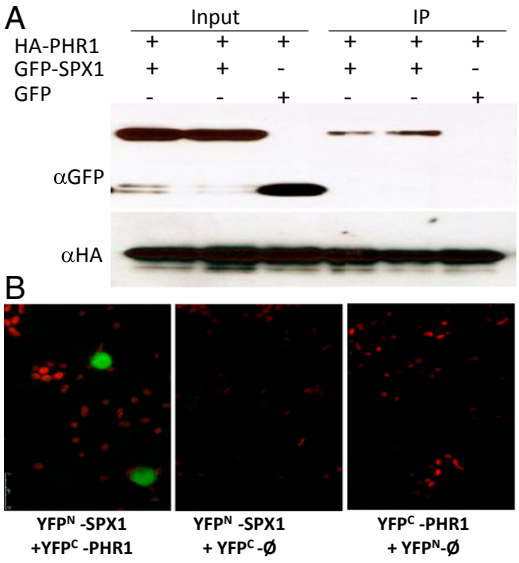

Fig. 1. SPX1 interacts with PHR1 *in planta*. (*A*) Co-IP of GFP-SPX1 and HA-PHR1. *N. benthamiana* leaves agroinfiltrated with HA-PHR1 and GFP-SPX1 or GFP-expressing constructs were treated with formaldehyde after harvest; protein extracts were immunoprecipitated with anti-HA antibody and detected in Western blots with anti-HA and anti-GFP antibodies. (*B*) Analysis of SPX1 and PHR1 interaction by BiFC. Confocal images of *N. benthamiana* epidermal cells expressing different construct combinations as indicated are shown. The interaction between SPX1 and PHR1 in the nucleus leads to reconstitution of YFP fluorescence in the nucleus of cells that coexpress the YFP^{N}-SPX1 and YFP^{C}-PHR1 constructs. (Scale bar: 10 μm.)

We also examined the effect of altered SPX1 activity on other physiological responses to −P, such as anthocyanin accumulation, root-to-shoot growth ratio, and root hair number and length (Fig. S4). The root-to-shoot growth ratio increased only in the *spx1spx2* double mutant compared with WT and was only significant when plants were grown at the highest Pi regimens (1,000 and 100 μM). Anthocyanin accumulation was higher in the *spx1spx2* double mutant and lower in *GFP-SPX1*–overexpressing plants compared with WT in all Pi regimens except the highest (2 mM). Significant alterations in root hair number and/or length [local Pi-controlled responses (43)] compared with WT plants were detected in *spx1spx2* in both +Pi and −Pi, and in the SPX1-overexpressing line when grown in −P. It is noteworthy that in + Pi conditions, the *spx1spx2* mutant showed reduced root hair size relative to WT plants. This reduction could be due to higher Pi levels in mutant plants than in WT plants, which would override the potentially positive effect of the *spx1spx2* mutation on root hair development. The results show that some effects of altered SPX1 activity are largely Pi-dependent (Pi accumulation and root-to-shoot growth ratio), whereas others appear to be less so (anthocyanin accumulation and root hair number and length). Thus, it seems that the SPX1 and SPX2 effect on certain responses is not fully Pi-dependent; alternatively, the effect on some responses (anthocyanin accumulation and root hair number and length) of SPX1 and SPX2 impairment or overexpression in −P conditions results from their altered activity at intermediate Pi levels before full Pi starvation conditions are reached.

We examined these possibilities relative to anthocyanin accumulation by analyzing two Pi starvation time points (10 and 20 d; Fig. S4*C*). The effect of altered SPX1 activity on anthocyanin accumulation was more pronounced at day 10 than at day 20 in Pi starvation. SPX1 function thus appears to be primarily Pi-dependent.

To determine the effect of the *spx1spx2* double mutation on gene expression and its possible Pi dependence, we analyzed transcriptomes of plants grown in +Pi and −Pi conditions. Given

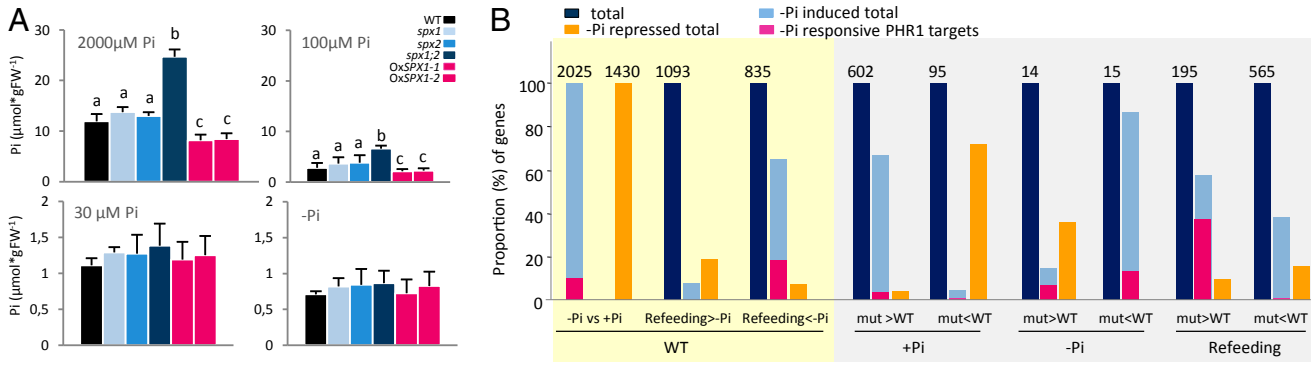

Fig. 2. Physiological and molecular effects of altering SPX1 and SPX2 activity, and the influence of the Pi growth regimen. (*A*) Pi levels in WT, *spx1* and *spx2* single-mutant plants, *spx1spx2* double-mutant plants, and two independent transgenic lines overexpressing *GFP-SPX1* (*OxSPX1-1, OxSPX1-2*), all grown in four Pi regimens (2,000, 100, and 30 µM, and −Pi) for 10 d. Data show mean ± SD ($n = 3$). Shared or different letters above bars indicate nonsignificant and significant differences between groups ($P < 0.05$) according to Student *t* tests. (*B*) Diagram showing transcriptomic analysis of the effect of Pi growth conditions on gene expression in WT and *spx1spx2* plants grown for 8 d in +Pi, in −Pi, or after brief Pi refeeding (4 h). The total number of genes whose expression is induced or repressed by Pi starvation in WT plants or is higher (Refeeding > −Pi) or lower (Refeeding < −Pi) in Pi-refed vs. Pi-starved WT plants is shown above bars (2× cutoff; false discovery rate is ≤0.05). The number of genes whose expression is higher [mutation (mut) > WT] or lower (mut < WT) in *spx1spx2* plants than in WT plants in each growth condition is also shown. The percentage of Pi starvation-responsive genes (−Pi-induced and −Pi-repressed) is indicated, as well as the percentage of PHR1 direct targets [as described by Bustos et al. (9)]. Three biological replicates were analyzed.

that *SPX1* and *SPX2* are Pi starvation-inducible, whereas most physiological effects of altering their activity require Pi (Fig. 2*A* and Fig. S4), we also analyzed transcriptomes of Pi-starved plants after short-term Pi refeeding. Results showed marked Pi dependence of the *spx1spx2* effects (Fig. 2*B* and Dataset S1). Although only 29 genes showed significant expression differences between the *spx1spx2* and WT plants grown in −Pi conditions (15 up-regulated and 14 down-regulated, twofold cutoff, false discovery rate ≤0.05), when these plants were grown in +Pi conditions or Pi-refed, this number was >20-fold higher (697 and 760 genes, respectively). In +Pi-grown plants, >65% of genes whose expression was higher or lower in *spx1spx2* than in WT plants were PSI or Pi starvation-repressed genes, respectively, which indicates that *SPX1* and *SPX2* are primarily regulators of PSRs. For *spx1spx2* double mutants in Pi-refeeding conditions, 58% and 38% of the up-regulated and down-regulated genes, respectively, were PSI. Of these up-regulated PSI genes, 65% were direct PHR1 targets, as described by Bustos et al. (9), whereas only 2.5% of the down-regulated PSI genes were direct PHR1 targets. Expression of PHR1 PSI targets is thus especially influenced by *SPX1* and *SPX2* after brief Pi refeeding. These transcriptomic phenotypes are consistent with the hypothesis that SPX1 and SPX2 are Pi-dependent inhibitors of PHR1 activity.

We compared our transcriptomic data with the data of Thibaud et al. (21), which dissected systemically and locally controlled molecular responses to Pi (Table S1). We found notable differences between the two studies with regard to the repression response. For example, the Pi starvation-repressed gene set reported by Thibaud et al. (21) shows greater overlap with the PSI gene set than with the Pi starvation-repressed gene set of our study; the repression response was therefore not considered further. There was nonetheless a good degree of coincidence between PSI genes in the study by Thibaud et al. (21) and our study, such that 85 of the 110 systemically controlled (Ind. S) and 181 of the 301 locally controlled (Ind. L) PSI genes were also induced in our study (total of 2,025 PSI genes). We also found similar relative representation of Ind. S and Ind. L in the gene set up-regulated in the *spx1spx2* mutant plants grown in +Pi (of 602 up-regulated genes in *spx1spx2*, 24 and 74 were Ind. S and Ind. L, respectively), which indicated that primarily *SPX1* and related genes control both types of responses at the molecular level. In short-term Pi refeeding, Ind. L genes were enriched in the gene set down-regulated in the *spx1spx2* double mutant. This result indicates that in contrast to its negative effect on Pi starvation induction after long-term growth in a Pi-rich regimen, SPX1-(related) activity slows repression of Ind. L genes after Pi refeeding in Pi-starved plants, and suggests that SPX1 regulates regulators of Pi starvation other than PHR1.

Pi-Dependent Interaction Between SPX1 and PHR1 in Vivo. We tested whether the SPX1 Pi-dependent inhibitory effect on PHR1 was due to Pi dependence of the SPX/PHR1 interaction itself, using co-IP experiments in transgenic plants that coexpressed HA-PHR1 and GFP-SPX1 grown in +Pi and −Pi conditions. To preserve the *in planta* SPX1/PHR1 interaction, we treated plants with the cross-linking agent formaldehyde immediately after harvest (44). The SPX1/PHR1 interaction was detected only in +Pi conditions (Fig. 3*A*). Confocal microscopy analysis of GFP-SPX1 in plants grown in +Pi and −Pi conditions showed that SPX1 is a nuclear protein, irrespective of Pi growth conditions (Fig. 3*B*). Because PHR1 is also constitutively located in the nucleus (7), we concluded that the Pi-dependent interaction of SPX1 and PHR1 is not due to altered subcellular localization of any PHR1 or SPX1 proteins in plants grown in −Pi conditions.

PHR1 Binding to Its Targets Is Low Pi-Dependent. Two alternative models could explain the inhibitory effect of SPX1 on PHR1. SPX1 could inhibit PHR1 binding to DNA or could act as a co-repressor, such that the PHR1/SPX1 complex functions as a repressor, in contrast to the primary role of PHR1 as a transcription activator (9). To discriminate between these models, we examined the Pi dependence of PHR1 binding to its cognate target elements in vivo, using ChIP coupled with PCR of PHR1 targets (Fig. 4). We found strong PHR1 binding to targets in plants grown in −P conditions, which was greatly reduced in plants grown in +Pi conditions or after refeeding of Pi-starved plants (Fig. 4). These results point to the second model, in which SPX1 inhibits PHR1 binding to DNA in a Pi-dependent manner. Given that Pi levels in Pi-refed plants are approximately one-half of Pi levels in plants grown in Pi-rich media (Fig. 4), a direct Pi effect on SPX1 is sufficient to explain the reduction observed in PHR1 binding to its targets in Pi-refed plants.

Pi-Dependent Inhibition of PHR1 Binding to DNA by SPX1. To confirm the possibility that SPX1 is a Pi-dependent inhibitor of PHR1 binding to its recognition sequence PHR1 binding site (P1BS) (7, 9), we performed in vitro DNA-binding assays using increasing

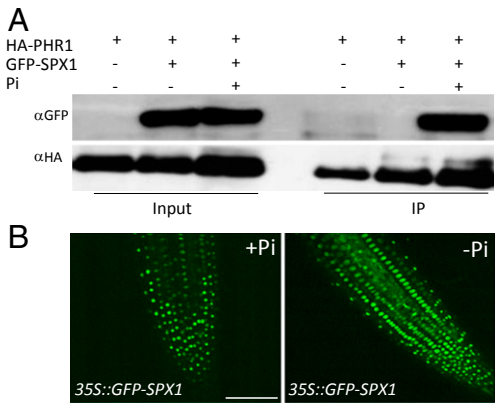

Fig. 3. Cellular Pi-dependent interaction between SPX1 and PHR1 *in planta*. (*A*) Co-IP assay of the *in planta* interaction between GFP-SPX1 and HA-PHR1 in plants grown in +Pi (2 mM) and −Pi conditions. *Arabidopsis* plants constitutively expressing GFP-SPX1 and HA-PHR1 were grown for 8 d in +Pi or −Pi conditions and prefixed with formaldehyde after harvest to preserve the *in planta* protein interaction status (44). Protein extracts were immunoprecipitated with anti-HA and detected by Western blotting using anti-GFP antibody. (*B*) Confocal microscopy images showing that GFP-SPX1 is located in the nucleus, irrespective of the Pi growth regimen of the plant. (Scale bar: 50 μm.)

amounts of SPX1 in binding buffer with or without Pi (15 mM). To distinguish direct from indirect Pi effects on inhibition, for DNA-binding assays, we used affinity-purified bacterially expressed ΔPHR1, whose DNA-binding specificity is similar to that of the full-sized protein (7) and SPX1 protein; these proteins were tagged with maltose-binding protein (MBP) and GST, respectively (Fig. S5). EMSAs showed that in the presence of Pi, GST-SPX1 efficiently displaced the ΔPHR1/P1BS interaction, whereas the SPX1 inhibitory effect was very weak when Pi was absent (Fig. 5*A*). Using EMSA, we examined the range of Pi concentrations in which SPX1 effectively inhibits PHR1 binding to P1BS (Fig. S6). SPX1 inhibitory activity showed a clear dose-dependent response to Pi levels, with optimal activity at 15 mM and 50% activity at ∼0.3 mM. This sensitivity of SPX1 inhibitory activity in vitro is compatible with physiological Pi levels in plants grown in Pi-rich media [10–15 mM total Pi, 0.5 mM cytosolic Pi (36)].

In pull-down assays, we analyzed P1BS competition for the ΔPHR1/SPX1 interaction. Reciprocal to the finding that SPX1 displaced P1BS binding to ΔPHR1 in the presence of Pi, P1BS competed with SPX1 for PHR1 binding only when Pi was lacking (Fig. 5*B*). These results show that SPX1 can interact with PHR1 in both +Pi and −Pi conditions in the absence of DNA; however, in the presence of excess DNA, the SPX1/PHR1 interaction is displaced. These data explain why in the *in planta* co-IP assay, which is performed in the presence of genomic DNA, only when Pi is present is the SPX1/PHR1 interaction detected. Because the in vitro data in Fig. 5 were obtained using purified bacterially expressed proteins, we conclude that Pi itself directly affects the SPX1 competition of the PHR1/P1BS interaction. We also tested the specificity of the Pi effect by analyzing whether other anions, such as nitrate, sulfate, and Phi, similarly affected the SPX1/ΔPHR1 interaction. Other than Pi, only Phi had an effect on the interaction (Fig. 5*C*). The fact that Phi represses PSRs has been considered evidence that Pi itself is a signal (39, 40), a concept that is strengthened by our data.

Discussion

In this study, we identify a mechanism for Pi-dependent negative control of PHR1 activity in *Arabidopsis*, based on a nuclear SPX domain that inhibits PHR1 DNA-binding activity in a Pi-dependent manner. This conclusion is substantiated by three lines of evidence. In the first, phenotypic effects of altering SPX1 (and SPX2) are largely Pi-dependent, particularly the transcriptomic phenotype, and affect systemically and locally controlled PSRs. Second, PHR1 binding to SPX1 and to its targets in vivo is Pi-sensitive. Third, SPX1 competes for PHR1 binding to its recognition sequence in a manner greatly dependent on the presence of Pi or of its nonmetabolizable analog Phi. PHL1 acts redundantly with PHR1 (9), and we show here that SPX1 and SPX2 are functionally redundant; it is thus likely that our findings for PHR1 and SPX1 can be extrapolated to PHL1 and SPX2.

The fact that Pi dependence on SPX1 inhibition of PHR1 DNA binding can be recreated in vitro with purified proteins indicates that the SPX1/PHR1 module links Pi perception and signaling, and further strengthens the idea that Pi itself acts as a signal, especially given the finding that Phi can replace Pi in the SPX1/PHR1 interaction (Fig. 5*C*). The Phi effect on the SPX1/PHR1 interaction provides a simple mechanistic explanation for the previously reported observation that Phi can repress PSRs. Results similar to the results reported here have been obtained in the rice system (*Oryza sativa*), which indicates the ubiquity of SPX1 function in plants [at least for angiosperm plants; see companion paper by Wang et al. (45)].

Several yeast SPX domain proteins have a role in Pi homeostasis, and there is evidence that two of them, PHO81 and PHO87, have Pi-sensing properties, although the precise mode of Pi sensing by the SPX domain in these yeast proteins is not yet known. In the case of the yeast PHO81 sensor, the SPX domain is dispensable for some of the sensing properties mediated by the P-rich compound *myo*-D-inositol heptakisphosphate, whose synthesis is increased by Pi starvation (29), although some PHO81 functions depend on its SPX domain (46). It thus appears that SPX domain proteins might have evolved additional Pi sensing mechanisms, mediated by domains other than SPX. Nevertheless, it will be interesting to evaluate whether the SPX domains of distinct proteins, from yeast to animals, share biochemical mechanisms with SPX1, which shows a Pi-sensitive affinity for PHR1. Rice SPX4, an SPX1 homolog in cytosol, was recently shown to inhibit traffic to the nucleus of the rice PHR1 homolog, PHR2; Pi levels control SPX4 protein

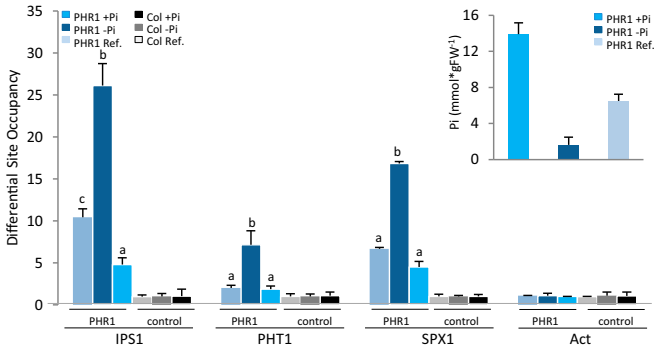

Fig. 4. Cellular Pi-dependent interaction between PHR1 and its targets *in planta*. ChIP and promoter PCR amplification analysis of PHR1 targets in plants grown in +Pi (2 mM), in −Pi, and after Pi refeeding (Ref.). Control Columbia (Col) and transgenic PHRI promoter ($PHR1_{pro}$)::*PHR1-MYC* plants were used in the experiment, in which three PHR1 targets (*SPX1, IPS1, and PHT1*) and one control [*ACT8* (Act)] were analyzed by quantitative PCR. Recovery of target by co-IP with anti-MYC antibody was compared with recovery of a nonbound control (Act) in the same immunoprecipitation. The Pi levels in plants used in the experiment are shown (*Upper Right*). Data show mean ± SD (n = 2). Shared or different letters above bars indicate nonsignificant and significant differences between groups (P < 0.05), respectively, according to Student t tests. FW, fresh weight.

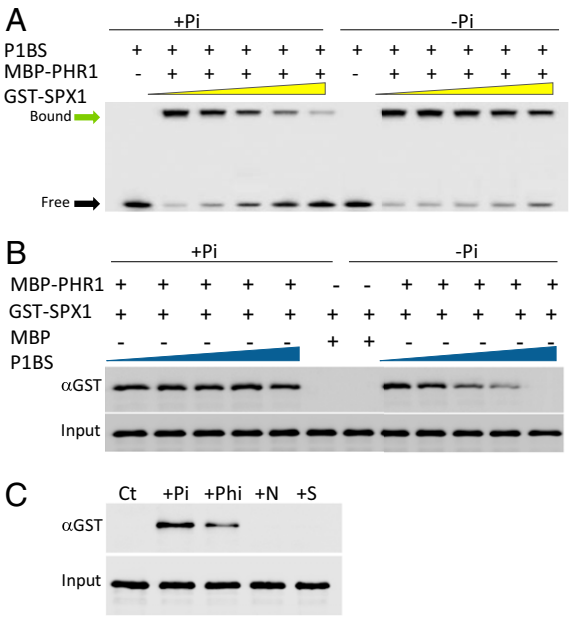

Fig. 5. Direct Pi effect on the SPX1/PHR1 interaction. (*A*) EMSA of the interaction between MBP-ΔPHR1 and P1BS, showing Pi-dependent inhibition of the MBP-ΔPHR1/P1BS interaction by GST-SPX1. The experiment was performed with 0.1 pmol of 4× P1BS; 0.3 pmol of MBP-ΔPHR1; and 0, 0.6, 1.2, 2.5, and 5 pmol of GST-SPX1. (*B*) Pull-down assays showing that the MBP-ΔPHR1/GST-SPX1 interaction is displaced by P1BS only when Pi is lacking in the incubation buffer. The experiment was performed with 1.5 pmol of MBP-ΔPHR1 or MBP; 12.5 pmol of GST-SPX1; and 0, 0.2, 0.5, 1.25, or 3 pmol of 4× P1BS probe. (*C*) Pull-down assays showing that only Phi can replace the Pi effect on the SPX1/PHR1 interaction. All reactions included fixed amounts of MBP-ΔPHR1, GST-SPX1, and P1BS (1.5, 12.5, and 3 pmol, respectively). The control (Ct) reaction contained 50 mM NaCl in pull-down buffer; in other cases, 45 mM NaCl was replaced by 15 mM NaH_2PO_4 (+Pi), 15 mM NaH_2PO_3 (+Phi), 45 mM $NaNO_3$ (+N), and 22.5 mM Na_2SO_4 (+S). Proteins were pulled down with dextrin Sepharose resin and detected in immunoblotting with anti-GST antibody. The tagged ΔPHR1 and SPX1 proteins used in these experiments were bacterially expressed and affinity-purified.

stability through an unknown mechanism (47). The possibility that Pi-mediated conformational effects underlie the Pi-dependent stability of SPX4 should be examined.

An important feature of *SPX1* action is its inducibility by Pi starvation, thereby forming a negative regulatory loop with PHR1 whose output is Pi-dependent. A model for the SPX1/PHR1 functional interplay is schematically shown in Fig. 6. Such a loop allows self-regulation of the strength of PSRs to meet the Pi demand of the plant. It is of interest that because SPX1 inhibition of PHR1 is Pi-dependent, prolonged Pi starvation provokes physiological and temporal uncoupling between SPX1 protein accumulation and activity. A possible explanation for the strong SPX1 induction by Pi starvation is that it allows rapid repression of PHR1 PSI targets after Pi refeeding. It thus appears that during Pi starvation, plants accumulate SPX protein to allow shutdown of direct PHR1 targets after Pi refeeding; the strength and speed of this repression depend on the severity of the Pi starvation stress. In contrast, PSI genes whose expression must be maintained during early stages of Pi refeeding are not under direct PHR1 control, and some are positively controlled by SPX1 and SPX2. This type of SPX1(-related) control might ultimately indicate that the greater the stress severity, the higher is the potential toxicity of a sudden Pi boost. For rapid shutdown of expression of direct PHR1 targets after refeeding, the nuclear localization of SPX1 is more appropriate than the cytoplasmic localization of SPX4, because SPX1 could inhibit nuclear PHR1 that is present and acting on its targets and not only PHR1 that would be newly synthesized.

Our findings in this study indicate that PHR1 is the main target of SPX1 inhibition, although we cannot rule out SPX1 control of other regulatory proteins. The down-regulation of some locally controlled PSI genes in the *spx1spx2* double mutant after Pi refeeding lends plausibility to this possibility. Although a large proportion of the genes whose expression is altered in the *spx1spx2* double mutant compared with WT plants are Pi starvation-responsive genes, there is still a considerable proportion of the genes with altered expression in the double mutant that are not Pi starvation-responsive (Fig. 2). This finding again raises the possibility of additional SPX1-controlled regulatory genes, which would broaden the potential role of SPX proteins in plant physiology, emphasizing the need for further research into SPX1 partners.

Materials and Methods

Plant Material and Growth Conditions. T-DNA insertional mutants (42) *spx1* (SALK-092030) and *spx2* (SALK-080503) were obtained from the Arabidopsis Biological Resource Center, and the double mutant *spx1spx2* was obtained by crossing the single mutants. Growth conditions and media were as described (48).

Yeast Two-Hybrid Screens. For yeast two-hybrid screening, we prepared a normalized cDNA library (49) in the pGADT7-Rec vector (Clontech) essentially following the Matchmaker protocol PT3529-1 (Clontech). Normalization was as described (49). RNA was isolated from a mixture of plants Pi-starved for different times. A PHR1 fragment (ΔPHR1) encompassing aa 208–362 and lacking transactivation domains was cloned into the pGBKT7 (Clontech) and used to screen for interactors.

Co-IP Assays. Co-IP assays to detect protein/protein interactions *in planta* included a formaldehyde cross-linking step after harvesting plant material (44). Cross-linked proteins in extraction buffer (*SI Materials and Methods*) were immunoprecipitated with anti-HA affinity matrix, and immunoblots were developed using anti-HA and anti-GFP antibodies. Competitive pull-down assays included fixed amounts of MBP-ΔPHR1 (1.5 pmol) and GST-SPX1 (12.5 pmol) and varying amounts of P1BS1 probe (0–3 pmol), and they included or did not include 15 mM Pi in the incubation buffer (Fig. 3). In some experiments, Pi was replaced by Phi, nitrate, or sulfate. For pull-down, we used MBP affinity resin, and immunoblots were developed with anti-GST.

EMSAs. EMSAs were performed with recombinant MBP-ΔPHR1 alone or with different amounts of GST-SPX1 protein as described (7), including or not including varying concentrations of Pi in the incubation buffer (0.1–15 mM). For protein expression in *Escherichia coli*, the PCR fragment encoding the full-sized SPX1 protein or ΔPHR1 was cloned in pGEX-4T-1 encoding a GST tag (GE Healthcare) and pDEST-TH1 with MBP tag (Clontech), respectively.

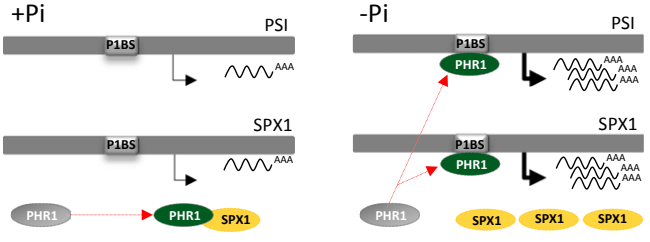

Fig. 6. Model for the negative regulatory loop between *SPX1* and *PHR1*, and its Pi dependence. *SPX1* is a target of PHR1. In the presence of Pi, SPX1 displays high binding affinity to and sequesters PHR1; thus, binding of PHR1 to its PSI targets via P1BS is inhibited, and their transcription, including that of *SPX1*, is just basal. In the absence of Pi, the affinity of the SPX1/PHR1 interaction is reduced and PHR1 interacts with its targets, resulting in their transcriptional induction. As a consequence, in –Pi-grown plants, there is increased *SPX1* expression and protein accumulation, although these plants lack inhibitory activity; however, high SPX1 protein levels allow rapid shutdown of PHR1 target gene expression after Pi refeeding. AAA, Poly A tail of mRNA.

Other Molecular, Cell Biology, and Physiological Analysis. Transcriptomic analysis was as described (9). ChIP-PCR was performed following an established protocol (50); the *ACT8* promoter was used as a negative control. The quantitative RT-PCR assay and measurement of cellular Pi concentration were performed as described. Agroinfiltration of *N. benthamiana* and confocal analysis for subcellular localization or for BiFC assays was as reported previously (19, 51). Determination of Pi and anthocyanin content was as described (52, 53). Details of constructs and plant materials are given in *SI Materials and Methods*, and primers used are listed in Table S2. More details are provided in *SI Materials and Methods*.

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