CONTROL OF PHOSPHATE STARVATION RESPONSES IN *Arabidopsis thaliana.* NEW REGULATORS AND REGULATORY INTERACTIONS

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Dedicated to,

&

My uncle

(Neyveli B. Venkatesh)

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ABBREVIATIONS

AGRIS : International System for Agricultural Science and Technology

A.thaliana : *Arabidopsis thaliana*

A.tumefaciens : *Agrobacterium tumefaciens*

ABRC : Arabidopsis Biological Research Centre

AD : Activation Domain

ADP : Adenosine diphoshate

ATP : Adenosine triphosphate

B : Boron

BD : Binding domain

bHLH : basic helix-loop-helix

BiFC : bimolecular fluorescence complementation

Ca : Calcium

CC : Coiled Coil

cDNA : Complementary Deoxyribonucleic acid

ChIP : Chromatin Immunoprecipitation

CID : Charge Induction Device

Cl : Chlorine

Col-0 : Columbia

Cr : Chromium

Cu : Copper

DNA : Deoxyribonucleic acid

E.Coli : *Escherichia coli*

E2 : Ubiquitin-conjugating enzyme

E3 : Ubiquitin-ligase

EMSA : Electrophoretic Mobility Shift Assay

Fe : Iron

GST : Glutathione S-transferase

ICP-MS : Inductively Coupled Plasma Mass Spectroscopy

ICP-OES : Inductively Coupled Plasma Optical Emission Spectroscopy
IPS : Induced by Phosphate starvation
K : Potassium
LB : Lysogeny broth
MBP : Maltose Binding Protein
MFS : Major Facilitator Superfamily
Mg : Magnesium
Mn : Manganese
Mo : Molybdenum
Na : Sodium
NaCl : Sodium Chloride
Ni : Nickel
NLA : Nitrogen Limitation Adaptation
NP40 : Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol)
ORF : Open Reading Frame
P : Phosphorous
PAGE : Polyacrylamide Gel Electrophoresis
P1BS : PHR1 binding sequence
PHL1 : PHR1 (-like)
PHO2 : Phosphate 2
PHR1 : Phosphate starvation Response 1
PHT1;1 : Phosphate transporter 1
Pi : Phosphate
PI : Protease Inhibitor
PMSF : phenylmethanesulfonylfluoride
PPCK : Phosphoenolpyruvate carboxylase kinase
PSI : Phosphate starvation induced
PSR : Phosphate starvation responses
RING : Really Interesting New Gene
RNA : Ribonucleic acid
RPM : Revolutions per minute

RSD	: Relative Standard Deviation
S	: Sulfur
S. cerevisae	: *Saccharomyces cerevisiae*
SDS	: Sodium Dodecyl Sulfate
SPX	: Syg1 Pho81 XPR1
Sr	: Strontium
T-DNA	: Transfer Deoxyribonucleic acid
TF	: Transcription Factor
TPT	: TRANSPLANTA
-WL	: -Tryptophan, -Leucine
-WLA	: -Tryptophan, -Leucine, -Adenine
-WLAH	: -Tryptophan, -Leucine, -Adenine, -Histidine
WT	: wild type
Y2H	: Yeast two hybrid
Zn	: Zinc
3AT	: 3-Amino-1,2,4-triazole

CONTENTS

SUMMARY

To cope with growth under Pi starvation conditions, plants have evolved a series of morphological and biochemical adaptations aimed to survive at best the stress situation. Pi starvation signaling mechanism in plants has been widely studied in the past two decades. However, there were some gaps in the knowledge of this pathway; for instance, on the mechanism of regulation of PHR1 (master regulator of Pi starvation responses) activity, on the properties of the Pi sensor, the complete TF set controlling the transcriptional networks underlying Pi ion homeostasis etc,.

In this study we have contributed to the knowledge of phosphate starvation signaling at 3 different fronts.

1) **Identification of candidate TFs controlling PSRs using ionomics** -we used a large scale ionomic profiling approach to study the elemental profile of the transgenic lines of TRANSPLANTA collection conditionally overexpressing TFs. In general, we observed that alterations in the ionome involved disturbances in the levels of many elements. Giving emphasis to P nutrient signaling, we selected 5 TF candidates (belonging to families of DREB, bZIP, NAC and KNAT) whose ionomic pattern indicated potential correlations between P and other elements like Zn, Fe and Mn.

2) **SPX1 is a Pi dependent inhibitor of PHR1**. Following a yeast two hybrid approach, we identified SPX1 as an interactor of PHR1. Subsequent characterization studies included physiological and transcriptomic analysis of *spx1spx2* mutants, Co-immunoprecipitation assay *in-planta* and *in-vitro,* as well as DNA binding assays. As a result of this characterization, we established that SPX1 is a Pi dependent inhibitor of PHR1, qualifying it as a sensor component.

3) **New roles of PHO2 and NLA in Pi starvation signaling**. In this study, we found that PHO2 and NLA interact with each other suggesting they act in concert in the ubiquitination pathway. In line with the previous finding that the negative growth regulators bHLH149 is a target of PHO2, bHLH149 is also shown to be regulated by NLA, reinforcing the link between Pi starvation signaling and growth control. In addition, SPX1 is also shown to be a PHO2/NLA target, contributing to form a negative regulatory loop in Pi starvation signaling involving PHR1, NLA, PHO2 and SPX1.

RESUMEN

Las plantas han desarrollado una serie de respuestas morfológicas y bioquímicas destinadas a adaptar su crecimiento en condiciones de bajo Pi, en el suelo. El mecanismo de señalización de ayuno de fosfato en plantas ha sido ampliamente estudiado en las últimas dos décadas. Sin embargo, todavía existían importantes lagunas en el conocimiento de esta ruta; por ejemplo, sobre cómo se regula la actividad de PHR1 (regulador maestro de las respuestas al ayuno de Pi) ; sobre la naturaleza y el modo de accion del sensor de Pi y sobre el conjunto de TFs de las redes de transcripción subyacentes a la homeostasis de Pi etc,.
En este estudio hemos contribuido en tres aspectos diferentes al conocimiento de la señalización del ayuno de fosfato.

1) **Identificación de nuevos TFs candidatos mediante aproximaciones ionómicas**. – Se ha realizado un analisis a gran escala del pefil ionómico de lineas TRANSPLANTA que sobreexpresan condicionalmente TFs de Arabidopsis, para detectar TFs cuya sobreexpresión altera el ionoma. En los casos encontrados, en general, se observó que las alteraciones en el ionoma implican alteraciones en los niveles de muchos elementos. Centrándonos en los TFs relacionados con la homeostasis de Pi, se seleccionaron 5 TF candidatos (pertenecientes a familias de DREB, bZIP, NAC y KNAT) cuyo patrón ionómico refleja las posibles correlaciones entre P y otros elementos como Zn, Fe y Mn.

2) **SPX1 es un inhibidor de PHR1 dependiente de Pi** - Siguiendo una aproximación basada en el método de los dos híbridos de levadura, se identificó SPX1 como un interactor de PHR1. Estudios de caracterización posteriores incluyeron análisis fisiológicos y transcriptómicos de mutantes *spx1spx2*, ensayos de co-inmunoprecipitación *in planta* e *in vitro*, así como ensayos de unión de ADN. Como resultado de esta caracterización, se estableció que SPX1 es un inhibidor PHR1 directamente dependiente de Pi, cualificándolo como un componente del sensor de Pi.

3) **Nuevas funciones de PHO2 y NLA en la vía de señalización de ayuno de Pi**. En este estudio, se encontró que PHO2 y NLA interaccionan entre sí lo que sugiere que actúan en concierto en la ruta de ubiquitinación implicada en la señalización de Pi. En línea con datos previos que establecieron que el inhibidor de crecimiento bHlH149, está controlado

por PH02, se ha comprobado que también está regulado por NLA, reforzando el vínculo entre la señalización del ayuno de Pi y el control del crecimiento. Además, también hemos demostrado que SPX1 es diana de PHO2 y NLA, lo que contribuye a formar un bucle regulador negativo en la señalización del ayuno de Pi que implica a PHR1, NLA, PHO2 y SPX1.

INTRODUCTION

1. Phosphorous

Phosphorous (P) is an essential macronutrient required for all living organisms, including plants. Next to Nitrogen, Phosphorous along with Potassium constitute the primary nutrients followed by the 3 secondary nutrients – Ca, Mg and S and the Micronutrients – B, Cl, Cu, Fe, Mn, Mo and Zn. P is a component of key macromolecules, such as nucleic acids and phospholipids, and as a constituent of ATP/ADP, is involved in the regulation of energy transfer reactions and determines a key regulatory event in the post-translational control of protein activity (i.e., phosphorylation).

Though the total phosphorous content in soils is quite high, P nutrition is frequently a factor, limiting crop productivity due to the fact that inorganic Phosphate (Pi), the form in which P is preferentially assimilated by plant roots, is quite unavailable because it gets adsorbed to soil particles and insolubilized in the presence of some cations (Fe^{3+}, Ca^{2+}, Mg^{2+} and Al^{3+}), eventually getting unavailable.

Conventional practices to cope with Pi limitation involve increased application of fertilizers to achieve maximum yield, but reduction of phosphorus fertilizer use is presently a main objective towards adopting sustainable agricultural practices, given the serious environmental problems associated with excess use (for instance, eutrophication) and the non-renewability of the P resources, which will dramatically reduce their availability in the next 50 years. Due to this, the adaptive system that allow plant to grow under Pi limiting conditions has gained considerable interest in recent years because of its potential for improving Pi acquisition and use efficiency in crops.

2. Plant adaptation to Pi starvation

To cope with fluctuations in externally available Pi, plants have developed adaptive mechanisms to balance external Pi levels with internal needs to maintain cellular P homeostasis in order to coordinate growth, development and reproduction (Lin *et al.*, 2013). These adaptive strategies involve morphological, biochemical and physiological

changes oriented towards increased Pi acquisition and use efficiency and to protect themselves from Pi starvation stress.

Morphological adaptations exhibited by plants during Pi starvation include changes in root architecture, by decreasing primary root length, increasing the number and length of the lateral roots & root hairs, leading to increased root to shoot ratio aimed at scavenging the Pi from the superficial soil surface (Fig1A). Some plants further modify the soil scavenging potential of their roots by forming lateral proliferations called proteoids (Massonneau *et al*., 2001) or establishing symbiotic associations with mycorrhizal fungi (for review, see Harrison *et al.,* 1999).

Biochemical and physiological adaptations include i) secretion of phosphatases, organic acids, and RNases from root exudates to facilitate Pi release from insoluble P pools in the rhizosphere, ii) increase in the accumulation of the photo protective anthocyanin pigment (Fig1B), decreased rate of photosynthesis, utilization of alternative glycolytic or respiratory pathways that circumvent steps requiring phosphate (such as increase in the synthesis of sulpholipids and galactolipids to substitute for phospholipids), contributing to improved plant survival during prolonged periods of phosphate deprivation.

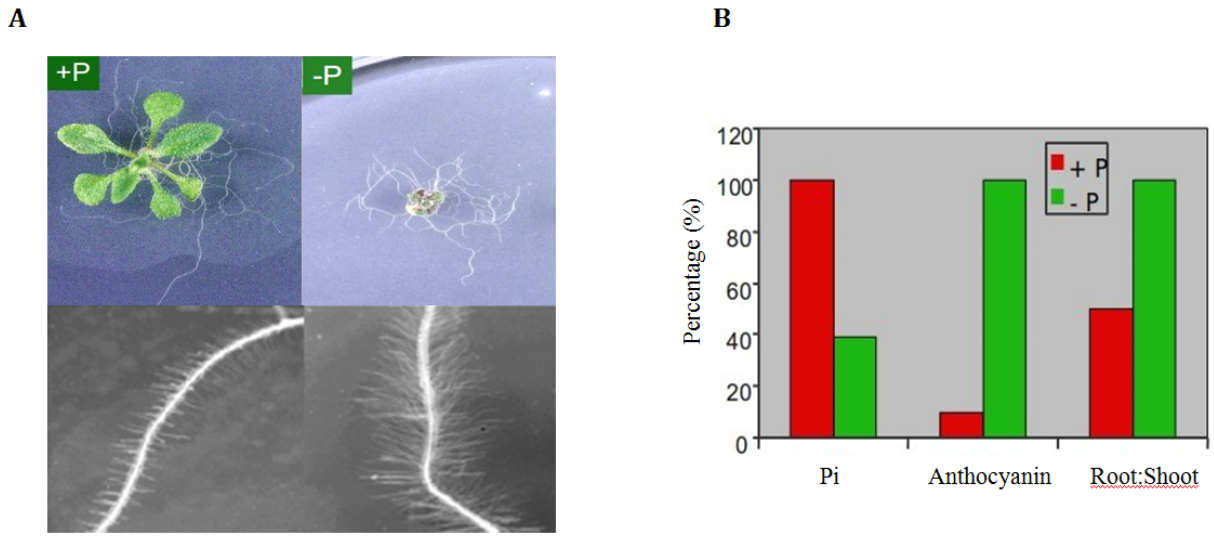

Fig 1: Morphological & physiological adaptations of plants to Pi starvation

A) Photographs of plants (upper panels) or plant roots response of plants grown at +P and –P conditions for 12 days.

B) Comparison of levels of Pi, anthocyanin and root: shoot growth ratio between plants grown at –Pi versus +Pi.

3 Key players in the control of Pi starvation responses

The variety of adaptive strategies evolved by plants to cope with Pi deficiency involves changes in the expression profiles of several hundreds of genes (Bustos *et al.*, 2010; Secco *et al.,* 2013). Significant progress has been made in the last 2 decades on the identification and characterization of regulators of Pi starvation responses that underlines the importance of transcriptional control in the regulation of these responses.

3.1 Transcriptional regulators of Pi starvation responses

Transcription factors (TFs) play a key role in regulating gene expression in all organisms by binding to target genes through short specific DNA motifs which eventually alter the ability of RNA polymerase to be recruited to this target. In *Arabidopsis*, transcriptional regulation of Pi deficiency responses is starting to get disclosed and already an array of transcription factors mainly belonging to MYB, bHLH, C2H2 and WRKY families have been implicated.

3.1.1 MYB family TFs

PHR1 (At4g28610) is the first TF identified to be involved in the control of Pi starvation response in a vascular plant (Rubio *et al.*, 2001). It contains a MYB domain specific for plants, and in addition it possesses the C-terminal coiled coil (CC) domain. *PHR1* by itself is not very responsive to Pi levels; it's a central positive regulator of most of the Pi Starvation Induced (PSI) genes. Independent of the Pi status of the plant, PHR1 is localized in the nucleus. PHR1 binds DNA as a dimer to an imperfect palindromic 8 base pair sequence (GNATATNC) namely the P1BS (PHR1 binding sequence) present in the promoters of many Pi starvation responsive genes. Interestingly, Pi starvation repressed genes do not show enrichment of P1BS motif in their promoters, indicating that the effect of PHR1 on Pi starvation repressed genes is indirect (Bustos *et al*., 2010).

PHL1 (At5g29000) - PHR1-LIKE 1 is a phylogenetically close relative of PHR1 with which it displays functional redundancy. Together, these two TFs act as key integrators of both specific and generic Pi starvation responses (Bustos *et al*., 2010).

MYB62 (At1g68320) is a member of R2R3 type MYB TF family (Stracke *et al*., 2001). *MYB62* expression is specifically induced in young leaves during Pi starvation. MYB62 is localized to the nucleus and exerts negative regulation on Pi starvation responses via changes in gibberellic acid metabolism and signaling (Devaiah *et al*., 2009).

3.1.2 C2H2 TF family

ZAT6 (At5g04340) is the first cysteine-2/histidine-2 (C2H2) zinc finger transcription factor reported to regulate root development and nutrient stress responses. ZAT6 is a nuclear localized protein whose synthesis is induced during Pi starvation. It represses primary root growth and regulates Pi homeostasis through the control of root architecture (Devaiah *et al*., 2007a).

3.1.3 WRKY TF 6 and 75

The C2H2 zinc finger domain of WRKY proteins regulates spatiotemporal expression of their target genes by binding to W box (TTGAC/T) elements. One of the characteristic features of WRKY proteins is the ability of auto and cross regulation of their own promoters and other WRKYs respectively (Rushton *et al*., 2010). WRKY75 is a positive regulator of several phosphate starvation induced (PSI) genes including phosphatases, *Mt4/IPS1* genes and high affinity Pi transporters. It also acts as a negative regulator of some components of root development, independent of Pi stress response (Devaiah *et al*., 2007b). WRKY6 and WRKY42 are involved in Arabidopsis responses to low Pi stress by regulation of *PHO1* expression (Chen *et al*., 2009). *WRKY6* has also been shown to be highly induced by Arsenate (As) and is proposed to inhibit Pi transporter *PHT1;1* to prevent Arsenic entry into the cell, particularly when Pi levels are low (Castrillo *et al*., 2013).

3.1.4 bHLH TF family

The basic helix-loop-helix (bHLH) proteins are a super family of TFs that bind as dimers to specific DNA target sites and are critical regulatory components in transcriptional networks. bHLH32 acts as a negative regulator of a range of Pi starvation-induced processes in *Arabidopsis*. Among the genes negatively regulated by bHLH32 are those encoding *PPCK* (phosphoenolpyruvate carboxylase kinase), which is involved in modifying metabolism so that Pi is spared (Chen *et al*., 2007).

Another member of this bHLH TF superfamily known to play a role in the control of phosphate starvation responses (PSR) is OsPTF1 (Yi *et al*., 2005). OsPTF1 is located in the nucleus independent of the Pi status of the plant and is induced in roots under Pi starvation conditions. Overexpression of *OsPTF1* enhances tolerance to Pi starvation in rice (Yi *et al*., 2005). bHLH149 also known as AIF4 (ATBS1 interacting factor 4) (At1g09250) is a negative regulator of cell elongation and growth (Ikeda *et al*., 2013). Unlike general bHLH TFs which binds to DNA to exert its transcriptional activity, *bHLH149* encodes a nuclear non-DNA binding protein. Previous experiments in our laboratory have shown efficient *in-vivo* interaction of bHLH149 with SPX1 under Pi starvation conditions and with PHO2 (see section 3.4.3) at Pi sufficient conditions (Isabel Mateos, PhD manuscript, 2010). The bHLH149 protein accumulation depends upon the phosphate content during growth and is regulated by SPX1 and PHO2. During normal phosphate supply, PHO2 would act as a negative regulator of bHLH149 protein activity by promoting its degradation, whereas during phosphate starvation, SPX1 would act as a positive regulator of its accumulation. In this way, low Pi levels have negative impact on plant growth (Isabel Mateos, PhD manuscript, 2010).

3.2 Role of SPX domain containing proteins in Pi signaling

Among the many and diverse proteins involved in the plant response to Pi starvation, proteins containing the SPX domain are key players controlling a set of processes involved in the maintenance of an internal steady state of Pi ions at the level of the cell, defined as Pi homeostasis (for review, see Secco *et al*., 2012). SPX domain is named after the Suppressor of yeast *gpa1* (Syg1), the yeast Phosphatase 81 (Pho81), and the human Xenotropic and polytropic retrovirus receptor 1 (XPR1). This hydrophilic domain is found at the N-termini of various proteins in all major eukaryotes, from *Caenorhabditis elegans* and Drosophila to mammals (Stefanovic *et al*., 2011). In yeast, proteins containing the SPX domain are involved in Pi transport and sensing, or the sorting of proteins to endomembranes (Wang *et al*., 2004). Most of the SPX-domain proteins with known functions in plants are involved in the regulation of either nutritional homeostasis or the response to environmental cues. Studies in yeast and *Arabidopsis* have also suggested that the SPX domain itself could be involved in the fine tuning of Pi transport and signaling

through mechanisms such as physical interactions with other proteins (Duan *et al*., 2008; Hurlimann *et al*., 2009; Zhou and Ni, 2010).

The Arabidopsis genome encodes 20 genes with the SPX domain, classified into four sub-families based on the presence of additional domains in their structure namely the SPX, SPX-EXS, SPX-MFS and SPX-RING families. Proteins exclusively harboring the SPX domain, referred to as SPX proteins constitute 4 and 6 members in *Arabidopsis* (AtSPX1-AtSPX4) and rice respectively (OsSPX1-OsSPX6) (Duan *et al*., 2008). All the SPX genes, with the exception of *AtSPX4* and *OsSPX4*, are highly induced by Pi starvation. In addition, studies in *Arabidopsis* showed that these responses were under the control of PHR1 and its closest family member PHL1 (Bustos *et al*., 2010).

3.2.1 SPX proteins have a broad range of subcellular localization. The SPX1 and SPX2 isoforms from both *Arabidopsis* and rice are targeted to the nucleus. While AtSPX3 is reported to be localized to unidentified cytoplasmic speckles, AtSPX4, OsSPX3, OsSPX4 and OsSPX5 are localized in nucleus and cytoplasm (Duan *et al*., 2008; Shi J *et al*., 2014; Lv Q *et. al*, 2014). The different sub-cellular localization of the SPX family proteins attributes to their diversified functions.

3.2.2 SPX-EXS proteins: The PHO1 family members are the only proteins in eukaryotes that contain both the SPX and EXS domains. PHO1, one of the well characterized members of this family is involved in regulation of Pi homeostasis in *Arabidopsis* (Wang *et al*., 2004). *PHO1* is expressed in roots and is involved in long distance Pi transport from root to shoot (Hamburger *et al*., 2002). PHO1 also plays a crucial role in Pi efflux out of cells to maintain intracellular Pi homeostasis (Stefanovic *et al*., 2007). Recently Khan *et al*., (2014) have demonstrated that PHO1 with its close homolog PHO1; H3 regulate transfer of Pi to shoot in response to Zn limitation providing a link between P and Zn homeostasis in *Arabidopsis*.

3.2.3 SPX-MFS sub-family proteins: The Major Facilitator Superfamily (MFS) represents the largest group of transport carriers in all organisms, which are often coupled to the movement of another ion. Proteins of this family can function as uniporters, symporters or antiporters, and have a diverse range of substrates, such as ions, sugars, nucleosides, amino acids and peptides. Based on the properties of the SPX and MFS domains, it has been

hypothesized that proteins harboring these two domains could be involved in both transport and signaling (Lin *et al*., 2010). This family includes *OsSPX-MFS1–OsSPX-MFS4* with *OsSPX1-MFS 1* and *OsSPX1-MFS 3* repressed by Pi starvation and *OsSPX-MFS2* induced by Pi starvation (Lin *et al*., 2010).

3.2.4 SPX-RING proteins: In both *Arabidopsis* and rice, only 2 proteins possess the RING and the SPX domains. To date, the only characterized member of the SPX-RING family is the Nitrogen Limitation Adaptation (*NLA- At1g02860*) gene (Peng *et al*., 2007), also called benzoic acid hypersensitive 1 (*BAH1*), for its role in the immune response (Yaeno & Iba, 2008). The *nla* mutant was first identified for its altered growth response on nitrogen (N) starvation, being unable to accumulate anthocyanin, resulting in an early senescence phenotype (Peng *et al*., 2007, 2008). A recent study has demonstrated the involvement of NLA (an E3 Ub ligase) in phosphate homeostasis wherein *nla* mutant showed increased Pi uptake and content leading to Pi toxicity, under low-nitrate and high-phosphate availability (Kant *et al*., 2011).

3.3 miRNA and non-coding RNAs in post transcriptional regulators of Pi signaling

microRNAs (miRNAs) are a small non-coding RNA molecules containing about 22 nucleotides found in plants, animals, and some viruses, which functions in RNA silencing and post-transcriptional regulation of gene expression (Ambros *et al*., 2004 and Bartel *et al*., 2004). miRNAs silence genes that have complementary or partially complementary sequences to the miRNAs by causing mRNA cleavage or translational repression (Ambros *et al*., 2004; Bartel *et al*., 2004 and He *et al*., 2004). In *Arabidopsis*, a limited number of miRNA molecules have been shown to be specifically and strongly induced by Pi limitation, including miR399, miR778, miR827, and miR2111 (Fuji *et al*., 2005; Hsieh *et al*., 2009; Pant *et al*., 2009). miR399 targets the transcript of *PHO2*, an E2 ubiquitin conjugase (Lin *et al*., 2008; Pant *et al*., 2008) thereby causing an increase in the expression of root Pi-uptake transporters (e.g. *PHT1;8 and PHT1;9*), and hence in the acquisition of Pi by the roots and its translocation to the shoot.

In addition to miR399 and *PHO2*, this particular Pi-signaling network also involves Induced by Pi Starvation *(IPS)* genes (Burleigh and Harrison, 1999; Liu *et al*., 1997). These

Pi starvation induced transcripts were characterized by an unusual feature in that they do not have long open reading frames; instead they contain a conserved 23-bp region complementary to miRNA399. *IPS* transcripts are thought to operate by a mechanism called 'target mimicry' to fine-tune the *PHO2*–miRNA399 pathway by inhibiting the action of miRNA399-charged silencing complexes on *PHO2* mRNA, and adjusting the transcript levels of *PHO2*, which is a key player in balancing Pi with respect to its supply and demand (Franco-Zorilla *et al*., 2007).

miR827, induced by low Pi regulates expression of *NLA* which is a positive regulator of nitrogen starvation responses (Kant *et al*., 2011). miRNA2111, specifically regulated by Pi starvation, was shown to target *At3g27150*, which encodes a kelch domain containing F-box protein (Hsieh *et al*., 2009). Suppression of miR395 during Pi limitation is suggested to up-regulate the expression of *APS4* (ATP sulfurylase) and *SULTR2;1* (Sulfate transporter) which could serve to increase sulfate translocation to meet the augmented demands of Sulfur towards sulfolipid synthesis during Pi starvation stress (Hsieh *et al*., 2009).

3.4 Post translational regulators

Post-translational modifications controlling Pi signaling include Phosphorylation, Ubiquitination and Sumoylation. Phosphorylation involves the addition of a covalently bound phosphate group to a serine, threonine or a tyrosine residue of a protein by a protein kinase. Ubiquitination and sumoylation of proteins involve the attachment of a small peptide, Ubiquitin or SUMO (small ubiquitin-like modifier) respectively, to proteins substrates (for review, see Rojas *et al*., 2014). These modifications either target proteins to degradation through the 26S proteosome or alter protein activity, localization or interaction abilities (Moon *et al*., 2004; Ulrich *et al*., 2005; Colby *et al*., 2006).

3.4.1 Protein Phosphorylation

Protein phosphorylation is a well-known type of posttranslational modification that is involved in the regulation of numerous cellular processes. Besides modulating protein activity, an important role of protein phosphorylation is to regulate protein targeting. For example, the Arabidopsis nitrate transporter NRT1.1 (Martin *et al*., 2008) and the aquaporin PIP2;1 (Prak *et al*., 2008) both require phosphorylation to reach the plasma

membrane. Protein phosphorylation *in-vivo* in the C-terminal region of the Pi transporters - PHT1;1 (Ser-514 and Ser-520) and PHT1;4 (Ser-524) has been reported by Nuhse *et al*., (2004) and Bayle *et al*., (2011). Phosphorylation of PHT1;1 at Ser-514 inhibits its trafficking to its destination, the plasma membrane under non-limiting Pi conditions (Bayle *et al*., 2011).

3.4.2 Sumoylation in Pi signaling

SIZ1 (At5g60410), a SUMO E3 ligase is a focal controller of Pi starvation-dependent responses. T-DNA insertional mutated alleles of *SIZ1* exhibits exaggerated prototypical Pi starvation responses, including cessation of primary root growth, extensive lateral root and root hair development, increase in root/shoot mass ratio, and greater anthocyanin accumulation. In line with this, PHR1 has been shown to be an SIZ1 sumoylation target *in-vitro* (Miura *et al*., 2005).

3.4.3 Ubiquitination pathway components in Pi signaling

PHO2, also known as Ubiquitin Conjugase 24 (UBC24) is a negative regulator of PSRs. *pho2* mutant accumulate excessive Pi in shoots leading to Pi toxicity accompanied by leaf senescence (Aung *et al*., 2006). PHO2 localizes to the post- endomembranes compartments (Golgi apparatus) where it triggers ubiquitination and degradation of its target (PHO1). As *PHO2* is targeted to degradation by miR399 during Pi starvation conditions (see section 3.3), it acts on PHO1 only during Pi sufficiency where PHO2 interacts with PHO1 at the post-endomembranes system. This leads to targeting of PHO1 to degradation via multi-vesicular body mediated vacuolar proteolysis and results in inhibition of Pi transport from root to shoot (Liu *et al*., 2012). This action of PHO2 on PHO1 is aimed to prevent accumulation of toxic levels of Pi in plants grown in Pi rich soils. However, whether or not PHO1 or bHLH149 (another potential target of PHO2 as mentioned in section 3.1.4) is directly ubiquitinated by PHO2 is yet to be explored.

In addition to PHO2, another component of Ubiquitination pathway controlling Pi homeostasis in plants is NLA (as already mentioned in section 3.2.4). Interestingly, the Pi overaccumulator *pho2* mutant displays enhanced Pi levels at growth conditions of low nitrate similar to the case of the *nla* mutant (Kant *et al*., 2011). It appears that as a default,

low N would result in increased accumulation of Pi and the NLA/PHO2 provides a mechanism for preventing excess Pi accumulation in conditions of low N.

One recent study showed that PHO2 and NLA act in independent but cooperative fashion to mediate degradation of PHT1 transporters (Lin *et al*., 2013). However, Park *et al*., (2014) demonstrated that NLA specifically requires PHO2 for poly-ubiquitination of PHT1;4 in *A.thaliana*. Additionally, post-translational decay of PHT1;4 at high Pi is inhibited by MG132 (proteosome inhibitor), indicating the participation of 26S proteosome. Consistent with NLA/PHO2 function in PHT1;4 degradation, co-expression of NLA and PHO2, which occurs at high Pi, causes a decrease in PHT1;4 levels (Park *et al*., 2014).

4. Local and systemic Pi sensing and signaling

In order to adapt to the heterogeneous nutrient availability in soils, plants have developed complex mechanisms that not only integrate information of Pi levels in the soil (local Pi sensing and signaling) but also considers Pi levels in the whole plant (systemic Pi signaling) (Zhang *et al*., 2014). Roots perceive fluctuations in extracellular nutrient levels and send signals to the shoot, via the xylem, as a warning of impending limitation in the supply of the particular nutrient. Shoots sense these root derived nutrient signals and send signals both to the shoot apices and roots, via the phloem, to adjust developmental processes and nutrient uptake (Lough and Lucas, 2006; Liu *et al*., 2009; Lucas *et al*., 2013). Local Pi sensing and signaling can initiate adjustments in root system architecture (RSA) to enhance Pi acquisition, whereas the systemic, or long distance signaling pathways act to regulate Pi uptake, mobilization and redistribution (Linkohr *et al*., 2002; López-Bucio *et al*., 2003; Svistoonoff *et al*., 2007; Thibaud *et al*., 2010; Chiou and Lin, 2011; Nagarajan and Smith, 2012). Pi itself, the phytohormones- auxin, ethylene, cytokinins, abscisic acid, gibberellins, and the strigolactones, along with sugars, miRNAs and Ca^{2+} have all been implicated in Pi local and systemic sensing and signaling pathways (Chiou and Lin, 2011).

5. Limitations of genetic analysis towards dissecting signaling pathways and potential strategies to overcome these limitations

Functional characterization of genes is a required step towards understanding its biological significance. It has benefited from the application of reverse genetics tools either

based on the knock out (T-DNA insertion mutant) as well as knock down (artificial microRNA) or on the use of constructs overexpressing a particular gene of interest. Despite having considerable information and numerous genetic tools, researchers working on the functional characterization of genes are often faced with problems caused by functional redundancy between members of large gene families (Coego *et al*., 2014). For instance, Transcription factors (TFs) are often part of large families, in which closely related members display functional redundancy, thereby hindering their precise characterization.

Overexpression of individual members of redundant TF families may be used as an approach to overcome redundancy problems; however, this strategy is hampered by the fact that ectopic overexpression of TFs often results in deleterious effects or it potentially causes off-target effects thus causing misleading phenotypes (Kasuga *et al*., 1999). To overcome this problem, chemically inducible conditional overexpression of TFs offers a potential useful solution. The possibility of conditional overexpression allows to control the time and place of overexpression of the desired gene thereby, helping in the assessment of gene-triggered effects.

5.1 TRANSPLANTA

Using the approach of conditional overexpression system, TRANSPLANTA consortium generated a collection of homozygous Arabidopsis TRANSPLANTA (TPT) lines, in which the expression of TFs encoded by full-length cDNAs in a Gateway-compatible pER8GW vector (Papdi *et al*., 2008) derived from the original pER8 vector (Zuo *et al*., 2000) is inducible by β–estradiol. Thus far, 1636 independent homozygous lines, representing an average of 2.6 lines for every TF, have been produced for the inducible expression of 634 TFs. The whole TF collection includes members of all TF families defined in AGRIS (http://arabidopsis.med.ohio-state.edu/AtTFDB), except for the small RKD, BRR-BPC, CAMTA, GeBP, GRF, PHD and WHIRLY families. The representation of TFs from each family ranges from 10 to 100%, although for most of the families over 30% of their members are present in the TRANSPLANTA collection. Moreover, some of the most important families including AP2/EREBP, ABI3/VP1, Alfin-like, BZR, C2C2 CO-like, DOF, YABBY, CCAAT, E2F-DP, G2-like, GRAS, HSF, MADS, MYB, NAC, RAV, TCP, WRKY and b–ZIP are represented with more than 50% of their members in the TRANSPLANTA collection (Coego *et al*., 2014). To

document the versatile applicability and usefulness of the TPT lines, β-estradiol induced proliferation of root hairs, dark-induced senescence, anthocyanin accumulation and dwarfism were confirmed in lines conditionally expressing full-length cDNAs encoding RHD6, WRKY22, MYB123/TT2 and MYB26, respectively, in agreement with previously reported phenotypes conferred by these TFs (Coego *et al*., 2014). This powerful resource of conditionally over-expressing TFs could also be used to identify novel regulators of Pi starvation signaling pathway.

6. Elemental profiles reflect plant adaptations to the environment

The majority of the elements that make up a plant, with the exception of carbon and oxygen, are obtained from soil through the roots. These soil derived elements are required for plant structure, metabolism, protein function, signaling, and proper osmotic and electrochemical potential (Baxter *et al*., 2009). The accumulation of a given element is a complex process controlled by a network of gene products critical for uptake, binding, transportation, and sequestration. Many of these genes and physiological processes affect more than one element. Therefore, to get a complete picture of the gene networks involved in the maintenance of a nutrient's homoeostasis, it would be necessary to simultaneously study as many of the elements contained in a cell, tissue or organism as possible. The complete elemental profile is termed as the ionome which includes both the mineral ions and the trace elements compositions and reflects the physiological state of an organism (Baxter *et al*., 2009).

6.1 Ionomics

The study of the ionome termed as Ionomics, is defined as the quantitative and simultaneous measurement of the elemental composition of living organisms and changes in this composition in response to physiological stimuli, developmental stage, and genetic modifications (salt *et al*., 2008). Ionomics requires the application of high-throughput elemental analysis techniques, together with the incorporation of Bioinformatics (Fig 2). For achieving successful elemental profiling, multi element analysis of plant samples as a rapid, robust, sensitive and precise analytical system needs to be established. Various techniques including flame atomic absorption spectroscopy and inductively coupled

plasma mass spectroscopy (ICP-MS) or inductively coupled plasma optical emission spectroscopy (ICP-OES) have been used for both single and multiple element analysis of plant samples. ICP-OES has the advantage of lower cost and simplicity over ICP-MS. Although ICP-OES is less sensitive than ICP-MS, some of this sensitivity is won back by the robustness of ICP-OES in more concentrated sample matrices. Whereas ICP-MS struggles with sample matrices with greater than 0.1% solids, ICP-OES can handle up to about 3% dissolved solids (Salt *et al*., 2008). To optimize for high-throughput, cost and precision, we chose to use ICP-OES as our analytical tool.

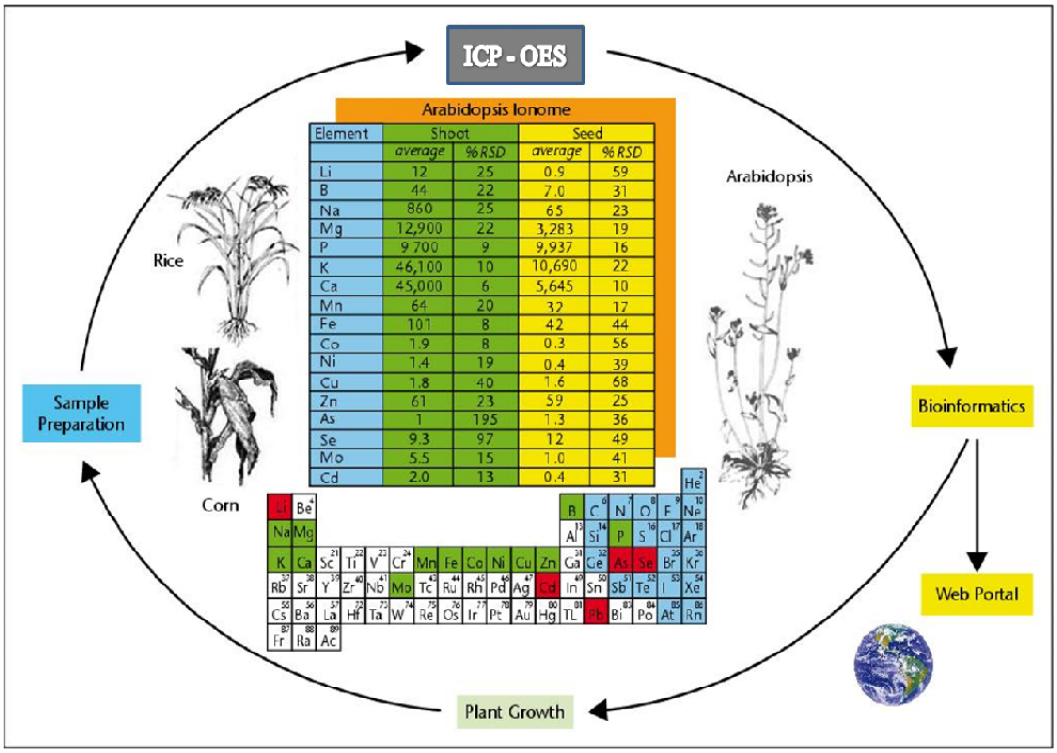

Fig 2: High throughput Ionomics *Picture modified from Salt et al., 2004.*
Diagram represents the steps involved in Ionomics. Elements in the periodic table highlighted in green are examples of essential elements for plants and those in red represents nonessential trace elements. The table represents Arabidopsis (Col-0) shoot and seed ionome, all elements presented as µg/g dry weight. %RSD refers to Relative Standard Deviation.

6.2 ICP-OES

The ICP-OES is composed of two parts: the ICP and the optical spectrometer. ICP is a type of plasma source that ionizes the analyte atoms for their detection by ICP-OES or ICP-MS. The plasma is generated by a silica torch that consists of 3 concentric quartz glass

tubes. The output or "work" coil of the radio frequency (RF) generator surrounds part of this quartz torch. Argon gas is typically used to create the plasma. Flowing Argon is introduced into the plasma torch and the radio frequency field ionizes the gas, making it electrically conductive. Plasma at up to 8000K, is insulated both electrically and thermally from the instrument, and maintained in position by a flow of coolant (argon) gas. The samples to be analyzed are digested with nitric acid at high temperature in a termostatized bath and pumped into the nebulizer via a peristaltic pump where it is converted into an aerosol, which passes into the spray chamber with the carrier argon gas. On introduction into the plasma, atoms in the sample are ionized, generally into singly charged positive ions. When the ionized analyte atoms in the ICP plasma fall back to ground state, they emit photons at wavelengths characteristic of a particular element. The resulting light from the plasma, representing a summation of emitted light from all the atoms introduced to the plasma is focused and passed through optical slits into a spectrophotometer. An optical fibre within the spectrophotometer separates the collected photons by wavelength, and a charge injection device (CID) detector simultaneously measures the intensities of photons at multiple wavelengths. By comparing these energy intensities to reference standards a quantitative measurement of each element in the sample can be obtained (Fig 3).

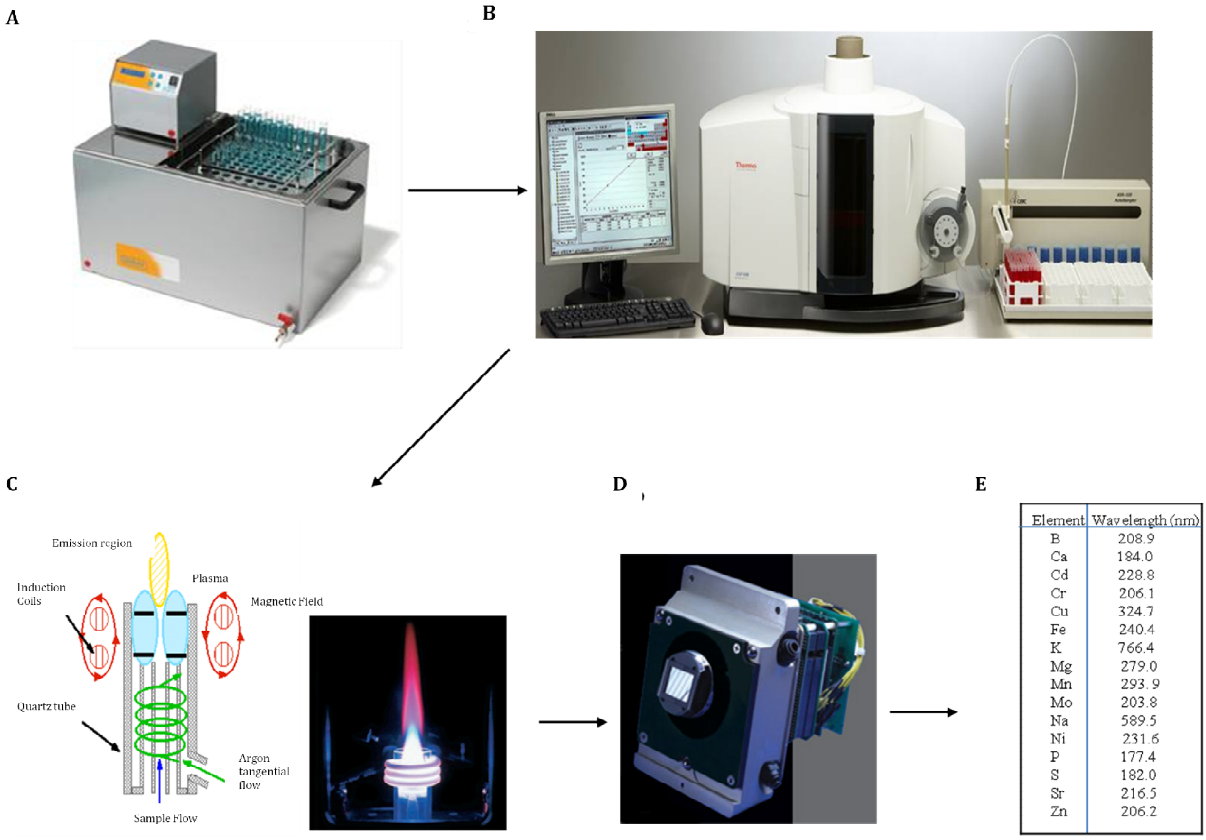

Fig 3: Schematic representation of the steps involved in ICP-OES

Samples are digested with HNO_3 at 100 ºC in a termostatized bath and are introduced to ICP-OES. On the plasma and at high temperature (8000 K), all elements get excited and emit energy as light at a characteristic wavelength. The CID (charge injection device) detector acts as a photographic plate that can acquire data for large parts of the spectrum simultaneously. Each element emits light energy at a characteristic wavelength. Intensity of the light emitted is directly proportional to its concentration.

OBJECTIVES

Considerable advances in the past two decades has made Pi starvation signaling one of the best studied system among those of different mineral nutrients in plants. However, there were many gaps in the knowledge of Pi starvation signaling, when this thesis was initiated. For instance, on the regulation of PHR1 activity, on the nature of the Pi sensor, or regarding the downstream targets of the PHO2-NLA ubiquitination system components, as well as on the transcriptional networks underlying Pi ion homeostasis and the complete TF set controlling these networks.

The objective of my PhD project was to contribute to the further understanding of the Pi starvation signaling pathway. Towards this, the following specific objectives were pursued:

1. Identification of novel TFs involved in Pi starvation signaling (Taking advantage of the TRANSPLANTA collection of transgenic plants conditionally over expressing TFs of *Arabidopsis thaliana*).
2. Identification of novel Pi signaling components interacting with PHR1. Sensor properties of SPX1, a Pi dependent inhibitor of PHR1.
3. New light on NLA (E3 ubiquitin ligase) and PHO2 (E2 ubiquitin conjugase) function in Pi signaling. Role in growth control and in a novel feed-back loop in Pi starvation signaling.

MATERIALS AND METHODS

1. Microbiological strains

Escherichia coli (E. coli) DH5α (Woodcock *et al*., 1989),

Escherichia coli DB3.1: (Invitrogen),

E. coli BL21pLysS (Novagen),

Agrobacterium tumefaciens C58C1 (Yanofsky & Nester, 1986) and

Yeast strain - *Saccharomyces cerevisae* AH109 (Clontech).

2. Plasmids

Vectors used for cloning: pDON201, pDON207 and pDON221 (Invitrogen)

Destination vectors for Y2H: pGADT7 and pGBKT7 (Clonetech).

Binary vectors: pGWB2, pGWB6, pGWB21 (Nakagawa *et al*., 2007),

pBHA (Parcy F., personal communication).

Vectors used for BiFC assay - pBiFP 1,2,3,4 (Azimzadeh *et al*., 2008).

3. Plant material

All *Arabidopsis thaliana* plants used in this study, including mutants and transgenic plants were on the ecotype Columbia (Col-0). Tobacco plants used for transient expression were *Nicotiana benthamiana*. T-DNA insertional mutants (Alonso *et al*., 2003) *spx1* (SALK-092030) and *spx2* (SALK-080503), *TF-182* (SALK_103716C) and *TF137* (salk_057190) were obtained from the Arabidopsis Biological Resource Center (ABRC) and the double mutant *spx1spx2* was obtained by crossing the single mutants *spx1* and *spx2* (Puga *et al*., 2014). Mutants *phr1* (Rubio *et al*., 2001), *pho2* (Delhaize y Randall, 1995) were available in laboratory, *nla* mutant (Peng *et al*., 2007) was kindly provided by Dr. Steve Rothstein (university of Guelph, Canada). Seeds of the transgenic plants over expressing TFs for elemental profile analysis were used from TRANSPLANTA (TPT) collection (Coego *et al*., 2014). Other transgenic lines used in this study were (PHR1pro)::PHR1-MYC, 35S::GFP-SPX1 (Puga *et al*., 2014), 35S::HA-PHR1 (Bustos *et al*., 2010), 35S::HA-bHLH149, 35S::HA-

bHLH149_*pho2* (Isabel Mateos, PhD manuscript, 2010). 35S::HA-bHLH149_*nla* and 35S::GFP-SPX1_*nla* transgenic lines were developed by crossing the respective overexpressing line with *nla* mutant .

4. Culture methods

4.1 Bacterial culture methods

Lysogeny broth (LB) medium was used for all the bacterial cultures (10 g/L Tryptone, 5g/L yeast extract and 10 g/L NaCl, pH7.0) (Sambrook *et al*., 1989). *E. coli* and *A. tumefaciens* were cultured at 37 ºC and 28 ºC respectively with agitation at 250 r.p.m (revolutions per minute). Concentration of antibiotics used include: ampicilin (100µg/mL), gentamicin (25 µg/mL), hygromycin (40 µg/mL), kanamycin (50 µg/mL), rifampicin (50 µg/mL), spectinomycin (50 µg/mL).

4.2 Yeast culture methods

For routine growth of AH109 *S.cerevisae* strain, YPAD (20 g/L peptone/tryptone, 10 g/L yeast extract, 40 % glucose, 40 mg/L adenine, pH 5.8) was used (Clontech Yeast Protocols Handbook). For the selective growth, Yeast nitrogen base (YSD) and YSD supplemented with different amino acid drop outs of clonetech (-WL, -WLA, -WLHA) were used.

5. Plant Growth conditions

Plants were grown in complete medium (+Pi) as described by Bates and Lynch, (1996) using one strength nutrient salts (Johnson *et al*., 1957). +Pi conditions involved 1mM KH_2PO_4. For the Pi deficient medium (−P), KH_2PO_4 was replaced by equimolar amounts of KCl_2. Seeds were surface sterilized using 75% bleach before plating and stratified at 4°C for 3 days. Growth chamber conditions were 22°C, 60% humidity, and 16-h light/8-h dark photoperiod with 100 µmols / m^2 s^1 of fluorescent light. Short day conditions had 8 h light/16h dark period.

For the phenotypic screening of TPT lines, seeds were directly germinated on Johnson one strength plates with 5µM β-estradiol. For the Elemental profile analysis, TPT

collection plants were initially grown for 8 days in Johnson one strength plates and then passed to 10μM β-estradiol supplemented conditions for next 6 days.

6. Binary constructs

The ORFs of SPX1, PHR1, NLA were amplified from corresponding fullsized cDNAs [SPX1, (ABRC clone 184E21); PHR1 (AJ310799), NLA (RAFL16-64-D14)] and cloned into pDONR201 (Invitrogen). The SPX1 and PHR1 entry clones were mobilized to destination vectors pBiFP 2 and pBiFP 3 to obtain translational fusions YFP^N-SPX1 and YFP^C-PHR1 under the control of the 35S promoter for BiFC assay. SPX1 and NLA entry clones were also mobilized to pGWB6 and pGWB21 respectively to obtain translational fusions of GFP (35S::GFP-SPX1) and MYC (35S:: MYC-NLA) under the control of the 35S promoter. The ORF of PHR1 was cloned to pBHA vector containing the 3×HA epitope (kindly provided by F. Parcy, Centre National de la Recherche Scientifique, Grenoble, France) to yield 35S::HA-PHR1. 35S::PHO2 was developed by Isabel Mateos (PhD Manuscript., 2010).

To prepare the PHRI promoter (PHR1pro)::PHR1-MYC construct, a DNA fragment containing six MYC repeats was obtained by BamHI/SalI digestion of the pGEM-6×MYC plasmid and cloned into pBIB. A 3,847-bp DNA fragment containing the PHR1 genomic region (including a 1,787 bp upstream of the first ATG) was PCR-amplified using Expand High Fidelity Polymerase (Roche) and primers gPHR1-F/gPHR1-R, digested with SalI, and cloned into the previously obtained pBIB-6×MYC vector.

7. Constructs and Yeast two hybrid (Y2H) assay

For the screening of NLA interactors, the entry clones of full length NLA and Δnla (lacking the RING domain) were mobilized to yeast destination vectors pGBKT7 to use as bait. PHR1, SPX1, PHO2, bHLH149 in pGADT7 (available in laboratory) were used as prey proteins. NLL-pGADT7 was developed by cloning ORF of NLL (U61217) to pDON201 and eventually mobilizing to destination vector, pGADT7.

For the screening of PHR1 interactors, a normalized *Arabidopsis* cDNA library was constructed from phosphate (Pi)-starved plants. Plants were grown for 7 d in complete medium and transferred to Pi-depleted media for 0.5, 1, 2, 4, and 7 d. Plants were mixed, and RNA was obtained with TriReagent (Ambion) and used to construct a normalized

cDNA library. Double-stranded cDNA (ds cDNA) was prepared using the Matchmaker protocol PT3529-1 (Clontech) with minor modifications. Normalization was as described by Sommer *et al*., (1990). After separation of single-stranded cDNA (ss cDNA) and ds cDNA on a hydroxyapatite column, normalized ss cDNA was converted into ds cDNA, cloned into pGADT7-Rec.

A PHR1 fragment (aa 208–362, ΔPHR1) lacking transactivation domains was fused to the Gal4 DNA-binding domain and used as bait to screen the yeast library for PHR1 interactors. A deletion series corresponding to SPX1 was generated for interaction assays with ΔPHR1 by amplification of full-length SPX1 cDNA with oligonucleotide pairs, as indicated in (Table M2), and was cloned to pGADT7.

Y2H experiments were done according to the Matchmaker GAL4 Two-Hybrid System (Clontech). Bait and Prey proteins co-transformed to AH109 (yeast strain). Positive clones were selected on media lacking tryptophan, leucine, histidine, and adenine and in the presence of 5 mM 3-amino-1,2,4-triazole, a competitive inhibitor of the product of the *HIS3* gene which is involved in histidine biosynthesis in *S. cerevisae*.

The list of the primers and the corresponding destination vectors used are shown in Table M1 and M2.

Primer name	Primer sequence (5'-3')	Application
NLA _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAAGTTTTGTAAGAAGTATG	To clone to pDON201
NLA_attB2	GGGGACCACTTTGTACAA GAAAGC TGGGTC TCATATTCCAGTGAAGCTTCGGCATTGG	To clone to pDON201
PHO2_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAATGTCCCTTACTGACTCTG	To clone to pDON201
PHO2-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATGATTCTGGTCCAATCTCTTG	To clone to pDON201
NLL_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAAGTTTGGTGAGACGTTCAC	To clone to pDON201
NLL_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATAACCGATCATATGCTTCG	To clone to pDON201
UBC8_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCTTCGAAACGGATCTT GA	To clone to pDON201
UBC8_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGCCCATGGCATACTTCTGAG	To clone to pDON201
TF-137 k.o_LP	ACATCAACACGGCCAAGTAAC	Genotyping TF-137 mutant
TF-137 k.o_R	TCTCTCCTCACTCCCTCCTTC	Genotyping TF-137 mutant
TF-182 k.o_F	AATATCCTGTATTCGTCGCCC	Genotyping TF-182 mutant
TF-182 k.o_R	ATGAACGGAAGTCATGCATTC	Genotyping TF-182 mutant

Table M1: List of primers used in NLA and TRANSPLANTA project

Primer Name	Primer Sequence (5'-3')	Application
SPX1-F	AGAGATAGAATTGCGAAAGC	Genotyping spx1-1 and spx1,spx2
SPX1-R	CTATTTGGCTTCTTGCTCC	Genotyping spx1-1 and spx1,spx2
SPX2-F	CACCATCAATCCTGTAACCAA	Genotyping spx2-1 and spx1,spx2
SPX2-R	TGGCCGGAGTCATTCGTCAT	Genotyping spx2-1 and spx1,spx2
SPX1.1F	CGAAAGCTAAGGATTCAATGGAG	spx1 TDNA; genotyping
SPX1.1R	GGATGAAAGGTAAACGCATGAG	spx1 TDNA; genotyping
SPX2.1F	ATGAAGTTCGGCAAGAGCCT	spx2 TDNA; genotyping
SPX2.1R	GCTCCATAAGCTTGAGCTTC	spx2 TDNA; genotyping
LB1	GCGTGGACCGCTTGCTGCAACT	Left border TDNA; genotyping
SPXB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGTTTGGTAAGAGTCTCAGC	ORF SPX1 in pDONR201
SPXB2-STOP	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATTTGGCTTCTTGCTCCAACAATGG	ORF SPX1 in pDONR201
SPX1-FL-F	CCCAGAATTCAAAAGAGCTATGAAGTTTG	Y2H
SPX1-FL-R	GTCGGATCCTCTATTTGGCTTCTTG	Y2H
SPX1-del1-F	CCCAGAATTCAAAAGAGCTATGAAGTTTG	Y2H
SPX1-del1-R	TAGGGATCCAAGGTTGCTAAAGAACTTTC	Y2H
SPX1-del2-F	CCCAGAATTCAAAAGAGCTATGAAGTTTG	Y2H
SPX1-del2-R	TCAGGATCCGCTAGGCAGCGATTGTG	Y2H
SPX1-del3-F	CAACGAATTCATGATCAGACTAAAGG	Y2H
SPX1-del3-R	GTCGGATCCTCTATTTGGCTTCTTG	Y2H
SPX1-del4-F	CTGGTGAATTCATGCGTTTACCT	Y2H
SPX1-del4-R	GTCGGATCCTCTATTTGGCTTCTTG	Y2H
P1BS 4X F	GAATTGAATATGCAATGGAATATGCTTAGGCATATTCCATAGAATATTCCTAGA	4xP1BS; Competitive Co-IP
P1BS 4X R	TCTAGGAATATTCTATGGAATATGCCTAAGCATATTCCATTGCATATTCAATTC	4xP1BS; Competitive Co-IP
mP1BS 4X F	GATTTTAAGCTGAAATGTAAGCTGATTAGTCATCGTACATATAAGCGTACTAGA	4xmP1BS; Competitive Co-IP
mP1BS 4X R	TCTAGTACGCTTATATGTACGATGACTAATCAGCTTACATTTCAGCTTAAAATC	4xmP1BS; Competitive Co-IP
P1BS 1x F	GTTATCCGCGGCAAAAGAAACTGTTAGAATATTCCTGA	P1BS; EMSA
P1BS 1x R	TCAGGAATATTCTAACAGTTTCTTTTGCCGCGGATAAC	P1BS; EMSA
dPHR1-B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGCGGTGGGGGAGGCATGGAATTGCG ACCTGTTAGCACAAC	deltaPHR1 in pDONOR201
dPHR1-B2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACCCTTTGGTAAGACCAGAGTTTTGC	deltaPHR1 in pDONOR201
PHT1;1-F	CCTCAACTCTCCAGAGAAGTTCTTA	qPCR
PHT1;1-R	TTCGGCCATTTCCTAGAGC	qPCR
SQD1-F	CATCCTCTAAACCAAAGCGTGT	qPCR
SQD1-R	AGTAGCCCAACCGCAATAAC	qPCR
ACP5-F	CAGTTTCTAACTAGTGGTGCTGGA	qPCR
ACP5-R	GCTTGGGATTGATGGTCACT	qPCR
ACT8-F	GACTCAGATCATGTTTGAGACCTTT	qPCR
ACT8-R	CCAGAGTCCAACACAATACCG	qPCR
ChIP-ACT8-F	CCCGCCTATATAAATAGTTCAACAC	ChIP-PCR
ChIP-ACT8-R	GACGACGAGGCAATTCAAAG	ChIP-PCR
ChIP-SPX-F	TCACCCACAGATAACCACGA	ChIP-PCR
ChIP-SPX-R	GGGGAAGAGGTTTAGAGATATAAAAG	ChIP-PCR
ChIP-IPS1-F	AAACTGAAAAGGCAATTTTGG	ChIP-PCR
ChIP-IPS1-R	AAGTGGAAGCAGATGATGGAA	ChIP-PCR
ChIP-PHT1-F	GCTTATGTTCTCGCGAATATCC	ChIP-PCR
ChIP-PHT1-R	CATTTGAGGAGTGACAATCAGG	ChIP-PCR
gPHR1-F	CAACGAAGATTACGAAGCTCGAAAGTACCG	PHR1pro:PHR1-6myc
gPHR1-R	AAAAGTCGACATTATCGATTTTGGGACGCTTTGGC	PHR1pro:PHR1-6myc
SPX1-GST-F	AAAGAATTCATGAAGTTTGGTAAGAGTCTCAGCAAT	GST-SPX1
SPX1-GST-R	AAAGTCGACCTATTTGGCTTCTTGCTCCAACAATGG	GST-SPX1

Table M2. Oligonucleotides used in SPX1 project.

8. Transformation methods

8.1 Bacterial transformation

Transformation of competent DB3.1 and DH5α *E. Coli* cells was carried out by heat-shock method as described in (Sambrook *et al*., 1989) or by electroporation as described in (Chassy and Flickinger, 1987). Transformation of competent C58C1 *A. tumefaciens* cells was performed as described in (Weigel and Glazebrook, 2002). Transformed *E. Coli* and *A. tumefaciens* cells were plated to selective media (LB with corresponding antibiotics) and incubated overnight at 37 ºC or 48 hours at 28 ºC, respectively.

8.2 Transformation of *Arabidopsis thaliana*

A. thaliana plants were grown in soil for 20-25 days in long-day growth conditions. Young inflorescences were infiltrated by inversion during 10 minutes with a suspension of *A.tumefaciens* carrying the binary construct of interest (floral dip method, Clough *et al*., 1998), in Murashige and Skoog (MS) medium (3.67 g of MS from Duchefa Bochemie per 1.5 L bidistilled water) supplemented with 5 % of sucrose and 0.02 % of the surfactant Silwet L77 (Bechtold *et al*., 1993). Seeds obtained were selected in Johnson medium supplemented with 50 µg/mL carbenicilin (used to inhibit *A. tumefaciens* growth) and the corresponding antibiotic for selection.

9. Transient expression assay

3 weeks old *N.benthamiana* plants were used for transient expression analysis. Agroinfiltration of the desired constructs was usually accompanied with P19, the suppressor of gene silencing (Voinnet *et al*., 2003) according to Sparkes *et al*., (2006). 3 days post agroinfiltration, samples were either used for western blot or confocal analysis.

10. RNA extractions, qRT-PCR and transcriptomic assays

For quantitative RT-PCR gene expression analysis, total RNA was extracted with TriReagent (Ambion) and treated with DNase I Turbo (Ambion). RNA (1 µg) was used for first-strand cDNA synthesis with the High-Capacity cDNA Archive Kit (Applied Biosystems), and a 1:10 dilution was used as a template for PCR amplification. Triplicate reactions were

carried out in an ABI7500 Real-Time PCR System thermocycler using SYBR Green PCR Master Mix (both from Applied Biosystems). Oligonucleotides used are listed in (Table M2).

For transcriptomic analyses, plant material from three independent replicates was obtained. RNA was extracted as indicated and purified with RNeasy Mini Kit columns (Qiagen). Total RNA (5 μg) was used to obtain biotinylated cRNA utilizing the 3′ Amplification One-Cycle Target Labeling Kit and for hybridization of Arabidopsis ATH1 arrays (Affymetrix). Arrays were hybridized, washed, stained, scanned, and analyzed as in the study by Bustos *et al*., (2010).

11. Protein Extraction and 2D Electrophoresis

Proteins were extracted in buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 0.1% Nonidet P-40, and 1× complete protease inhibitor (Roche) and centrifuged (16,000 × g, 4 °C, 30 min), and supernatants were collected. This step was repeated twice. Proteins were precipitated with 60% (wt/vol) trichloroacetic acid and resuspended in sample buffer. Samples were loaded directly on 10% SDS/PAGE for 1D separation or on Immobilized pH gradient (pH 3–5.5) strips for initial separation, followed by 10% (v/v) SDS/PAGE 2D electrophoresis. After separation, proteins were immunoblotted and detected with corresponding antibodies.

12. ChIP and PCR Amplification

ChIP assays were performed as described (Kaufmann *et al*., 2010). PHR1pro:PHR1-MYC and Col-0 plants were grown on +Pi medium for 7 d and transferred to +Pi or −Pi medium for 5 d; plants were then placed in +Pi or −Pi for 4 h. Seedlings (1.5–2 g) and 2.5 μL of anti-MYC antibody (Sigma) were used for ChIP. Precipitated DNA was dissolved in 30 μL of ultrapure H_2O, and 1 μL was used for quantitative PCR amplification as above. Amplicons corresponded to the promoter regions of the genes *IPS1, SPX1,* and *PHT1;1*, each spanning a PHR1 Binding Site. Relative enrichment of immunoprecipitated fragments of targets was compared with the immunoprecipitated fragment of non-target control (ACT8) in the same immunoprecipitation, as described by Zheng *et al*., (2009). Two biological replicates were analyzed.

13. Expression of Recombinant Proteins in *Escherichia coli*

SPX1 and ΔPHR1 were expressed in *Escherichia coli* as fusions with GST (Glutathione S-transferase) and maltose-binding protein (MBP) tags, respectively. To obtain a ΔPHR1 fragment fused to MBP, ΔPHR1-pENTR201 was transferred to pDEST-TH1 to yield MBP-ΔPHR1. Recombinant GST-tagged SPX1 was obtained by amplification of full length SPX1 and cloning in pGEX-4T-1 (GE Healthcare). For expression, *E. coli* cultures at OD 0.5 were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (18 °C, overnight). Recombinant proteins were purified using amylose resin (New England BioLabs) or glutathione Sepharose 4 Fast Flow (GE Healthcare).

14. Co-immunoprecipitation assays

To assay the interaction between PHR1 and SPX1 *in planta*, we crossed 35S::HA-PHR1 and 35S::GFP-SPX1 transgenic plants and analyzed the F2 generation. Plants were grown for 8 d in +P and −P conditions and cross-linked in 1% formaldehyde (30 min) to preserve in vivo interactions, and proteins were extracted with lysis buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.25% Nonidet, 5% (v/v) glycerol] supplemented with 1 mM PMSF and protease inhibitors (Roche). The soluble extract (400 μg) was used to immune-precipitate the HA-PHR1 protein using anti-HA Affinity Matrix (Roche; 4 °C, 2 h). GFP-SPX1 was detected with HRP–anti-GFP antibody (Miltenyi) and the SuperSignal Chemiluminescence kit (Pierce). Procedures were identical for co-immunoprecipitation of proteins expressed in *N. benthamiana*.

Competitive pull-down assays were performed with fixed amounts of MBP-ΔPHR1 and GST-SPX1 (1.5 pmol and 12.5 pmol, respectively) in binding buffer [10 mM Tris·HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5 mM EDTA, 0.05% Nonidet P-40, 2.5% (v/v) glycerol] in a total volume of 200 μL (4 °C, 2 h). A NaH_2PO_4 solution was added to pull-down buffer to a final concentration of 15 mM Pi, where indicated. Proteins were incubated with increasing amounts of a DNA fragment containing four PHR1-binding sites (4× P1BS: 0, 0.2, 0.5, 1.25, or 3 pmol). To examine the effect of anions other than Pi on the SPX1/PHR1 interaction using pull-down assays, all reactions included fixed amounts of MBP-ΔPHR1, GST-SPX1, and P1BS (1.5, 12.5, and 3 pmol, respectively). The control

reaction contained 50 mM NaCl in pull-down buffer; in other cases, 45 mM NaCl was replaced by 15 mM NaH_2PO_4, 15 mM NaH_2PO_3, 45 mM $NaNO_3$, and 22.5 mM Na_2SO_4. Proteins were pulled down with dextrin Sepharose resin and detected in immunoblots with anti-GST antibody (Sigma).

15. EMSA

MBP-ΔPHR1 (0.3 pmol) was assayed for binding to the 4× P1BS probe (0.1 pmol) in the presence of increasing concentrations of bacterially expressed GST-SPX1 (0, 0.6, 1.2, 2.5, and 5 pmol) in a total volume of 20 μL. The 4× P1BS probe was generated by annealing biotin-labeled semicomplementary primers, as described by Becker *et al*., (1990). For EMSA, we used the LightShift Chemiluminescent EMSA Kit (Pierce). A solution of NaH_2PO_4 was added to EMSA buffer to a final concentration of 0.1–15 mM Pi in the indicated samples, and 0.3–45 mM NaCl replaced 0.1–15 mM Pi in –P samples.

16. Microscopy

Confocal microscopy was performed as described by González *et al*., (2005) to detect protein/protein interactions using bimolecular complementation assays in agroinfiltrated *N. benthamiana* leaves and for analysis of GFP-SPX1 localization in *Arabidopsis*.

17. Elemental profile analysis

Plant material was analyzed for Ionomic profiling (at Timac Agro, Pamplona) following Lahner *et al*., (2003) and Baxter *et al*., (2008) with some minimal variations. Briefly, weighed Shoot samples of TPT lines along with Col-0 were dried at 65°C for 2 days. Digestion was carried out in around 40 mg dry weight with 3.5 mL of HNO_3 (Sigma-Aldrich Trace Metal grade) at 100 ºC in a termostatized bath for 4 h. Each sample was diluted to 7 mL with 18 MΩ water.

Samples were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) on a Thermo Scientific iCAP 6500. On the plasma and at high temperature (8000 K) all elements are excited and emit energy as light at a characteristic wavelength. The CID detector acts as a photographic plate that can acquire data for large

parts of the spectrum simultaneously. A number of elements in one determination for each sample are obtained. For ionomic analysis these wavelengths were used for each element: B, 208.9 nm; Ca, 184.0 nm; Ca, 317.9 nm; Cd, 228.8 nm; Cr, 206. 1 nm; Cu, 324.7 nm; Fe, 240.4 nm; Fe, 259.9 nm; K, 766.4 nm; Mg, 279.0 nm; Mg, 285.2 nm; Mn, 293. 9 nm; Mo, 203.8 nm; Na, 589.5 nm; Ni, 231.6 nm; P, 177.4 nm; P, 178.2 nm; S, 182.0 nm; Sr, 216.5 nm; Zn, 206.2 nm.

18. Physiological assays

Cellular Pi levels were determined in 12 day-old seedlings grown under different Pi conditions in solid media, as described in (Ames, 1966). Anthocyanin content was measured as described (Swain *et al*., 1959). Mean values were compared using the Student t-test.

19. Accession numbers

PHR1 (At4g28610), *SPX1 (At5g20150)*, *SPX2 (At2g26660)*, *PHO2 (At2g33770)*, *bHLH149 (At1g09250)*, *NLA (At1g02860)*, *NLL (At2g38920)*, *TF-117 (At2g40340)*, *TF-137 (At1g68640)*, *TF-182 (At2g24430)*, *TF-220 (At1g71450)*, *TF-252 (At1g70510)*.

RESULTS

1. Identification of Novel TFs involved in Pi starvation signaling

1.1 Screening of TRANSPLANTA lines for identification of Pi starvation signaling related TFs

To identify novel TFs involved in the Pi starvation signaling pathway, we decided to screen the collection of transgenic lines conditionally overexpressing TFs of *Arabidopsis thaliana*, developed in the TRANSPLANTA (TPT) project. We carried out visual phenotypic screens of the TPT lines by directly seeding on +Pi and –Pi media supplemented with β-estradiol to induce the transgene overexpression. Pi regime media without β-estradiol was used as control to check for any off-target effects.

For the visual phenotypic screening, we focused mainly on 2 criteria, namely changes in root architecture and anthocyanin pigment accumulation of TF candidate genes compared to wild type (WT) Col-0. Out of 560 overexpressing lines corresponding to 280 TFs screened in Pi regime conditions, only 2 TFs (*TCP16* and *NAC1*) overexpression displayed reproducible phenotypic alterations compared to WT.

1.1.1 *OxTF-TCP16 (OxAt3g45140)* and *OxTF-NAC1* (*OxAt1g01010)* plants display reduced growth in Pi sufficient conditions.

When grown along with WT under +Pi conditions, *OxTF-TCP16* and *OxTF-NAC1* displayed reduced growth accompanied with leaf chlorosis; this effect was highly pronounced in the case of *OxTF-NAC1* transgenic plants (Fig 4 and 5). This growth impairment effect was alleviated at Pi deficiency conditions in both *OxTF-TCP16* and *OxTF-NAC1* transgenic plants (Fig 4 and 5). To check whether the leaf chlorosis at high Pi growth conditions was caused by the accumulation of high phosphate levels, we quantified the shoot phosphate levels. *OxTF-NAC1* had increased Pi levels (40%) in comparison to wild type plants, whereas *TCP16* overexpression had no significant effect on Pi levels (Fig 6).

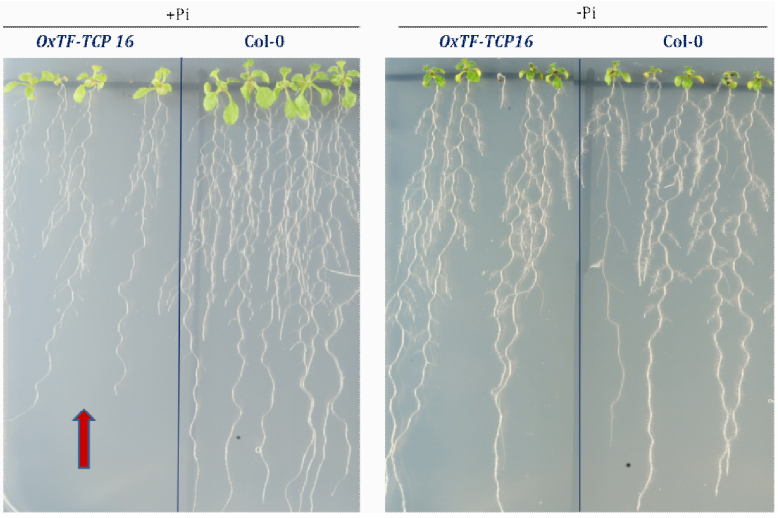

Fig 4: *OxTF-TCP 16* (*OxAt3g45150*) plants display reduced growth in Pi sufficiency

Col-0 and *OxTF-TCP16* plants were grown on +Pi and –Pi medium supplemented with β-estradiol for 12 days. Arrow in red points to the *OxTF-TCP16*.

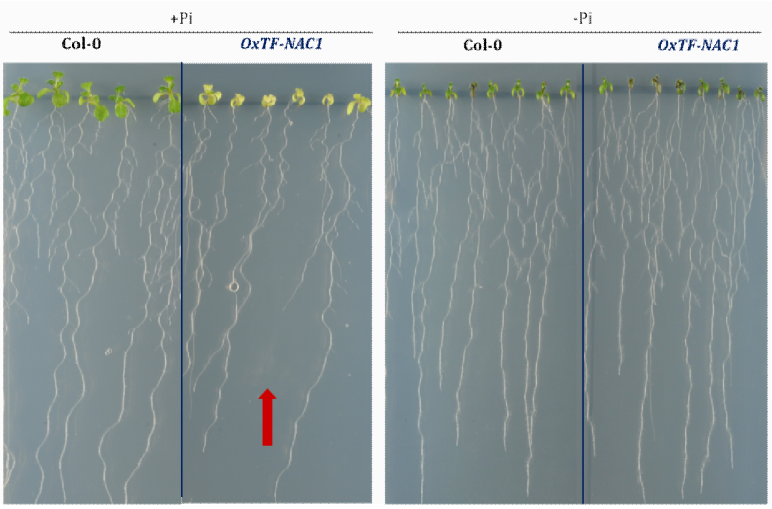

Fig 5: *OxTF-NAC1* (*OxAt1g01010*) display reduced growth in +Pi

Col-0 and *OxTF-NAC1* plants were grown on +Pi and –Pi medium supplemented with β-estradiol for 12 days. Arrow in red points to the *OxTF-NAC1*.

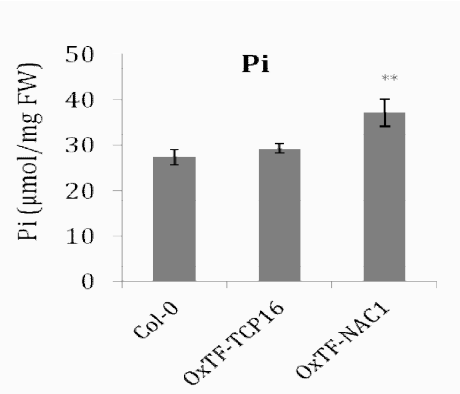

Fig 6: Pi levels of the *OxTF-TCP16* and *OxTF-NAC1*
Col-0, *OxTF-TCP* and *OxTF-NAC1* plants were grown on +Pi medium supplemented with β-estradiol for 12 days. Data represents average of 3 biological replicates. ** indicate significance difference (P ≤0.01) according to student t-test.

Thus, this phenotypic screening rendered only 1 TF whose overexpression effect reflects a potential role of the TF in Pi homeostasis; the potential role in Pi signaling of the

second TF whose overexpression does not alter Pi levels is still uncertain. As an alternative approach, we decided to continue the search for novel TFs of Pi starvation signaling, based on the ionomic analysis.

1.2 Elemental profile analysis

Towards the large scale elemental profile analysis of the TPT lines conditionally overexpressing TFs, we used ICP-OES (performed at TIMAC AGRO facilities (www.timacagro.es). In order to obtain sufficient material for analysis and to avoid the agar contamination from the plant growth media, we decided to analyse the shoots for the elemental profile measurement.

Out of the 450 TFs (each with 1-3 overexpressing independent lines) provided by TRANSPLANTA consortium, we selected one independent line for 320 TFs for shoot ionome analysis. Dried shoot samples (see Methods section-17) of the selected TPT lines, grown in +Pi medium supplemented with β-estradiol were used for shoot ionome quantification. We measured the amounts of 16 elements namely B, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, Sr, and Zn. Shoot ionomics was performed in batches of 14 TFs along with Col-0, to feed the system with internal controls. As most of the candidates are expected to have elemental profiles similar to the Col-0, experiment execution in batches allows to robustly define the average levels of each element. Of the 16 elements measured, we observed that in general, B, Cr and Ni had high standard deviation with lower sensitivity. Therefore, these elements were not considered for analysis.

Out of the 320 TFs expressing transgenic TPT lines analyzed, overexpression of 34 TFs showed significant alterations in its ionomic profile compared to Col-0. The ratio of the total number of TFs to that of the sum of the number of elements altered in these TFs (34/87) in Table1 indicates that ionomic alterations generally concern many elements (approximately 3 elements as average). Indeed, overexpression of only 14 TFs had alterations in a single element.

Number of TF overexpressors altered in the composition of different elements													
Element	Ca	Cd	Cu	Fe	K	Mg	Mn	Mo	Na	**P**	S	Sr	Zn
No. of TFs	6	3	2	18	3	7	16	8	5	**5**	2	2	10

Table 1: Number of TFs whose overexpression results in alterations in the levels of each element

Giving emphasis to P element, we selected the following TFs whose overexpression had alterations in P levels, for further analysis (Table 2).

TF Description	Gene locus	TF label in graph
DREB 2C	At2g40340	TF-117
bZIP TF (PERIANTIA)	At1g68640	TF-137
ANAC 38	At2g24430	TF-182
Member of a DREB TF	At1g71450	TF-220
KNAT2	At1g70510	TF - 252

Table 2: Overexpression of 5 TFs significantly altered P levels.

The ionomic profiles of the selected five TFs overexpressing TPT lines are shown for each element in Fig 7 (left bars). Following the 1st round of ionomic profile analysis of the TPT lines, we decided to repeat the elemental profile analysis of these 5 TFs overexpressing TPT lines (which showed altered Pi levels), to check for data reproducibility. For this second round of ionomic analysis, we used all the independent overexpressing transgenic lines of these 5 TFs available in the TPT collection. Only one line was available for *TF-117* and *TF-182*; two lines for *TF-137* and 3 independent overexpressing lines for each of *TF-220* and *TF-252*. However, we observed that only one overexpressing TPT line per TF, showed altered elemental profile identical to the 1stscreening, while the other lines/TF had elemental levels similar to Col-0. The line that showed alteration is represented by right vertical bars for each element in (Fig 7).

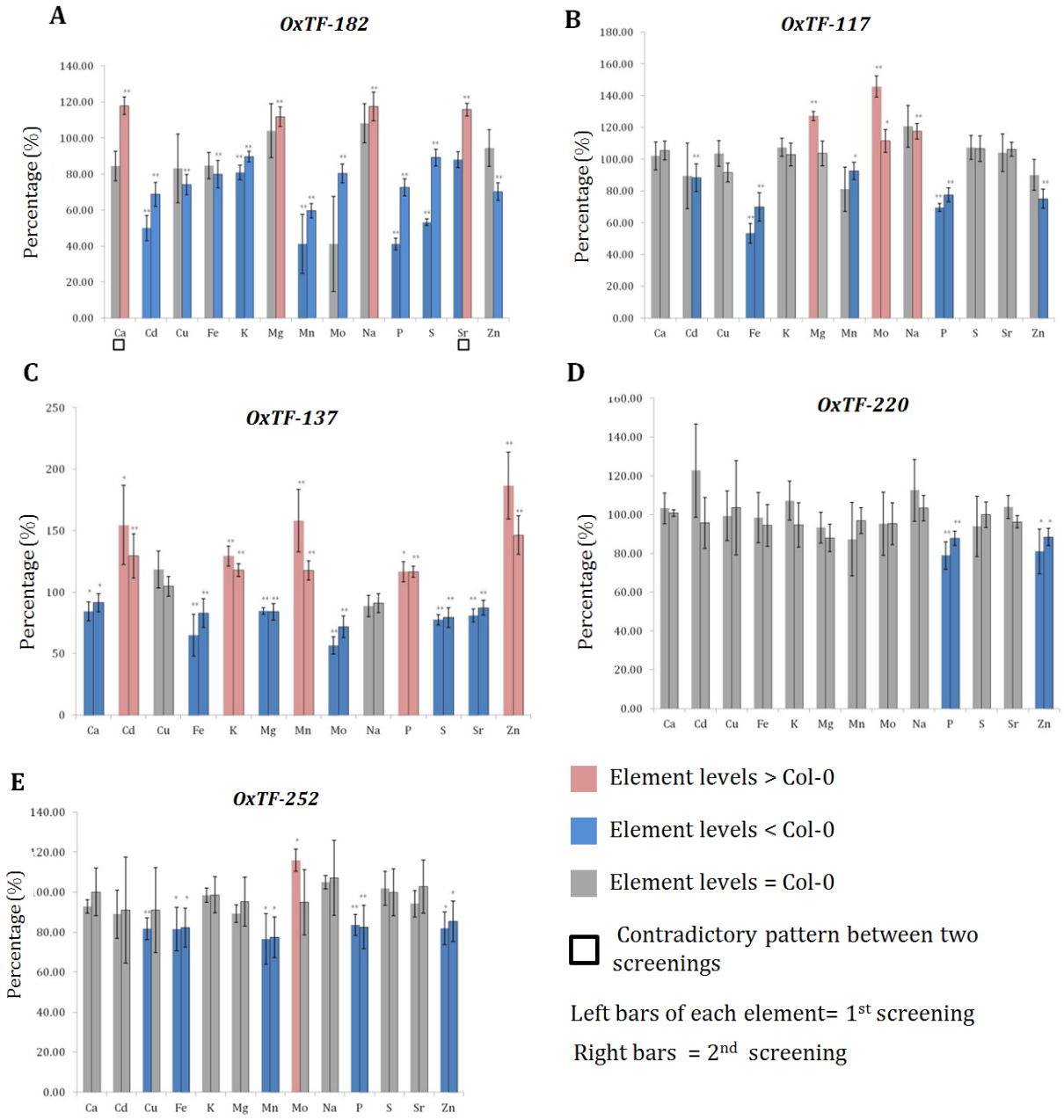

Fig 7: Ionomic profile pattern of *OxTF-117*, *OxTF-137*, *OxTF-182*, *OxTF-220* and *OxTF-252*. The bars represented in the graph are normalized to Col-0. X-axis corresponds to different elements quantified and the Y-axis represents the percentage level of each element relative to Col-0. Values for each TF represents the average of 4 independent biological replicates in the case of 1st screening (left bars) and 8 biological replicates in the case of 2nd screening (right bars). Significant difference of $P \leq 0.05$ calculated according to Student t-test is represented by single * symbol and P value ≤ 0.01 is represented by ** symbol.

In order to check whether the observed ionomic alterations were specific to TF overexpression (i.e, dependent on β-estradiol treatment) or were due to some off-target

effects, we checked the effect of β-estradiol on intracellular Pi levels in lines displaying alterations in ionomic profiles corresponding to *TF-117, TF-137* and *TF-182*; we had no sufficient seeds for analysis of *TF-220* and *TF-252*. While β-estradiol had no significant effect on Col-0 Pi levels, in the TPT lines alterations in Pi levels were β-estradiol dependent proving that altered ionomic profiles obtained in candidate TF genes were due to transgene overexpression (Fig 8).

We observed a good level of concordance in the pattern of elements profile between the 2 rounds of ionomics screening. For instance, *OxTF-137* showed exactly similar ionomics profile in both rounds of screening (Fig 7C) and among these, only in the case of *OxTF-182*, for unknown reasons, Ca and Sr accumulated levels were in opposite direction between the two screenings (marked in Fig 7A with □ symbol). On comparing the 2 rounds of the ionomic profiling results of the candidate TFs overexpressors, we noticed that the statistical significance of the 2nd screening was generally higher compared to the 1st screening, possibly due to the fact that larger numbers of replicates were analyzed.

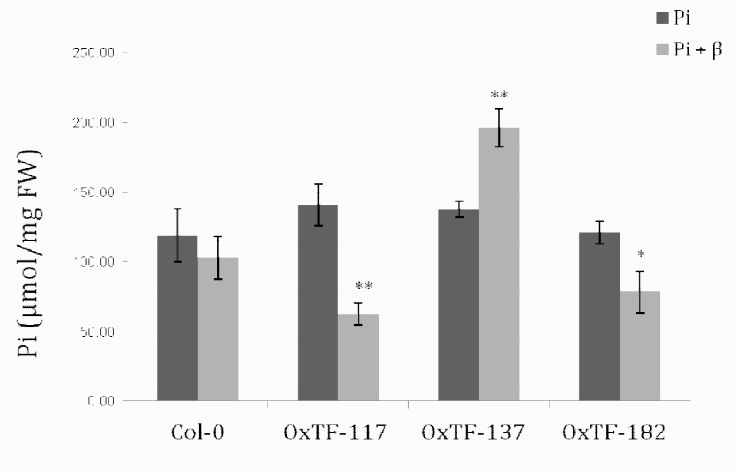

Fig 8: Alterations in Pi levels of the selected TPT lines is dependent on β-estradiol treatment

Overexpressing lines of *TF-117, TF-137* and *TF-182* along with Col-0 were grown in +Pi medium supplemented with or without β-estradiol. Data represents mean ± S.D of 3 biological replicates. Based on the student's t-test calculation, $P \leq 0.05$ is marked with * symbol and $P \leq 0.01$ is marked with ** symbol

In addition to the TFs shown above, we also found a candidate TF (described here as *TF-113 – ANAC30*) whose overexpression resulted in reduced accumulation in all elements examined (Fig 9B). However, *TF-113* overexpression had deleterious effect on plant growth leading to premature death (Fig 9A). Thus alteration of ionomic profile was likely due to plant mis functioning. Therefore, this line was not considered for further analysis.

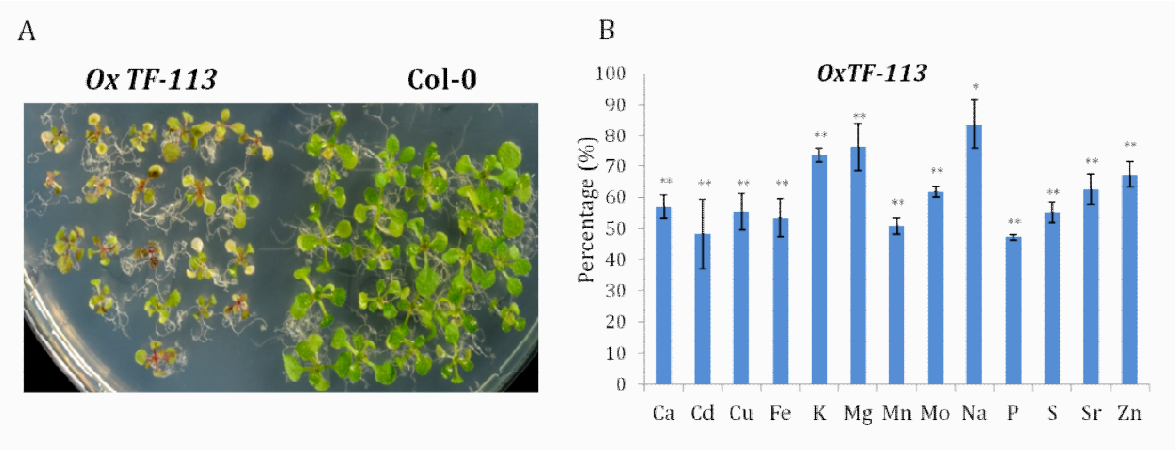

Fig 9: *OxTF-113* showed symptoms of lethality

A) Phenotype displayed by *OxTF-113*. Transgenic plants overexpressing *TF-113* and Col-0 were grown on +Pi medium supplemented with β-estradiol for 12 days.

B) Ionomic profile of *OxTF-113*. Values represented here are normalized to Col-0. ** indicate significance difference ($P \leq 0.01$) according to student t-test, n=4.

Unaltered Pi levels in knock out mutants of two selected TF candidates

Following the shoot ionomics analysis of the selected TFs overexpressing TPT lines, we wanted to find whether Pi levels were also affected in these selected TF mutant plants. Towards this, we searched for mutants in stock centres and found k.o. mutants for 4 TFs (*TF-137*, *TF-182, TF-220 and TF-252*). However, due to time restrictions, we focused on mutants for two TFs (*TF-137* and *TF-182)* that corresponded to Pi starvation inducible genes (Bustos *et al., 2010*). We quantified free intracellular Pi level of the TFs overexpressors (*OxTF-137* and *OxTF-182*) and their corresponding knock out mutant (*137* k.o. and *182* k.o.) compared to Col-0. Consistent with our previous results of shoot ionomics analysis (Fig 7A, C), overexpression of *TF-137* resulted in increased Pi and that of *TF-182* overexpression lead to decreased Pi levels. However, the k.o. mutants of these 2 TFs had Pi levels similar to Col-0 (Fig10). These results showing undisturbed Pi content in k.o. mutants could reflect functional redundancy.

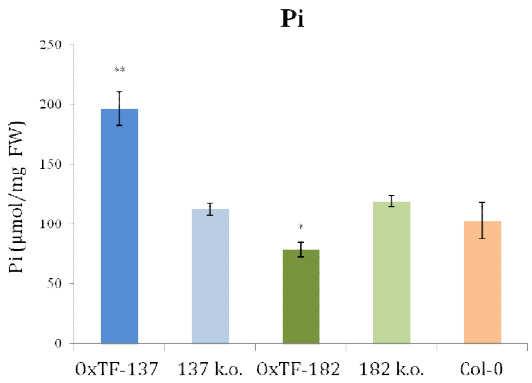

Fig 10) Pi levels of overexpressing lines and knock out mutants of selected TFs compared to Col-0. Data represents mean ± S.D of 3 biological replicates. Plants were grown for 12 days in +Pi medium, (supplemented with β-estradiol in the case of *OxTF-137* and *OxTF-182*). Based on the student's t-test calculation, $P \leq 0.05$ is marked with * symbol and $P \leq 0.01$ is marked with ** symbol.

2. Identification of novel Pi signaling components interacting with PHR1. Sensor properties of SPX1, a Pi dependent inhibitor of PHR1

2.1 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 (Rubio *et al*., 2001). We screened a normalized yeast two-hybrid cDNA library, using as bait a truncated derivative of PHR1 (ΔPHR1, amino acids 208-362) that lacks transcription activation domains. One candidate PHR1 partner was SPX1 (Fig. 11A), described as a nuclear protein involved in Pi signaling (Duan *et al*., 2008), which has an SPX domain also present in yeast Pi sensors. We determined that SPX1 interacts with PHR1 *in planta* using a co-immunoprecipitation (co-IP) assay in *Nicotiana benthamiana* plants (Fig. 11B). 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. 11C). 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. 11A).

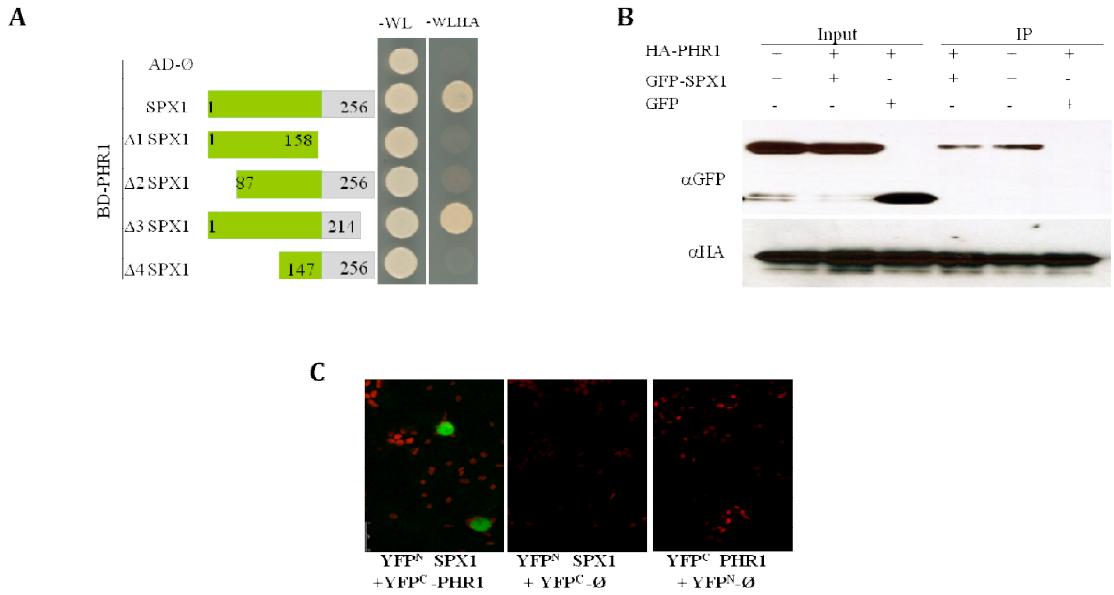

Fig 11: PHR1 interacts with SPX1

A. SPX1 interacts with PHR1 in yeast. The interaction between ΔPHR1 and SPX1 or its deletion derivatives is indicated by yeast growth in selective media lacking tryptophan, as well as leucine (−WL) and leucine, histidine, and adenine (−WLHA). AD, GAL4 activation domain; BD, GAL4 DNA-binding domain.

B. Co-immunoprecipitation of GFP-SPX1 and HA-PHR1. *Nicotiana 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 blot with anti-HA and GFP antibodies.

C. 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 co-express the YFP^N-SPX1 and YFP^C-PHR1 constructs. Bars=10μm.

2.2 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 (Duan *et al*., 2008). We identified single *spx1* and *spx2* mutants in the Salk collection (Alonso *et al*., 2003) and used them to generate a double mutant. We also generated transgenic plants that over-expressed 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 over-expressing lines (Fig. 12A). 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. 12A). 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. 12A).

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. 12B, C). 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 OxGFP-SPX1 plants compared to WT in all Pi regimens except the highest (2mM). Significant alterations in root hair number and/or length (local Pi-controlled responses, Bates *et al*., 1996) compared with WT plants were detected in *spx1spx2* in both +Pi and –Pi, and in the SPX1-over-expressing line when grown in –P (Fig 12C). It is noteworthy that in +Pi conditions, the *spx1spx2* mutant showed reduced root hair size relative to WT plants. This 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) 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 examining two Pi starvation time points (10 and 20 d; Fig. 12D). 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.

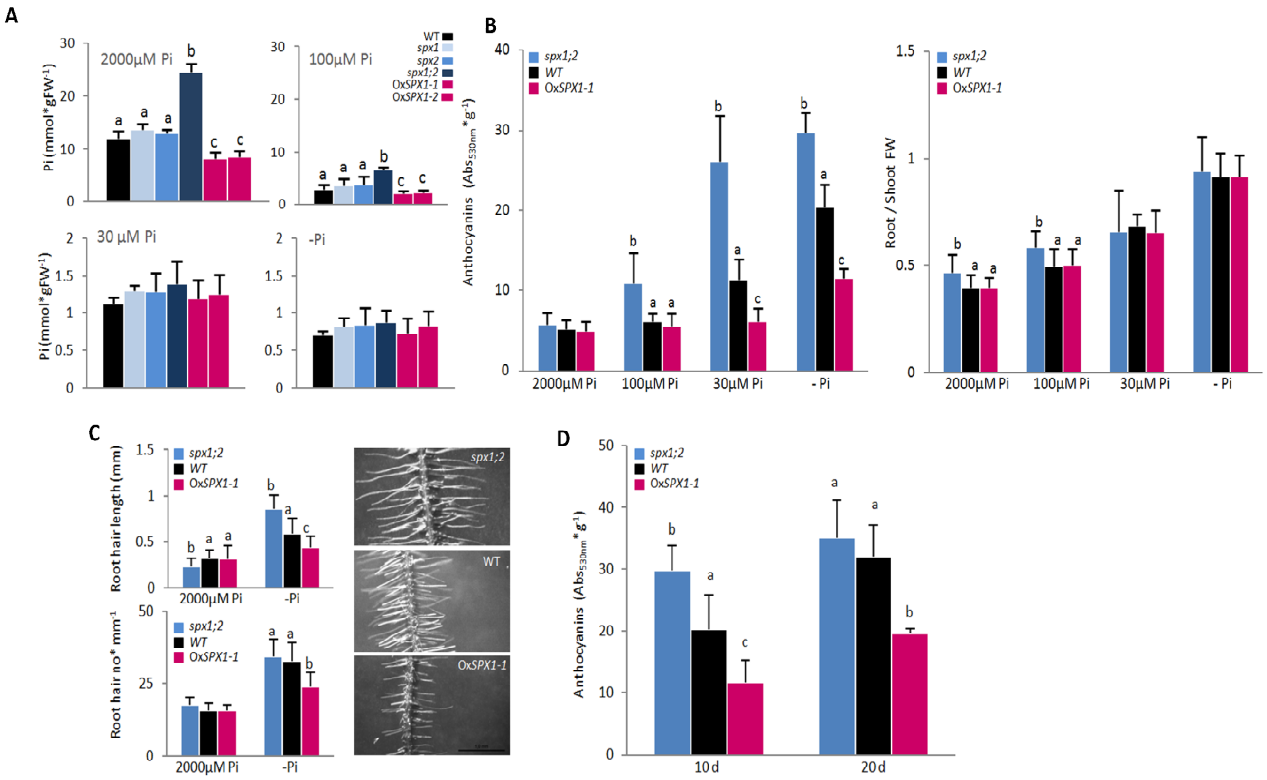

Fig 12: Effects of altering SPX1 and SPX2 activity in physiological/morphological responses to Pi starvation.

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.

B. Histograms of anthocyanin content and of root-to-shoot fresh weight (FW) ratio of WT and *spx1spx2* double mutant plants and an OxGFP-SPX1 transgenic plant, all grown in four Pi regimens (2,000, 100, and 30 µM and −Pi) for 10 d.

C. Histograms of root hair length and number in WT, *spx1spx2*, and OxGFP-SPX1 plants grown in the +Pi (2 mM) or −Pi conditions (Left), and a detail showing root hairs of these genotypes grown in −Pi conditions (Right).

D. Anthocyanin content of WT, *spx1spx2*, and OxGFP-SPX1 plants grown in −P conditions and harvested at two different times after germination (10 and 20 d).

In all cases, data show mean ± SD (n = 3). Shared or different letters above bars indicate non significant and significant differences between groups ($P < 0.05$) according to Student t tests.

To determine the effects 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 that *SPX1* and *SPX2* are Pi starvation-inducible, whereas most physiological effects of altering their activity require Pi (Fig. 12), we also analyzed transcriptomes of Pi-starved plants after short-term Pi refeeding. Results showed marked Pi dependence of the *spx1spx2* effects (Fig. 13 and Table 3; microarray data has been deposited in the Gene Expression omnibus database, www.ncbi.nlm.nih.gov/geo; accession number GSE52046). 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, 2-fold cut-off, FDR ≤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; this 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*., (2010), 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 (Fig 13).

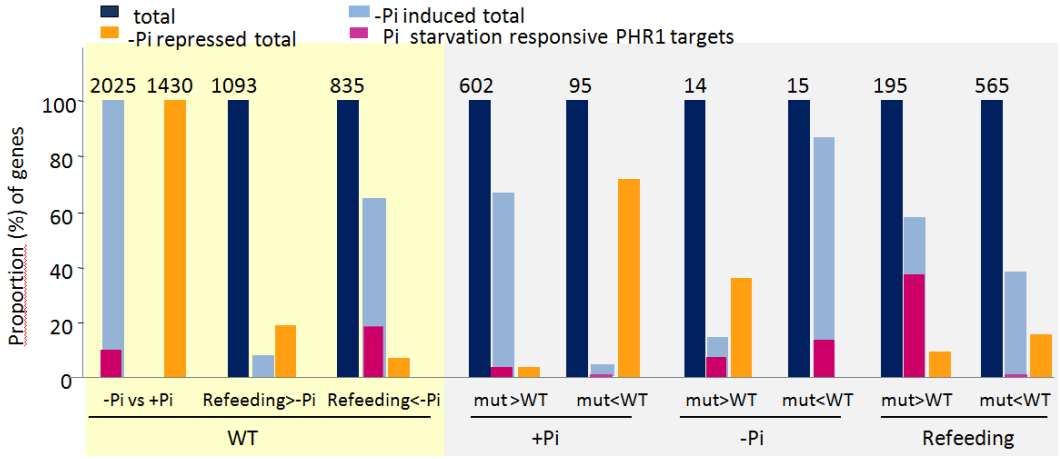

Fig 13: Diagram showing transcriptomic analysis of the effect of Pi growth conditions on

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×cut-off; 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*., 2010). Three biological replicates were analyzed.

We compared our transcriptomic data with the data of Thibaud *et al*.,(2010), which dissected systemically and locally controlled molecular responses to Pi (Table 3). 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*., (2010) 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*., (2010) and our study, such that 85 of the 110 systemically controlled induced (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 downregulated in the *spx1spx2* double mutant. This 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.

		WT −P		WT refeeding		*spx1;2* +P		*spx1;2* refeeding	
	Total genes	Ind (2025)	Rep (1430)	Ind (1093)	Rep (835)	Up (602)	Down (95)	Up (195)	Down (565)
Ind. S	110	85	2	2	60	24	0	42	4
Ind. L	301	181	6	15	53	74	1	4	32
Rep. S	111	16	19	16	6	6	2	1	1
Rep. L	238	61	19	13	10	0	4	19	3

Table 3: Effect of *spx1spx2* on systemically and locally controlled transcriptomic responses to Pi starvation: Numbers in the second column (Total genes) indicate the number of genes that are

systemically controlled Pi starvation-induced (Ind. S) or -repressed (Rep. S) and locally controlled Pi starvation-induced (Ind. L) or -repressed (Rep. L) genes in the study of Thibaud et al., (2010). The remaining columns indicate, out of these total genes in Thibaud et al., (2010) gene sets, the number of genes that coincide with the genes in the groups identified in this study: induced and repressed by Pi starvation (Ind. –P and Rep. –P, respectively) or after Pi refeeding (Ind. refeeding and Rep. refeeding, respectively) in wild type plants (WT), and upregulated or downregulated in Pi sufficient conditions (Up +P and Down +P, respectively) or after refeeding of *spx1spx2* plants compared with WT plants (Up refeeding and Down refeeding, respectively). The number of genes for each set of our study is shown in parentheses.

2.3 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 SPX1/PHR1 interaction itself, using Co-IP experiments in transgenic plants that co-expressed HA-PHR1 and GFP-SPX1 grown in +Pi and −Pi conditions. The SPX1/PHR1 interaction was detected only in +Pi conditions (Fig. 14A). 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. 14B). Because PHR1 is also constitutively located in the nucleus (Rubio *et al*., 2001), 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.

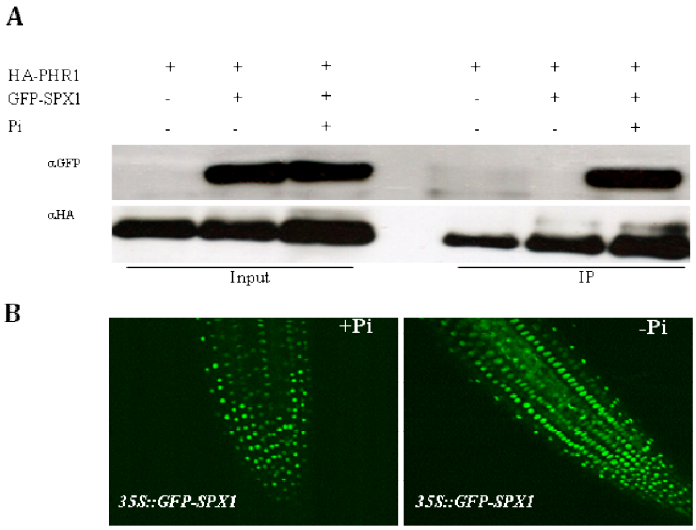

Fig 14: Cellular Pi-dependent interaction between SPX1 and PHR1 *in planta*.

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 (Serino *et al*., 2003). 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.

2.4 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 (Bustos *et al*., 2010). 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. 15). 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. 15). These results point to the first 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. 15), a direct Pi effect on SPX1 is sufficient to explain the reduction observed in PHR1 binding to its targets in Pi-refed plants.

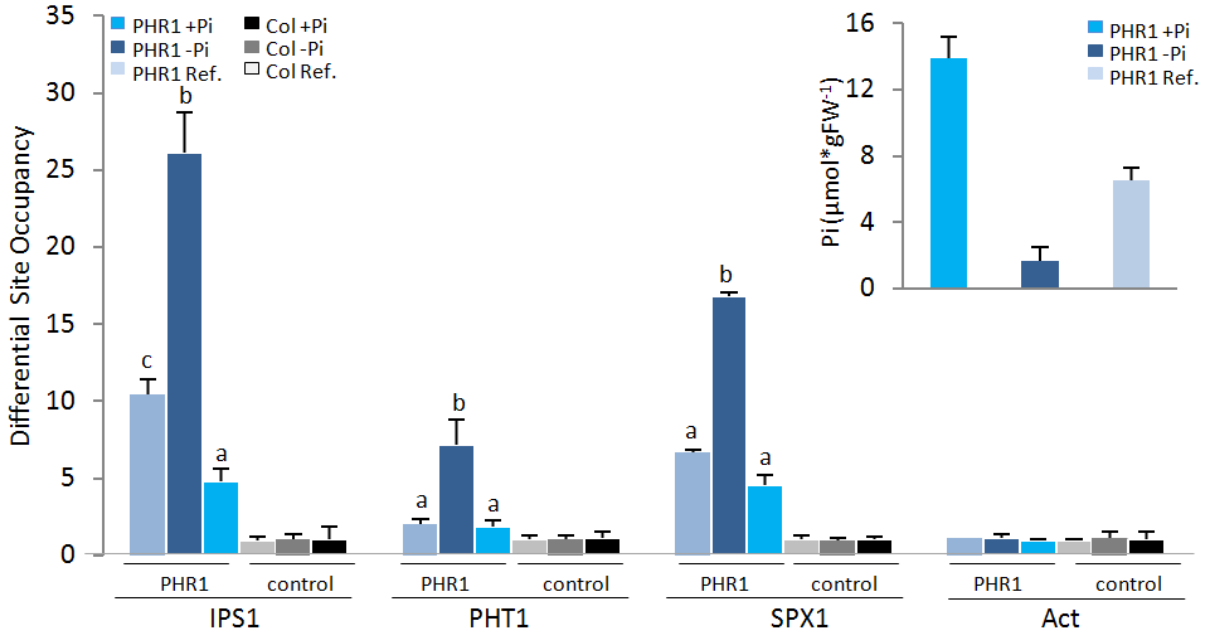

Fig 15: Cellular Pi-dependent interaction between PHR1 and its targets in planta

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-0) and transgenic PHRI promoter *(PHR1pro)::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 qPCR. Recovery of target by Co-IP with anti-MYC antibody was compared with recovery of a non-bound 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 non-significant and significant differences between groups ($P < 0.05$), respectively, according to Student t tests. FW refers to fresh weight.

2.5 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 P1BS (Rubio *et al*., 2001 and Bustos *et al*., 2010), we performed *in-vitro* DNA-binding assays using increasing 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 (Rubio *et al*., 2001) and SPX1 protein; these proteins were tagged with MBP and GST, respectively. 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. 16A). Using EMSA, we examined the range of Pi concentrations in which SPX1 effectively inhibits PHR1 binding to P1BS (Fig. 16B). 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 (Rouached *et al*., 2011)].

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. 16C). 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. This explains why in the *in planta* co-IP assay (Fig 14A), 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. 17) 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. 16D). The fact that Phi represses PSRs has been considered evidence that Pi itself is a signal (Carswell *et al*., 1996 and Ticconi *et al*., 2001), a concept that is strengthened by our data.

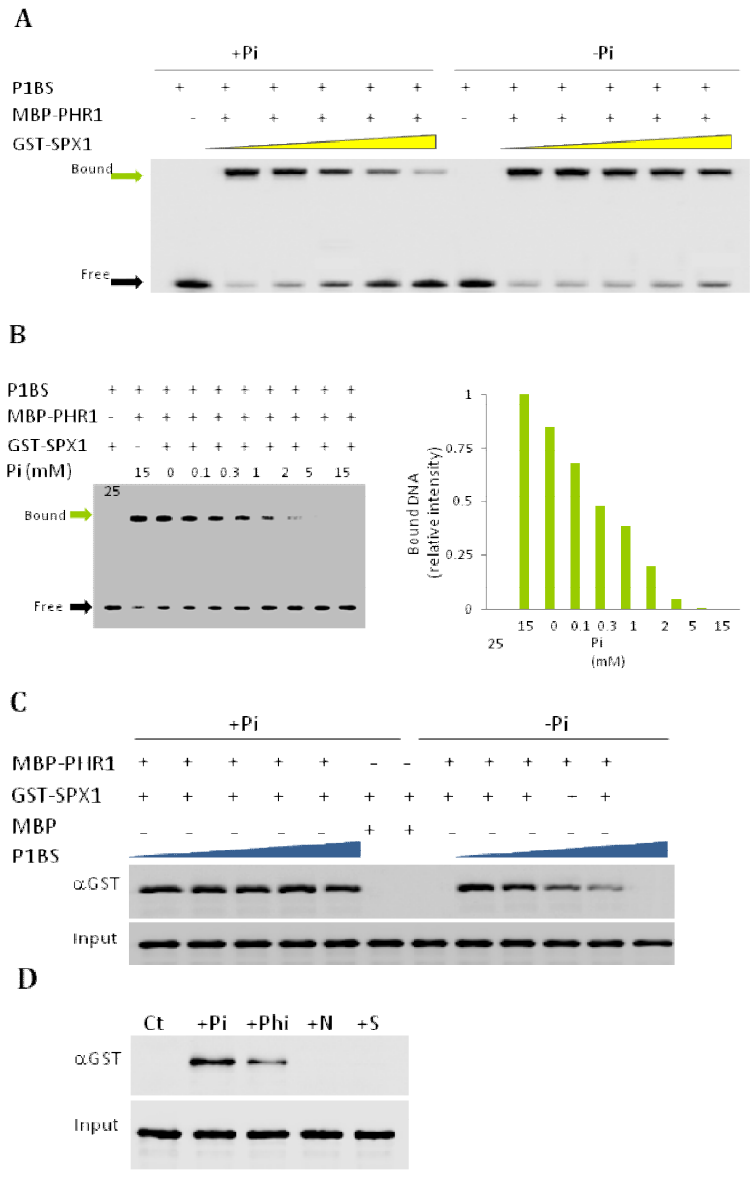

Fig 16: Direct Pi effect on the SPX1/PHR1 interaction

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. EMSA of the interaction between MBP-ΔPHR1 and P1BS in the presence of SPX1, showing that GST/SPX1 inhibition of the MBP-ΔPHR1/P1BS interaction is Pi dose-dependent. The experiment was performed with 0.1 pmol of 4× P1BS, 0.3 pmol of MBP-ΔPHR1, and 5 pmol of GST-SPX1 with varying Pi concentrations (0–25 mM), as indicated. Binding inhibition was normalized to the sample lacking SPX1. The tagged ΔPHR1 and SPX1 proteins used in these experiments were bacterially expressed and affinity-purified

C. 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.

D. 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 50mM 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.

3. New light on NLA (E3 ubiquitin ligase) and PHO2 (E2 ubiquitin conjugase) function in Pi signaling. Role in growth control and in a novel feed-back loop in Pi starvation signaling.

As already mentioned, *nla* displays Pi toxicity symptoms similar to *pho2* under low nitrate and high Pi conditions (see introduction section 3.2.4 and 3.4.3), suggesting that both NLA and PHO2 may function in the same pathway. To test this possibility, we first decided to examine whether NLA interacts with PHO2 and other components of Pi starvation signaling pathway.

3.1 NLA interacts with PHO2, SPX1 and bHLH149

Full length NLA and a RING domain deletion mutant of NLA (described here as Δnla) were used as baits for Y2H assay (Fig 17A). PHR1, SPX1, PHO2, bHLH149 and NLL (NLA-like, a close homolog of NLA) were used as prey proteins. UBC8 (Ubiquitin conjugase 8) was used as a positive control as it was demonstrated to interact with NLA by Peng *et al*., (2007). Intact NLA (possessing both SPX and RING domain) was able to interact with PHO2, SPX1 and bHLH149; the interaction being strong with SPX1, intermediate with PHO2 and

weak in the case of bHLH149 (Fig 17B). The loss of these positive interactions in the case of Δnla implied that NLA interacted with SPX1, bHLH149 and PHO2 via its RING domain. Concordant to the recent results of Park *et al*., (2014), we also confirmed that NLA interacts with itself via its RING domain. No interaction was found between NLA and PHR1 or NLL (Fig 17B).

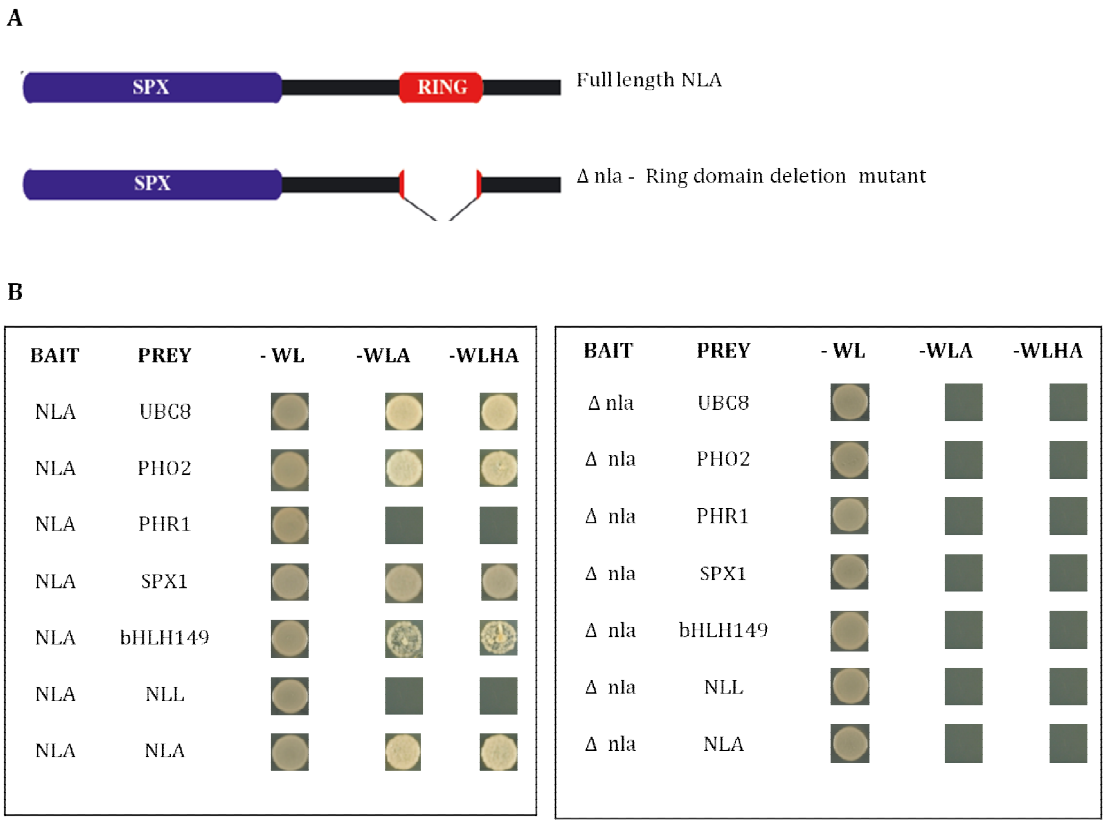

Fig 17: NLA interacts with PHO2, SPX1 and bHLH149 in Y2H assay

A. Schematic representation of structure of bait proteins: full length NLA (E3 ubiqutin ligase) with N-terminal SPX domain, represented in blue and C-terminal RING domain in red; and deletion mutant (Δnla) lacking the RING domain. This figure is taken from Peng *et al*., (2007).
B. Yeast cells co-transformed with bait and prey proteins were selected and subsequently grown on yeast synthetic dropout lacking Trp and Leu (-WL) as a transformation control and on selective media lacking Trp, Leu, His and Ade (-WLHA) to test protein interactions.

The finding that PHO2 interacts with NLA in Y2H assay (Fig 17 B) gave evidence to our hypothesis that PHO2 and NLA participate in the same pathway. This finding was confirmed by data from Chua laboratory (Park *et al*., 2014) demonstrating that NLA

interacts with PHO2 in yeast and *in planta*. Next we wanted to verify whether NLA interacts with bHLH149 and SPX1 *in planta.* Towards this, we performed Co-immunoprecipitation (Co-IP) assay by transient expression in *N.benthamiana*. Both SPX1 and bHLH149 were able to interact *in-vivo* with NLA (Fig 18A, B and C).

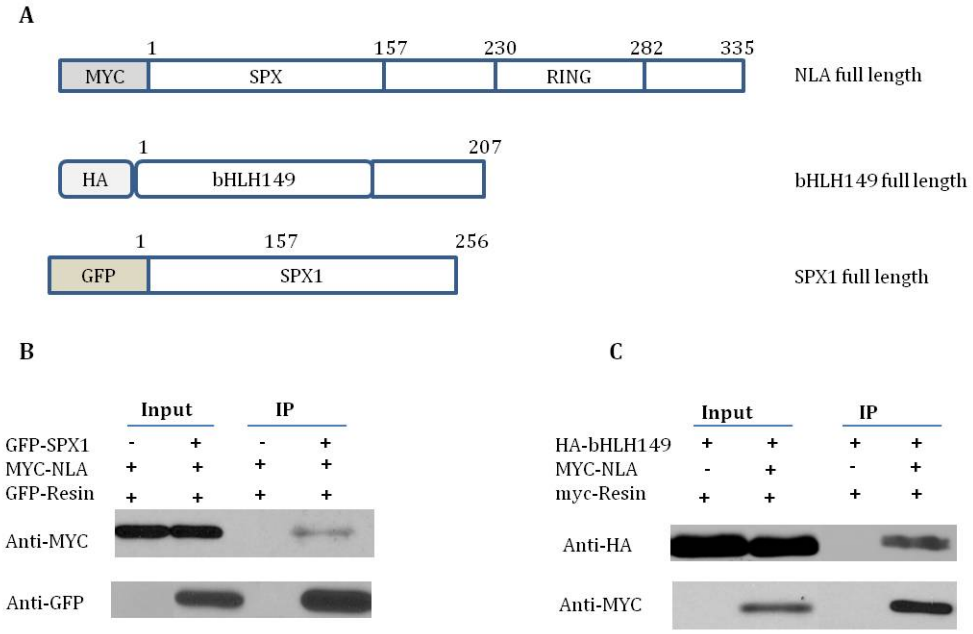

Fig 18: NLA interacts with bHLH149 and SPX1 *in planta*

A. Schematic diagram showing the constructs used for the Co-IP assay.
B. Co-IP assay of GFP-SPX1 and MYC-NLA: Transient expression of 35S::GFP-SPX1 and 35S::MYC-NLA was performed in *N.benthamiana* using agro-infiltration. GFP-SPX1 was immunoprecipitated using anti-GFP antibody with subsequent detection in western blots using anti-GFP and anti-MYC antibodies.
C. Co-IP of MYC-NLA and HA-bHLH149 : 35S::MYC-NLA and 35S::HA-bHLH149 constructs were transiently expressed in *N.benthamiana* leaves and plants extracts were Immunoprecipitated with anti-MYC antibody and detected in western blots using anti-MYC and anti-HA antibodies.

3.2 NLA and PHO2 mediate degradation of SPX1 and bHLH149

Based on our Y2H and Co-IP assays, we hypothesized that SPX1 and bHLH149 could be targets of PHO2 and NLA. To test if SPX1 is regulated by PHO2 or NLA, we first performed transient expression assay in *N.benthamiana* plants, agroinfiltrated with either 35S::GFP-SPX1 alone or together with 35S::PHO2 and 35S::MYC-NLA. We observed a minor

effect of PHO2 or NLA alone on SPX1. However, the combination of PHO2 and NLA greatly reduced the level of SPX1 (Fig 19A). To verify that SPX1 degradation by PHO2 and NLA was at protein level, we checked *SPX1* transcripts by qRT-PCR to exclude an artifact due to transgene silencing (Fig 19B). In Parallel, we generated *Arabidopsis thaliana* transgenic plants overexpressing SPX1 in WT, *pho2* and *nla* mutant backgrounds to check the effects of these mutations on SPX1 level. We observed that SPX1 protein level was higher in *nla* mutant background compared to WT (Fig 19C). Unfortunately, due to transgene silencing in the *pho2* background, we could not evaluate the effect of *pho2* mutation on SPX1 level. The combined effect of *nla* mutation and SPX1 overexpression on Pi levels is currently being examined.

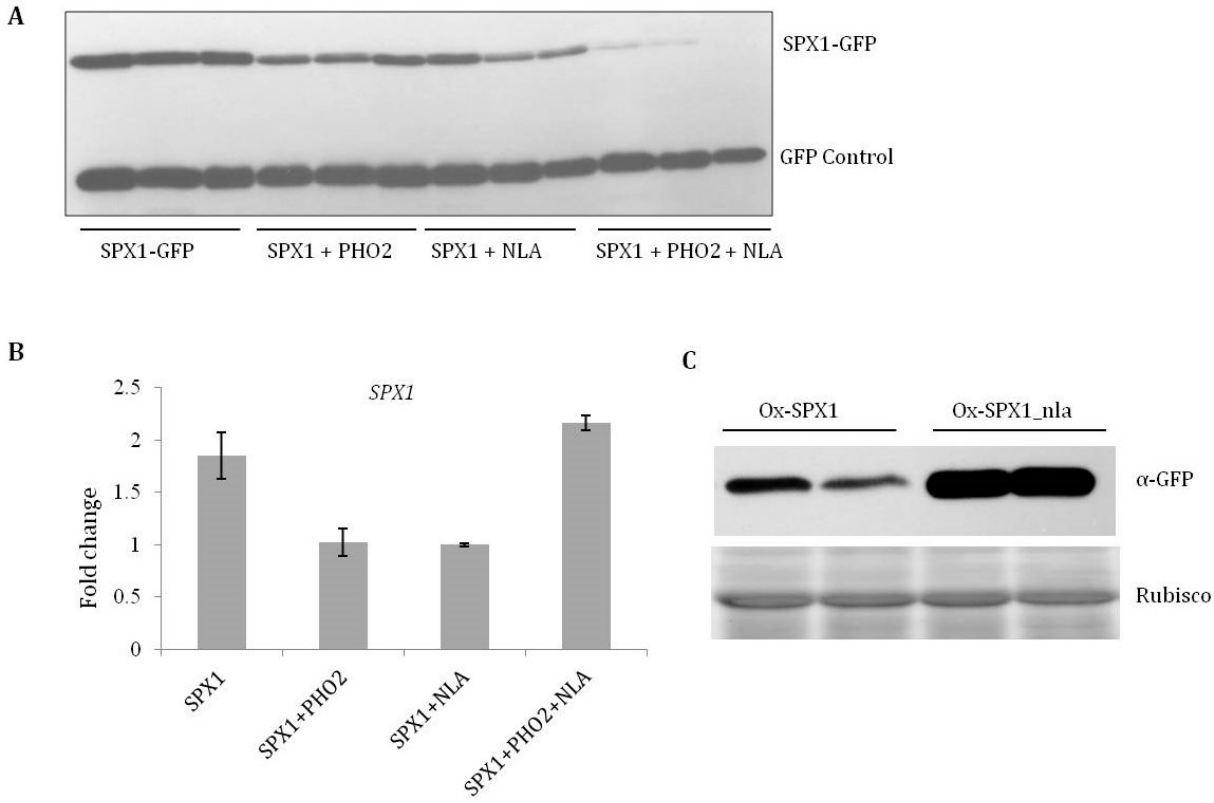

Fig 19: PHO2 and NLA mediate degradation of SPX1 *in planta*

A. *N.benthamiana* plants were agro-infiltrated with 35S::GFP-SPX1 alone or together with 35S::PHO2, 35S::MYC-NLA; samples were collected after 3 days and used for western blot analysis. 35S:GFP was used as internal control.
B. qRT-PCR expression analysis of *SPX1* in the samples of (A). 35S::GFP co-infiltrated with the samples was used as the endogenous control. Data represents mean ± S.D of 3 independent biological replicates.

C. Effect of *nla* mutation on SPX1 protein level in *Arabidopsis*: OxGFP-SPX1 and OxGFP-SPX1_*nla* transgenic plants were grown on complete nutrient conditioned soil. Rubisco was used as loading control. 2 independent transgenic lines were analyzed.

To assess if bHLH149 is also a target of PHO2 and NLA, we transiently expressed 35S::HA-bHLH149 alone or together with 35S::PHO2 and 35s::MYC-NLA in *N.benthamiana.* Contrary to our expectation, bHLH149 stability was unaffected by PHO2 or NLA (Fig 20A). There are many possibilities that could explain this negative result, since a transient assay in a heterologous system does not perfectly mimic the situation in the homologous system. Therefore, we decided to examine the effects of *pho2* and *nla* mutation on bHLH149 protein stability in *Arabidopsis.* We observed increased level of bHLH149 protein in *pho2* or *nla* background compared to WT background (Fig 20B). Interestingly, protein levels of bHLH149 in mutant backgrounds had a positive correlation with plant size reduction. i.e., OxbHLH149_*pho2* and to a lesser extent OxbHLH149_*nla* had clearly a smaller plant size compared to OxbHLH149_WT (Fig 20C). The combined effect of *pho2* or *nla* mutation and bHLH149 overexpression on Pi levels is currently being characterized.

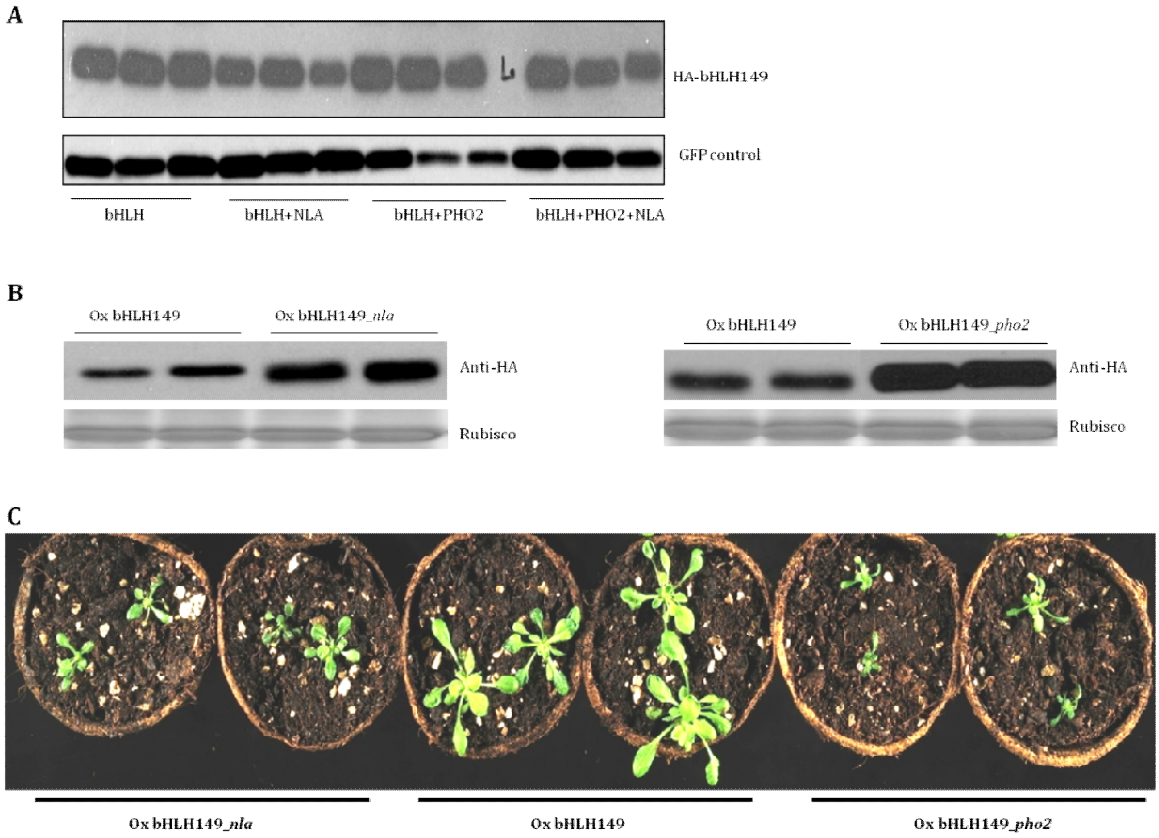

Fig 20: Effect of PHO2 and NLA on bHLH149

A. Western blot analysis showing the effect of PHO2 and NLA on bHLH149. *N.benthamaiana* was agroinfiltrated with 35S::HA-bHLH149 alone or with 35S::PHO2 and 35S::MYC-NLA and samples were collected after 3 days. 35S: GFP used as the internal control. Each sample includes 3 independent biological replicates.
B. Activity of PHO2 and NLA on bHLH149 in stable *Arabidopsis* transgenic lines. 2 individual transgenic plants for each line are displayed. Rubisco is shown as loading control.
C. Picture represents 3 weeks old OxbHLH149, OxbHLH149*_pho2* and OxbHLH149*_nla* transgenic plants grown in complete nutrient soil under short day conditions.

DISCUSSION

Plants have evolved a set of adaptive responses including developmental and biochemical/metabolic changes to cope with growth under Pi limiting conditions. Considerable information have been gathered in the past decade on the components of the Pi starvation signaling pathway including (i) identification of PHOSPHATE STARVATION RESPONSE 1 (PHR1) and related transcription factors as master regulators of Pi starvation responses (PSRs) (Rubio V *et al.,* 2001; Zhou J, *et al.,* 2008; Bustos R, *et al.,* 2010; Ren F, *et al.,* 2012; Wang J, *et al.,* 2013) (ii) demonstration of the involvement of ubiquitination system components, including PHO2 and NLA in Pi signaling (Bari R *et al*., 2006; Kant S *et al*., 2011; Huang TK, *et al.,* 2013; Lin WY *et al*., 2013; Park BS *et al*., 2014) (iii) identification of miRNAs as mobile signals in Pi homeostasis (Pant BD *et al*., 2008; Kuo HF and Chiou TJ, 2011); and (iv) identification of Pi starvation-induced (PSI) riboregulators of miRNA activity, based on target mimicry (Zorrilla JM, *et al.,* 2007) and natural antisense RNA that activates translation of PHO1 mRNA (Jabnoune M, *et al.,* 2013).

In this study, we have contributed to the knowledge on the control of these responses in three aspects:

1. Identification of five new candidate TFs controlling PSRs.
2. Identification of SPX1 as a Pi dependent inhibitor of PHR1, qualifying it as a component of the Pi sensor.
3. Uncovering new roles of PHO2 and NLA in Pi starvation signaling regarding growth control and in the generation of a feedback loop concerning SPX1.

1. Identification of Novel TFs involved in Pi starvation signaling

In this project we report data that establishes the use of ionomic profiling as a potential functional genomics tool for the identification of genes involved in the accumulation of mineral nutrients and trace elements in plants. In our first round of elemental profile screening involving 320 TFs overexpressing TPT lines, we identified in total 34 TFs overexpressing TPT lines with altered elemental profiles using ICP-OES, out of

which overexpression of 5 TFs showed significant alterations in P levels (shown by left vertical bars for each element in Fig 7)

Repetition of the ionomic analysis of these selected 5 TFs overexpressing TPT lines (which included analysis of all the available overexpressing transgenic TPT lines of these TFs) gave a good level of concordance in the ionomic profile pattern with the 1st screening However, the alteration in ionomic profile could be observed only in one transgenic line per TF (represented by right vertical bars for each element in Fig 7), despite that for 3 out of 5 TFs, we examined atleast two independent lines. Nonetheless, we showed that the effect on the ionome of the lines showing alterations was only found upon β-estradiol treatment (Fig 8), indicating that the ionome alterations in the selected lines is dependent on TF overexpression.

With regard to the ionomic signatures obtained among the selected 5 TFs, overexpression of one of these TFs (*TF-137)* resulted in increased level of P, while the overexpression of the other 4 TFs (*TF-117, TF-182, TF-220* and *TF-252*) caused decrease in the level of P suggesting that among these 5 TFs, TF-137 would probably act as positive regulator of Pi starvation responses (Fig 7).

In addition to P, we also observed some interesting correlations in the type of changes in the accumulation pattern of P and those of other elements. P accumulation exhibited a positive correlation with accumulation of Zn, Mn, Cd and to a lesser extent with that of K. Thus, in all four OxTF lines (*TF-117, TF-182, TF-220 and TF-252*), displaying lower P than WT plants, they showed lower Zn and except for *OxTF-220*, lower Mn (Fig 7A, B, D and E); whereas *OxTF-137* had increased levels of P as well as of Zn and Mn (Fig 7C). Similar correlations were observed between P and Cd in the case of three TFs overexpressors; wherein *OxTF-182* and *OxTF-117* showed reduced levels of these 2 elements (Fig 7A and B) and *OxTF-137* caused increase in the accumulation of these 2 elements (Fig 7C). In addition, P also exhibited a positive correlation in the accumulation pattern with K, but only in the case of *OxTF-137* and *OxTF-182* (Fig 7A and C). These correlations in the accumulation of several ions informs of the existence of positive cross talks of different strengths between the signaling pathways of these ions.

A potential crosstalk between P and Zn has already been documented. For instance, Zn concentration in wheat and maize plants was reported to decrease with the application

of P and vice versa (Robson and Pitman, 1998; Verma and Minhas, 1987). In barley, Zn-deprivation resulted in over-accumulation of Pi in shoots (Huang *et al*., 2000). Recently, it was demonstrated that Zn deficiency caused an increase in shoot Pi content in the WT and *pho2* mutant, but not in the *phr1* and *pho1* mutants indicating the role of PHR1 and PHO1 in the co-regulation of Zn and Pi homeostasis. In addition, PHO1; H3 (close homolog of PHO1) was also found to be upregulated in response to Zn deficiency (Khan *et al*., 2014). These observations can be explained in the framework of our findings that indicate that Zn and P uptake systems are co-regulated.

In our ionomic profiling analysis, we also observed that, P accumulation displayed a moderated negative correlation with some of the elements like Na, Mg and Mo. For example, low P levels were accompanied with high Mg in *OxTF-182* and vice versa in *OxTF-137* (Fig 7A and C). Similarly, low P levels in *OxTF-117* and *OxTF-182* plants caused increase in the accumulation of Na levels (Fig 7A and B). In case of Mo, the pattern of accumulation of P with Mo in these TFs overexpressing lines were different; while *OxTF-182* showed reduced levels of both P and Mo (Fig 7A), *OxTF-117*, *OxTF-252* and *OxTF-137* had the accumulation of these 2 elements oriented in opposite directions; *OxTF-117* and *OxTF-252* had decreased P accompanied with increased Mo and vice-versa in the case of *OxTF-137* (Fig 7B, E and C).

A more complex situation concerns Pi: Fe inter-relationships. In the recent years many research groups have studied the gene networks that control Fe homoestasis (Lahner *et al*., 2003) and the links between P and Fe nutrition (Baxter *et al*., 2008; Bournier *et al*., 2014). Studies have shown that increased Fe uptake by IRT1 (Iron tranporter) in plants was accompanied with side effects of increased accumulation of Mn, Co, Zn and Cd (Vert *et al*., 2002, Guerinot *et al*., 1996 and Rampey *et al*., 2006). In our ionomic profile analysis, we also observed similar side effects associated with Fe levels although not in a strict manner. In the overexpressing lines of *TF-117* and *TF-182*, decreased Fe levels were accompanied with decreased levels of Cd, Zn, and P (Fig 7 A and B). But in the case of *OxTF-137*, we observed a negative correlation between these elements wherein decreased Fe content was accompanied by increased Cd, P and Zn (Fig 7C). It would be important to check with a larger number of TFs to establish the strictness of these correlations.

Altogether, our ionomic profile data has given the first evidence for the involvement of these five TFs in P starvation signalling and its cross talk with those of other nutrients. Moreover, based on transcriptomic data of Pi starvation by Bustos *et al*., (2010), we found that out of these 5 TFs studied, two of them (belonging to families bZIP and NAC) were induced by Pi.

Recently, Lahner *et al*., (2003), based on their mutant's analysis estimated that 2-4% of the genome of *A.thaliana* contributes to controlling the rosette leaf ionome of unstressed plants. Our data points to a larger proportion of TF (10%) affecting the ionome which indicates that changes in TFs activity have a higher impact on the ionome than changes in other types of genes, in line with the more important role of TFs in the control of gene expression.

2. SPX1 is a Pi dependent inhibitor of PHR1

In this study, we identify a mechanism for Pi-dependent negative control of PHR1 activity in Arabidopsis, based on a nuclear SPX domain protein 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 (Bustos *et al*., 2010), 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. 16D). 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*., (2014).

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 (Lee *et al*., 2007), although some PHO81 functions depend on its SPX domain (Swinnen *et al*., 2005). 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 stability through an unknown mechanism (Lv Q *et al*., 2014). 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. 21. 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 cytoplasm 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.

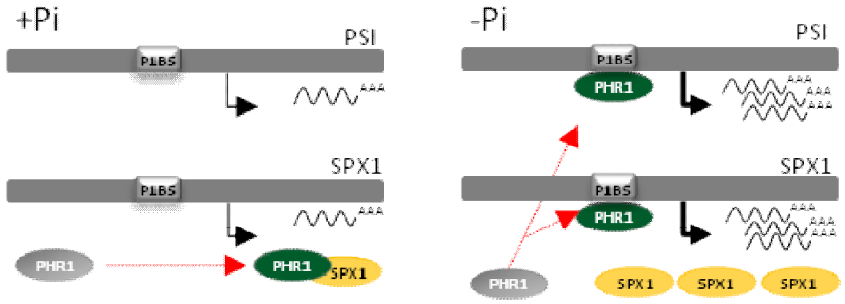

Fig 21) Model for the negative regulatory loop between SPX1 and PHR1, and its Pi dependence. *SPX1* is a target of PHR1. In the presence of Pi, SPX1 displays high binding affinity to and sequesters PHR1; thus, binding of PHR1 to its PSI targets via P1BS is inhibited, and their transcription, including 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.

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. Indeed, our transcriptomic data indicated that upon refeeding, locally controlled genes display induced expression in the mutants, which reveals that SPX1 positively regulates these genes transiently upon refeeding. One model to explain this is shown in Fig 22. 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. 14). 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.

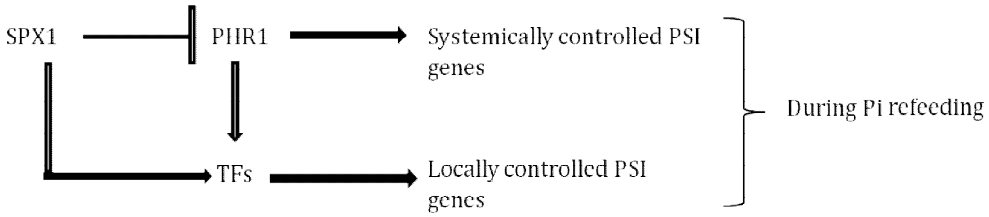

Fig 22: Model to explain the role of SPX1 in early stages of Pi refeeding
During Pi refeeding conditions, SPX1 inhibits PHR1 thereby eventually repressing direct targets of PHR1 mostly corresponding to systemically controlled Pi starvation induced genes. However, locally controlled Pi starvation induced genes, although indirectly controlled by PHR1, at early stages of refeeding their expression depends on additional TFs that are positively controlled by SPX1.

3. New light on NLA and PHO2 function in Pi signaling. Role in growth control and in a novel feed-back loop in Pi starvation signaling

The *nla* mutant was initially identified in a genetic screen for impaired growth under nitrogen limiting conditions (Peng *et al*., 2007). Subsequently, it was found that this growth defect was due to high Pi levels indicating the system of crosstalk between nitrogen and Pi starvation signaling (Kant *et al*., 2011). High accumulation of Pi in *nla* resembles *pho2* mutant which also accumulates high phosphate under phosphate sufficient conditions. In addition, the fact that both are components of ubiquitination pathway, suggested they share functions in mediating ubiquitination. In line with this, in our study, we found that PHO2 and NLA interact and cooperate at least in the degradation of some targets (e.g., SPX1). This finding has been recently confirmed by Park *et al*., (2014), that demonstrated that NLA interacts with PHO2 *in-vitro* and *in-vivo,* and the PHO2/NLA pair together polyubiquitinates the high affinity Pi transporter PT2 (PHT1;4) and targets it for degradation. In addition to our finding that PHO2 and NLA interact with each other we also showed that they control the accumulation of SPX1 and of the negative regulator of growth bHLH149.

3.1 Control of SPX1 by PHO2 and NLA – a new feedback loop in Pi signaling

We have shown here that SPX1 is efficiently degraded by the co-expression of PHO2 and NLA in transient expression assays in *N.benthamiana* (Fig 19A). Moreover, studies in homologous system show that SPX1 displays enhanced accumulation in *nla* mutant

background Vs that in the WT (Fig 19C). Altogether, this data indicates that SPX1 stability is regulated by PHO2 and NLA.

The finding that SPX1 interacts with PHO2 and NLA both in yeast two hybrid assays (Fig 17 B) and *in-planta*, as shown by Co-IP (Fig 18B) suggest that the effect of PHO2 and NLA on SPX1 is direct. i.e, SPX1 is a substrate of PHO2 and NLA. However, the possibility of SPX1 being a substrate of PHO2 and NLA is in apparent contradiction to its localization; SPX1 is in the nucleus while PHO2 and NLA are shown to be localized to endomembranes and to plasma membrane respectively. One possible explanation to conciliate this paradox is to invoke that SPX1 may be recycling between nucleus and cytoplasm and, out of the nucleus; it could be degraded by PHO2 and NLA.

The existence of this negative control of SPX1 by PHO2 and NLA generates a negative feedback loop in Pi signaling (Fig. 23).

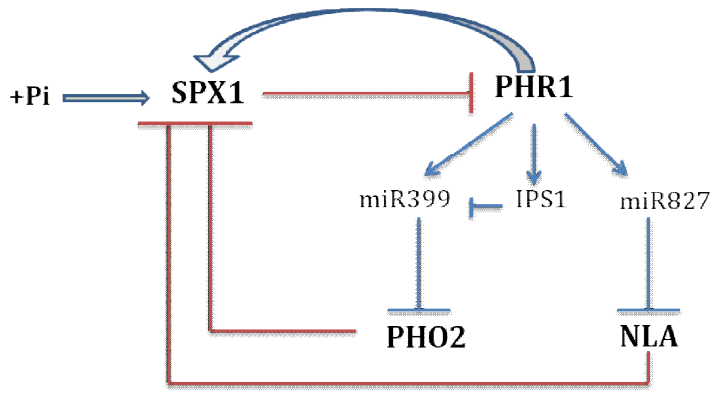

Fig 23: Model representing the regulatory feedback loop between PHR1, SPX1, PHO2 and NLA SPX1 at +Pi conditions inhibits PHR1 activity. As a result, the inhibitory effect on PHO2 and NLA accumulation by miRNAs is relieved; leading to the degradation of SPX1 by PHO2/NLA. Blue lines refer to pretranslational control; and red refers to regulation involving protein-protein interactions.

3.2 Control of bHLH149 by PHO2 and NLA. Integration of growth control in Pi signaling

Previous experiments in our laboratory have found that bHLH149 (a negative regulator of cell growth and elongation) was regulated by PHO2 in a Pi dependent manner (Mateos, PhD manuscript, 2010). Given this, we were interested to find whether bHLH149 was regulated by NLA also. Towards this, we first checked the activity of PHO2 and NLA on bHLH149 in *N.benthamiana* by transient expression. Though, we were not successful in demonstrating the degradation of bHLH149 by PHO2 and NLA in *N.benthamiana* (Fig 20A),

which could be attributed to the fact that transient expression does not exactly mimic the *in-vivo* situation, our results in the homologous system were quite conclusive (Fig 20B). Notably, the increased protein levels of bHLH149 in *pho2* and to a lesser extent in *nla* mutant backgrounds compared to WT had a positive correlation with plant size reduction (Fig 20C).

The direct interaction of bHLH149 with PHO2 (Mateos, PhD manuscript, 2010) and NLA (Fig 17B and 18C) suggests that bHLH149 is a most likely a direct target of PHO2 and NLA. Like with SPX1, the possibility of bHLH149 as direct target of PHO2 and NLA requires an explanation for the different localization.

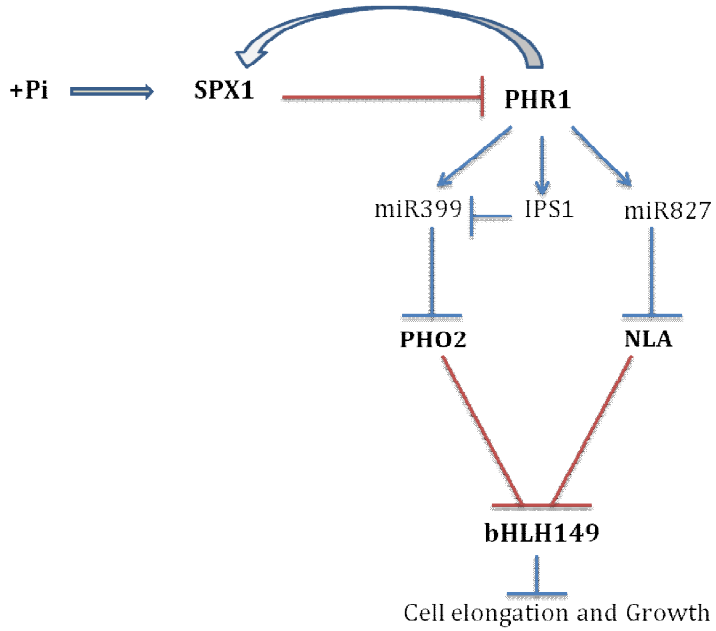

Fig 24: Model linking growth control with Pi starvation signaling via the action of NLA and PHO2 on bHLH149. The regulation of bHLH149 by PHO2 and NLA provides a mechanism for growth control by Pi starvation. Blue lines refer to pretanslational control; and red lines refer to control involving protein-protein interactions.

The regulation of bHLH149 by PHO2 and NLA provides a mechanism for Pi starvation control of growth (Fig 24), beyond the simplistic expectation that growth reduction is a direct consequence of starvation. Indeed growth reduction will lower the Pi demand and the reduction in cell size increases the surface/volume ratio providing a suitable geometry to adapt to low nutrient availability. In Fig 25, it is provided a schema integrating our findings in the Pi starvation signaling pathway.

The finding of the link between PHO2/NLA and bHLH149 informs on a possible biotechnological strategy to uncouple Pi starvation and growth control, so that by reducing

the negative control of PHO2 and NLA by miR399 and miR827 respectively, or by impairing bHLH149, we could limit the negative impact of reducing Pi supply on growth.

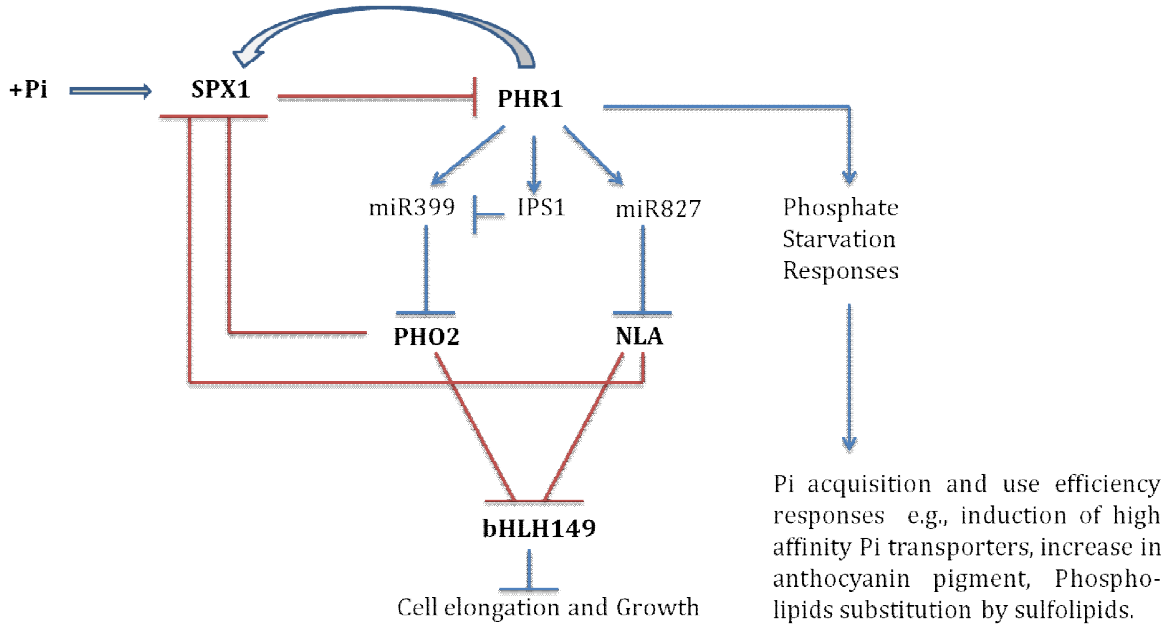

Fig 25: Schematic representation of the Pi starvation signaling pathway

CONCLUSIONS

1. The TRANSPLANTA collection of transgenic plants conditionally overexpressing TFs has served as a powerful resource for the identification of TFs involved in Pi starvation signaling pathway.
2. We provide data that establishes the use of elemental profiling as a practical functional genomics tool for the identification of genes involved in the accumulation of P mineral ion and other elements. In general, alterations in the level of one element normally disturbed levels of many elements giving evidence that ion signaling pathway in plants are linked.
3. We have identified 5 TFs [(two DREB family TFs At2g40340 and At1g71450), bZIP TF (At1g68640), ANAC38 (At2g24430), and KNAT2 (At1g70510)] whose over expression resulted in significant alterations in the level of P and other elements. Two of them (ANAC38 and bZIP TF) correspond to Phosphate starvation induced genes.
4. In a study aimed at finding the potential interactors/regulators of PHR1, we identified SPX1 and found it is a Pi dependent inhibitor of PHR1 in *Arabidopsis*.
5. Intact SPX domain is necessary for the interaction of SPX1 with PHR1.
6. SPX1 and PHR1 form a negative feedback loop that allows self-regulation of the strength of Phosphate Starvation Responses to meet the Pi demand of the plant.
7. In a study to find targets of PHO2 and NLA in Pi starvation signaling pathway, we found bHLH149 and SPX1 as potential targets of PHO2 and NLA.
8. PHO2 and NLA mediate degradation of bHLH149 (negative regulator of cell elongation) possibly at +Pi revealing a novel growth control mechanism.
9. PHO2 and NLA together mediate degradation of SPX1. This provides the basis for a negative feedback regulatory loop between PHO2, NLA, SPX1 and PHR1 aimed to maintain Pi homeostasis.

CONCLUSIONES

1. La colección TRANSPLANTA de líneas transgénicas que expresan condicionalmente diferentes factores de transcripción (FTs), ha resultado una herramienta idónea para la identificación de FTs implicados en la ruta de señalización de la carencia de fosfato.
2. Aportamos datos que establecen que la caracterización de los perfiles de acumulacion de elementos constituyen una herramienta de genómica funcional para la identificación de los genes implicados en la acumulación del P mineral y de otros elementos. Las alteraciones en el nivel de un elemento concreto, generalmente, están asociadas a alteraciones en los niveles de varios elementos, lo que evidencia la relación ión-homeostasis en plantas.
3. Hemos identificado 5 FTs [dos DREB FTs (At2g40340) y (At1g71450), bZIP TF (At1g68640), ANAC38 (At2g24430) y KNAT2 (At1g70510)] cuya sobrexpresión provocó alteraciones significativas en los niveles de P y otros elementos. Cabe resaltar que dos de estos FTs (ANAC38 y bZIP TFs) corresponden a genes inducidos por ayuno de fosfato.
4. En otro estudio encaminado a identificar potenciales interactores/reguladores de PHR1, hemos identificado a SPX1, habiendo establecido que es un inhibidor de PHR1 dependiente de Pi en *Arabidopsis*.
5. La interacción entre SPX1 y PHR1, requiere el dominio SPX intacto.
6. SPX1 y PHR1 conforman un bucle de retroalimentación negativo que permite la autorregulación de las respuestas frente al ayuno de fosfato, para adaptarse a la demanda de fosfato por parte de la planta.
7. En otro estudio encaminado a conocer dianas de PHO2 y NLA en la ruta de señalización del ayuno de fosfato, hemos encontrado que bHLH149 y SPX1 son dianas de PHO2 y NLA.

8. PHO2 y NLA posiblemente determinan directamente la degradación de bHLH149 (un regulador negativo de la elongación celular) en +Pi, evidenciando un nuevo mecanismo de control del crecimiento.
9. PHO2 junto con NLA median la degradación de SPX1. Esto contribuye a la formación de un bucle de retroalimentación negativo entre PHO2, NLA, SPX1 y PHR1 para el mantenimiento de la homeostasis de Pi.

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