



# Study of Physiological and molecular mechanisms underlying the co-regulation between phosphate and zinc homeostasis in plants

Mushtak Kisko

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE MONTPELLIER SUPAGRO

En Ecophysiologie et Adaptation des plantes

École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau  
Portée par l'Université de Montpellier

Unité de recherche B&PMP  
Laboratoire de Biochimie et Physiologie Moléculaire des Plantes

## Study of Physiological and molecular mechanisms underlying the co-regulation between phosphate and zinc homeostasis in plants

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Le 08/03/2018

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## *Dedication*

*This thesis is dedicated to the spirit of my  
father, who lives in my mind and my heart,  
To my mother, who was the constant source of  
support and encouragement,  
To my brothers & my sisters and their families,  
To my beloved wife, and to my sweet children  
Iden & Eveen.*

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Mushtak KISKO

# **Study of Physiological and molecular mechanisms underlying the co-regulation between phosphate and zinc homeostasis in plants**

## **Abstract**

In plants, while it is clear the homeostasis of different nutrients is highly dependent on each other, they are usually studied independent of each other. Given the paucity of past studies assessing the biological significance of mineral nutrient homeostasis interaction, very little is known about the genetic and molecular basis of such interactions. During my thesis, we made significant progress in going towards a more integrative comprehension of the problem and identify the molecular and genetic bases for a highly important and conserved nutrients interaction: the interaction of zinc and phosphate. First, using the phosphate transporter PHO1;H3 as entry molecular point, and by combining system biology and functional genomics approaches we have identified the functional module (four transcription factors) that regulates the expression and activity of PHO1;H3 under zinc deficiency leading to control Pi accumulation in shoots. Second, following our discovery of *Lyso PhosphatidylCholine (PC) AcylTransferase1 (LPCAT1)* using genome-wide association studies (GWAS), we determined complete molecular pathway controlling the expression of this gene. We further uncovered a fundamental link between phospholipid metabolism and phosphate-zinc homeostasis interaction via *LPCAT1*, which lays the foundations to explore a new role for Lyso-PC and PC in control of macro- and micronutrients homeostasis interaction. Taken together, our discoveries offer a new perspective on how to improve phosphate content in plants, as our findings suggests that modulating the zinc-deficiency signalling pathway might be a good and simple approach for that.

# Étude des mécanismes physiologiques et moléculaires impliqués dans la co-régulation entre l'homéostasie du phosphate et celle du zinc chez les plantes

## Résumé

Chez les plantes, malgré qu'il est reconnu que les homéostasies des différents nutriments sont fortement dépendantes les unes des autres, elles sont généralement étudiées indépendamment les unes des autres. Vu le manque des études antérieures évaluant la signification biologique de l'interaction entre les homéostasies des nutriments minéraux, rares sont nos connaissances sur les bases génétique et moléculaire de ces interactions. Au cours de ma thèse, je me suis focalisé de manière approfondie sur une compréhension plus intégrative de ce sujet en identifiant les bases moléculaires et génétiques d'une interaction nutritive très importante et conservée ne serait-ce que l'interaction entre l'homéostasie du zinc et celle du phosphate, et dans laquelle les gènes *PHO1;H3* et *Lyso PhosphatidylCholine (PC) AcylTransferase1 (LPCAT1)* jouent des rôles primordiaux. En combinant des approches de la biologie des systèmes et de la biologie fonctionnelle, j'ai identifié un module fonctionnel (comprenant quatre facteurs de transcriptions) qui régule l'expression de *PHO1;H3* en condition de déficience en zinc. Suite à une étude de génétique d'association via l'approche GWAS, j'ai découvert un nouveau rôle du gène *LPCAT1* dans l'accumulation du phosphate en conditions de déficience en zinc, ce qui a facilité, par la suite, la détermination d'une nouvelle voie moléculaire complète contrôlant l'expression de ce gène. Ce travail m'a permis de mettre en évidence un lien fondamental entre le métabolisme des phospholipides et l'interaction entre l'homéostasie des éléments phosphate-zinc, ainsi que de proposer un nouveau rôle des *lyso phosphatidylcholine* et *phosphatidylcholine* dans le contrôle de l'interaction entre l'homéostasie des macro- et des micronutriments chez les plantes. Les derniers résultats offrent une nouvelle perspective vers le développement des nouvelles stratégies d'amélioration de l'accumulation de phosphate dans les plantes à travers la modulation de la voie de signalisation de la déficience en zinc.

## Abbreviation

<sup>31</sup> P NMR	<sup>31</sup> Phosphorus nuclear magnetic resonance	EXS	(ERD1/XPR1/SYG1) domain in the conserved C-terminal hydrophobic portion
<i>A. halleri</i>	<i>Arabidopsis halleri</i>	Fe	Iron
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>	GAL4	Galactose-responsive TF
AD	Activation Domain	Gb	Giga base pairs
Ade	Adenine	GFP	Green Fluorescent Protein
AMM	algorithm mixed model	GRNs	Gene Regulatory Networks
ANOVA	Analysis of variance	GST	Glutathione S-transferase
<i>Bam</i> HI	<i>Bacillus amyloliquefaciens</i> type II restriction enzymes	GUS	β-glucuronidase
BD	Binding Domain	GWAPP	GWAS-Web-App
bHLH	basic helix-loop-helix TF	GWAS	Genome Wide Association Studies
Biotin-TEG	Biotin-tetra-ethyleneglycol	H/His	Histidine
bp	Base pairs	HMA	Heavy Metal ATPase
bZIP	Basic Leucine Zipper TF	ICE1	Inducer of CBP Expression 1
CaMV 35S	Promoter of the 35S RNA of the Cauliflower mosaic virus	IFDC	International Fertilizer Development Center
cDNA	Complementary deoxyribonucleic acid	IPTG	Isopropyl-β-D-thiogalactoside
CDS	Coding DNA sequence	IRT1	Iron-Regulated Transporter1
ChIP	Chromatin Immuno-Precipitation	kbp	Kilo base pairs
Col-0	Columbia-0	KO	Knock-Out
CPUs	Central Processing Units	<i>LPCAT1</i>	<i>Lyso-PhosphatidylCholine AcylTransferase 1</i>
C <sub>T</sub>	Threshold cycle	Mb	mega base pairs
DAP-seq	DNA affinity purification sequencing	<i>MBOAT</i>	<i>Membrane Bound O-Acyl Transferase</i>
dI-dC	deoxyinosinic-deoxycytidylic	miR399	microRNA399
DNA	Deoxyribonucleic acid	mRNA	Messenger ribonucleic acid
DOF53	DNA Binding with One Finger 53	MYB	MYelo Blast (R2R3-type) TF
DTT	Dithiothreitol	NASC	Nottingham Arabidopsis Stock Centre
E2Fc	<i>A. thaliana</i> homolog of E2F C TF	ng	Nanogram
EMSA	Electrophoretic Mobility Shift Assay	NLA	Nitrogen Limitation Adaptation
EPO	Evolutionary Pre-breeding population	NS	Non-selective medium
		OD660	Optical density at a wavelength of 660 nm
		P	Phosphorous
		PA	Phytic Acid
		PBS	Phosphate-buffered saline
		PC	Phosphatidyl Choline
		PCR	Polymerase Chain Reaction

PHF1	Phosphate Transporter Traffic Facilitator1	TFs	Transcription Factors
PHO1	Phosphate 1	TLC	Thin Layer Chromatography
PHO1;H1	PHO1 closest homologue	Tris	Tris(hydroxymethyl)aminomethane
PHO1;H3	PHO1 homologue	Tukey HSD	Tukey honest significant difference
PHO2	Phosphate2	UBQ10	Ubiquitin10
PHR1	Phosphate Respose1	UTR	Untranslated region
PHT1	Inorganic Phosphate Transporter 1	VND7	ASCULAR RELATED NAC- DOMAIN PROTEIN 7
PHT2	Chloroplasts located Phosphate Transporter 2	W	Tryptophan
PHT3/MPT	Mitochondrial Phosphate Transporter 3	WT	Wild-Type
PHT4	Golgi-apparatus located Phosphate Transporters 4	Y1H	Yeast One-Hybrid
PHT5/VPT	Vacuolar Phosphate Transporter 5	Y2H	Yeast Two-Hybrid
Pi	inorganic Phosphate	ZDRE	Zinc Deficiency Response Element
PLs	Phospholipids	ZIP	Zrt/IRT-like protein
<i>Pst</i> I	<i>Providencia stuartii</i> type II restriction enzymes	Zn	Zinc
PYE	Iron transport (POPEYE)	zntA	Zinc/cadmium/lead-transporting P- type ATPase
qRT-PCR	Quantitative real time PCR		
QTL	Quantitative Trait Locus		
R	r-programming language		
R <sup>2</sup>	R squared (coefficient of determination)		
<i>RegMap</i>	Regional Mapping		
REV	REVOLUTA		
Rf	Retention factor		
RNA	Ribonucleic acid		
RT-qPCR	Real-time quantitative reverse-transcription PCR		
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>		
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis		
SNP	Single Nucleotide Polymorphism		
SPX	(SYG1/Pho81/ XPR11) tripartite domain in the N- terminal hydrophilic portion		
SPX-MFS	SYG1/PHO81/XPR1- Major Facility Superfamily		
SSC	Saline-Sodium Citrate		
T-DNA	transfer DNA		



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## **Chapter I.**

### **General introduction**

# Chapter I. General introduction

## I. Introduction générale

Pendant ces dernières cinquante années, la population mondiale a connu une augmentation considérable (deux fois environ) et s'est accompagnée d'une forte augmentation (3 fois) de la production céréalière qui atteint 2.5 milliards de tonnes (FAO 2016). Toutefois, cette population continuera certainement à croître pour atteindre les 9.7 milliards d'habitants en 2050 (DESA 2015); et le problème est qu'il faudra doubler voire même tripler la production agricole mondiale, surtout dans les pays africains. Ceci pose un problème pour la sécurité alimentaire qui, d'après la FAO (2009), est basée non seulement, sur une alimentation suffisante en quantité, mais aussi, suffisamment nutritive pour permettre une vie saine et active à l'Homme. Par conséquent, assurer une augmentation de cette production au rythme de la croissance de la population demeure un défi important auquel les chercheurs doivent agir, surtout que nous sommes conscients que les solutions connues pour augmenter la productivité de l'agriculture au XXe siècle, dont l'utilisation intensive des engrais, montrent actuellement leurs limites.

La fertilisation fait partie des enjeux qualitatif et quantitatif de la production végétale sur lesquelles repose le système alimentaire mondial. Par exemple, en absence d'un apport important en engrais azotés, la récolte mondiale n'atteindrait que 50 % de son niveau (Erisman *et al.*, 2008). Un autre élément indispensable à la croissance et au développement des plantes est le phosphore (P). La faible disponibilité de cet élément dans la plupart des sols tropicaux représente souvent le premier facteur limitant la production agricole. La première révolution verte, était à l'origine d'une utilisation intensive des engrais phosphatés dont l'objectif était d'assurer une forte production des plantes. Néanmoins, en même temps que la fabrication des engrais phosphatés exerce des pressions croissantes, la réserve mondiale en P est susceptible de s'épuiser à échéance de quelques décennies, d'ailleurs il semblerait que le pic de production de phosphate est proche. Une étude relativement récente laisse entendre que celui-ci se produira entre 2030 et 2040 (Cordell *et al.*, 2009), ce qui impliquerait une baisse de la production alimentaire, qui, devrait normalement augmenter pour nourrir les 9 milliards d'habitants sur terre, prévus par les démographes en 2050. Un scénario plus optimiste, présenté par l'IFDC (International Fertilizer Development Center) repousse la disparition du phosphore à 300 ans. Une diminution des ressources de P limiterait la productivité de l'agriculture, et les rendements agricoles chuteraient à un niveau qui ne lui permettra pas de nourrir correctement l'Homme en 2050. Aujourd'hui, les rendements annuels de céréales dans les pays industrialisés dépassent



les cinq tonnes par hectare, alors qu'elle ne dépassait pas une tonne de céréales par hectare avant l'utilisation d'une source externe de phosphate. La production et l'utilisation responsable d'engrais phosphatés dans l'agriculture sont des éléments essentiels dans la sécurité alimentaire mondiale. C'est pour cette raison que nous considérons nécessaire et urgent de transformer les pratiques actuelles étant à l'origine des pertes importantes de phosphate dans l'environnement par le développement de plantes plus économes en terme d'utilisation des engrais phosphatés. En effet, la redéfinition des paramètres de sélection qui visent à obtenir des variétés présentant une utilisation optimum du Pi absorbé pourrait être une des solutions qui résoudrait ce problème. Ceci permettrait non seulement de réduire le cout de production des plantes d'intérêt agronomique, mais aussi d'être respectueux de cet environnement

Le Pi est un composant essentiel de l'agriculture durable, d'où la nécessité de développer une meilleure compréhension des mécanismes qui régulent l'homéostasie de cet élément chez les plantes. Ceci permettrait de proposer des stratégies qui visent à améliorer l'utilisation efficace du P. Durant ces dernières années, les résultats obtenus par plusieurs laboratoires de recherche ont permis d'avancer nos connaissances sur les mécanismes physiologiques utilisés par les plantes pour faire face à une déficience en phosphore. Cependant, au niveau moléculaire, la régulation du transport et de l'accumulation du Pi dans la plante en réponse à une carence en Pi, n'a été étudiée en profondeur que chez la plante modèle *Arabidopsis thaliana*. Ceci s'explique principalement par la disponibilité des données dès la séquence génomique de cette plante et par les avancées technologiques importantes permettant le développement des outils de biologie moléculaires adaptés pour ce genre d'études. Néanmoins, bien que plusieurs gènes impliqués dans le transport de Pi aient été identifiés, les approches utilisées à nos jours sont loin de nous renseigner sur les réseaux de gènes impliqués dans la régulation de l'homéostasie de Pi en réponses à un stress multi-nutritionnel. Nous avons maintenant des évidences sur l'existence de nombreuses interactions reliant la régulation de l'homéostasie du Pi à celles des autres éléments nutritifs, tel que les métaux essentiels, par exemple, une carence en Zn cause une augmentation de l'accumulation de Pi dans la plante, par contre, un excès de Zn provoque une réduction de l'accumulation de cet élément. Donc, il existe, évidemment, un lien entre l'homéostasie de ces deux éléments.

Malgré l'importance de ces interactions entre éléments en agronomie, les bases moléculaires ainsi que leur signification biologique demeurent peu connues. Pour cela, nous considérons que la recherche dans la compréhension des bases ainsi que les acteurs

moléculaires mis en jeu au niveau de l'interaction (Pi/Zn) pour réguler l'homéostasie du Pi dans la plante, constitue une nouvelle frontière dans ce domaine scientifique et tend à l'ouverture de nouvelles perspectives qui pourraient améliorer la nutrition phosphatée rien qu'en manipulant la signalisation de la carence en Zn chez les plantes.

Dans ce contexte, s'intègre le travail de ma thèse qui a été un terrain ayant permis de combiner des approches de physiologie végétale, de biologie moléculaire, de génétique d'association (GWAS), et de biologie des systèmes pour identifier les bases moléculaires impliquées dans l'interaction entre les voies de signalisation du P et celles du Zn chez les plantes. Comme c'est détaillé ci-après, mon travail de thèse est dicté par la volonté de conduire une recherche fondamentale de haut niveau sur la compréhension des mécanismes de régulation de l'homéostasie du Pi, et aussi envisager ensuite l'application sur des plantes d'intérêt agronomiques (exemple blé).

## II. Bibliographic introduction

### Introduction

Food security is a pressing global issue. Crop production has to double by 2050 to keep pace with the rapid increase in world population up to 9 billion. This target is challenging given the impact of the drive to reduce fertilizer inputs to make agriculture more environmentally sustainable. Developing crops with improved nutrient use efficiency would provide a solution. In frame of my thesis work, I focussed in discovering new molecular mechanisms that help improving P use efficiency in plants. Studying the regulation of phosphorus (P) homeostasis was motivated by the fact that global P reserves are becoming increasingly scarce, and consequently a potential phosphate (Pi) crisis looms for agriculture in the 21<sup>st</sup> century. In contrast to previous research work that focused on improving Pi homeostasis by modulating Pi deficiency response genes, I studied the regulation of P nutrition from a new perspective of improving Pi nutrition in plants by modulating the Zn-deficiency signalling pathway by investigating the Pi-Zn interactions in plants.

### A. Phosphorus.

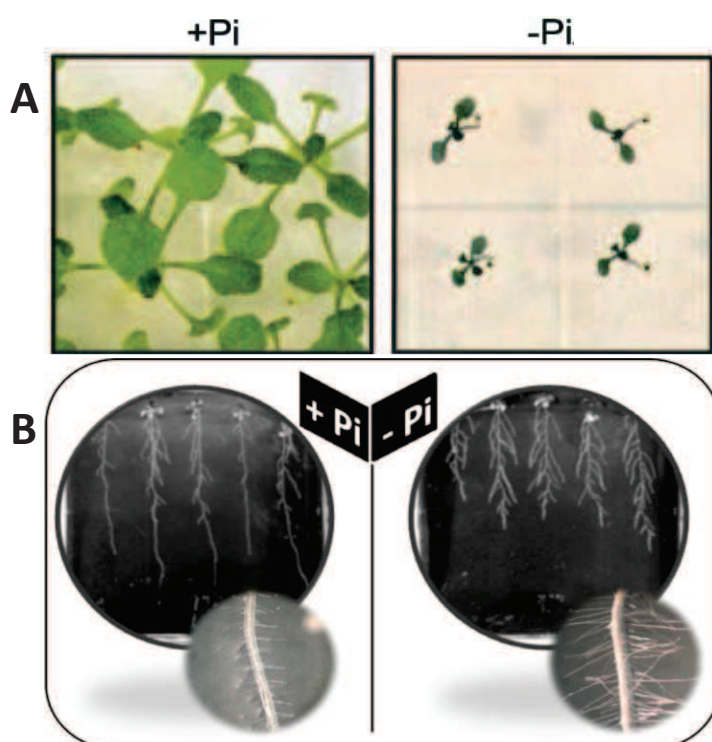
Phosphorous (P) is an essential macronutrient for plant growth and production, and it cannot be substituted with any other element to sustain plant life (Dessibourg 2010, Heuer *et al.*, 2017). The macronutrient Pi is an essential structural component of RNA and DNA, as well as phospholipids (Poirier and Bucher 2002). Pi is involved in many key biological processes in the cell, including numerous enzymatic reactions (Westheimer 1987, Poirier and Bucher 2002, Rouached *et al.*, 2010). Several signal transduction cascades also rely on Pi *via* the modulation of enzyme activity by protein phosphorylation, which can be adversely altered in situations where Pi is present in too low a concentration (Poirier and Bucher 2002).

P deficiency is widespread stress in natural and agricultural environments. Consequently, the global agricultural crop production is severely affected (Lott *et al.*, 2000, Heuer *et al.*, 2017). It is estimated that crop yield on 30–40% of the world's arable land is limited by inorganic phosphate (Pi) availability (Runge-Metzger 1995, MacDonald *et al.*, 2011). Many causes could explain the low availability of Pi for plants. First, the major form of P in soil is the inositol hexaphosphate, known as phytic acid (PA), which is not taken up by plant roots (Vance *et al.*, 2003, Belgaroui *et al.*, 2014, Secco *et al.*, 2017). Second, Pi ( $\text{HPO}_4^{2-}$ ) interacts with soil cations such as zinc ( $\text{Zn}^{2+}$ ) or ( $\text{Fe}^{2+}$ ) forming an insoluble complexes (Bouain *et al.*, 2014,

Mongon *et al.*, 2017, Rouached and Rhee 2017). Third, and more importantly, global Pi reserves are rapidly decreasing due to increase in demand (Van Kauwenbergh 2010, Van Vuuren *et al.*, 2010, Heuer *et al.*, 2017). Analysis of data collected over 14 years revealed that the usage of world P fertilizer considerably increased at the rate of about 357,000 t/ annum (i.e. an annual increase of 2.4%) (Lott *et al.*, 2011, Walan *et al.*, 2014). Experts agreed on that the world is facing serious P crisis (Abelson 1999). Noteworthy, global P reserve is not distributed uniformly, with a great discrepancy between countries (MacDonald *et al.*, 2011), which could have geopolitical implications. Taken together, these issues constitute compelling evidences justifying a direct link between Pi availability issues and the overwhelming world food security in coming few years. Therefore, developing a better understanding on how plants regulates Pi uptake, their transport in different parts and organs such including in seeds will help in designing new strategies to increase crop yield while reducing P input.

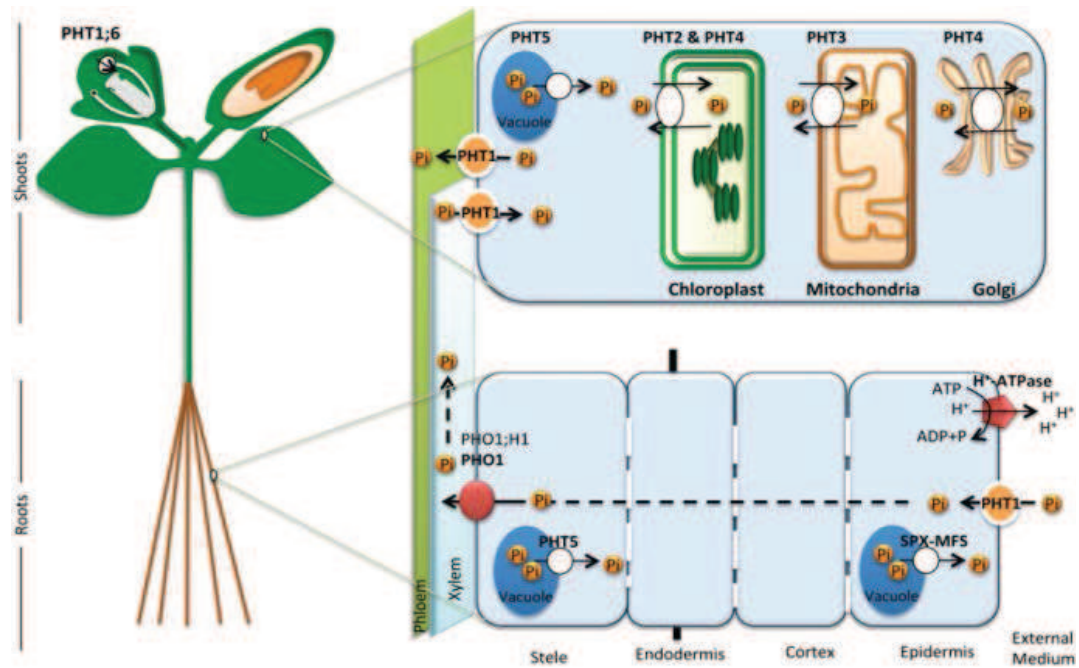
### 1. Phosphate uptake and transport in Arabidopsis.

In plants, Pi deficiency affects growth that manifest itself at phenotypic level by affecting the above- and underground tissues biomass (Figure I.1).



**Figure I.1. (A)** Growth of *Arabidopsis thaliana* plants on media containing phosphate deficiency and phosphate sufficiency. Seeds were germinated and grown for 18 d in media (Ticconi *et al.*, 2001) **(B).** Changes in primary root length and root hairs in grown either in presence (1 mM) or in absence of inorganic phosphate (Pi) for two weeks. (Bouain *et al.*, 2016).

Pi is acquired by root system, which is characterized by its great plasticity observed through the important changes of root architecture presumably to better explore soil surface (for review; (Bouain *et al.*, 2016). These morphological changes are concomitant with an increase of Pi uptake capacity through an induction of Pi uptake and transport (Shin *et al.*, 2004). Arabidopsis genome contain many *PHOSPHATE TRANSPORTERS* known as PHT (Poirier and Bucher 2002), and most of them are controlled by the Pi status of the plant. PHT family can be divided into five groups (PHT1-5) mainly based on their cellular localization and functional properties. Research results on this subject are summarized elegantly in many reviews (*e.g.* Nussaume *et al.*, 2011). In Arabidopsis, PHT1 subfamily contains 9 members and are reported to be preferentially expressed in roots, and function as a high-affinity Pi uptake transporter (Muchhal *et al.*, 1996, Misson *et al.*, 2005, Bayle *et al.*, 2011, Nussaume *et al.*, 2011, Remy *et al.*, 2012). (Shin *et al.*, 2004) provided genetic evidences proven PHT1.1 and PHT1.4 play crucial role in Pi transport in roots during growth under both low- and high-Pi environments. The double mutant shows a 75% reduction in Pi uptake capacity relative to wildtype (Shin *et al.*, 2004). While PHT2 subfamily members are localized in chloroplasts, PHT3/MPT proteins are mostly mitochondrial membrane transporters. PHT4 subfamily members are present on the golgi-apparatus located transporters. Recently, novel vacuolar Pi transporter was identified in the Arabidopsis and designated as SPX-MFS (SYG1/PHO81/XPR1-Major Facility Superfamily) proteins referred as PHT5 sub-family (for review, (Versaw and Garcia 2017), and PHT5/VPT/SPX-MFS (vacuoles/ tonoplast) (Liu *et al.*, 2015). Figure I.2.



**Figure I.2.** Tissue and subcellular localization of the Arabidopsis Pi transporters including PHTs and PHO1 protein families. Illustration of the proposed uptake and transport of phosphate in plant. Movement of phosphate is indicated by arrows. (Secco *et al.*, 2017)

## 2. Phosphate root-to-shoot translocation in Arabidopsis.

Pi have to go outside the cell, and then distributed between different plant organs. A Pi exporter referred as *Phosphate 1 (PHO1)* gene family comprises of 11 members mostly involve in transportation of Pi outside the cell (Hamburger *et al.*, 2002, Stefanovic *et al.*, 2007). It is believed that Pi loading into the root xylem in *Arabidopsis thaliana* is mainly achieved by PHO1 (Poirier *et al.*, 1991, Hamburger *et al.*, 2002) and PHO1;H1, as the mutation of both genes results in a very low phosphate level in the shoot, which severely compromises the plant life cycle (Stefanovic *et al.*, 2007). This extreme phenotype can be alleviated by supplying the double mutants with high Pi (Stefanovic *et al.*, 2007).

PHO1 does not show any structural homology with any other Pi or ion transporters. The PHO1 protein family harbours an SPX tripartite domain in the N-terminal hydrophilic portion, and an EXS domain in the conserved C-terminal hydrophobic portion (Hamburger *et al.*, 2002, Wang *et al.*, 2004). *Arabidopsis pho1* null mutants display several features associated with Pi deficiency, including severe reduction in shoot growth and accumulation of anthocyanins (Poirier *et al.*, 1991). Compared to the wild-type plant, *pho1* mutants have a normal Pi uptake rate, although there is a strong reduction (90%) in Pi transfer from the roots to the shoot; this



results in very low Pi levels in shoots (Poirier *et al.*, 1991). The *pho1* mutation was mapped using a positional cloning strategy in the gene AT3G23430 (Hamburger *et al.*, 2002). The activity of the *PHO1* promoter fused to a reporter gene (GUS) revealed that the *PHO1* promoter is predominantly expressed in the root vascular system, which is consistent with its implied role in Pi loading into the xylem (Hamburger *et al.*, 2002). At the protein level, the subcellular PHO1-GFP expression pattern was associated with similar punctate structures that co-localised with the Golgi/trans-Golgi network and uncharacterised vesicles (Arpat *et al.*, 2012). Experimental evidence showing that PHO1 specifically exports Pi from the cell has been provided through the use of transgenic plants expressing *PHO1* under the control of an inducible promoter (Arpat *et al.*, 2012). *In vivo* <sup>31</sup>P NMR experiments demonstrated that PHO1 overexpression causes a rapid loss of the vacuolar Pi pool, reflecting a dramatic efflux of Pi out of cells into the infiltration medium. Together, these results strongly indicate that PHO1 is a Pi exporter (Stefanovic *et al.*, 2011).

The *PHO1* gene is slightly up-regulated at the transcriptional level by Pi deprivation stress (Hamburger *et al.*, 2002). This regulation involves members of the *AtWRKY* family (<http://www.Arabidopsis.org/browse/genefamily/WRKY-Som.jsp>). Indeed, (Chen *et al.*, 2009) revealed that the regulation of *PHO1* expression in response to low-Pi stress involves *WRKY6* and *WRKY42*. *WRKY6* can repress the expression of *PHO1* in a Pi-dependent manner by binding to two W-boxes present in its promoter. The accumulation of the WRKY6 protein is diminished in the low Pi condition, which is likely caused by 26S proteasome-mediated proteolysis (Chen *et al.*, 2009). Consequently, *PHO1* expression is released by reducing WRKY6 binding to its promoter. In contrast to wild-type seedlings and the *wrky6-1* mutant, WRKY6 overexpression lines and the *pho1* mutant accumulate low Pi content in shoots (Chen *et al.*, 2009).

The Arabidopsis genome contains 10 additional genes that show homology to *PHO1* (Wang *et al.*, 2004). Phylogenetic analysis indicates that the *PHO1* family is subdivided into at least three clusters. However, the functional complementation of the *pho1* mutant by expression of the *PHO1* homologous genes under the control of the *PHO1* promoter revealed that only *PHO1* and its closest homologue *PHO1;H1* (At1g68740) could rescue the phenotype of the *pho1* mutant, in terms of shoot growth and shoot Pi content (Stefanovic *et al.*, 2007). Like *PHO1*, the *PHO1;H1* promoter is active in the vascular cylinder of roots. However, the *pho1pho1;h1* double mutant displayed a strong reduction in growth and in the capacity to

transfer Pi from the root to the shoot, as compared to *pho1*. *PHO1;H1* expression was very low in Pi-sufficient plants, although it was strongly induced under Pi-deficient conditions; this appears to be largely controlled by the transcription factor *PHOSPHATE RESPONSE 1* (PHR1) (Rubio *et al.*, 2001, Stefanovic *et al.*, 2007).

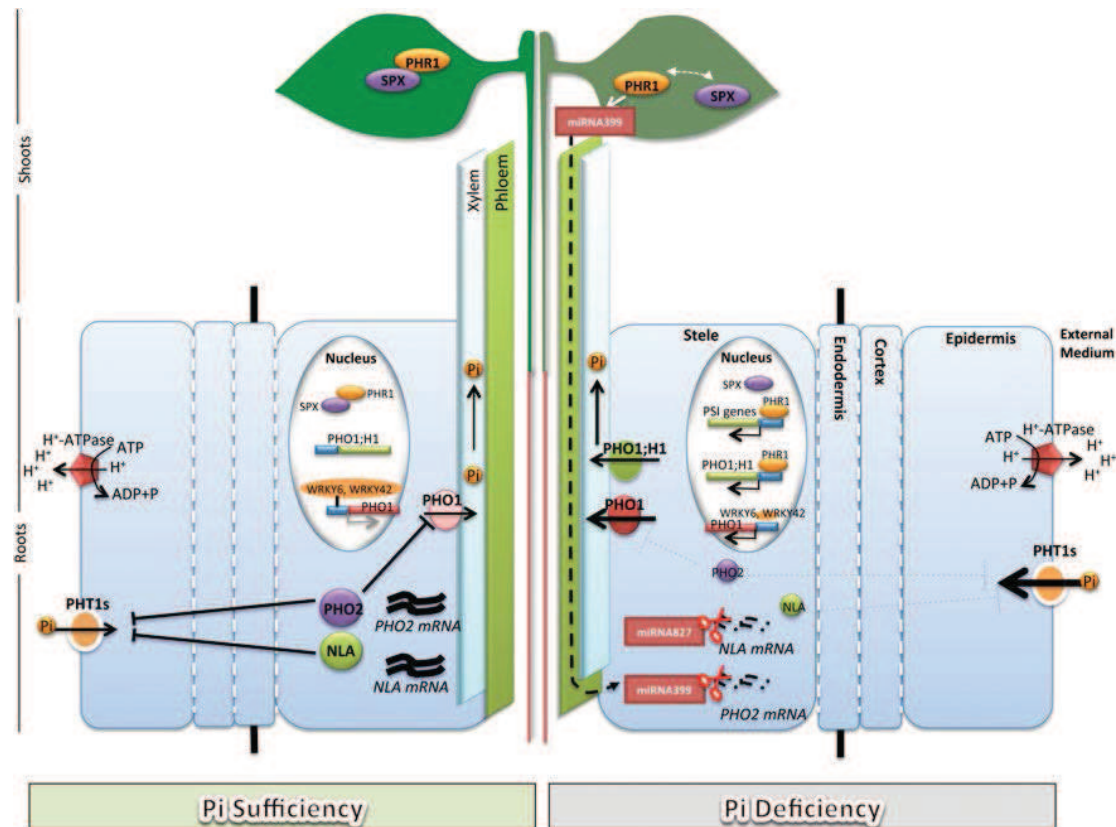
Together, these data reveal that PHO1 and PHO1;H1 are the key contributors for Pi loading into the root xylem, and that they are regulated by Pi deficiency through distinct signal transduction pathways.

### **3. Phosphate sensing and signaling in Arabidopsis.**

How plants sense and signal Pi deficiency is long standing question. In Arabidopsis, decades of research and only one complete Pi starvation signaling pathway has been identified so far, which can be defined as *SPX1-PHR1-miR399-PHO2-PHT1/PHO1* (for review; (Secco *et al.*, 2017). Key genes encoding for SPX proteins was initially revealed via transcriptome being up-regulated in response to P deficiency (Misson *et al.*, 2004). *SPX* genes ensure diverse functions in plant tolerance to Pi starvation (Duan *et al.*, 2008), and play an important role in sensing P concentration in cytosol through its capacity to bind the inositol polyphosphate signaling molecules (Wild *et al.*, 2016). SPX1 interact with the transcription factor Phosphate Response 1 (PHR1) in presence of Pi, and this interaction was dissociated under Pi deficiency (Wild *et al.*, 2016). Free PHR1 regulates many Pi-related genes (Bustos *et al.*, 2010). Among these genes, we can distinguish those that encodes for miRNA399 that targets *Phosphate2* (*PHO2*) transcripts. In response to Pi deficiency, *miRNA399* is transcriptionally regulated by PHR1, and then translocated from shoot to root by the phloem, where it targets the *PHO2* transcript (Bari *et al.*, 2006, Lin *et al.*, 2008, Pant *et al.*, 2008). The reduction of PHO2 protein abundance leads to the accumulation of PHT1 and PHO1 proteins (Liu *et al.*, 2012), and consequently an increase of the plant capacity to uptake Pi and translocate it to the shoot (Lin *et al.*, 2008, Briat *et al.*, 2015). Noteworthy, the proper functioning of this signaling pathway require the contribution of many others genes such the *SUMO E3 ligase SIZ1* (Miura *et al.*, 2005), *PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1* (PHF1, (González *et al.*, 2005) and *NITROGEN LIMITATION ADAPTATION* (NLA, (Lin *et al.*, 2013). SIZ1 is involved in the regulation of PHR1 by sumoylation (Miura *et al.*, 2005). PHF1 is required for the trafficking of Pi transporters to the plasma membrane (González *et al.*, 2005). NLA is proposed to function at plasma membrane to direct the degradation of PHT1s (Lin *et al.*, 2013), fine tuning thus the Pi uptake capacity of plants. The Pi long-distance signalling network includes



the MYB transcription factor PHR1, the ubiquitin E2 conjugase PHO2, and the miRNA399 (Pant *et al.*, 2008). (Figure I.3).



**Figure I.3.** Schematic representation of the regulatory pathways involved in Pi homeostasis in Arabidopsis. In +Pi, Phosphate Starvation Response 1 (PHR1), the main transcription factor is bound to an SPX protein, preventing the induction of Pi starvation induced genes (PSI) genes. In addition, the negative regulators PHO2 and NLA repress phosphate transporters such as PHT1s and PHO1. In -Pi, PHR1 is free and can activate PSI genes, such as PHO1:H1 and SPX genes. Concomitantly, miR399 and miR827 are induced and degrade transcripts from PHO2 and NLA, thus leading to increased uptake through increase activity of PHT1s and PHO1. (Secco *et al.*, 2017)

## B. Zinc.

The micronutrient Zn is required for proper cell functioning (Berg and Shi 1996, Salgueiro *et al.*, 2000, Sinclair and Krämer 2012), as it is a highly effective cofactor for hundreds of enzymes, the structural Zn-finger domains that mediate DNA-binding of transcription factors, and protein–protein interactions (Coleman 1998, Shahzad *et al.*, 2014). In plants, Zn is taken up at the root-soil interface, predominantly as free ions (Guerinot 2000, Milner *et al.*, 2013, Shahzad *et al.*, 2014). In recent years, significant progress has been made in our knowledge of the regulation of Zn acquisition in plants, and this phenomenon has been documented in many research publications and elegantly summarised in multiple reviews (e.g. (Sinclair and Krämer 2012). Many Zn uptake transporters have been identified and belong to the Zrt/IRT-like protein (ZIP) family of zinc transporters. In *A. thaliana*, the ZIP family contains 15 members (Mäser *et al.*, 2001), including the AtIRT1 which localizes preferentially to the plasma membrane of root epidermal cells (Vert *et al.*, 2002, Barberon *et al.*, 2011). It has been reported that *irt1* mutant accumulates less Zn as compared to wild type revealing its implication in Zn uptake (Henriques *et al.*, 2002). For ample information on the regulation of Zn uptake in Arabidopsis readers are referred to (Sinclair and Krämer 2012). After their acquisition at the root periphery, Zn can be fixed into the root *via* transport into vacuoles. Alternatively, their symplastic journey, thought to be mediated by the plasmodesmata, ends with their loading into root xylem. Zn transport, two members of the Arabidopsis P<sub>1B</sub>-ATPase subfamily: *HMA2* and its most closely related sequence in the *HMA* cluster, *HMA4* play crucial role in Zn loading into xylem (Hussain *et al.*, 2004, Verret *et al.*, 2004, Hanikenne *et al.*, 2008, Wong *et al.*, 2009, Siemianowski *et al.*, 2011). The most recent reports on their biological functions and the molecular mechanisms of their regulation in *A. thaliana* will be presented below.

### 1. Zinc root-to-shoot translocation in Arabidopsis.

In Arabidopsis, *HMA4* and *HMA2* gene encodes for a metal transporter located in the plasma membrane, with a very similar structure. HMA2 and HMA4 proteins play key roles in Zn loading into the root xylem, even though the signalling pathways that regulate their expression and activity remain poorly understood.

The Arabidopsis genes *HMA2* (At4g30110) and *HMA4* (At2g19110) are found in the Zn/Co/Cd/Pb subclass of the P<sub>1B</sub>-ATPases, and have well-documented roles in Zn loading into xylem (Hussain *et al.*, 2004, Verret *et al.*, 2004). In Arabidopsis, none of the individual *hma* mutants exhibit an observable or distinctive morphological phenotype when grown in soil, as

compared to the wild-type (Hussain *et al.*, 2004). The mutation of both genes results in a drastic Zn reduction in the shoot. This severely compromises the plant life cycle through visible morphological alterations, a stunted phenotype, and the formation of sterile flowers that lack pollen (decreasing plant fertility). This severe phenotype can be alleviated by supplying double mutants with high Zn.

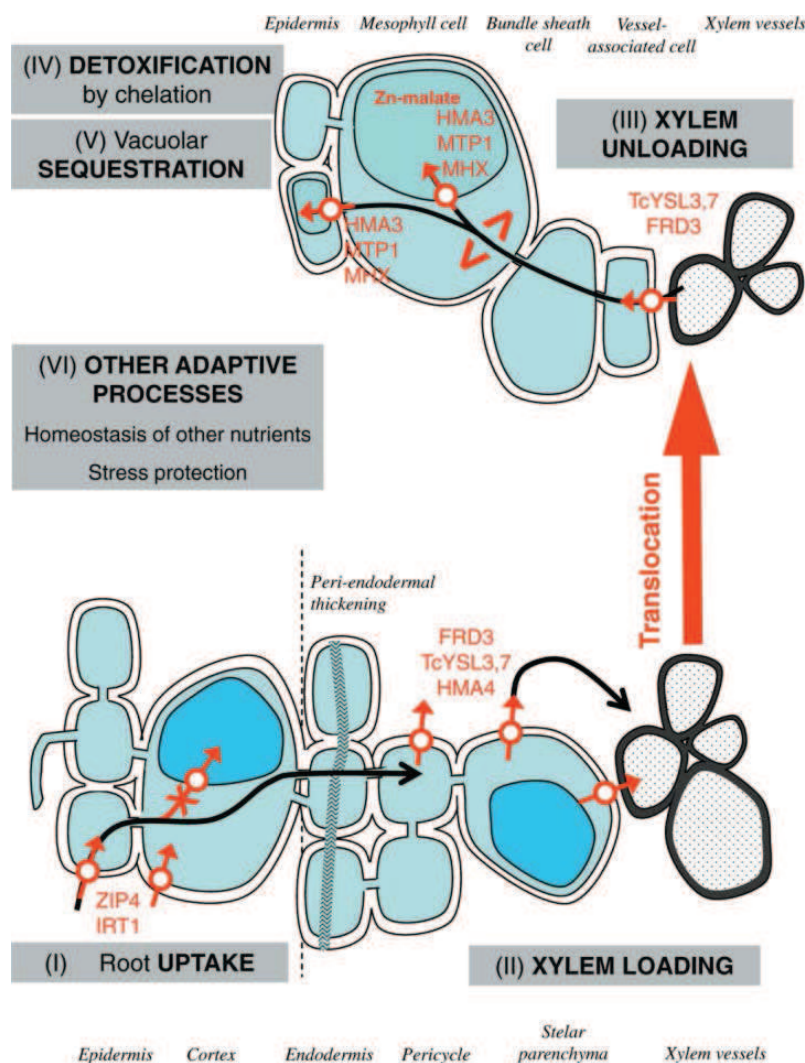
**HMA4:** *Arabidopsis thaliana* HMA4 was originally identified as a gene with increased expression in the Zn hyperaccumulator *Arabidopsis halleri*, as compared to its non-hyperaccumulator relative *A. thaliana* (Hanikenne *et al.*, 2008). The Zn content in the aerial parts of *hma4* mutant plants is decreased at a Zn concentration of 3  $\mu$ M, although it is not significantly affected at higher concentrations (100  $\mu$ M). Overexpression of *AtHMA4* in *A. thaliana* resulted in a 2-fold increase in Zn content in leaves, whereas no significant change was observed in root Zn content (Verret *et al.*, 2004).

**HMA2:** Expression of HMA2 promoter was observed in vascular bundles, and appeared to be expressed in components of both the xylem and the phloem (Hussain *et al.*, 2004). No decrease in the Zn content is observed in the single mutant *hma2*. The *A. thaliana hma2hma4* double mutant shows a 2-fold Zn increase in root pericycle cells, which causes a 2-fold decrease in shoots. This *hma2hma4* double mutant Zn-deficiency phenotype can be restored with the expression of full-length AtHMA2 (Wong *et al.*, 2009).

In *Arabidopsis* roots, HMA4 expression levels appeared to be enhanced by Zn (Hanikenne *et al.*, 2008), but it is still unclear whether the transcript level is modulated directly or indirectly by Zn. The specific mRNA activity of HMA4 in *A. halleri* and *A. thaliana* was observed in root pericycle and xylem parenchyma (Hanikenne *et al.*, 2008). When the  $\beta$ -glucuronidase (GUS) reporter gene was placed under the control of the HMA4 promoter in *A. thaliana*, expression was predominantly observed in root stellar cells located at the periphery of the xylem (Verret *et al.*, 2004). This expression pattern is consistent with the involvement of AtHMA4 in Zn loading into the xylem (Hussain *et al.*, 2004). A possible role for AtHMA4 in Zn efflux from the cytoplasm is also supported by its ability to restore growth to the *zntA* mutant in *S. cerevisiae*, which is defective in its endogenous Zn efflux pump at high Zn concentrations (Rensing *et al.*, 1997, Mills *et al.*, 2003, Verret *et al.*, 2004, Mills *et al.*, 2005).

In future, it will be interesting to identify the signalling pathways that regulate HMA2 and HMA4 at transcriptional and protein levels. Zinc sensing and signaling in *Arabidopsis*.

The Zn deficiency appears to be first sensed in shoots; the signal is then transmitted to the roots, where these cation transporters function (Assunção *et al.*, 2010b, Assunção *et al.*, 2013). This suggests the presence of long-distance Zn deficiency signalling molecules (which are yet to be identified). A recent working model of Zn deficiency signalling (Assunção *et al.*, 2013) proposes that the Arabidopsis transcription factors bZIP19 and bZIP23 play important roles in the response to Zn deficiency by regulating downstream genes, including ZIP members (*i.e.* the Zrt/Irt-like proteins, candidates that mediate root Zn uptake and transport) (Guerinot 2000, Assunção *et al.*, 2010b). (Figure I.4).



**Figure I.4.** Overview of current understanding of Zn transporters & accumulation. Those adaptations are highlighted in red. (I) Enhanced Zn uptake into root cells is thought to be driven by ZIP4 and to some extent by IRT1. (II) Active xylem loading depends on reduced vacuolar root sequestration (main Zn storage in the cortex) and enhanced activity of HMA4 in stelar parenchyma, leading to efficient efflux of Zn from symplasm. Efflux of citrate driven by FRD3 seems to play a role in Zn translocation. Loading of Zn in the xylem can also occur as Zn-nicotianamine complex by YSL proteins. (III) In the leaves, Zn is unloaded in vessel-associated cells by YSL proteins, and possibly by FRD3 in Arabidopsis halleri. Enhanced uptake of Zn in shoot symplastic pathway may be favored by ZIP6 in A. halleri. (IV) Detoxification is assumed to be operated by chelation of metals. <

> refers to chelation. Possible ligands of Zn in the cytoplasm are histidine and nicotianamine. (V) Vacuolar sequestration in the leaves is the main pathway of detoxification of metals. Zn is mainly stored in vacuoles of mesophyll (A. halleri) and/or epidermal cells (Thlaspi caerulescens), through the activity of MTP1, and possibly HMA3 and MHX. In the vacuoles, a large pool of malate favors the formation of Zn-malate complexes. (VI) Other adaptive processes include homeostasis of other nutrients, in particular Fe, P (PHT1-4), enhanced stress responses/protection (higher Glutathione GSH level, etc.). (Verbruggen *et al.*, 2009).

## **C. Phosphate and zinc homeostasis interaction in plants.**

Due to the central roles of Pi and Zn in numerous aspects of plant metabolism, it is not surprising that plants are profoundly affected by Pi or Zn starvation, and that their deficiencies provoke a coordinated series of morphological, physiological and biochemical adaptations (Mikulska *et al.*, 1998, Poirier and Bucher 2002, Misson *et al.*, 2005, Rouached *et al.*, 2010, Jain *et al.*, 2013). It is therefore of great importance for cells to tightly control Pi and Zn homeostasis, which likely interact *via* a complex process (Cakmak and Marschner 1986, Khan *et al.*, 2014).

Interactions between Pi and Zn in plants have been reported in numerous plant species (Reed 1946, Loneragan *et al.*, 1982, Verma and Minhas 1987, Webb and Loneragan 1988, Tagwira *et al.*, 1993, Gianquinto *et al.*, 2000, Huang *et al.*, 2000, Zhu *et al.*, 2001, Shi *et al.*, 2008). Such interactions are integrative as a plant loses its capacity to regulate Pi transport under Zn deficiency, despite the presence of an adequate Pi supply. Zn deficiency is associated with over-accumulation of Pi in the shoots of both dicotyledons and monocotyledons (Huang *et al.*, 2000, Misson *et al.*, 2004, Khan *et al.*, 2014). This interaction is of agronomic importance and can account for the shortcomings of current models that are typically focused on improving the assimilation of the individual elements. Earlier aforementioned studies have provided physiological evidence for the importance of Zn deficiency in Pi translocation to the shoots. More recently, (Khan *et al.*, 2014) have provided direct molecular evidence for the crosstalk between Pi and Zn nutrition in *A. thaliana*, by identifying genes involved in this crosstalk.

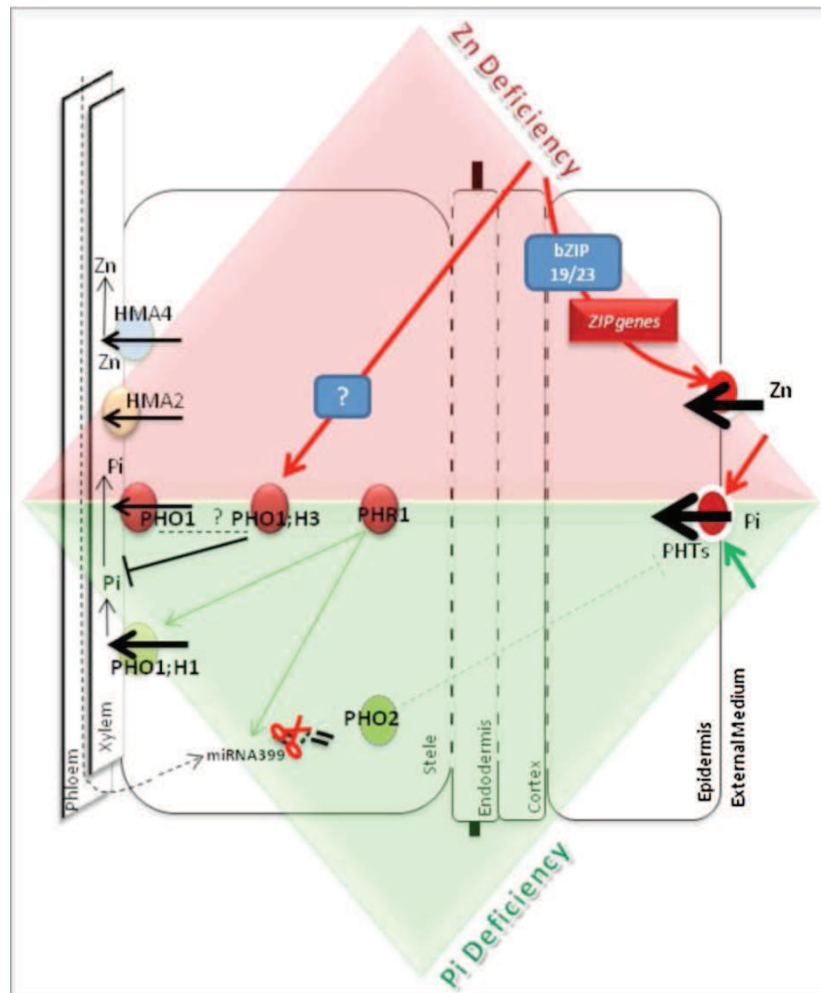
### **1. Role of PHT1 genes in regulating Pi homeostasis under Zn deficiency.**

Zn deficiency has a limited effect on the expression of high-affinity Pi transporters PHT1 in Arabidopsis. Analysis of the influence of Zn deficiency condition on the expression of the Pi transporters PHT1s revealed that only PHT1;1 was induced in shoots with concurrent slight changes in roots in comparison with Zn sufficiency (Jain *et al.*, 2013, Khan *et al.*, 2014). Worth noting, in monocotyledons, Zn deficiency has been shown to cause the upregulation of a high-affinity Pi uptake transporter, leading to the overaccumulation of Pi in the shoots (Huang *et al.*, 2000).



## 2. Emerging role for PHO1;H3 in Pi loading into the root xylem.

(Khan *et al.*, 2014) identified genes that are necessary for the increase in Pi over-accumulation in response to Zn deficiency in Arabidopsis. These genes include *PHR1*, *PHO1* and its homologue *PHO1;H3*. *PHR1* was already known as a major regulator of Pi deficiency signalling through its involvement in the so-called *PHR1-miRNA399-PHO2* regulatory pathway (Bari *et al.*, 2006). However, this regulatory pathway is not involved in the over-accumulation of Pi in the shoot in response to Zn deficiency (Khan *et al.*, 2014), and therefore a Zn-responsive signalling pathway involving *PHR1* remains to be elucidated. *PHO1* is most likely one of the final targets of the Zn-deficiency signalling pathway. Since its expression level does not change in response to Zn deficiency, it is likely that its activity is regulated through a protein-protein interaction, considering that a similar mechanism involving *PHO1* and *PHO2* has already been reported (Liu *et al.*, 2012). Finally, *PHO1;H3* is involved in the control of Pi accumulation in response to Zn deficiency, and thus appears to be involved in the regulation of Pi transport (Khan *et al.*, 2014), although no biological function has been identified so far. Nevertheless, it was recently reported that it is specifically and strongly induced by Zn deficiency, and that its expression pattern is similar to *PHO1*: both are expressed in cells of the root vascular cylinder and are localised to the Golgi when expressed transiently in tobacco cells (Khan *et al.*, 2014). When grown in Zn-free medium, *pho1;h3* mutant plants displayed higher Pi contents in the shoots than wild-type plants. However, this was not observed in a *pho1 pho1;h3* double mutant, suggesting that *PHO1;H3* restricts root-to-shoot Pi transfer that requires *PHO1* function for Pi homeostasis in response to Zn deficiency (Khan *et al.*, 2014). This makes *PHO1;H3* an interesting entry point to study Pi-Zn crosstalk in the root xylem. Future research to examine these questions should be aimed at uncovering the biological function of *PHO1;H3* in the regulation of Pi transport under Zn limitation, and identifying the gene regulatory network that regulate the expression of *PHO1;H3* (Figure I.5).



**Figure I.5.** Pi and Zn transport in plant roots. Pi and Zn are respectively conveyed into the symplast by PHT1 and ZIP transporters in the root epidermis. Transport into the xylem mainly (but not exclusively) involves PHO1 and PHO1;H3 for Pi, and HMA2 and HMA4 for Zn. The presence of *PHO1;H1* under Pi deficiency is detectable through the action of the transcription factor PHR1. In low Pi conditions the action of miRNA399 leads to the reduction of PHO2 transcript (and consequently its protein level), which causes an increase in the uptake and translocation of Pi by PHTs and PHO1, respectively. Zn deficiency leads to an increase in Zn uptake and distribution in the plant. This phenomenon involves the transcription factor bZIP19/23, which likely targets many ZIP transporters. Zn deficiency also leads to the upregulation of *PHO1;H3*, which negatively regulates Pi loading into the root xylem. The transcription factor that regulates *PHO1;H3* is unknown. (Kisko *et al.*, 2015).

### III. Objective of the thesis

Our current understanding of mechanisms regulating Pi homeostasis in plants come from investigations conducted mainly in model plants grown under Pi limitation. The genes discovered so far are specifically induced during Pi deficiency, and are not operating in conditions where Pi homeostasis is altered such as the increase of Pi accumulation in Zn-deficient plants. Despite its primary importance no investigation of the molecular basis of Pi-Zn interaction in plants was conducted so far.

It is clear that conducting a research program to dissect the Pi-Zn interaction in plants is original and timely. It holds a promise to discover new signalling pathway to enhance Pi nutrition in plants by modulating a Zn-deficiency signalling pathway. Thus, there is here a great opportunity to go beyond mainstream thinking that consists to focus only on Pi-starvation related genes, and we propose to identify a new route for Pi transport and accumulation in plants in Zn-dependent manner. This was the aim of my thesis, for which we have setted-up two main objectives that we have fulfilled using cutting-edge approaches and technologies:

1- We first developed a new computational biology tool to predict transcription factors (TFs) involved in the regulation of the expression of *PHO1;H3*. Then, we used reverse genetic approach to functionally validate these TFs with regards to their roles in regulating *PHO1;H3* expression, as well as their role in the accumulation of Pi in shoots of plants grown in presence or absence of Zn. Overall, this work lead to establish a new regulatory network for Pi accumulation under Zn deficiency in plant through *PHO1;H3*. These results are presented in Chapter 2 as manuscript accepted in Plant Physiology.

2- Using Genome-Wide Association Study (GWAS) to identify genes and natural variant that play key roles in the regulation of Pi accumulation under Zn deficiency. This approach (GWAS) emerged as a powerful tool for resolving genetic architecture of complex traits and for identification of underlying genetic determinants. We have successfully developed GWAS approach in Arabidopsis and results are presented in Chapter 3 as manuscript accepted in eLife.

The aforementioned chapters are preceded by a general introduction and review of the literature (Chapter I). Material and Methods used in the thesis are presented as part of each manuscript (Chapters II and III). My thesis ends with a general conclusion and perspective (Chapter IV). Two accepted review papers are included in the annex section.



## **Chapter II.**

# **TransDetect identifies a new regulatory module controlling phosphate accumulation in Arabidopsis**

## Chapter II. TransDetect identifies a new regulatory module controlling phosphate accumulation in *Arabidopsis*

### Résumé

Le phosphate (Pi) est un élément indispensable à la croissance et au développement des plantes. Etant une ressource naturelle non renouvelable, l'épuisement des gisements de Pi est devenu une préoccupation majeure du monde entier. De ce fait, réduire le volume d'engrais phosphaté, tout en maintenant la capacité productive des plantes cultivées, est devenu un objectif prioritaire.

Chez les plantes, le transport et l'accumulation de Pi impliquent de nombreux acteurs moléculaires qui sont principalement actifs en réponse à une carence en Pi. En revanche, plusieurs autres contraintes nutritionnelles sont connues également pour modifier l'homéostasie du Pi chez les plantes, comme la carence en zinc (Zn) dans le milieu. Les gènes qui permettent la régulation de l'accumulation du Pi en condition de carence en Zn demeurent inconnus jusqu'à ce jour.

Une interaction entre l'homéostasie du Pi et celle du Zn a été observée chez plusieurs végétaux, rapportant qu'une carence en Zn entraîne une suraccumulation de Pi dans les feuilles. Cependant, malgré leur importance agronomique, la signification biologique ainsi que les bases génétiques de ces interactions demeurent inconnues. Utilisant l'approche des gènes candidats chez *Arabidopsis*, notre équipe a identifié trois gènes importants dans la co-régulation de l'homéostasie du Pi et celle du Zn, dont le gène *PHO1;H3* (homologue du transporteur du phosphate PHO1) (Khan *et al.*, 2014). L'expression de *PHO1;H3* est induite spécifiquement dans les racines, en condition de carence en Zn, et sa mutation affecte l'accumulation du Pi dans les feuilles. *PHO1;H3* constitue pour nous un bon « point d'entrée », au niveau moléculaire, pour étudier l'interaction Pi-Zn chez les plantes. Nous nous sommes fixés comme objectif de découvrir les voies de signalisation de carence en Zn régulant l'accumulation du Pi. Nous nous sommes appuyés sur la biologie computationnelle, qui nous a permis de prédire/révéler de nouveaux facteurs de transcriptions (FTs) pouvant individuellement, ou en interagissant entre eux, réguler l'expression de *PHO1;H3*. En combinant cette approche avec la génétique reverse, et l'approche de génomique fonctionnelle, nous avons validé de nouveaux FTs impliqués dans la régulation de l'homéostasie du Pi chez *Arabidopsis*, en condition de carence en Zn.. Des expériences de simple et double hybride nous a permis de préciser d'une part les liens existants entre ces FTs et le promoteur *PHO1;H3* et les liens entre ces FTs d'autre

part. L'ensemble de ce travail a permis d'avoir accès à de nouveaux régulateurs de l'accumulation du Pi dans la plante, en condition de carence en Zn, et a été récemment publié dans le journal Plant Physiology:

TransDetect identifies a new regulatory module controlling phosphate accumulation in Arabidopsis

Pal S<sup>¶</sup>, Kisko M<sup>¶</sup>, Dubos C, Lacombe B, Berthomieu P, Krouk G, Rouached H. TransDetect Identifies a New Regulatory Module Controlling Phosphate Accumulation. Plant Physiol. 2017 Oct;175(2):916-926. doi: 10.1104/pp.17.00568. Epub 2017 Aug 21.

<sup>¶</sup> Authors equally contributed to this work

## Article: **TransDetect identifies a new regulatory module controlling phosphate accumulation in Arabidopsis**

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## Abstract

Identifying transcription factors (TFs) cooperation controlling target gene expression is still an arduous challenge. The accuracy of current methods at genome scale significantly drops with the increase in number of genes, which limits their applicability to more complex genomes, like animals and plants. Here, we developed an algorithm, TransDetect, able to predict TFs combinations controlling the expression level of a given gene. TransDetect was used to identify novel TFs module regulating the expression of Arabidopsis phosphate transporter *PHO1;H3* comprising MYB15, MYB84, bHLH35 & ICE1. These TFs were confirmed to interact between themselves and with the *PHO1;H3* promoter. Phenotypic and genetic analyses of TF mutants enable the organization of these four TFs and *PHO1;H3* in a new gene regulatory network controlling phosphate accumulation in zinc-dependent manner. This demonstrates the reliability of TransDetect to extract directionality in non-dynamic transcriptomes and to provide blueprint to identify gene regulatory network involved in a given biological process.

**Key words:** Computational biology, transcription factor, mineral nutrition, phosphate, zinc

**Short title:** TransDetect reveals key transcription factors involved in Pi homeostasis

## Introduction

Transcription factors are recognized as important orchestrators of living organisms response to environmental stimuli. Emerging experimental data indicates that a given gene in the eukaryotic genome is controlled by a high number of TFs (e.g. (Kaufmann *et al.*, 2010a, Xu *et al.*, 2013). In plants, techniques including Chromatin Immuno-Precipitation (ChIP, (Kaufmann *et al.*, 2010b, Nagel *et al.*, 2015), DAP-seq (O'Malley *et al.*, 2016), TARGET (Bargmann *et al.*, 2013, Para *et al.*, 2014, Medici *et al.*, 2015, Doidy *et al.*, 2016), or Y1H (Brady *et al.*, 2011, Gaudinier *et al.*, 2011, Taylor-Teeple *et al.*, 2015) are consistent with this potential high number of regulators, define some of the inner features of Gene Regulatory Networks (GRNs) topology. Knowing for instance that an eukaryotic genome possess ~2500 TFs, this presupposes that ~250,000 regulatory connections are pointing towards 30,000 genes. Thus, in average, a gene is likely to be under the direct influence of ~6 to 40 TFs. Hence complex interactions between TFs could determine the amplitude of the responses to different stress conditions. Despite its primary importance, so far our capacity to predict how specific TFs interact and form functional networks to regulate gene expression level is limited. Very few computational tools are able to predict TFs combinatorial effects so far. It is worth noting that while the current computational techniques are mainly based on the detection of co-occurrence of cis-regulatory elements in promoters as well as study of protein-protein interaction or Chip-Seq data none of these techniques use gene expression *per se* to extract such potential cooperation (GuhaThakurta and Stormo 2001, Nagamine *et al.*, 2005, Chang *et al.*, 2006, Yu *et al.*, 2006, Datta and Zhao 2007, Qin *et al.*, 2014).

In the last few years, attention has been stepped up to develop and use computational tools to decode complex TFs regulatory network involved in the regulation of complex biological process such as the coordination of nutrient signalling pathways (Rouached and Rhee 2017). For instance, an intriguing coordination between the homeostasis of a macronutrient (phosphate, Pi) and micronutrient (zinc, Zn) respectively has been recognized in plants: Pi accumulation in the shoots is increased by Zn deficiency (–Zn) (for review, (Bouain *et al.*, 2014, Kisko *et al.*, 2015). Nevertheless, despite its fundamental importance the molecular basis of the over-accumulation of Pi in –Zn conditions remains elusive. In Arabidopsis, the Pi transporter *PHO1;H3* was identified as an important player in the coordination of Pi and Zn homeostasis (Khan *et al.*, 2014). *PHO1;H3* transcript abundance specifically increases in roots upon –Zn treatment (Khan *et al.*, 2014). Plants that do not express *PHO1;H3* accumulate more Pi in the shoots than wild-type plants. *PHO1;H3* is proposed to be a negative regulator of Pi

translocation to the shoot in response to  $-Zn$  (Khan *et al.*, 2014). With these characteristics, *PHO1;H3* constitutes an entry point to extend our knowledge on the molecular network regulating Pi–Zn signalling crosstalk in plants.

In this study, we developed a computational tool, named TransDetect, able to predict TFs combination controlling a given gene. We used it to identify TFs involved in the regulation of the expression of *PHO1;H3*. A reverse genetic approach was then used to functionally validate the role of the identified TFs in regulating: (i) *PHO1;H3* expression (ii) Pi accumulation in shoots in presence or absence of Zn. The interaction between the TF themselves and with *PHO1;H3* promoter was assessed using yeast two- and one-hybrid assays, respectively. Overall, this work leads us to validate the TransDetect method, and to provide a backbone for the establishment of a new regulatory network for P accumulation under  $-Zn$  conditions in plants. The uncovered molecular network defines an independent path from the well-established Pi-starvation signalling pathway (Bari *et al.*, 2006, Lin *et al.*, 2008).

## Results

### TransDetect: a new algorithm identifying potential regulating pair of TFs

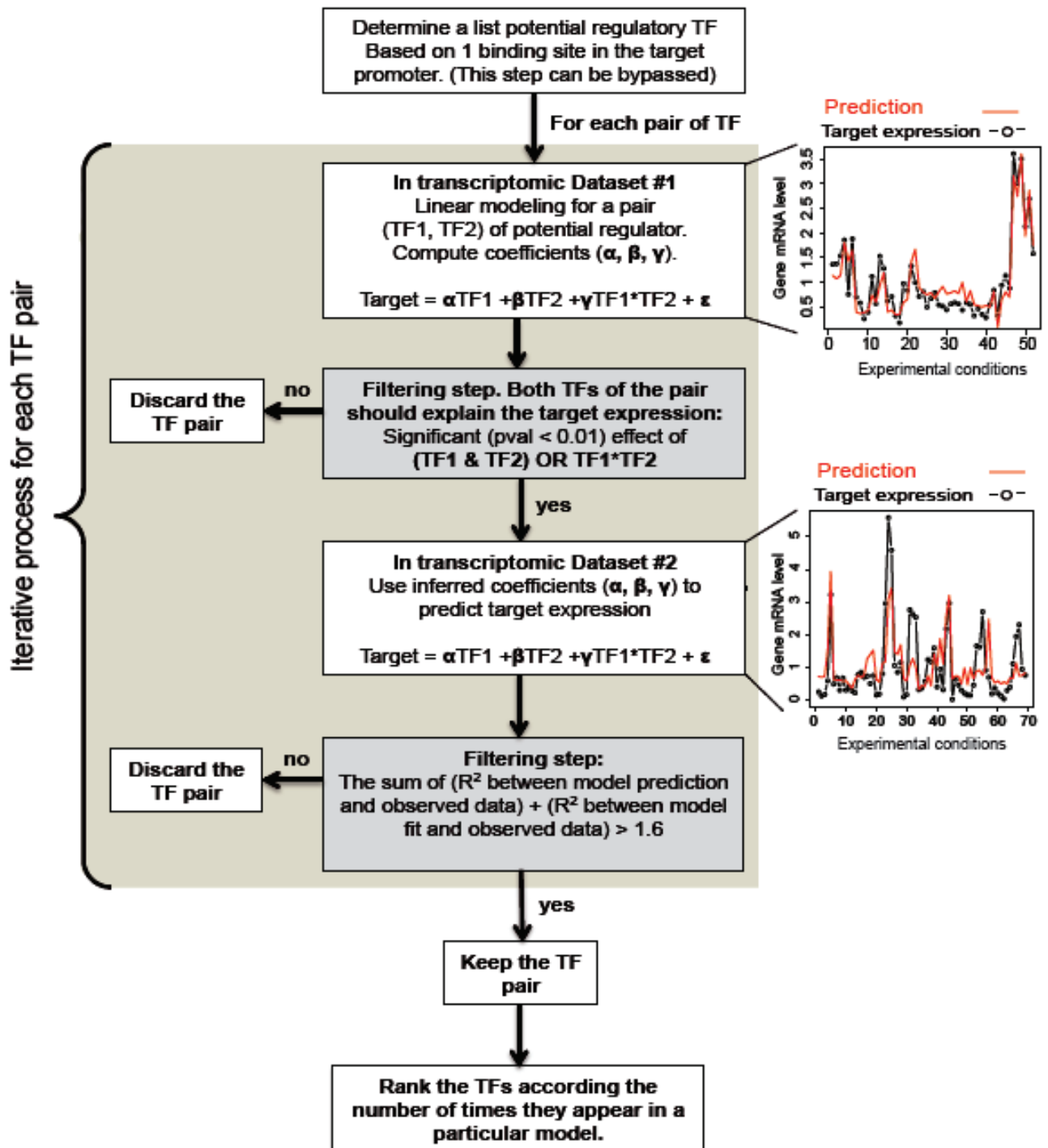
In this study, we first undertook a computational approach designed to extract, from transcriptomic data, potential information concerning TF interaction in the control of a particular gene. The scheme of the algorithm, written in R (<https://wwwr-project.org/>), is depicted in Figure II.1. The whole process starts with the selection by the user of a particular gene (here *PHO1;H3*). Then, the script selects all the TFs having a putative binding site in the target promoter as in (Katari *et al.*, 2010). This step can be easily bypassed in order to infer interactions of TFs that may not interact directly with the target gene or interact with a non-canonical DNA sequence. Following this, the algorithm enters into an iterative process (Figure II.1). Each pair of TFs expression in a first transcriptomic dataset (named data1) is used to fit the target expression following the equation:  $Target_{data1} = \alpha TF1_{data1} + \beta TF2_{data1} + \gamma TF1_{data1} * TF2_{data1} + \epsilon$ . In this equation,  $Target_{data1}$ ,  $TF1_{data1}$ ,  $TF2_{data1}$  represents the Target, TF1 and TF2 expression in the first dataset respectively  $TF1_{data1} * TF2_{data1}$  represents the potential combinatorial interaction of TF1 and TF2 expressions;  $\alpha$ ,  $\beta$ ,  $\gamma$  represents the coefficients of the linear modeling and  $\epsilon$  the non-explained variance. Each model is then evaluated based on two criteria. The first one is that both TF1 and TF2 have to explain the target expression additionally or in combination. This is performed by filtering on  $p$ values  $< 001$  on [the  $\alpha$  and

the  $\beta$  coefficients] OR the  $\gamma$  alone. Any model recording the effect of only one TF is discarded since the rationale is that it can be retrieved by simple correlation network studies. The second criterion is the capacity of the fitted model to predict the target expression in a second external dataset (named data2, see Figure II.1) that was not used to fit the model.

We thus use the fitted model coefficients to predict (without fitting) the Target expression, in the new conditions (data2), following the equation  $\text{Target}_{\text{data2}} = \alpha \text{TF1}_{\text{data2}} + \beta \text{TF2}_{\text{data2}} + \gamma \text{TF1}_{\text{data2}} * \text{TF2}_{\text{data2}} + \epsilon$ . It then evaluates the quality of the prediction by generating the  $R^2$  value for a linear regression between observed and predicted values (on the second dataset).

This process is iterated for all the potential TF pairs. This is necessary to find out the “best” pairs of TFs that: i) fit the training dataset ii) predict gene expression in the external dataset. These models are selected if they maximize the sum of the  $R^2$  values (default threshold 15 for the sum of the  $R^2$  0.75 for simple  $R^2$ ) in the fitted and in the predicted dataset. Thus it is possible to detect potential coordination of a gene expression by pairs of TFs. Finally each model (pair of TF) is saved. The TFs are finally ranked based of the number of times they have been found to participate in a model having passed the different criteria.





**Figure II.1. Scheme of TransDetect algorithm organization.** The algorithm is built on a inference iterative process. First the target gene transcript level is fitted by a linear combination of the transcript levels of two transcription factors (TFs). The resulting model is kept only if the two TFs significantly participate in the fit. The learnt coefficients are then used to predict the transcript levels of the target gene in an independent dataset. If the model is able to properly fit the transcript levels of the target gene in the first dataset and predict them in the second dataset, the corresponding TF pair is kept. A final list of selected TF pairs is generated and TFs are ranked based on the number of times they appear in this list.

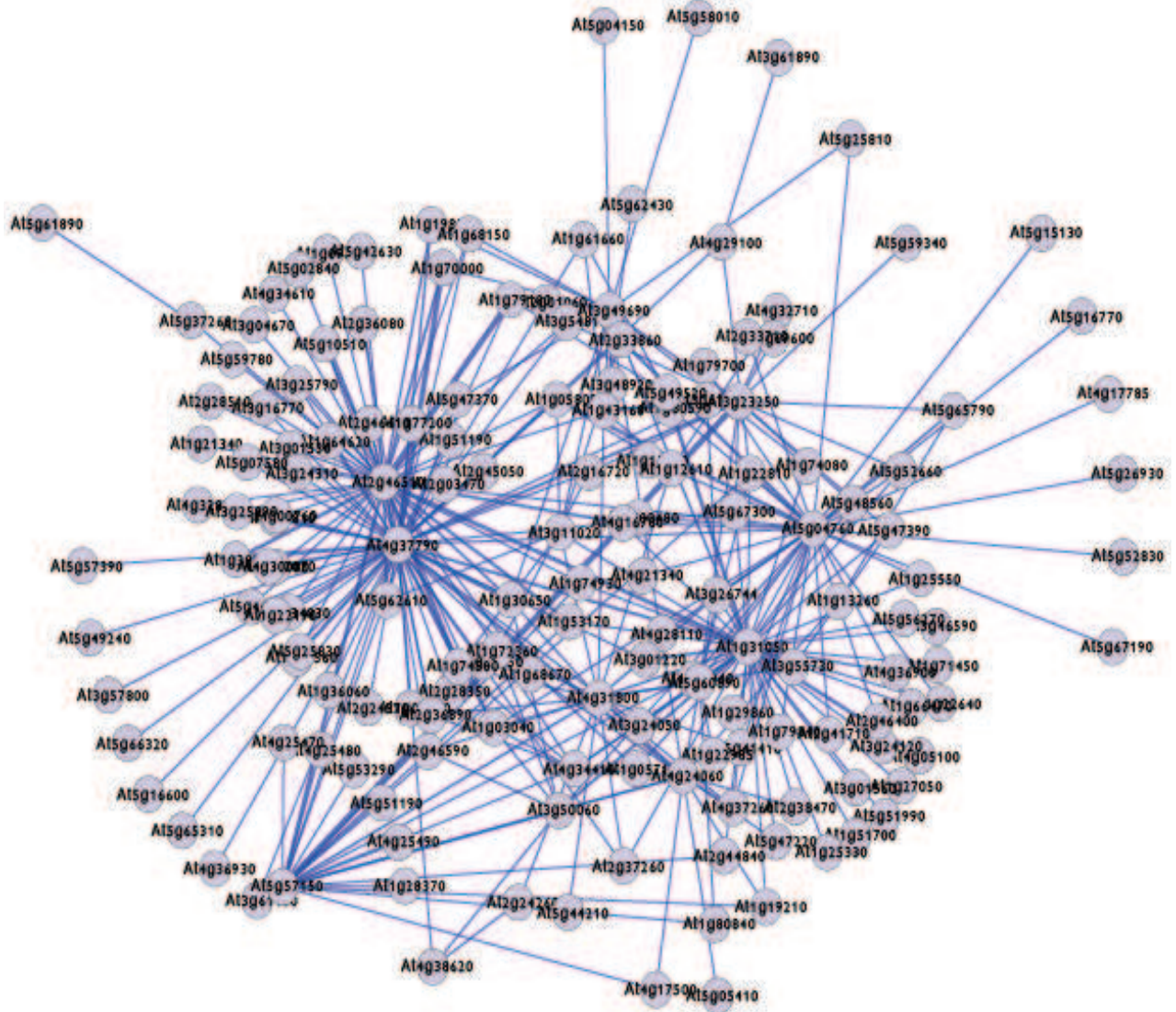
### **MYB15, MYB84 and bHLH35 regulate *PHO1;H3* expression under zinc deficiency**

TransDetect was used to identify TFs regulating the expression of the Pi transporter *PHO1;H3* in Arabidopsis roots. The root transcriptomic dataset used for the learning step (52 data points/Affy Chips) and the root transcriptomic dataset used for the validation was from (69 data points/Affy Chips) (Brady *et al.*, 2007, Dinneny *et al.*, 2008, Azevedo *et al.*, 2016). Our analysis identified a total of 165 TFs organized in pairs (supplementary table II.1) as potential regulators of *PHO1;H3*. The potential TFs network constructed based on TransDetect analysis (Figure II.2) illustrates the candidate TFs pairs to regulate *PHO1;H3*, and show number of TFs are involved in more than one connection. In this study, the candidate TFs were ranked based on the number of appearances in significant models. The top ten TFs were considered for further analyses (Figure II.3A)

*PHO1;H3* transcript abundance is known to increase in response to  $-Zn$  condition (Khan *et al.*, 2014). We therefore hypothesized that mutations of these TFs would affect the expression of *PHO1;H3* in response to  $-Zn$ . For each of the ten selected TFs *PHO1;H3* transcript accumulation was assessed by quantitative RT-PCR in roots of WT Col-0 plants and of two different T-DNA insertion mutant lines grown in the presence or absence of Zn for 18 days. As expected (Khan *et al.*, 2014),  $-Zn$  treatment caused a two-fold upper accumulation of *PHO1;H3* transcripts in WT Col-0 plants (Figure II.3A). Interestingly, among the twenty considered mutant lines the loss of function of members of the MYB15/MYB84 and MYB15/bHLH35 TFs pairs affect the *PHO1;H3* expression in  $-Zn$  conditions. Mutation of the two R2R3-MYB TFs *MYB15* (At3g23250) or *MYB84* (At3g49690) lead to an increase in *PHO1;H3* transcript accumulation in  $-Zn$  (Figure II.3A). This result suggests that these two TFs are negatively regulating *PHO1;H3* expression in response to a  $-Zn$ . In contrast, mutations in *bHLH35* (At5g57150) lead to a decrease in *PHO1;H3* transcript accumulation (Figure II.3A), revealing a positive regulatory role of *bHLH35* (activator) on *PHO1;H3* expression in  $-Zn$  condition. *PHO1;H3* transcript accumulation was not significantly altered in plants harbouring a mutation in any of the other 7 TFs (Figure II.3A). It is noteworthy that among these three TFs only *MYB15* was significantly ( $p<005$ ) induced in WT plants grown for 18 days in  $-Zn$  (Figure II.3B).

We then tested whether *MYB15*, *MYB84* and *bHLH35* could interact with the promoter of *PHO1;H3* (*pPHO1;H3*), using a yeast one-hybrid assay. Interestingly, MYB15 and MYB84 interacted with *pPHO1;H3* fragments, but bHLH35 did not (Figure II.3C). It thus cannot be

excluded that MYB15 and MYB84 regulate *PHO1;H3* expression independently from each other. However, bHLH35 may interact with *pPHO1;H3* through a partnership with another protein. Indeed, it cannot be ruled out that bHLH35 controls *pPHO1;H3* activity through the activation of an intermediate TF that was not identified using TransDetect.

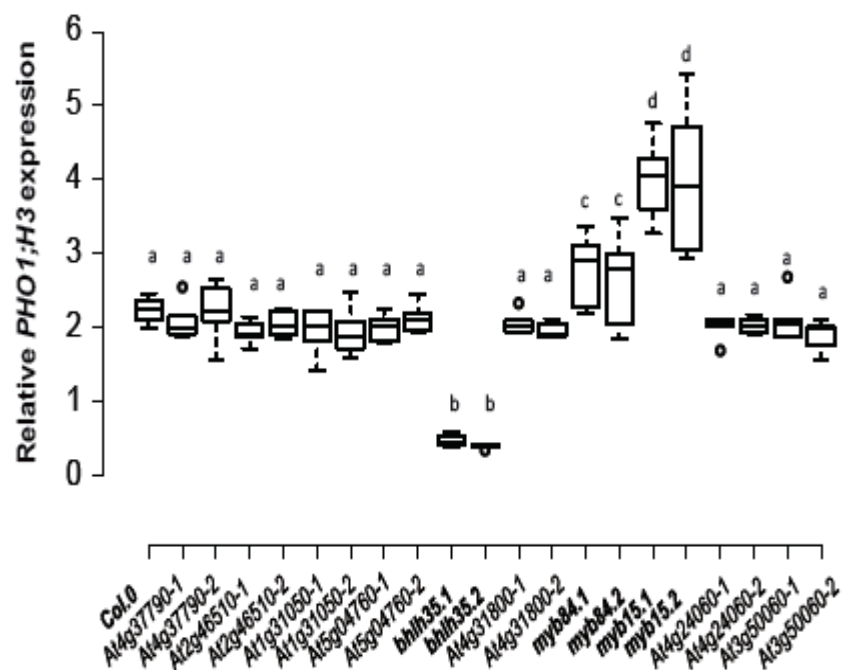


**Figure II.2. TransDetect network potentially influencing *PHO1;H3* gene expression.** Each node represent a potential *PHO1;H3* regulator based on the TransDetect criteria defined in the text. If a pair of TF is predicted to explain *PHO1;H3* expression it is linked by an edge. The edge width is proportional to the sum of the  $R^2$  for the fit and predicts processes (values are ranging from 16 to 176). The most influential factors are likely to be the most connected.

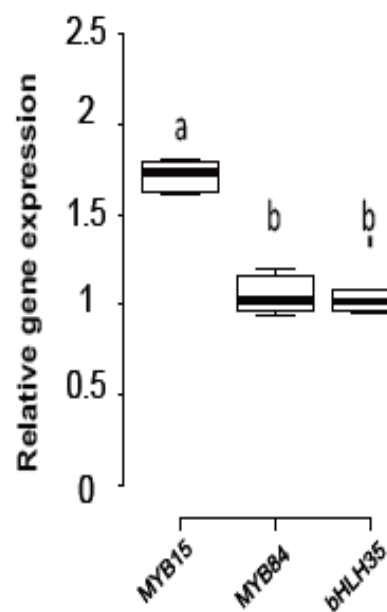
### **MYB15, MYB84 and bHLH35 influence Pi accumulation in the shoot under zinc deficiency**

*PHO1;H3* is known to regulate the accumulation of Pi in the shoot of Zn deficient plants (Khan *et al.*, 2014). When grown in Zn-free medium, *pho1;h3* mutant plants display a higher shoot Pi accumulation than wild-type plants, indicating that PHO1;H3 reduces Pi translocation to the shoot in response to –Zn (Khan *et al.*, 2014). To check whether changes in *PHO1;H3* transcript accumulation in *myb15*, *myb84*, and *bhlh45* mutant backgrounds would result in changes in Pi accumulation under Zn deficiency, we determined the Pi concentration in shoots of WT (Col-0), *myb15*, *myb84*, and *bhlh35* mutant lines grown in either +Zn or –Zn conditions for 18 days. As expected, Pi concentration was increased in the shoots of WT plants grown under –Zn (Figure II.3D). Then, while mutations in *myb15* and *myb84* resulted in reduced Pi accumulation in shoots, mutations in *bhlh35* caused an increase in Pi accumulation (Figure II.3D). These variations in shoot Pi concentration were consistent with the variations of *PHO1;H3* transcript abundance. Our results thus demonstrate the involvement of MYB15, MYB84 and bHLH35 in the regulation of Pi accumulation in the shoot of plants grown in –Zn conditions.

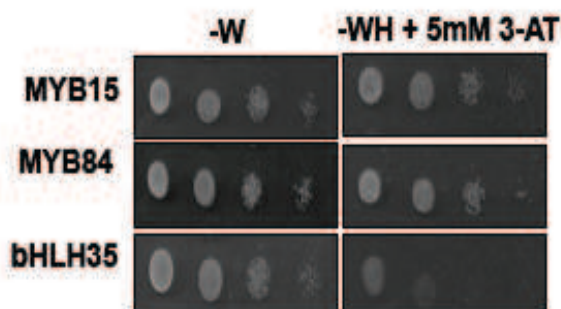
**A**



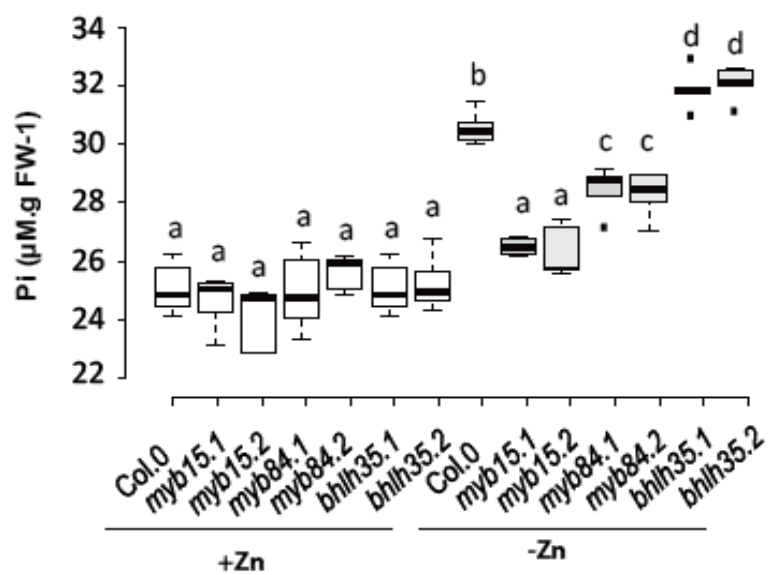
**B**



**C**



**D**



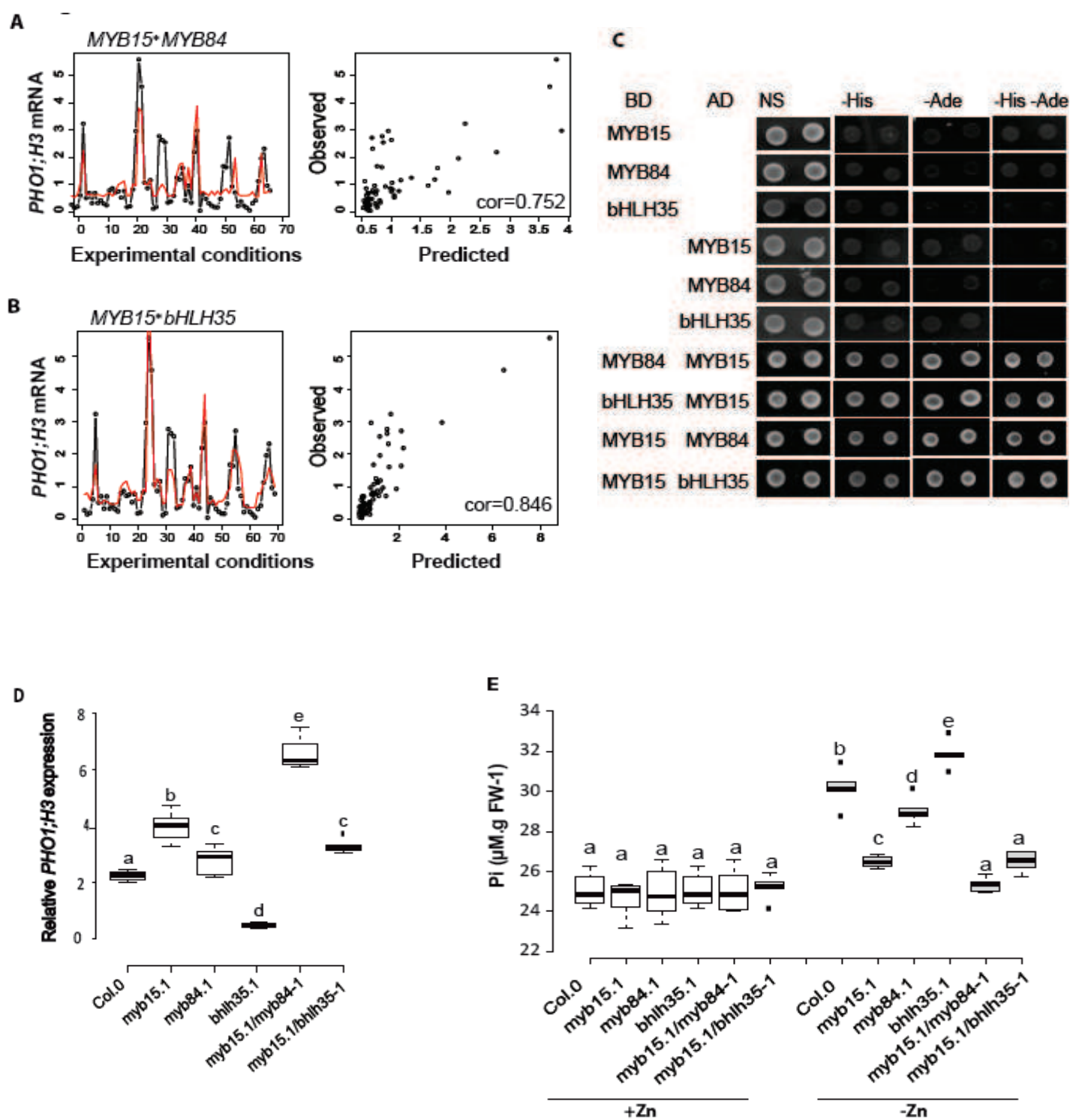


**Figure II.3. MYB15, MYB84 and bHLH35 regulate both the expression of *PHO1;H3* the accumulation of Pi in the shoot under zinc deficiency.** **A-** Relative *PHO1;H3* (At1g14040) transcript accumulation in the roots of wild-type plants (Col-0) and mutant lines harbouring loss-of-function mutations in the following transcription factors: At5g57150 (*bHLH35*), At3g23250 (*MYB15*), At3g49690 (*MYB84*), At4g37790 (*HAT22*), At2g46510 (*bHLH17*), At1g31050 (*bHLH111*), At5g04760 (*MYB-type*), At4g31800 (*WRKY18*), At4g24060 (*Dof46*) At3g50060 (*MYB77*). Plants were grown for 18 days in the presence (+Zn) or absence (–Zn) of zinc. *PHO1;H3* transcript abundance was measured by qRT-PCR normalized against *UBQ10* (At4g05320). **B-** *MYB15*, *MYB84* and *bHLH35* transcripts accumulation in response to Zn deficiency. Relative *MYB15*, *MYB84* and *bHLH35* transcript accumulation was quantified in roots of wild-type plants (WT) grown for 18 days in the presence or absence of Zn by qRT-PCR and normalized against *UBQ10*. **C-** Yeast one-hybrid assay. Sequences of the Arabidopsis *PHO1;H3* promoter fused to the *HIS3* auxotrophic marker were stably transformed into yeast. These different yeast strains were then co-transfected with *MYB15*, *MYB84* or *bHLH35*. Left panel, growth of the different yeast strains on control media deprived of tryptophan (-W), allowing the selection of yeast cells expressing the selected TFs. Right panel, growth of the different yeast strains on selective media deprived of tryptophan and histidine (-W -H). **D-** Pi concentrations measured in the shoots of wild type, *myb15*, *myb84*, or *bhlh35* plants grown for 18 days in the presence or absence of Zn. For A, C and D panels, central lines in the boxes show the medians; box limits indicate the 25<sup>th</sup> and 75<sup>th</sup> percentiles; whiskers extend 15 times the interquartile range from the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Letters a, b and c indicate significantly different values at  $p < 0.05$  determined by one-way ANOVA and Tukey HSD.

### **MYB15/MYB84 and MYB15/bHLH35 pairs interact regulate *PHO1;H3* expression Pi accumulation**

Since the MYB15/MYB84 and MYB15/bHLH35 TFs pairs were predicted by TransDetect to cooperatively regulate *PHO1;H3* expression (Figure II.4A-B), we tested whether the TFs constituting these pairs interact physically. Using a yeast two-hybrid assay, we found that MYB15 has the ability to physically interact with MYB84 and with bHLH35 (Figure II.4C). We then generated *myb15/myb84* & *myb15/bhlh35* double KO mutants in Arabidopsis by crossing single mutant lines. Interestingly, when grown in  $-Zn$ , the *myb15/myb84* double mutant showed an increase of the *PHO1;H3* expression level and a decrease of Pi accumulation in the shoots compared to *myb15*, *myb84* or WT plants (Figure II.4D-E). As already mentioned MYB15 and bHLH35 have opposite effects on both *PHO1;H3* expression and Pi accumulation. When grown in  $-Zn$ , the *myb15/bhlh35* double mutant showed an accumulation of *PHO1;H3* transcript (Figure II.4D) and Pi concentration in shoots similar to what was observed in the *myb15* single mutant (Figure II.4E). These results are indicative that the three TFs likely belong to the same molecular pathway regulating *PHO1;H3* in response to  $-Zn$ . It is likely that in this pathway MYB15 acts downstream bHLH35 (Figure II.4D-E).

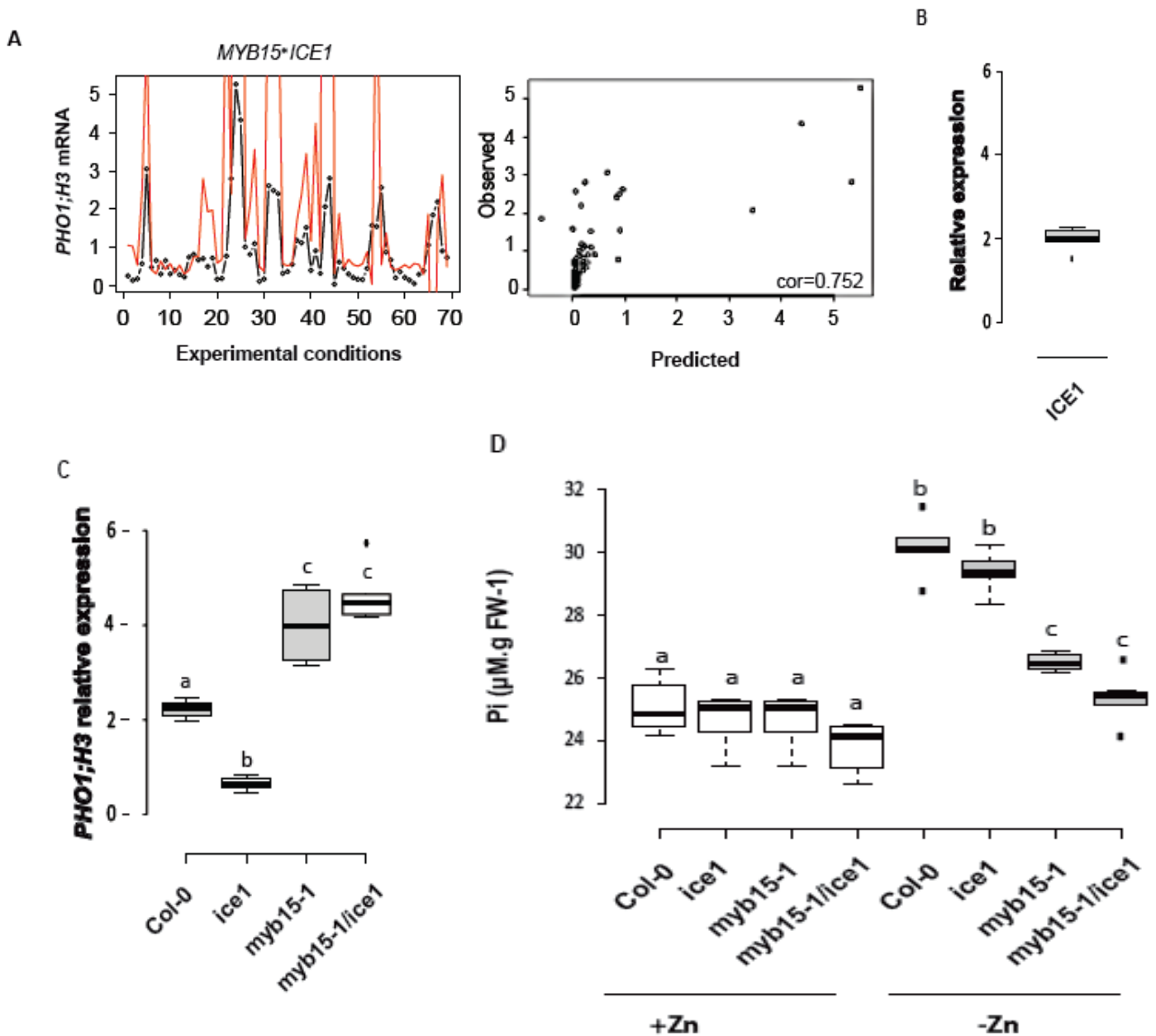




**Figure II.4. Interactions between MYB15 and MYB84 and between MYB15 and bHLH35 influence the expression of *PHO1;H3* Pi accumulation under zinc deficiency.** **A, B-** TransDetect prediction of correlation between the expression of the *MYB15* / *MYB84* and *MYB15* / *bHLH35* TF pairs and the *PHO1;H3* transcript level with  $R^2=0.75$  and  $R^2=0.84$  respectively. **C-** Yeast two-hybrid assay *bHLH35*, *MYB15* and *MYB84* were fused with either the GAL4 DNA binding domain (BD) or the GAL4 activation domain (AD) into appropriate expression vectors, which were then transferred into yeast. The different yeast strains were plated on non-selective medium (NS) or on selective media deprived of histidine (-His), adenine (-Ade) or both simultaneously (-His-Ade). **D-** Relative *PHO1;H3* transcript accumulation in roots of wild type (Col-0), *bhlh35*, *myb15*, *myb84*, *myb15/myb84* *myb15/bhlh35* mutant plants grown for 18 days in the absence of zinc (-Zn) compared to +Zn. *PHO1;H3* transcript abundance was measured by qRT-PCR and normalized against *UBQ10*. **E-** Shoot Pi concentrations measured in wild-type (Col-0), *bhlh35*, *myb15*, *myb84*, *myb15xmyb84* and *myb15xbhlh35* mutant plants grown on either +Zn or -Zn for 18 days. For D and E panels, Box central lines show the medians; box limits indicate the 25<sup>th</sup> and 75<sup>th</sup> percentiles; whiskers extend 15 times the interquartile range from the 25<sup>th</sup> and 75<sup>th</sup> percentiles Letters a, b and c indicate significantly different values at  $p < 0.05$  determined by one-way ANOVA and Tukey HSD.

### **ICE1 regulates Pi accumulation under Zn deficiency in a MYB15 dependent manner**

The role of MYB15 in regulating Pi accumulation is new. However, MYB15 has been shown to physically interact with the MYC-like bHLH TF ICE1 (INDUCER OF CBP EXPRESSION 1) to regulate the plant response to cold stress (Agarwal *et al.*, 2006, Miura *et al.*, 2007). Through this interaction, ICE1 suppresses the activity of MYB15 (Miura *et al.*, 2007). It is noteworthy that lowering the stringency of our TransDetect analysis revealed a potential effect of MYB15 and ICE1 as TFs pair on the expression of *PHO1;H3* (Figure II.5A). We thus tested whether ICE1, individually or cooperatively with MYB15, could be involved in the regulation of *PHO1;H3* expression. First *ICE1* transcript level was found to be significantly ~2 fold induced under  $-Zn$  (Figure II.5B). Then a KO mutation of *ice1* lead to a decrease in *PHO1;H3* transcript accumulation (Figure II.5C) and coupled with an increase in shoot Pi concentration under  $-Zn$  when compared to WT plants (Figure II.5D). The *myb15/ice1* double mutant displayed increased *PHO1;H3* transcript accumulation (Figure II.5C) and a decreased Pi concentration in shoots in response to  $-Zn$ , in a similar range as what was observed in the single *myb15* mutant (Figure II.5D). Our results thus indicate that MYB15 most probably acts downstream ICE1 to control Pi accumulation under  $-Zn$  via *PHO1;H3*.



**Figure II.5. The ICE1 / MYB15 transcription factor pair regulates both the expression of *PHO1;H3* and the accumulation of Pi under -Zn.** **A-** TransDetect's prediction of the correlation between the expression of the TF pair MYB15 and ICE1 and the *PHO1;H3* expression ( $R^2=0.73$ ). **B-** *ICE1* transcript accumulation. Expression of *ICE1* was quantified in wild type (Col-0) seedlings grown for 18 days in presence (+Zn) or absence (-Zn) of zinc. *ICE1* transcript abundance was measured by qRT-PCR normalized against *UBQ10*. **C-** *PHO1;H3* transcript accumulation. Expression of *PHO1;H3* gene was quantified in wild type (Col-0), *ice1*, *myb15* and *myb15/ice1* seedlings grown for 18 days in +Zn or -Zn. *PHO1;H3* transcript abundance was measured by qRT-PCR and normalized against *UBQ10*. **D-** Pi accumulations. Pi concentrations were measured from shoots of wild type (Col-0), *ice1*, *myb15*, and *myb15/ice1* seedlings grown for 18 days in presence +Zn or -Zn. For B, C and D, Box center lines show the medians; box limits indicate the 25<sup>th</sup> and 75<sup>th</sup> percentiles; whiskers extend 15 times the interquartile range from the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Letters a, b and c indicate significantly different values at  $p<0.05$  determined by one-way ANOVA and Tukey HSD.

## Discussion

Gene expression data has rapidly increased with the use of transcriptomic technologies, requiring the development of methods for their efficient analysis. Combination with functional genomics approaches these computational tools can help to gain new insight on the molecular basis of complex phenomenon such as the regulation of ions homeostasis in plants (Mongon *et al.*, 2017, Rouached and Rhee 2017). In this context, we developed and used an algorithm, TransDetect, to identify regulators (TFs) and to build a GRN that control the expression of a Pi transporter, *PHO1;H3*, in response to  $-Zn$  conditions.

In Arabidopsis, the *PHO1;H3* gene was demonstrated to act at the interface Pi-Zn homeostasis interaction (Khan *et al.*, 2014). The expression of *PHO1;3* is induced in response to  $-Zn$  treatments in roots (Khan *et al.*, 2014). Mutation of *PHO1;H3* causes an overaccumulation of Pi in Arabidopsis shoots (Khan *et al.*, 2014). Therefore, *PHO1;H3* gene was proposed to plays a negative regulatory role of Pi transfer from root to shoots in  $-Zn$  conditions (Khan *et al.*, 2014). Nevertheless, the GRN that regulates the expression of *PHO1;H3* in response to  $-Zn$  still unknown. Using TransDetect algorithm, we identified a list of candidate TFs pair for the regulation of the *PHO1;H3* expression. Among the top 10 candidates revealed by TransDetect, we tested 20 mutant lines (2 mutant per TFs) among which 3 display interesting phenotypes related to *PHO1;H3* expression (Figure II.3A-B). We believe that this relatively high level of success could be explained as follow: in general, algorithms focusing on correlation between genes do not provide any information concerning causality. Say that if 2 genes A and B are highly correlated, it does not imply if  $A \rightarrow B$  or if  $B \rightarrow A$ . Dynamic aspects can sort between the 2 situations (Krouk *et al.*, 2013). Unfortunately, the vast majority of the transcriptome in databases are not kinetics. We would like to mention here that, by the way TransDetect is working, it might extract some directionally information from static data. To clearly explain this, consider the ideal case where 2 TFs (TFA and TFB) control a Target gene following a logic gate (Figure II.6). In this particular case,  $\gamma$  coefficient of the linear model will be highly significant because it is the combination of TFA and TFB expression that is necessary to fully explain Target transcript level. On the other hand, it is not possible to infer TFA by a linear combination of Target and TFB, nor to explain TFB by a linear combination of Target and TFA. Thus, the term of the equation  $\gamma TFA * TFB$  intrinsically possess some directionality explanatory power. It is important to note that this directionality will occur only when some interactions between the explanatory variables exist. Since the TransDetect algorithm favours models having a significant interaction term between TFs, we

believe that this particularity might explain its availability to infer actual regulators. Thus, we propose that TransDetect is suitable to discover TFs that coordinate the expression of any gene or set of genes of interest. It worth noting that although microarray data were used in this study, TransDetect algorithm could also use value of mRNA level obtained from RNA-seq experiments. While ICE1 and MYB15 was not detected as TFs pair using our set-up of fitting and predicting dataset, it was detected by lowering the threshold. Therefore, testing different fitting and predicting dataset set-up on one hand, and changing threshold on another had, could help reaching conclusion on possible detection TFs pairs. In the frame of this study, beside *PHO1;H3*, the use of TransDetect enabled retrieving already known TFs and their targets, which are involved in the regulation of root development (e.g. DNA BINDING WITH ONE FINGER 53 (DOF53, At5g60200) its targets *REVOLUTA* (*REV*, At5g60690)) (Brady *et al.*, 2011), secondary cell wall synthesis (e.g. ARABIDOPSIS THALIANA HOMOLOG OF E2F C TF (E2Fc, At1g47870) and its target *ASCULAR RELATED NAC-DOMAIN PROTEIN 7* (*VND7*, At1g71930) (Taylor-Teeples *et al.*, 2015), and iron transport (POPEYE (PYE, At3g47640) and its target *IRON-REGULATED TRANSPORTER 1* (*IRT1*, At4g19690)) (Long *et al.*, 2010) (Figure II.8). These data further support the utility of TransDetect to detect TFs. The validation of detected TFs require *in planta* and biochemical testing, which would include the analysis of expression profiles of the target genes in different plant genetic backgrounds (eg wild-type plants, knock-out mutants /or overexpressing lines). As performed in this work, transcriptionally linked TFs and promoter of target genes could be further tested for their possible direct interaction.

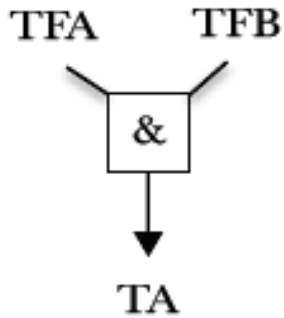
Current understanding of adaptive mechanisms regulating Pi homeostasis in plants comes from investigations conducted mainly in *Arabidopsis thaliana* under Pi limitation. From twenty years of research only a handful of TFs (for review (Jain *et al.*, 2012) and only one complete Pi signalling pathway, the “PHR1- miR399-PHO2” pathway (Bari *et al.*, 2006, Lin *et al.*, 2008) have been discovered. Nevertheless, as aforementioned, Pi accumulation in shoot is altered when plants are challenged by Zn limitation, and this alteration is not dependent from the “PHR1-miR399-PHO2” regulatory pathway (Khan *et al.*, 2014). These observations indicate the existence of specific regulatory pathway(s) underlying this Zn-Pi relationship (Khan *et al.*, 2014). Unfortunately, available tools do not offer the possibility to identify the TF pairs involved in such nutrient homeostasis coordination. Thanks to TransDetect used to search for TFs controlling Pi homeostasis through the examination of the publically available transcriptomic data sets with *PHO1;H3* as target gene we identified four new TF (MYB15,

MYB84, bHLH35 & ICE1) displaying a striking phenotype with regards to both *PHO1;H3* expression and shoot Pi accumulation in plant grown under  $-Zn$ . Considering that only a handful of TFs involved in the transcriptional control of plant response to Pi or Zn deficiency were described before this study (Assunção *et al.*, 2010a, Jain *et al.*, 2012, Khan *et al.*, 2014), one can consider that in this regard the success rate of this strategy - combining TransDetect together with functional genomics approaches - is high. More importantly, integration of these data provide blueprint for defining novel regulatory pathway controlling Pi homeostasis in plants. Using the phenotypic data obtained from the characterisation of single and double mutant TF mutants effect on the expression of *PHO1;H3* it was possible to propose a new regulatory transcriptional module regulating Pi accumulation in shoot of Zn-deficient plants (Figure II.7). In this module, MYB15, MYB84 are likely to play a negative regulatory role on the expression of *PHO1;H3*, while ICE1 and bHLH35 plays a positive regulatory role upstream MYB15 (Figure II.7). This work thus lead to the identification of new key players that act in the  $-Zn$  signalling pathways to control the expression of *PHO1;H3* and Pi accumulation in plants, which is indeed independent of the PHR1-miR399-PHO2 pathway constituents.

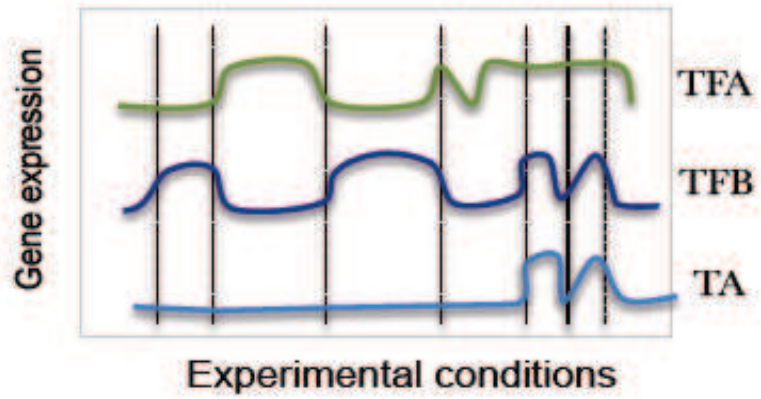
In conclusion, this work identified four new TFs acting to regulate Pi accumulation in Arabidopsis in response to Zn deficiency. Prediction using TransDetect was validated using three different strategies First, molecular and genetic evidences showing the involvement of the selected TFs in modulating *PHO1;H3* expression in response to  $-Zn$ . Second, yeast-one and two-hybrid experiments shows that these TFs can interact with the *PHO1;H3* promoter, that they are able to form TF pairs. Finally, these TF pairs are involved in the regulation of Pi accumulation in plants under  $-Zn$  conditions. The method developed in the frame of this work should benefit to other studies aiming at identifying TFs cooperatively regulating a gene(s) expression, and to dissect regulatory pathway(s) controlling an important biological phenomenon.



A



B

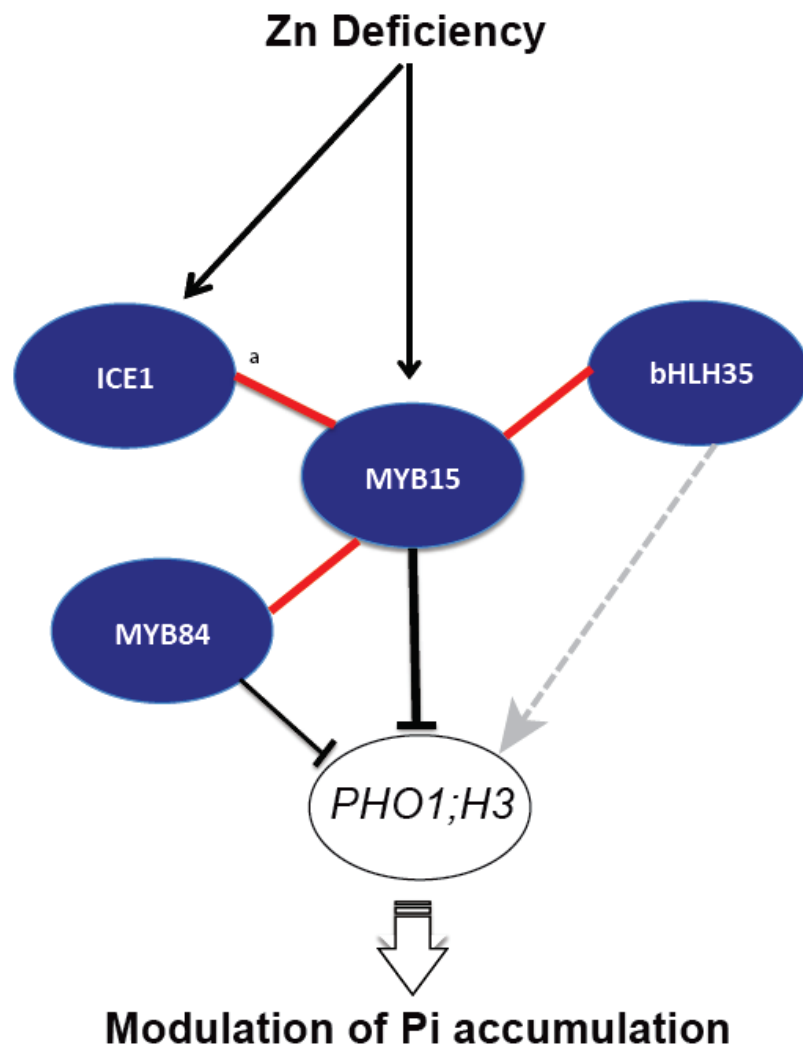


C

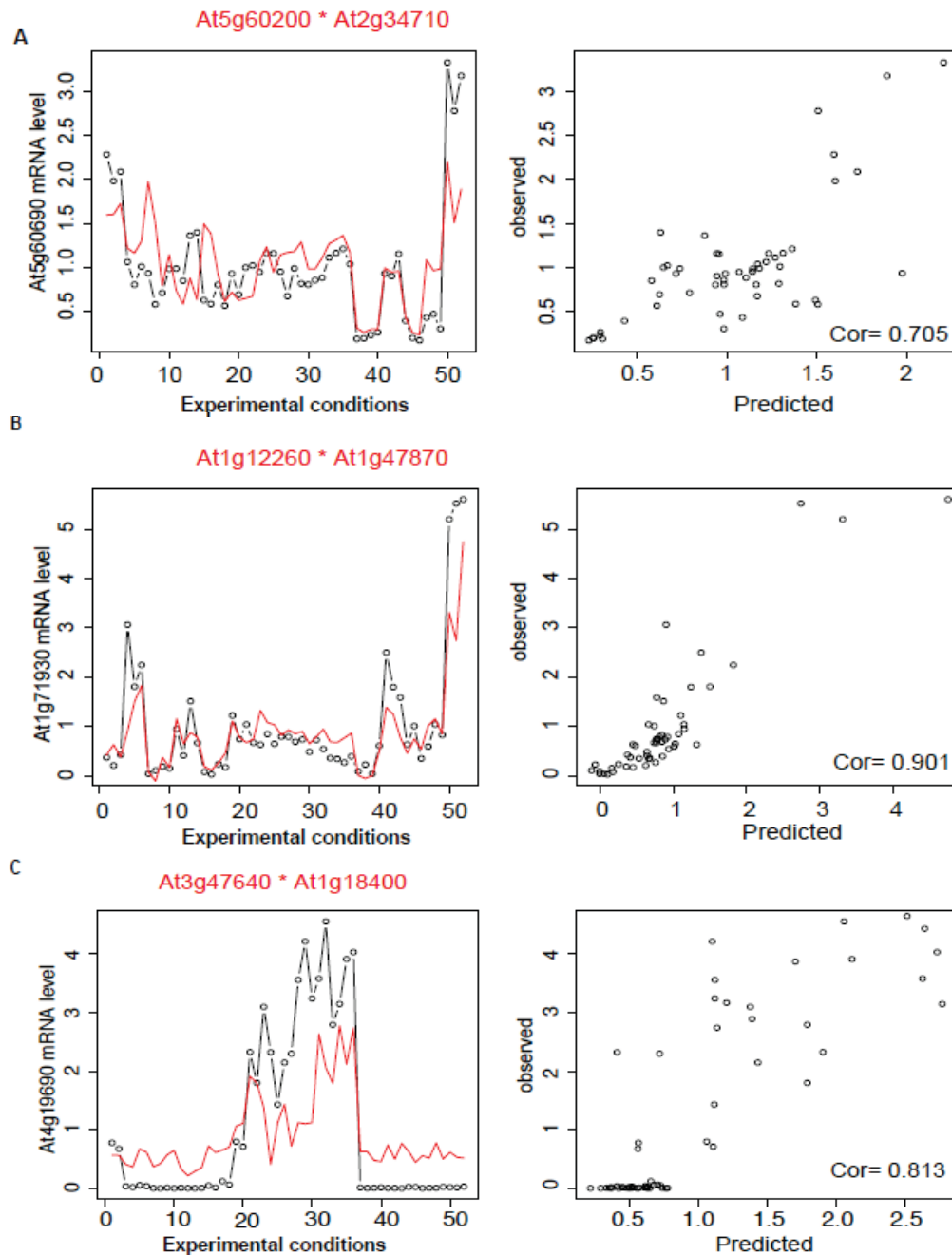
$$TA = \alpha TFA + \beta TFB + \gamma TFA * TFB + \epsilon$$

**Figure II.6. Idealized model to explain how TransDetect extract directionality in static data.** **A-** Two transcription factors TFA and TFB positively control the expression of a Target gene TA following a AND logic-gate. **B-** TA expression is induced only when TFA and TFB expression are both upregulated. **C-** Linear modelling of TA expression. Considering the ideal case where 2 transcription factors (TFA and TFB) control a target gene TA following a AND logic-gate. In this particular case,  $\gamma$  coefficient of the linear model will be highly significant because it is the combination of TFA and TFB expression that is necessary to fully explain TA expression. On the other hand, it is not possible to infer TFA by a linear combination of TA and TFB, nor to explain TFB by a linear combination of TA and TFA. Thus the term of the equation  $\gamma TFA * TFB$  intrinsically possess some directionality explanatory power in this case where both TFs interact in the control of TA.





**Figure II.7. Schematic representation of the MYB15, MYB84, bHLH35 and ICE1 regulatory module controlling *PHO1;H3* gene expression and Pi accumulation in shoots under zinc deficiency.** Phosphate concentration increases upon zinc deficiency. *PHO1;H3* plays a negative regulatory role in this process. Red solid lines indicate connections between MYB15, MYB84, bHLH35 and ICE1. Negative and positive regulatory effects of these transcription factors on *PHO1;H3* expression under zinc deficiency are indicated by flat-ended dashed lines and arrowheads, respectively. a indicates previous knowledge on ICE1 and MYB15 physical interaction.



**Figure II.8. TransDetect prediction of regulators for *REVOLUTA*, *ASCULAR RELATED NAC-DOMAIN PROTEIN 7* and *IRON-REGULATED TRANSPORTER 1*.** TransDetect was used to predict regulators for the following target genes **A)** *REVOLUTA* (*REV*, At5g60690), **B)** *ASCULAR RELATED NAC-DOMAIN PROTEIN 7* (*VND7*, At1g71930), and **C)** *IRON-REGULATED TRANSPORTER 1* (*IRT1*, At4g19690). Among number of TFs predicted, TransDetect retrieved known regulators for these genes, namely DNA BINDING WITH ONE FINGER 53 (DOF53, At5g60200) for *REV*, ARABIDOPSIS THALIANA HOMOLOG OF E2F C TF (E2Fc, At1g47870) for *VND7*, and POPEYE (PYE, At3g47640) for *IRT1*.

## Materials Methods

### Algorithm

The TransDetect algorithm as been written in R (<https://wwwr-project.org/>) and it follows the exact logic described in Figure II.1. The R code is available at <https://sites.google.com/site/gabrielkroukresearch/transdetect>. The running time is about 20 minutes per target gene on a desktop Apple Mac Pro computer with parallelized computation on 32 CPUs. The transcriptomic dataset used is from the Benfey lab (Brady *et al.*, 2007, Dinneny *et al.*, 2008, Azevedo *et al.*, 2016) and has been spited arbitrarily into fitting (52 chips) and predicting dataset (69 chips). The link to TransDetect algorithm is: <https://sites.google.com/site/gabrielkroukresearch/transdetect>

### Plant materials and growth conditions.

The *Arabidopsis thaliana* mutants used in all experiments were in the Columbia (Col-0) genetic background. The previously described *ice1-2* (At3g26744) mutant (Denay *et al.*, 2014) was provided by Dr Gwyneth Ingram (ENS, Lyon, France). T-DNA insertion mutant lines for considered TFs At5g57150 (line1: N516841 and line2: N536664); At3g23250 (line1: N651976 and line2: N491226); At3g49690 (line1: N641918 and line2: N612398); At4g37790 (N585964, N2100629); At2g46510 (N587068, N867699); At1g31050 (N595172; N545538); At5g04760 (N2101152, N2104259); At4g31800 (N550079, N871514); At4g24060 (N652104, N504243) and At3g50060 (N567655, N555373) were from Nottingham Arabidopsis Stock Centre (NASC) (Alonso *et al.*, 2003). The presence of a T-DNA insertion within the TF gene and absence of transcripts of the mutated TFs were checked using the appropriate PCR strategy using PCR primers listed in (supplementary Table II.2). Double mutant lines *myb15/myb84*, *myb15/bhlh35*, and *ice1/myb15* were generated through crossing. Homozygosity of the generated double mutants was confirmed through appropriate PCR strategy as performed for the identification of the single mutants. Plants were germinated grown in vertical position on 1% agar-solidified media (A1296, Sigma) The complete nutrient medium contained 0.5 mM KNO<sub>3</sub>, 1 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.25 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 100 μM NaFeEDTA, 30 μM H<sub>3</sub>BO<sub>3</sub>, 10 μM MnCl<sub>2</sub>, 1 μM CuCl<sub>2</sub>, 15 μM ZnSO<sub>4</sub>, 01 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 50 μM KCl. pH adjusted to 5.7. Zn-free medium was made by removing the only source of Zn (ZnSO<sub>4</sub>), by washing the agar Seeds were sown on the plates stratified at 4 °C in the dark for 3 d. Plates were then transferred in a growth chamber for 18 d, day 1 of growth being defined as the first day of exposure of stratified seeds to light. Plants were grown under long-day conditions (16/8h light/dark cycle, 250 μmol·m<sup>-2</sup>·s<sup>-1</sup>, 24/20°C).

### Phosphate concentration measurements.

Shoots and roots were collected separately. Shoots were weighed and ground into powder in liquid nitrogen, then incubated at 70 °C for ½ hour. The determination of Pi concentrations in these tissues was performed using the Ames methods (Ames 1966). For every measurement, three to five biological replicates were performed, leading to three to five corresponding samples, three plants per sample.

### Real-time quantitative reverse-transcription PCR.

Roots of 18 day old plants grown on different medium composition in the presence or absence of Zn were collected for gene expression analysis. Total RNA was extracted from 100 mg frozen roots using Plant RNeasy extraction kit (Qiagen) and RQ1 RNase-free DNase (Promega). Two µg of total RNA were used to synthesize cDNA using poly-A oligos. Real-time quantitative reverse-transcription PCR (RT-qPCR) was performed with a Light Cycler 480 Real-Time PCR System using SYBR green dye technology (Roche) as described previously (Rouached *et al.*, 2011). The *PHO1;H3* transcript abundance were quantified using quantitative real time PCR using specific primers listed in (s. table II.2), which showed an efficiency (E) of  $100\% \pm 3\%$ . E was determined after the analysis of serial 1:10 dilutions of a plasmidic solution of each target gene by using the equation  $E = [(10^{-1/s}) - 1] \cdot 100$ . In this equation “s” represent the slope of the linear regression of the threshold cycle ( $C_T$ ) values per the  $\log_{10}$  values of DNA copy numbers used for PCR reactions (Rouached *et al.*, 2008). Relative transcripts levels were quantified using the comparative threshold cycle ( $C_T$ ) method (Livak and Schmittgen 2001). For every data point,  $C_T$  value was the average of the  $C_T$  values obtained from the triplicate PCR analysis. For each gene, the relative amount of calculated mRNA was normalized to the level of the control gene *Ubiquitin10* (*UBQ10*: At4g05320) and expressed as relative values against wild-type plants grown in reference treatment (RT: +Zn) medium. For example, relative gene expression of the *PHO1;H3* genes  $\Delta C_{T,PHO1;H3}$  was expressed following normalization against the average of the  $C_T$  values obtained for the gene used for stardization:  $\Delta C_{T,PHO1;H3} = C_{T,PHO1;H3} - C_{T,UBQ10}$  (Livak and Schmittgen 2001). For treatment of interest (TOI: -Zn) was compared to a reference treatment (RT: +Zn), the relative expression of a *PHO1;H3* gene was expressed as a  $\Delta\Delta C_t$  value calculated as follows:  $\Delta\Delta C_t = \Delta C_{T,TOI} - \Delta C_{T,RT}$  (Livak and Schmittgen 2001). The fold change in relative gene expression was determined as  $2^{-\Delta\Delta C_t}$ . Using this method, +Zn values were normalized to 1. The methodology applies for the analysis of relative expression of the other genes: *MYB15*, *ICE1*, *BHLH35* and *MYB84*.

## **Yeast experiments.**

All the PCR products were obtained using high-fidelity Phusion DNA polymerase. The constructs were sequenced to ensure their integrity. All primers used for yeast one-hybrid (Y1H) and two-hybrid (Y2H) experiments are described in (supplementary table II.2). For Y1H experiments bHLH35 (At5g57150) cDNA was PCR-amplified from a pool of Columbia (Col-0) cDNA using the cbHLH35-B1 and cbHLH35-B2 primers, introduced into the pDONR207 vector (BP recombination, Gateway®), and then recombined into the pDEST22 vector (LR recombination, Gateway®) allowing the expression of bHLH35 fused to the GAL4 activation domain (AD) in yeast pDEST22. Clones containing MYB15 (At3g23250) and MYB84 (At3g49690) were obtained from a previous study (Kelemen *et al.*, 2015). In order to assess if bHLH35, MYB15 and MYB84 could interact with the different MYB (seven) and bHLH (one) putative binding site present on the *PHO1;H3* promoter, each one was separately cloned as hexamers into the pHis-LIC vector (Kelemen *et al.*, 2015). Subsequent interaction assays were carried out as described in (Dubos *et al.*, 2014). For Y2H experiments, bHLH35, MYB15 MYB84 were LR recombined into pDEST32 allowing fusion with the GAL4 DNA binding domain (BD). Each pDEST22 and pDEST32 vector containing either bHLH35, MYB15 or MYB84 were transformed alone or in two-by-two combination into yeast (AH109 strain, Clontech). Subsequent steps were carried out accordingly to manufacturer's instructions using the ADE2 HIS3 reporter genes (Clontech).

## **Statistical analysis.**

Statistical differences between genotypes were calculated using t-test analyses and ANOVA with subsequent post hoc tests using Graphpad Prism (GraphPad Software Inc, San Diego, CA, USA) or Microsoft Excel (Microsoft, USA).

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Sikender Pal, **Mushtak Kisko**, Christian Dubos, Benoit Lacombe, Pierre Berthomieu, Gabriel Krouk, Hatem Rouached

## Contributions

HR and GK designed the research. GK wrote the TransDetect code. HR supervised this project and analysed the data. SP, MK and CD performed most experiments. CD, PB and BL helped conducting data analysis and critically revised the manuscript. HR and GK wrote the manuscript.

## Declaration of interest

The authors declare no competing financial interests.

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## **Chapter III.**

### ***LPCAT1* controls phosphate homeostasis in a zinc-dependent manner**

## Chapter III. *LPCAT1* controls phosphate homeostasis in a zinc-dependent manner.

### Résumé

Pour assurer leur croissance et développement, les plantes doivent conserver une quantité constante en phosphate inorganique (Pi) et en zinc (Zn), éléments, disponibles en très faibles quantités dans le sol. Une relation inverse entre l'accumulation du Pi et du Zn *in planta* a été décrite sans être expliquée, indiquant, vraisemblablement, l'existence d'une interconnexion entre les homéostasies en Pi et en Zn. Nos connaissances sur cette relation sont cependant limitées, malgré son importance fondamentale dans l'amélioration de la nutrition minérale des plantes. Le projet se propose de déterminer les bases génétiques de l'interconnexion entre les voies de signalisation du Pi et celles du Zn, chez *Arabidopsis thaliana*. Pour atteindre cet objectif, nous avons utilisé l'approche de génétique d'association (GWAS, Genome Wide Association Studies). Cette approche a évolué, au cours des dix dernières années, pour devenir un outil puissant permettant d'étudier l'architecture génétique des caractères « trait » quantitatifs dans plusieurs organismes. Dans notre étude, en explorant la variation de l'accumulation du Pi dans 223 accessions d'*Arabidopsis*, GWAS nous a permis d'associer la variation de la teneur en Pi (phénotypes) aux loci (régions des génomes ; génotypes). La comparaison des résultats GWAS obtenus en présence ou en absence de Zn, nous a permis d'identifier des loci associés spécifiquement à l'accumulation de Pi en condition de carence en Zn. La validation fonctionnelle d'un de ces gènes, nommé *Lyso-PhosphatidylCholine (PC) AcylTransferase 1 (LPCAT1)*, a été réalisée par sa caractérisation au niveau physiologique et moléculaire en utilisant des mutants d'insertion ADN-T dans ce gène. Le dosage des phospholipides, dosage du Pi et détermination de niveaux d'expressions des gènes clés dans des plantes sauvages (différents écotypes) et mutants poussés en présence ou en absence du Zn. Par la suite, nous avons déterminé la cause de la variation allélique du *LPCAT1*, ce qui nous a amené à identifier un nouveau site de fixation pour l'un des facteurs de transcription impliqué dans la réponse à la carence en Zn, qui est le bZIP23. L'ensemble de ce travail permet de proposer une voie de signalisation complète dans laquelle *bZIP23*, *LPCAT1*, et le transporteur de Pi *PHT1;1* jouent un rôle important. En plus, cette voie de signalisation se caractérise par sa nouveauté car elle n'est active qu'en condition de carence en Zn pour réguler l'accumulation du Pi. Enfin, ceci ouvre de nouvelles perspectives pour améliorer la nutrition phosphatée chez les plantes en modulant la voie de la signalisation de la carence en Zn et dans lequel les



phospholipides jouent un rôle central. Les résultats de ce travail sont publiés dans le journal eLife:

LPCAT1 controls phosphate homeostasis in a zinc-dependent manner.

**Mushtak Kisko**, Nadia Bouain, Alaeddine Safi, Anna Medici, Robert C. Akkers, David Secco, Gilles Fouret, Gabriel Krouk, Mark G.M. Aarts, Wolfgang Busch, Hatem Rouached. *LPCAT1* controls phosphate homeostasis in a zinc-dependent manner. eLife 2018;7:e32077. DOI: <https://doi.org/10.7554/eLife.32077>.

## Article: *LPCAT1* controls phosphate homeostasis in a zinc-dependent manner.

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## Abstract

All living organisms require a variety of essential elements for their basic biological functions. While the homeostasis of nutrients is highly intertwined, the molecular and genetic mechanisms of these dependencies remains poorly understood. An intriguing interdependency links the micronutrient zinc (Zn) and the macronutrient phosphorus (P). In plants, Zn deficiency causes an overaccumulation of P in shoots, which can lead to an important yield loss. Here, we report a discovery of a molecular pathway that control phosphate (Pi) accumulation plants in Zn deficiency. Using genome-wide association studies and reverse genetics we first identified allelic variation of the *Lyso-PhosphatidylCholine (PC) AcylTransferase 1 (LPCAT1)* gene as the key determinant of shoot Pi accumulation, specifically under Zn deficiency. We then show that regulatory variation at the *LPCAT1* locus contributes significantly to this natural variation and we further demonstrate that the regulation of *LPCAT1* expression involves bZIP23, a major regulator of Zn deficiency signalling, for which we identify a new specific *cis*-binding site. Finally, we show that in Zn deficient conditions loss of function of *LPCAT1* increases the phospholipid Lyso-PhosphatidylCholine/PhosphatidylCholine ratio, the expression of the main Pi transporter *PHT1;1* via the transcription factor Phosphate Response 1 (PHR1), and that this leads to shoot Pi accumulation. Taken together, we have identified a novel pathway that controls Pi homeostasis in response to the Zn status of the plant in which *LPCAT1* and phospholipids have central roles.

## Introduction

All living organisms require an adequate supply of nutrients for growth and survival. Nutrient deficiencies lead to decreased plant survival and lower nutritional value of foods, which has a profound impact on human health (Myers *et al.*, 2014). In particular zinc (Zn) and iron (Fe) deficiencies affect up to 2 billion people worldwide (Hilty *et al.*, 2010). According to the World Health Organization, about 800,000 child deaths per year are attributable to Zn deficiency alone (Akhtar 2013). The widespread occurrence of deficiencies in micronutrients such as Zn and Fe in human populations is due to low dietary intake (Rouached 2013) (Myers *et al.*, 2014) (Shahzad *et al.*, 2014). In the light of crop optimization for yield and nutritional quality, it is therefore an important goal to understand the genetic and molecular basis of plant nutrition. A complicating circumstance is that plant uptake, storage and use of these nutrients are partly dependent of each other (Rouached and Rhee 2017). For instance, physiological Zn deficiency leads to over-accumulation of phosphorus (P) in the shoots (for review (Bouain *et al.*, 2014) (Kisko *et al.*, 2015). Noteworthy, when the Zn supply is low, increasing P supply caused reduction of plant height, delayed development and caused more severe leaf symptoms including chlorosis and necrosis (Ova *et al.*, 2015). At high P supplies, Zn deficiency associated with elevated shoot P levels causes P toxicity (Marschner 2012). Interestingly, this P-Zn interaction is also recognized in a wide variety of other biological systems, including rats (Wallwork *et al.*, 1983), human cells (Sandström and Lönnerdal 1989), and multiple fungal species (Freimoser *et al.*, 2006). In *Saccharomyces cerevisiae* yeast, the Zn status acts as a major determinant of the ability to store P (Simm *et al.*, 2007). Much like Zn nutrition, P homeostasis is of global relevance as current agricultural practices require large amounts of P. At the same time, world-wide P reserves are becoming increasingly scarce and a potential P crisis looms for agriculture at the end of this 21<sup>st</sup> century (Abelson 1999) (Neset and Cordell 2012). How P and Zn homeostasis are coordinated is therefore not only a fundamental biological question but has also serious implications for global agronomic and biotechnological applications.

P is critical component of many metabolites and macromolecules, including nucleic acids and phospholipids (PLs) (Poirier and Bucher 2002, Rouached *et al.*, 2010). Of an equal importance, Zn provides chemical, structural and regulatory functions in biological systems (Christianson 1991), for instance as cofactor for hundreds of enzymes, or by binding to PLs to maintain membrane structure (Binder *et al.*, 2001) (Sinclair and Krämer 2012). Plants have evolved the ability to adjust to large fluctuations in external P or Zn supply. P is taken up by the

root system in the form of inorganic phosphate (Pi). In *Arabidopsis thaliana* (Arabidopsis), this uptake relies on members of the high affinity Pi transporter family (PHT1) (Sinclair and Krämer 2012), of which PHT1;1 is the major contributor (Ayadi *et al.*, 2015). Upon P deficiency, the expression of some *PHT1* transporters increases as a result of the activation of the “PHR1-miR399-PHO2” signalling pathway (Bari *et al.*, 2006) (Lin *et al.*, 2008, Pant *et al.*, 2008), causing a strong increase in the acquisition of Pi and its subsequent translocation to the shoots (Lin *et al.*, 2008, Pant *et al.*, 2008). In contrast to our understanding of the molecular mechanisms involved in sensing and signalling of Pi abundance (Chiou and Lin 2011, Zhang *et al.*, 2014), little is known about how plants sense and signal Zn deficiency. A putative working model of Zn deficiency signalling was proposed by (Assunção *et al.*, 2013), which is centred around two essential members of the bZIP transcription factor (TF) family in Arabidopsis, bZIP19 and bZIP23, without which plants are unable to respond to Zn starvation by inducing the expression of genes involved in Zn uptake and distribution (Assunção *et al.*, 2010a). Beyond common set of genes targeted by these two TFs, each TF could regulate distinct genes (Inaba *et al.*, 2015), but the identity of distinctive binding site recognized by each remains poorly unknown. Identifying such binding motif is necessary to better understand how plants regulate Zn homeostasis.

The interaction between Zn and Pi homeostasis in plants (for reviews (Bouain *et al.*, 2014) (Kisko *et al.*, 2015) is also obvious at the molecular level. For instance, Zn deprivation causes an up-regulation of *PHT1;1* and consequently an over-accumulation of Pi in *Arabidopsis thaliana* (Jain *et al.*, 2013) (Khan *et al.*, 2014). The expression of Pi uptake transporters is normally tightly controlled in roots in response to the P status of the plant, but it is clear that this tight control is lost under Zn deficiency. Remarkably, although the involvement of PHOSPHATE RESPONSE1 transcription factor (PHR1) in the coordination of Pi-Zn homeostasis has been demonstrated (Khan *et al.*, 2014), the Zn deficiency-induced Pi uptake transporter expression is independent of the aforementioned canonical “PHR1-miR399-PHO2” signalling pathway (Khan *et al.*, 2014), indicative of room for new discoveries in Pi homeostasis under Zn deficiency in plants.

In this study we set out to identify the genes controlling such novel mechanisms to cause Pi accumulation in shoots of Zn-deficient Arabidopsis plants. Genome wide association (GWA) mapping was employed using a subset of 223 Arabidopsis accessions from the RegMap panel (Horton *et al.*, 2012), which enabled us to demonstrate that there is heritable natural variation

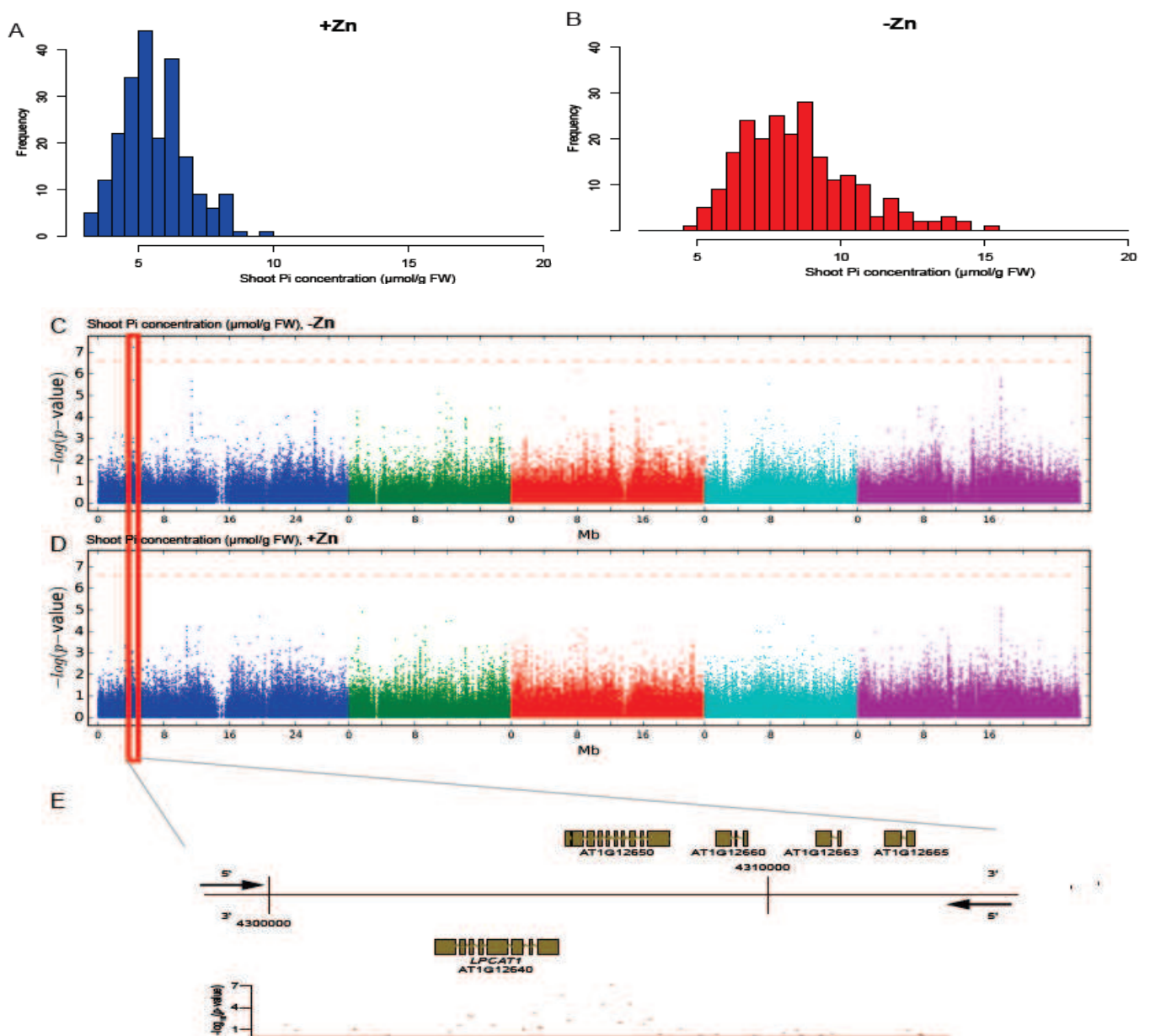
of Pi accumulation in responses to Zn deficiency and that one major locus governing this is the *LysoPhosphatidylCholine AcylTransferase 1* (*LPCAT1*) gene. Under Zn deficiency, *lpcat1* mutants showed an alteration in the phospholipids *Lyso-PhosphatidylCholine/PhosphatidylCholine* (Lyso-PC/PC) ratio, and an up-regulation of the expression of the main high affinity Pi transporter gene, *PHT1;1*, which involves the transcription factor PHR1. Finally, we demonstrate that *LPCAT1* acts downstream of one of the two key Zn starvation signalling TFs, bZIP23, for which we identified a new binding site sequence. Overall, this study uncovered a novel pathway, in which *LPCAT1* plays a key role in the coordination of Pi homeostasis and Zn deficiency response in plants through modulation of phospholipid metabolism and Pi transporter expression.

## Results

### **GWAS identify two candidate genes involved in the accumulation of Pi in the shoot under Zn deficiency.**

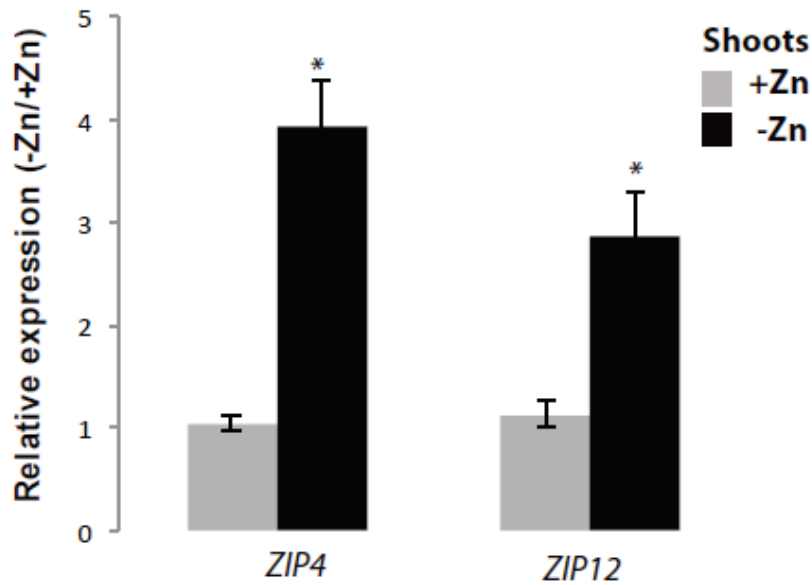
To identify genes regulating shoot Pi concentration under Zn deficiency, genome wide association studies (GWAS) were conducted. To do so, a diverse set of 223 Arabidopsis accessions, selected from the RegMap panel (Horton *et al.*, 2012) was grown on agar medium supplemented with (+Zn) or without Zn (–Zn) for 18 days, before assessing their shoot Pi concentration (supplementary table III.1). Transcript analyses of two Zn-deficiency marker genes, *ZIP4* and *ZIP12* (Jain *et al.*, 2013) in shoots of Col-0 plants demonstrated that plants were responding to the Zn limitation stress (Figure III.2). Under the +Zn condition, shoot Pi concentration varied across the 223 accessions from 3 - 10  $\mu\text{mol}$  of Pi per gram of fresh weight (median  $\sim 5.45 \mu\text{mol}.\text{gram}^{-1}$  fresh weight of Pi) (Figure III.1A) while in –Zn, it increased to 4 - 16  $\mu\text{mol}$  of Pi per gram of fresh weight (median  $\sim 8.23 \mu\text{mol}.\text{gram}^{-1}$  fresh weight of Pi) (Figure III.1B). The broad-sense heritability ( $H^2$ ) of the shoot Pi concentrations was high (0.63 under +Zn and 0.47 under –Zn condition), suggesting this trait to be largely governed by genetic factors. Using the genotype and the shoot Pi concentration as input, we performed a mixed model (AMM method (Seren *et al.*, 2012)) GWAS that corrects for population structure (Korte *et al.*, 2012) for both Zn conditions (Figure III.1C, D). Using the highly conservative 0.05 Bonferroni multiple testing correction threshold, we identified a single significant SNP to be associated with Pi concentration in the shoots, which was specific for the –Zn condition (Figure III.1C, D) ( $P\text{-value} = 5.86 \times 10^{-8}$ ; FDR  $< 0.01$ ). This SNP explained 11% of the variation in shoots Pi under –Zn that was explained by the model (the model itself explained 49% of the

total Pi variation). The significantly associated SNP was located at the upstream and coding regions of two candidate genes, namely *Atlg12640* and *Atlg12650* (Figure III.1E). *Atlg12650* encodes an unknown protein likely to be involved in mRNA splicing via the spliceosome, and *Atlg12640* encodes a member of the *Membrane Bound O-Acyl Transferase (MBOAT)* gene family known as *LysoPhosphatidylCholine AcylTransferase 1 (LPCAT1)*, (Wang *et al.*, 2012)). *LPCAT1* is an evolutionarily conserved key enzyme that is involved in phospholipid metabolism and more precisely in the Lands cycle (Lands 1960). In Arabidopsis *LPCAT1* has been shown to catalyze the conversion of lysophosphatidylcholine (Lyso-PC) to produce phosphatidylcholine (PC) (Zheng *et al.*, 2012).



**Figure III.1. Genome-wide association (GWA) analysis of Arabidopsis shoot Pi concentration.** 223 *Arabidopsis thaliana* accessions were grown supplemented with zinc (+Zn) or without zinc (-Zn) for 18 days under long day conditions, upon which shoot inorganic phosphate (Pi) concentrations were determined. (A, B) Histogram of the frequency distribution of mean shoot Pi concentration of Arabidopsis accessions in +Zn (A) and -Zn (B). (C, D) Manhattan plots of GWA analysis of Arabidopsis shoot Pi concentration in -Zn (C) and +Zn (D). The five Arabidopsis chromosomes are indicated in different colours. Each dot represents the  $-\log_{10}(P)$  association score of one single nucleotide polymorphism (SNP). The dashed red line denotes the Bonferroni 5% correction threshold. Boxes indicate the location of the *LPCAT1* (red) quantitative trait loci (QTL). Mb, megabase pairs. (E) Gene models (upper panel) and SNP  $-\log_{10}(P)$  scores (lower panel) in the genomic region surrounding the GWA QTL at the *LPCAT1*; 5' and 3' indicate the different genomic DNA strands and orientation of the respective gene models.





**Figure III.2. mRNA abundance of Zn-responsive genes *ZIP4* and *ZIP12* in roots of Col-0 plants grown in presence and absence of Zn.** Reverse transcriptase qPCR analyses of transcript levels changes in response to Zn-deficiency of the genes *ZIP4* (At1g10970) and *ZIP12* (At5g62160) in shoots of Arabidopsis (Col-0). Seedlings were grown on vertical agar plate in presence or absence of Zn for 18 days. Transcript levels of these genes are expressed relative to the average transcript abundance of the *UBIQUITIN10* (*UBQ10*; At4g05320) that was used as an internal control. Every data point was obtained from the analysis of shoots collected from a pool of six plants. Data presented are means of three biological replicates  $\pm$  SE. Asterisks indicate statistically significant differences compared to the +Zn condition for each gene analyzed ( $P < 0.01$ ).

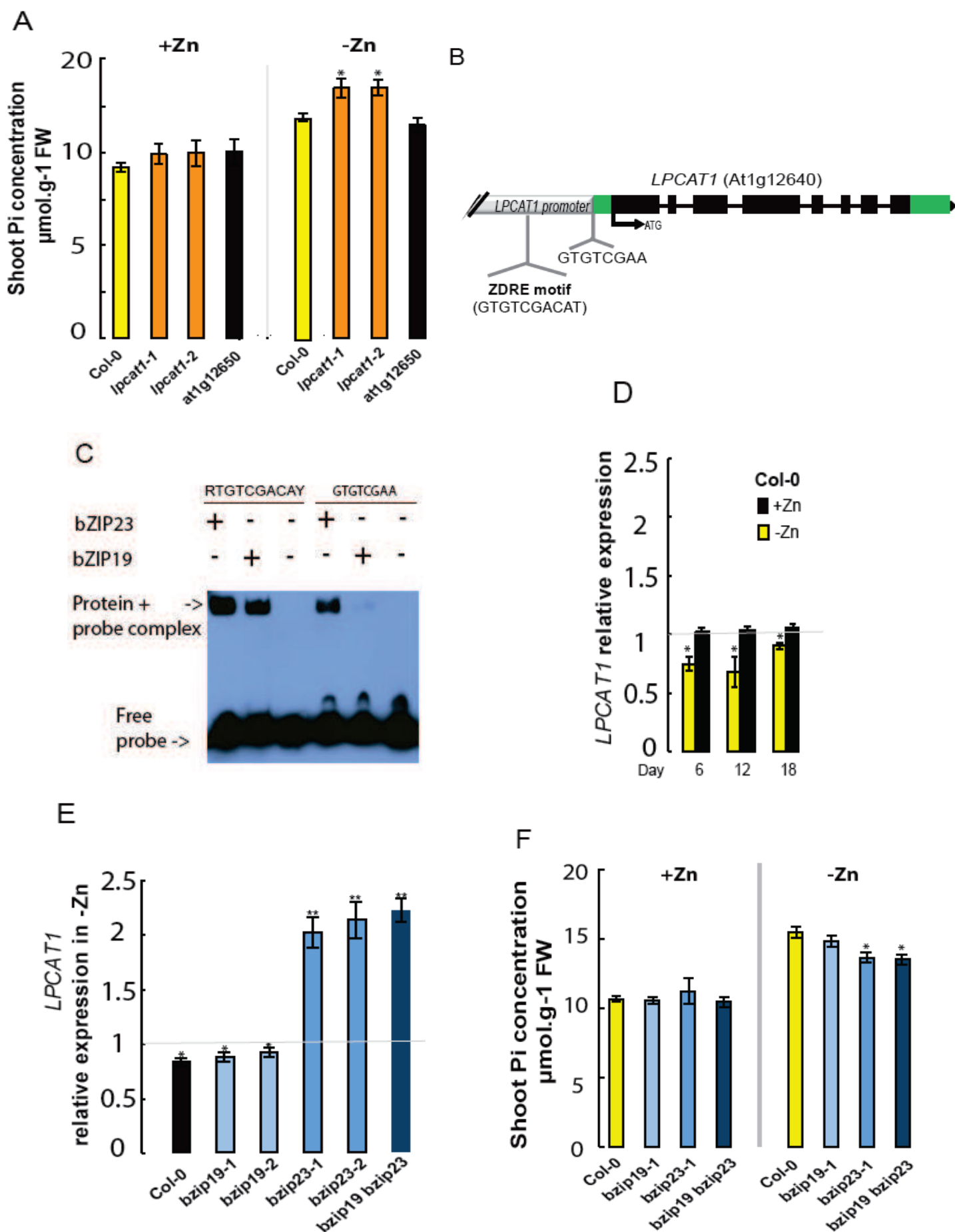
### ***LPCAT1* is involved in regulating shoot Pi concentration in Zn deficiency.**

In order to determine the causal gene underlying the shoot Pi accumulation Quantitative Trait Locus (QTL) in  $-Zn$ , we used a reverse genetic approach. The first thing we studied was to test if any of these two genes is indeed involved in the  $-Zn$  specific variation in shoot Pi concentration. Therefore, wild-type Arabidopsis (Columbia-0, Col-0), T-DNA insertion mutant lines for *LPCAT1* (At1g12640) (Wang *et al.*, 2012) and for *At1g12650* gene were grown for 18 days on  $+Zn$  or  $-Zn$  media before assessing their shoot Pi concentration. In response to  $-Zn$ , Col-0 plants showed a significant increase ( $\sim 29\%$  increase,  $P\text{-value} < 0.05$ ) in their shoot Pi concentration compared to  $+Zn$  conditions (Figure III.3A), which is in line with a previous report (Khan *et al.*, 2014). Importantly, while Pi accumulation in response to  $-Zn$  in *At1g12650* mutants was indistinguishable from Col-0, *lpcat1* mutants displayed a significant increase in shoot Pi concentration ( $\sim 36\%$  increase,  $P\text{-value} < 0.05$ ) (Figure III.3A). We confirmed that this increase in shoot Pi concentration in the *lpcat1* mutants is specific to the  $-Zn$  treatment as no significant differences were observed in the  $+Zn$  condition compared to Col-0. These results showed that *LPCAT1*, and not *At1g12650*, is involved in regulating shoot Pi concentration in response to Zn deficiency in Arabidopsis. Our further efforts were therefore directed at understanding the transcriptional regulation of *LPCAT1* by  $-Zn$ , and then at resolving how allelic variation at the *LPCAT1* gene contributes to the variation in shoot Pi concentration.

### ***LPCAT1* acts downstream of bZIP23 transcription factor.**

To understand the regulatory context of *LPCAT1*, we used the search tool AthaMap (Bülow *et al.*, 2010) to identify *cis*-regulatory elements within the 1500-bp region upstream of the *LPCAT1* start codon (in Col-0 background). Among the numerous potential transcription factor-binding sites, we identified the presence of a single copy of the 10-bp Zinc Deficiency Response Element (ZDRE, RTGTCGACAY)(Assunção *et al.*, 2010a), located 377 bp upstream of the ATG (Figure III.3B). This motif is a known binding site for the bZIP19 and bZIP23 transcription factors, the key transcriptional regulators of the  $-Zn$  response (Assunção *et al.*, 2010a). Given the presence of the ZDRE, we hypothesized that the expression of *LPCAT1* under  $-Zn$  could be controlled by the bZIP19 or bZIP23 TFs. An electrophoretic mobility shift assay (EMSA) was performed, using a 30-bp promoter fragment containing the 10-bp potential ZDRE, which confirmed that both bZIP19 and bZIP23 could bind to this *cis*-regulatory element (Figure III.3C), as had already been shown by (Assunção *et al.*, 2010a).

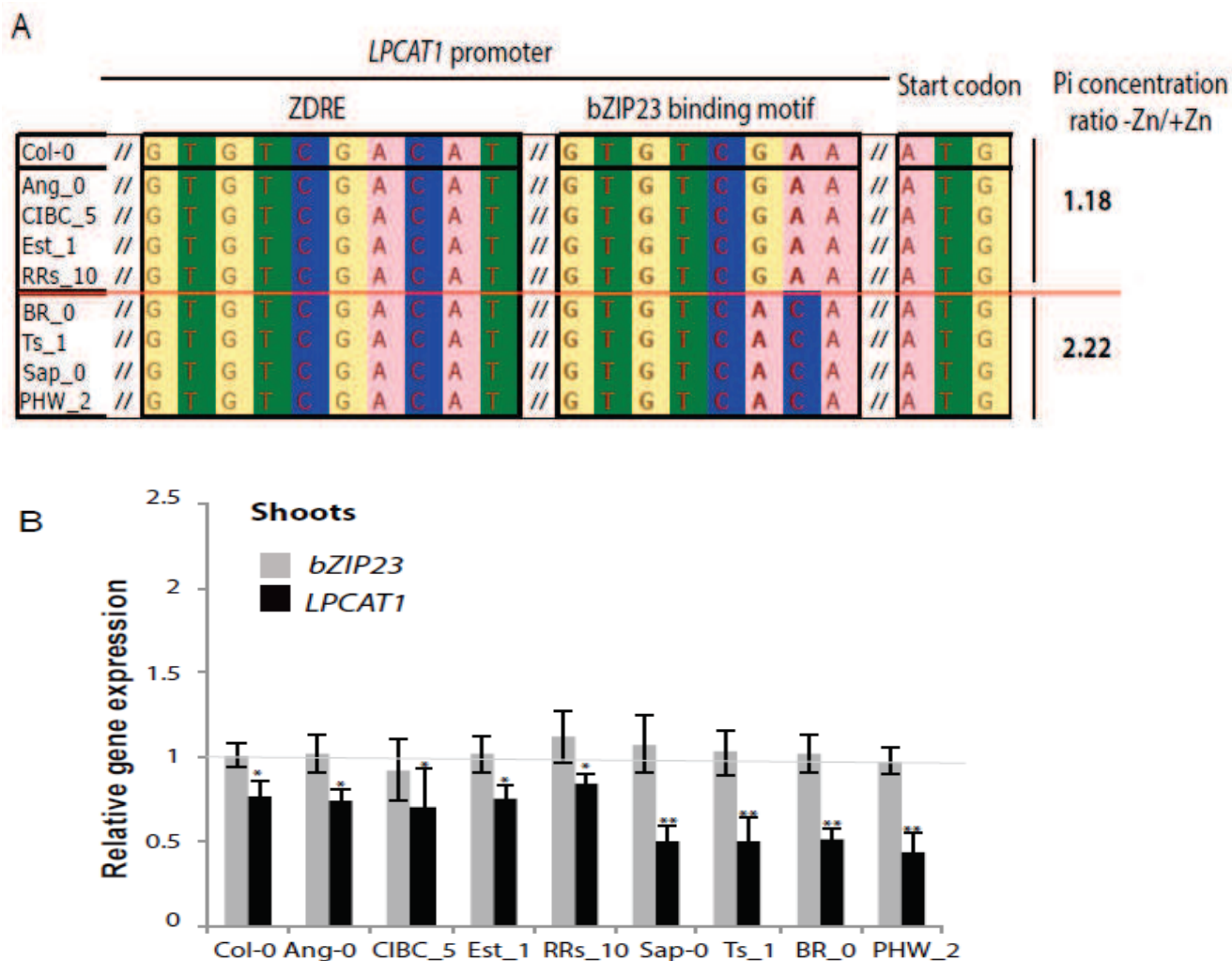
Further analysis of the regulatory regions of *LPCAT1* led us to identify a new motif GTGTCGAA (5' untranslated region of *LPCAT1*), very similar to that of the ZDRE motif (RTGTCGACAY) (Figure III.3B). Due to the sequence similarity of this newly identified motif to that of ZDRE, we first tested the capacity of bZIP23 or bZIP19 to bind to this motifs. Interestingly, EMSA analysis revealed that bZIP23 could bind to the newly identified motif, while bZIP19 showed an extremely weak (if any) binding capacity to new motif (Figure III.3C). These findings strongly support the Zn-dependency of *LPCAT1* expression. We therefore determined the transcript abundance of *LPCAT1* in shoots of Arabidopsis wild-type plants (Col-0) grown in  $-Zn$  for 6, 12 and 18 days. In response to  $-Zn$ , transcript accumulation of *LPCAT1* was changed, showing significant down-regulation compared  $+Zn$  conditions (Figure III.3D). This result shows that repression of *LPCAT1* upon low  $-Zn$  is associated with higher Pi levels and suggests that transcriptional regulation of *LPCAT1* is important for its involvement in Pi homeostasis. We next tested whether these bZIP TFs could be involved in regulating the expression of *LPCAT1* in  $-Zn$ . To test this, we determined the expression levels of *LPCAT1* in the *bzip19* and *bzip23* single and *bzip19/bzip23* double knock-out mutant lines and WT plants (Col-0) grown for 18 days in  $+Zn$  and  $-Zn$  conditions. The *LPCAT1* transcript was significantly up-regulated in the *bzip23* and *bzip19/bzip23* mutant lines, compared to Col-0 and *bzip19* in  $-Zn$ , which showed a significant down-regulation (Figure III.3E). This indicates that bZIP23, but not bZIP19, is involved in negatively regulating the expression of *LPCAT1* under  $-Zn$ . We therefore hypothesized that *bZIP23* but not bZIP19 are necessary for the downregulation of *LPCAT1* in  $-Zn$  and subsequent Pi accumulation and there assessed the capacity of the mutants to accumulate Pi when grown with or without Zn for 18 days. While in  $+Zn$ , all plants showed similar shoot Pi content, we observed a significant decrease in shoot Pi content in the *bzip23* and *bzip19/bzip23* mutants compared to Col-0, confirming the regulatory role of bZIP23 and not bZIP19 (Figure III.3F). Taken together, this suggests that bZIP23 represses *LPCAT1* upon  $-Zn$  and this repression leads to the over-accumulation of Pi in shoots in Arabidopsis grown under  $-Zn$  condition.



**Figure III.3. Loss of function mutation of *Lyso-PhosphatidylCholine AcylTransferase 1 (LPCAT1)*, and not *Atlg12650*, affects shoot Pi concentration in a Zn supply and bZIP23 dependent manner.** (A) Shoot Pi concentration of 18-days-old Col-0 wild-type plants, *lpcat1* and *Atlg12650* mutants grown in +Zn or -Zn conditions. (B) Gene structure of *LPCAT1*. The grey box represents the promoter region, green boxes are 5' and 3' untranslated regions, black boxes represent exons, and black lines represent introns, the arrow head indicates the direction of transcription, ATG indicates the start codon. The Zinc Deficiency Response Element (ZDRE) binding site for bZIP19 and bZIP23, and the newly identified binding site for bZIP23 are indicated. (C) Differential binding of bZIP19 and bZIP23 to two promoter regions of *LPCAT1* gene. EMSA analysis on 30-bp promoter fragments from motif present in *LPCAT1* promoter of contrasting accessions showed in (A). (D) Relative *LPCAT1* transcript abundance (-Zn/+Zn) in Col-0 wild-type plants grown on +Zn or -Zn agar medium for 6, 12 and 18 days. (E) Relative *LPCAT1* transcript abundance in Col-0 wild-type plants, *bzip19*, *bzip23*, and *bzip19/bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. The relative mRNA levels was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). (F) Shoot Pi concentration in Col-0 wild-type plants, *bzip19* and *bzip19/bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. Values are means of three to six biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of 10 plants. Error bars indicate SD; one and two asterisks indicate a significant difference with WT plants (ANOVA and Tukey test) of  $P < 0.05$  and  $P < 0.01$ , respectively.

**Allelic variation of *LPCAT1* determines natural variation of Pi content under zinc deficiency.**

We next wanted to test whether allelic variation of *LPCAT1* is causal for the observed differences in Pi accumulation under  $-Zn$ . For this, we selected two contrasting groups of accessions with either a high ratio (Br-0, Ts-1, PHW-2 and Sap-0) or a low ratio (Ang-0, CIBC-5, Col-0, EST-1, RRS-10) of Pi accumulated in shoots of  $-Zn$  plants compared to  $+Zn$  plants (Figure III.4A). Interestingly, comparative sequence analysis of the regulatory regions of *LPCAT1* of these accessions revealed that the common ZDRE motif (Figure III.3B) didn't display any variation between these two groups of accession (Figure III.4A), and that the newly identified *bzip23* specific motif (Figure III.3B) showed clear variation between the two groups of accession with the accessions with low Pi ratio exhibiting a Col-0 like GTGTCGAA motif (Figure III.4A) and the high Pi accumulating accession displaying a GTGTCACA motif. Consistent with the hypothesis that *bZIP23* regulation of *LPCAT1* is affected by these polymorphisms, *LPCAT1* was downregulated by a larger extent in accessions that accumulated more Pi upon  $-Zn$  (Figure III.4B) while the expression of *bZIP23* remained unchanged in all accessions and growth conditions tested.

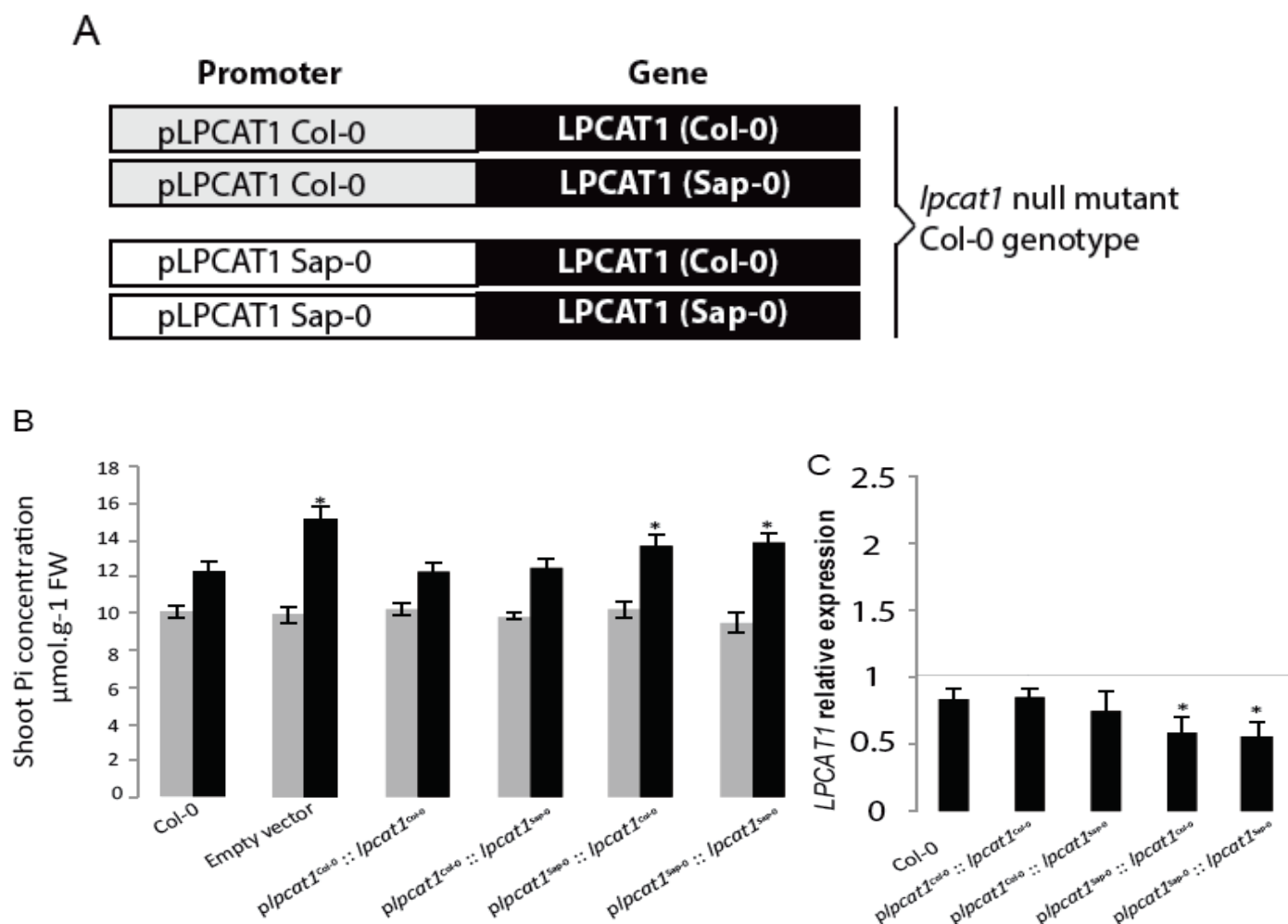


**Figure III.4. Identification of a new binding motif specific for bZIP23, and the variation of *LPCAT1* gene expression between genotypes in -Zn condition.** (A) Sequence comparison for ZDRE and the new binding site motif for bZIP23 in the promoter of accession with high ratio of Pi accumulation in -Zn/+Zn (Col-0, Ang-0, CIBC-5, Est-1, RRS-10) and low Pi accumulation ratio -Zn/+Zn (Sap-0, Ts-1, Br-0 and PHW-2). (B) Relative *bZIP23* and *LPCAT1* transcripts abundance in -Zn and +Zn conditions. Col-0, Ang-0, CIBC-5, Est-1, RRS-10, Sap-0, Ts-1, Br-0 and PHW-2 genotypes were grown on +Zn or -Zn agar medium. The relative mRNA level was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). Values are means of three to biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of 10 plants. Error bars indicate SD; one and two asterisk indicates a significant difference with Col-0 plants (ANOVA and Tukey test) of  $P < 0.05$  and  $P < 0.01$ , respectively.



To test whether the difference in *LPCAT1* expression was due to the natural allelic variation in the regulatory regions and whether this was causal for the Pi accumulation, we focused on only two contrasting accessions, Sap-0 and Col-0, which displayed a significantly different capacity to accumulate shoot Pi in –Zn. Noteworthy, the *LPCAT1* promoter and predicted amino acid coding sequences of Col-0 and Sap-0 displayed 97.9% and 99.4% sequence identity respectively (data not shown). The *lpcat1* knock-out mutant (in Col-0 background) was then transformed with either an empty vector (control) or one of four constructs containing 1.5 kbp of the promoter (immediately upstream of the start codons) of either p*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup> respectively fused to either the coding region of *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup> (Figure III.5A). Three independent, single locus insertion lines (based on segregation of the insertion in progeny of a hemizygous plant) were considered for the analysis. When expressed under the p*LPCAT1*<sup>Col-0</sup> promoter, *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup> complemented the *lpcat1-1* knock-out mutant phenotype and showed a similar Pi content to WT (Col-0) plants in both +Zn and –Zn conditions (Figure III.5B). This indicates that the polymorphisms in the coding region are not responsible for the change in Pi content in –Zn conditions. In contrast, lines complemented with the p*LPCAT1*<sup>Sap-0</sup>:*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup>:*LPCAT1*<sup>Sap-0</sup> transgenic lines showed significantly higher Pi content compared to p*LPCAT1*<sup>Col-0</sup>:*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Col-0</sup>:*LPCAT1*<sup>Sap-0</sup> lines or WT (Col-0) in –Zn conditions (Figure III.5B). This result demonstrates that regulatory variation in of the *LPCAT1* promoter determines Pi accumulation and favours the model that variation in the expression level of *LPCAT1* as the cause of the variation in Pi accumulation in –Zn. Therefore, we assessed *LPCAT1* mRNA accumulation in in WT (Col-0) and all transgenic lines grown in both +Zn and –Zn conditions. Our result showed that while *LPCAT1* is down-regulated in all tested lines by –Zn treatments, the lines complemented with the *LPCAT1* driven by p*LPCAT1*<sup>Sap-0</sup> accumulates significantly lower *LPCAT1* mRNA than that of those under the control of p*LPCAT1*<sup>Col-0</sup> and WT (Col-0) (Figure III.5C). Taken together, our results indicate that the allelic variation between Col-0 and Sap-0 in the promoter of the *LPCAT1* gene causes the difference in *LPCAT1* expression, and confirm that this difference leads to the difference in Pi accumulation under –Zn. Importantly, the polymorphisms in the *bzip23* binding site in the promoter of *LPCAT1* suggest a potential cis-regulatory mechanism for this.

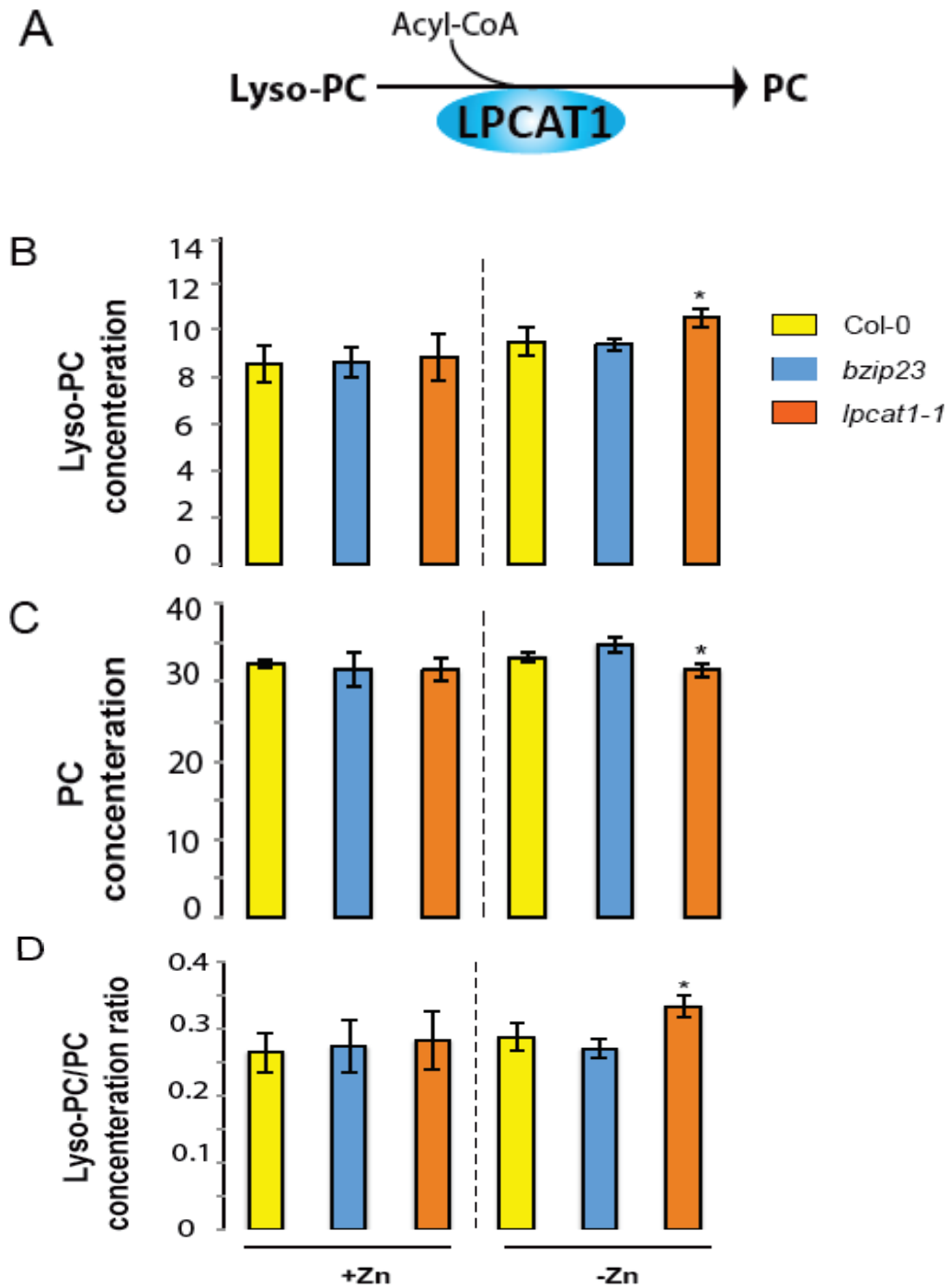




**Figure III.5. Natural allelic variation of *LPCAT1* locus causes phenotypic variation of Pi accumulation in Zn deficiency conditions.** (A) Schematic representation of the transgenic constructs used to complement the *lpcat1* null mutant (Col-0 background). (B) Shoot Pi concentration (–Zn / +Zn) of 18-days-old Col-0 wild-type plants, *lpcat1* mutant transformed with empty vector, or with constructs schematized in (A) grown in +Zn or –Zn conditions. (C) Relative *LPCAT1* transcript abundance in wild-type plants (Col-0 background) and the transgenic lines generated using the construct schematized in (A) grown on +Zn or -Zn agar medium. The relative mRNA levels was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). Values are means of three to biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of six plants. Error bars indicate SD; asterisks indicate a significant difference with Col-0 plants (ANOVA and Tukey test) of  $P < 0.05$ .

### ***LPCAT1* mutation impacts phospholipid concentrations in –Zn.**

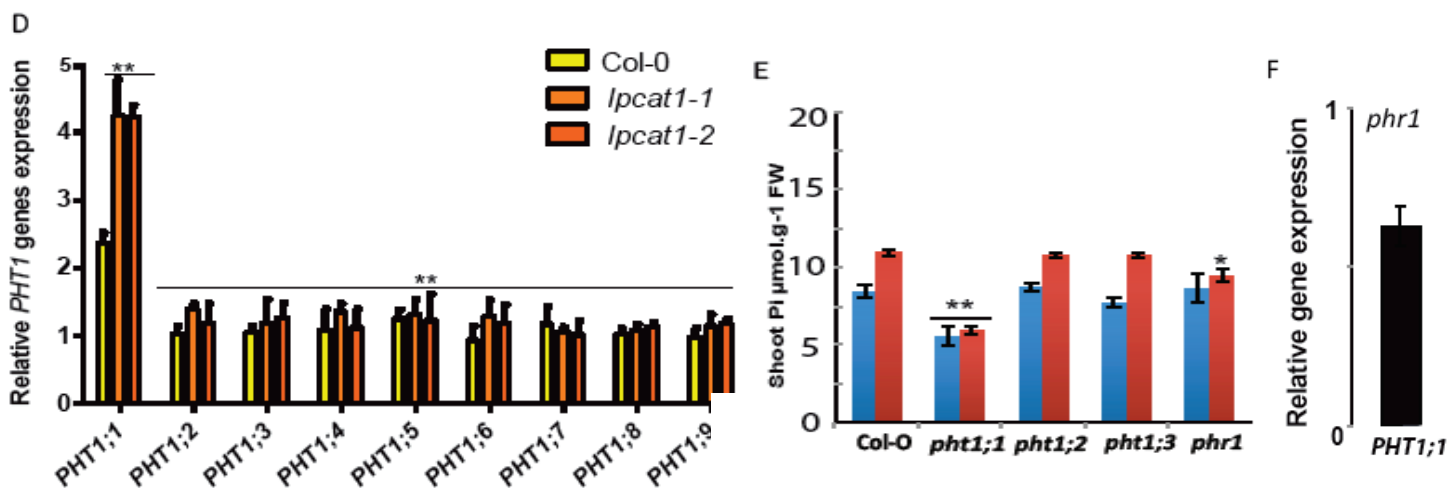
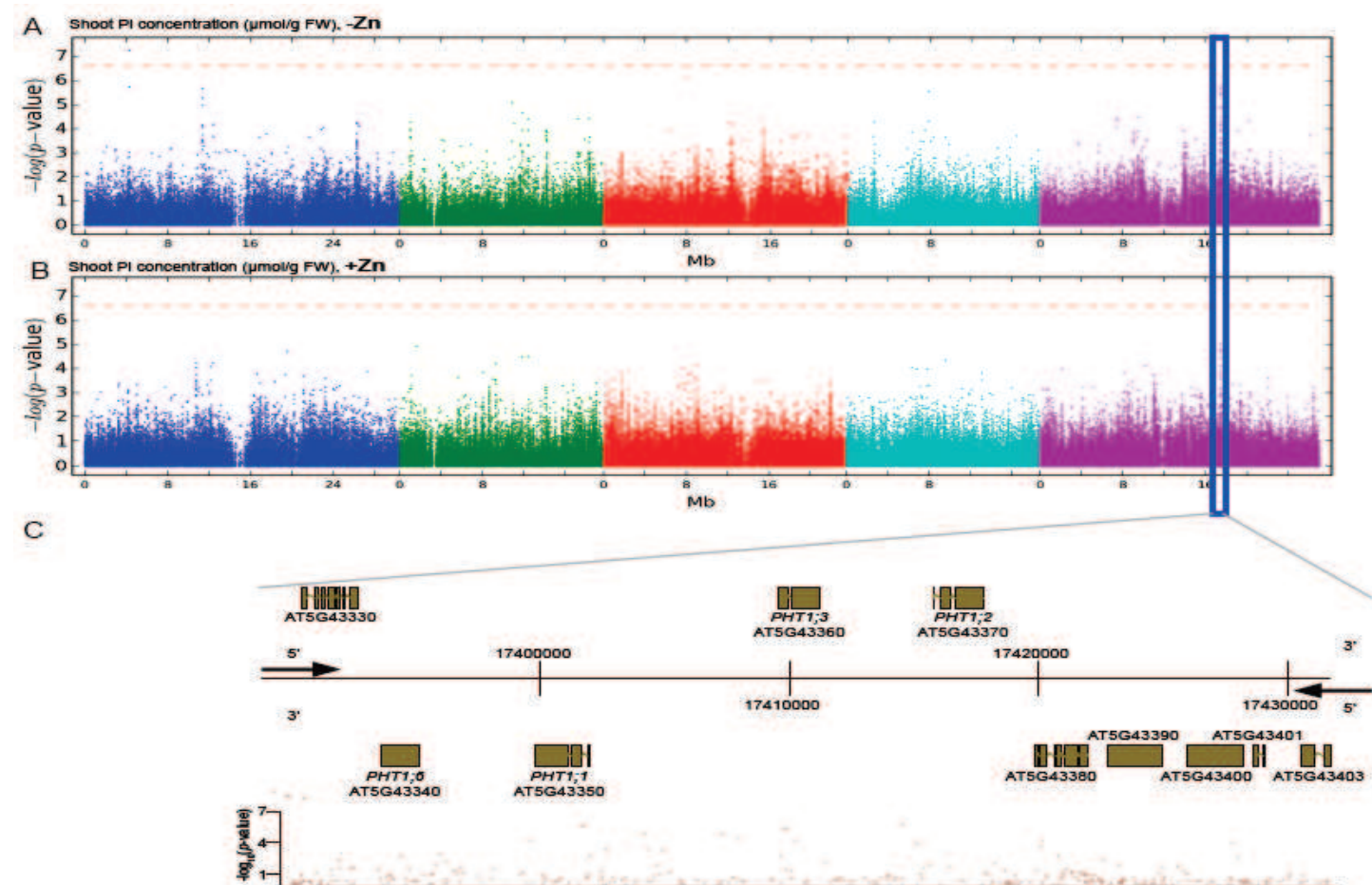
While *LPCAT1* had not been implicated in any known process involving Zn, it is known to catalyse the conversion of lyso-phosphatidylcholine (Lyso-PC) to phosphatidylcholine (PC) in the remodelling pathway of PC biosynthesis (Figure III.6A) (Lands 1960) (Chen *et al.*, 2007) (Wang *et al.*, 2012). Consequently, we hypothesized that a mutation in *LPCAT1* or *bZIP23* would affect the Lyso-PC and PC under –Zn conditions. To test this, we measured the composition of these two phospholipid classes in the shoots of the Col-0 wild type and the *bzip23* and *lpcat1* mutants, in +Zn and –Zn conditions. In +Zn, no significant changes in the Lyso-PC and PC levels in the three different genotypes were observed (Figure III.6B, C). However, under –Zn, *bzip23* showed a modest (but non-significant) decrease in the Lyso-PC/PC ratio while the mutation in *LPCAT1* resulted in a significant increase of Lyso-PC and a decrease of PC, resulting in an increase of the Lyso-PC/PC ratio (~1.2 fold, *P-value* < 0.05) compared to Col-0 plants (Figure III.6D). These results demonstrate that the *LPCAT1* function is required to maintain the shoot Lyso-PC/PC ratio under –Zn.



**Figure III.6. Loss of function mutations of *LPCAT1* affect the lyso-PC/PC ratio in  $-Zn$  conditions.** (A) Schematic representation of the biochemical function of *LPCAT1*, which catalyses the formation of phosphatidylcholine (PC) from lyso-PC and long-chain acyl-CoA. (B) Lyso-PC concentration (C) PC concentration (D) Lyso-PC/PC concentration ratios of Col-0 wild-type plants, *bzip23* and *lpcat1* mutant lines grown in  $+Zn$  or  $-Zn$  conditions for 18 days. Individual measurements were obtained from the analysis of shoots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.05$ ) between mutants and Col-0 are indicated with asterisks.

### **Accumulation of Pi in *lpcat1* involves the HIGH AFFINITY PHOSPHATE TRANSPORTER *PHT1*;1.**

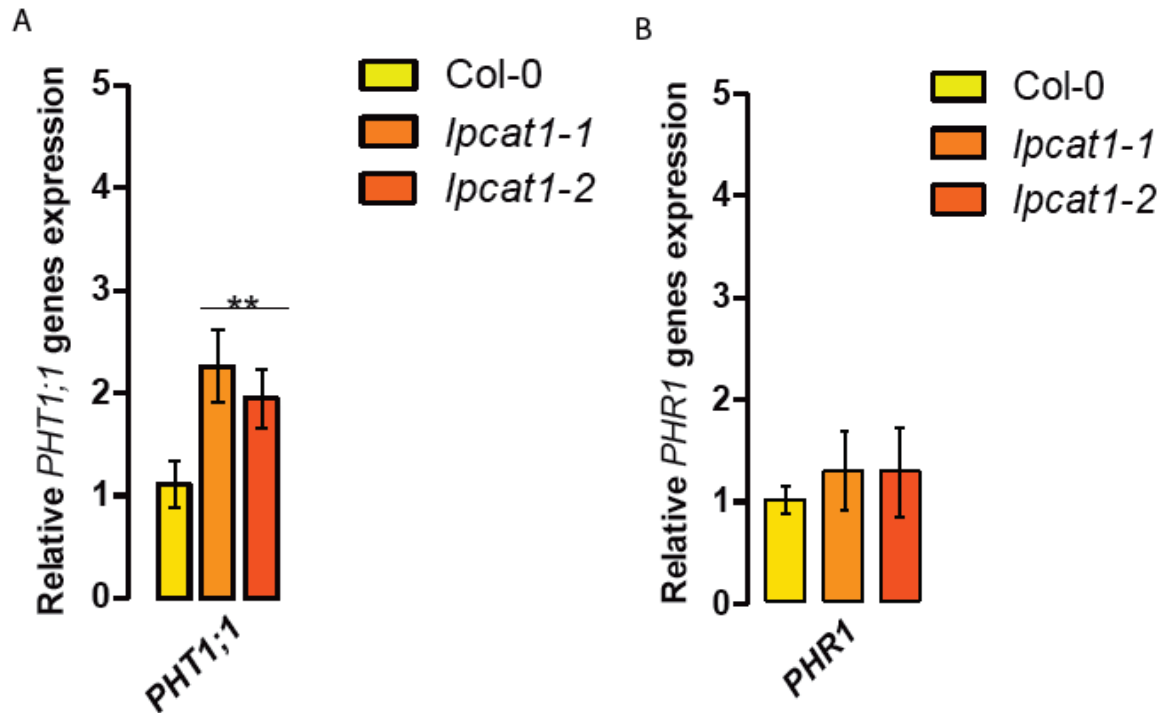
While the molecular function of *LPCAT1* is related Lyso-PC/PC homeostasis, it doesn't answer the question how it might cause Pi levels to increase under  $-Zn$  conditions. A first hint towards answering this question came from our GWAS data: While not above the Bonferroni threshold, the most significant association under  $+Zn$  conditions and the 3<sup>rd</sup> most significant association under  $-Zn$  conditions were both located in a region of chromosome 5 containing members of the high affinity Pi transporters *PHT1* gene family, namely *PHT1*;1, *PHT1*;2, *PHT1*;3 and *PHT1*;6 (Figure III.7A-C). The association with this locus highlights the effectiveness of our genetic association study in identifying genes involved in shoot Pi accumulation under different Zn regimes. Except for *PHT1*;6, the role of these genes in Pi uptake, transport and accumulation in Arabidopsis is well documented (Nussaume *et al.*, 2011, Ayadi *et al.*, 2015). To test, the activity of one of these genes might be related to the *LPCAT1* dependent Pi accumulation under  $-Zn$ , we assessed the expression of the *PHT1* transporter genes in the shoots of *lpcat1* mutant and WT (Col-0) plants grown in  $+Zn$  or  $-Zn$  for 18 days. In all genotypes, *PHT1*;1 was the only member of the *PHT1* gene family to be significantly up-regulated in the  $-Zn$  condition (Figure III.7D). Zn deficiency induces transcription of *PHT1*;1 already  $\sim 2.2$  fold ( $P < 0.05$ ) in WT (Col-0) and this induction was further increased by 2-fold ( $P < 0.01$ ) in *lpcat1* mutants, when compared to  $+Zn$  (Figure III.7D). The expression of the *PHT1*;1 was thereafter tested for responsiveness to  $-Zn$  in roots of WT (Col-0) and the *lpcat1*-1 mutant. While  $-Zn$  caused no significant change in expression of the *PHT1*;1 in roots of WT, it increased its expression by  $\sim 2$ -fold in roots of *lpcat1* mutant (Figure III.8A). We next determined the effects loss of function for each phosphate transporter located under the second GWAS peak (*PHT1*;1, *PHT1*;2 and *PHT1*;3) for the accumulation of Pi in  $-Zn$  in 18-day-old plants. The *pht1*;1 mutant showed low Pi accumulation in presence of Zn compared to WT plants (Figure III.7E) consistently with (Shin *et al.*, 2004) that reported that the *pht1*;1 mutant showed a reduction in Pi content of the shoots relative to wild type plants grown under control condition ( $+Pi+Zn$ ). Importantly, no increase of Pi concentration was observed in the shoots of *pht1*;1 grown in  $-Zn$ , which contrast with the Pi accumulation in *pht1*;2 and *pht1*;3 that was in a similar range to WT plants in presence or absence of Zn. These results show the involvement of *PHT1*;1 in the overaccumulation of Pi in the shoot of *lpcat1* grown in  $-Zn$ , and further supports a second, marginal peak of the GWAS on the chromosomal region of *PHT1* genes.



**Figure III.7. Loss of function mutations of *LPCAT1* show enhanced expression of *PHT1;1* when compared to Col-0 wild-type plants.** (A, B) Genome-wide association (GWA) analysis of Arabidopsis shoot Pi concentration. 223 *Arabidopsis thaliana* accessions were grown agar medium supplemented with zinc (+Zn) or without zinc (-Zn) for 18 days under long day conditions, upon which shoot inorganic phosphate (Pi) concentrations were determined. Manhattan plots of GWA analysis of Arabidopsis shoot Pi concentration in -Zn (A) and +Zn (B). The five Arabidopsis chromosomes are indicated in different colours. Each dot represents the  $-\log_{10}(P)$  association score of one single nucleotide polymorphism (SNP). The dashed red line denotes the Bonferroni 5% correction threshold. Boxes indicate the location of the PHT1 (blue) quantitative trait loci (QTL). Mb, megabase pairs. (C) Gene models (upper panel) and SNP  $-\log_{10}(P)$  scores (lower panel) in the genomic region surrounding the GWA QTL at the or PHT1 locus ; 5' and 3' indicate the different genomic DNA strands and orientation of the respective gene models. (D) Relative expression level of all members of the Arabidopsis *PHT1* gene family in shoots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on +Zn or -Zn agar medium. (E) Shoot Pi concentration of 18-days-old Col-0 wild-type plants, *pht1;1*, *pht1;2*, *pht1;3* and *phr1* mutants grown in +Zn or -Zn conditions. (F) Relative expression level of *PHT1;1* gene in roots of 18-days-old *phr1* mutant grown on +Zn or -Zn agar medium. mRNA accumulation was quantified by RT-qPCR, normalized to the mRNA level of the *UBIQUITIN10* reference gene (*UBQ10*: At4g05320) and expressed as relative values against Col-0 grown in +Zn medium (control). Individual measurements were obtained from the analysis of shoots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.05$  and  $P < 0.01$ ) are indicated by one or two asterisks.

**The induction of PHT1;1 in *lpcat1* involves the PHOSPHATE RESPONSE1 transcription factor (PHR1).**

While we had shown that *PHT1;1* is downstream of *LPCAT1*, it remained unclear how it would be regulated. A known regulator of *PHT1;1* is *PHR1* (Bustos *et al.*, 2010), a key transcription factor involved in the regulation of Pi homeostasis. *PHR1* was already shown to be involved in the coordination of Pi-Zn homeostasis as no change in Pi concentration was observed in the shoots of *phr1* grown in presence or absence of Zn (Khan *et al.*, 2014). Therefore, we first determined the expression level of *PHR1* in WT and *lpcat1* mutant grown for 18 days in  $-Zn$ . *PHR1* expression showed no change in WT plants, and slightly increase but not significant in *lpcat1* mutant grown in Zn deficiency (Figure III.8B), which is consistent with previous results showing that regulation of *PHR1* does not occur transcriptional level but rather at posttranslational level (Rubio *et al.*, 2001) (Miura *et al.*, 2005). We then determined the expression level of *PHT1;1* in *phr1* mutant in  $-Zn$ . *PHT1;1* expression level significantly decreased in *phr1* mutant in  $-Zn$  compared to WT plants (Figure III.7F). No change in Pi concentration was observed in the shoots of *phr1* regardless the Zn regime consistently with (Khan *et al.*, 2014) (Figure III.7E). Taken together, these results provides evidence for a model that *PHR1* regulates the expression of *PHT1;1* in  $-Zn$  in an *LPCAT1* dependent manner.



**Figure III.8. High Affinity of Phosphate Transporter (*PHT1;1*) and Phosphate Response 1 (*PHR1*) gene expression analysis.** **A)** Loss of function mutations of *LPCAT1* show enhanced expression of *PHT1;1* when compared to Col-0 wild-type plants. Relative expression level of *PHT1;1* gene in roots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on +Zn or -Zn agar medium. mRNA accumulation was quantified by RT-qPCR, normalized to the mRNA level of the *UBIQUITIN10* reference gene (*UBQ10*: At4g05320) and expressed as relative values against Col-0 grown in +Zn medium (control). **B)** Loss of function mutations of *LPCAT1* did not changed the expression of *PHR1* when compared to Col-0 wild-type plants. Relative expression level of *PHR1* gene in roots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on +Zn or -Zn agar medium. mRNA accumulation was quantified by RT-qPCR, normalized to the mRNA level of the *UBIQUITIN10* reference gene (*UBQ10*: At4g05320) and expressed as relative values against Col-0 grown in +Zn medium (control). Individual measurements were obtained from the analysis of roots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.01$ ) are indicated by two asterisks.



## Discussion

Understanding how Zn and Pi homeostasis are wired to regulate growth is crucial to offer a new perspective of improving Pi nutrition in plants by modulating the Zn-deficiency signalling pathway. Our study provides a first insight into the genetic and molecular mechanism that controls shoot Pi concentration under  $-Zn$  in plants by discovering a pathway which includes the  $-Zn$  response TF *bZIP23* that target the *LPCAT1* and the TF *PHR1* that targets the Pi transporter *PHT1;1*.

In *A. thaliana*, GWAS has been shown to be a powerful approach to detect loci involved in natural variation of complex traits including variation in the accumulation of non-essentials or toxic elements in plants, such as sodium (Baxter *et al.*, 2010), cadmium (Chao *et al.*, 2012) or arsenic (Chao *et al.*, 2014). Here we used GWAS to identify genes involved in the regulation of the essential macronutrient (P) concentration in its anionic form (Pi) in plants grown under control conditions (+Zn) and  $-Zn$ . In both conditions, our GWA analysis reveals that there is widespread natural variation in shoot Pi concentration, and supports the existence of genetic factors that affect this trait (Figure III.1). The GWAS data support the  $-Zn$  specificity of this response, since no association was detected around the *LPCAT1* locus in our control condition (+Zn) (Figure III.1). The presence of the Zinc Deficiency Response Element (ZDRE) (Assunção *et al.*, 2010a) in the promoter of *LPCAT1* and more particularly the newly identified binding motif specific for bZIP23 (Figure III.3, III.4) in the 5' untranslated leader of *LPCAT1* is a strong argument supporting the Zn-dependency of this response.

A ZDRE is present in the promoter regions of many genes targeted by bZIP19 and bZIP23 (Assunção *et al.*, 2010a). In addition to their positive regulatory role by inducing several Zn deficiency related genes, publicly available microarray showed that bZIP19 and bZIP23 may have a negative regulatory role as many genes were induced in the *bzip19/bzip23* mutant background compared to WT plants grown in  $-Zn$  (Azevedo *et al.*, 2016). A functional redundancy of these two TFs was proposed based on the oversensitivity of the *bzip19* and *bzip23* double mutant to  $-Zn$ , which was not observed with either *bzip19* or *bzip23* single mutants (Assunção *et al.*, 2010a). This redundancy may not be absolute, as recent physiological and genetic evidence indicates that bZIP19 and bZIP23 are not completely redundant and they not only regulate the same, but also separate sets of genes in Arabidopsis (Inaba *et al.*, 2015). Our results support this finding by showing that only bZIP23 is involved in regulating *LPCAT1* in response to  $-Zn$ . bZIP23 is likely to do so through two *cis*-elements in the non-coding part of

the *LPCAT1* gene. One being the aforementioned ZDRE, which can also bind the bZIP19 paralogue of bZIP23, the other a novel binding motif, GTGTC[A/G] [C/A]A, which is specific to bZIP23. This latter element resides in the 5'-untranslated leader of *LPCAT1*. Binding of bZIP23 to this element therefore might physically block the transcription of the *LPCAT1* gene under Zn deficient conditions. This is further supported by the repressive role for bZIP23 on the expression of *LPCAT1* under Zn deficiency. Genomic sequence surveys screening for this new TF-binding site promise to further help identifying a complete list of genes potentially regulated by bZIP23 in order to fully understand the involvement of bZIP23 in the -Zn response in a genome-wide manner.

Of a particular interest is that our study revealed that this novel bZIP23-interacting sequence motif is subject to natural variation in *A. thaliana* (Figure III.4), and its alteration may be associated with changes in the binding capacity of bZIP23. There are several ways that genetic variants can mechanistically contribute to plant adaptation. Many reported examples with regards to nutrient accumulation involve a change in the coding sequence of a gene that then alters the amino acid sequence of the encoded protein, thus leading to the disruption of gene function and a phenotypic change (Baxter *et al.*, 2010) (Chao *et al.*, 2012) (Chao *et al.*, 2014). Reports on the role of specific regulatory element polymorphisms in the regulation of complex traits such as nutrient homeostasis crosstalk are less common, also because it is difficult to identify these relevant sequence changes. In our study we demonstrated that allelic variation (SNPs) in the novel bZIP23 binding motif upstream of the *LPCAT1* gene is associated with variation in *LPCAT1* expression levels, which in turn results in variation in Pi accumulation in -Zn conditions. The *LPCAT1* natural variants such as found in this study offer new inspiration for agronomical and biotechnological applications to optimize Pi use efficiency in plants.

Mutation of *LPCAT1* results in altered Lyso-PC and PC concentrations; an altered Lyso-PC/PC ratio; increased *PHT1;1* expression levels; and ultimately an over-accumulation of Pi under Zn deficiency (Figure III.6, III.7). The induction of the expression of genes encoding P uptake transporters under Zn deficiency has been reported in crop plants as demonstrated in barley (*Hordeum vulgare*) (Huang *et al.*, 2000); and Arabidopsis (Jain *et al.*, 2013) (Khan *et al.*, 2014), but the molecular mechanism that govern this induction remained obscure. Our study showed that the induction of *PHT1;1* in plants grown under Zn deficiency, WT or *lpcat1*, requires the action of PHR1 TF. The increase in *PHT1;1* expression levels is likely to explain

the increased shoot Pi concentration in *lpcat1* since it is known that CaMV 35S promoter driven overexpression of this Pi transporter significantly increases shoot Pi concentration (Mitsukawa *et al.*, 1997) (Shin *et al.*, 2004) (Catarcha *et al.*, 2007). Moreover, our finding provides evidence supporting a role for a Lyso-PC/PC-derived signal in regulating Pi homeostasis under –Zn. Until recently our knowledge on PL-derived signals in plants was scarce; however, physiological and molecular studies have shown that some PL classes could serve as precursors for the generation of diverse signalling molecules (Spector and Yorek 1985) (Testerink and Munnik 2005). For instance, Lyso-PC was shown to act as a signal for the regulation of the expression of arbuscular mycorrhiza (AM)-specific Pi transporter genes in potato, tomato and recently in *Lotus japonicas* (Drissner *et al.*, 2007, Vijayakumar *et al.*, 2016). In addition to the involvement of individual PLs in specific physiological processes in plants (e.g ion transport), a broader importance of changes in Lyso-PC/PC ratio for the regulation of plants development and basic cell biology is emerging. For instance, in *Arabidopsis* alteration of the Lyso-PC/PC ratio shortens the time to flower (Nakamura *et al.*, 2014). In human cells, the Lyso-PC/PC ratio was also associated with an impairment of cell function, signalling and metabolism (Mulder *et al.*, 2003) (Klavins *et al.*, 2015). Our data now demonstrate a fundamental link between PL metabolism, particularly Lyso-PC/PC, and Pi accumulation in –Zn condition, and lays the foundation for exploring the role of Lyso-PC/PC-derived signal in controlling ion homeostasis and response to environmental changes not only in plant cells but also in other organisms. In this context, knowing that PHR1 is expressed irrespective of P- (Rubio *et al.*, 2001) or Zn-regime (Khan *et al.*, 2014), and that its regulation at post-translational level has been proposed (Miura *et al.*, 2005), an attractive hypothesis would be that Lyso-PC/PC-derived signal plays a role in the posttranslational regulation of PHR1 under Zn limitation to control Pi transporter. Testing this hypothesis will deserve further investigation.

Overall, our study shed light on molecular mechanism underlying an old observation made as early as 1970s, namely P-Zn interaction in plants (Warnock 1970, Marschner and Schropp 1977) (Loneragan *et al.*, 1979). By combining GWAS and functional genomics approaches, we discovered a complete pathway involved in the regulation of shoot Pi accumulation in –Zn that can be defined as bZIP23-LPCAT1(Lyso-PC/PC)-PHR1-PHT1;1. Beyond its fundamental importance, our study could have a direct impact on plants growth in field by improving plant growth while reducing P supply, and will help meeting one of challenges facing agriculture in the 21<sup>st</sup> century.

## Materials and Methods

### Plant materials and growth conditions

A subset of 223 *Arabidopsis thaliana* accessions of the RegMap panel (Horton *et al.*, 2012) was used for genome-wide association studies. The names of accessions are provided in table III.1. All lines were used side by side in the same growth chambers under the same conditions, 22 °C under long days (16 h light and 8 h dark). *Arabidopsis* mutants used in this study are in the Columbia-0 genetic background. The *phr1* mutant was previously described in (Khan *et al.*, 2014). The *bzip19bzip23* mutant previously described by (Assunção *et al.*, 2010a) was used in this work. T-DNA insertion mutant lines for the At5g43350 (N666665, *phr1;1*), At5g43360 (N661080, *phr1;2*), At5g43370 (N448417, *phr1;3*), At1g12640 (N686743 (*lpcat1-1*, (Wang *et al.*, 2012)), N442842) and At1g12650 (N526222) genes were obtained from the European *Arabidopsis* Stock Centre (arabidopsis.info; University of Nottingham, UK). Plants were germinated and grown on vertically positioned agar-solidified media (A1296, Sigma). The complete nutrient medium contained: 9.5 mM KNO<sub>3</sub>, 10.3 mM NH<sub>4</sub>NO<sub>3</sub>, 1.5 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 100 µM FeNaEDTA, 100 µM MnSO<sub>4</sub>, 30 µM ZnSO<sub>4</sub>, 100 µM H<sub>3</sub>BO<sub>3</sub>, 5 µM KI, 1 µM Na<sub>2</sub>MoO<sub>4</sub>, 0.1 µM CuSO<sub>4</sub> and 0.1 µM CoCl<sub>2</sub> (adapted from (Murashige and Skoog 1962). Zn-deficient medium was made by omitting ZnSO<sub>4</sub>. Seeds sown on plates were stratified at 4 °C for 3 days. Plates were then transferred to a growth chamber for 18 days set at the following conditions: 16/8h light/dark cycle, 250 µmol m<sup>-2</sup> s<sup>-1</sup> light, and 24/20 °C (light/dark).

### Plasmid construction and plant transformation.

The *LPCAT1* coding region driven by its native promoter (1.5 kbp fragment immediately upstream of the start codon including the 5' untranslated region (5'-UTR)) from Col-0 and Sap-0 accessions were amplified using PCR and the following primers p*LPCAT1*<sup>Col-0</sup>-forward 5'-cgctgcagggtgtcgaaaacccgtttt-3'; p*LPCAT1*<sup>Col-0</sup>-reverse 5'-cgggatcctgatcagagagtacaacaggagag-3'; p*LPCAT1*<sup>Sap-0</sup>-forward 5'-cgctgcagggtgtcacaacccgggt-3' and p*LPCAT1*<sup>Sap-0</sup>-reverse 5'-cgggatccatgatcagatagttacaacaggagagg-3', and then cloned into the binary vector pCambia1301 by restriction enzymes *Bam*HI and *Pst*I (site underlined). The *LPCAT1* coding regions were amplified using PCR and the following primers p*LPCAT1*<sup>Col-0</sup>-forward 5'-cgctgcagttattcttctttacgcggtttt-3'; p*LPCAT1*<sup>Sap-0</sup>-forward 5'-cgctgcagttattcttctttacgtggtttt-3' and p*LPCAT1*<sup>Col-0/ Sap-0</sup>-reverse 5'-cgctgcagatggatgatgagttcaatggctg-3'. *Pst*I was used for the fusion of p*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup> promoters to either *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup>. The

constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and then used for Arabidopsis transformation by the floral dip method (Clough and Bent 1998). Transgenic plants were selected by antibiotic resistance, and only homozygote descendants of hemizygote T2 plants segregating 1:3 for antibiotic resistance: sensitivity was used for analysis.

### **Inorganic phosphate concentration measurements and GWA Mapping**

All accessions were grown in the presence or absence of zinc for 18 days. Shoots were collected, weighed and ground into powder in liquid nitrogen. An aliquot (30 mg) was incubated at 70 °C in NanoPure water, for 1 hour. Inorganic phosphate (Pi) concentrations were determined using the molybdate assay as previously described by (Ames 1966). The shoot Pi concentrations across the analysed accessions was used as phenotype for GWA analysis. The GWA analysis was performed in the GWAPP web interface using the mixed model algorithm (AMM) that accounts for population structure (Seren *et al.*, 2012) and using the SNP data from the RegMap panel (Atwell *et al.*, 2010) (Brachi *et al.*, 2010, Horton *et al.*, 2012). Only SNPs with minor allele counts greater or equal to 10 (at least 10 out of 223 accessions contained the minor allele) were taken into account. To correct for multiple testing, a Bonferroni correction with a threshold of 0.05 was used to detect significant associations.

### **Gene expression analysis by quantitative RT-PCR**

For expression analysis, the Plant RNeasy extraction kit (Qiagen) was used to extract total RNA free of residual genomic DNA from 100 mg frozen shoot material. Total RNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific). Two µg of total RNA was used to synthesize cDNA. Reverse transcriptase PCR (RT-qPCR) was performed with a Light Cycler 480 Real-Time PCR System (Roche) using SYBR green dye technology (Roche) as described by (Khan *et al.*, 2014). The primers used in this study are *LPCAT1*-forward 5'-ggtgtaagcttgacgaaac-3'; *LPCAT1*-reverse 5'-agagaacaagaaccgga-3' and *UBQ10*-forward 5'-aggatggcagaactcttgct-3'; *UBQ10*-reverse. 5'-tcccagtcacgtcttaacg-3'. The primers used to quantify *ZIP4* are *ZIP4*-forward 5'-cggtaaacaataagaatcaggagc-3'; *ZIP4*-reverse 5'-taaatctcgagcgttgatg-3'; and for *ZIP12* are *ZIP12*-forward 5'-aacagatctcgcttggcg-3'; *ZIP12*-reverse 5'-aatgtgatcatcatcttggg-3'. Primers used to quantify the *PHT1* gene family member are designed according to (Khan *et al.*, 2014). Quantification of mRNA abundance was performed in a final volume of 20 µL containing 10 µL of the SYBR Green I master mix, 0,3 µmol primers, and 5 µL of a 1:25 cDNA dilution. PCR conditions were as 95°C for 5 min, and followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, 72 °C for 25 s. One final cycle was

added in this program: 72 °C for 5 min. For every reaction, the cycle threshold (Ct) value was calculated from the amplification curves. For each gene, the relative amount of calculated mRNA was normalized to the calculated mRNA level of the *Ubiquitin10* control gene (*UBQ10*: At4g05320) and expressed as relative values against wild-type plants grown in the presence or absence of Zn in the medium. Quantification of the relative transcript levels was as described in (Rouached *et al.*, 2008). The mRNA abundance of each genes was expressed following normalization against the CT values of *Ubiquitin10* mRNA, for instance  $\Delta Ct, LPCAT1 = Ct, LPCAT1 - (Ct, UBQ10)$ . Quantification of the relative transcript levels was performed as following, Zn deficiency treatment (-Zn) was compared to +Zn treatment (+Zn), the relative mRNA accumulation of each gene was expressed as a  $\Delta\Delta Ct$  value calculated as follows:  $\Delta\Delta Ct = \Delta Ct, LPCAT1(-Zn) - \Delta Ct, LPCAT1(+Zn)$ . The fold change in relative gene expression was determined as  $2^{-\Delta\Delta Ct}$ .

### **Expression and purification of bZIP19 and bZIP23 proteins.**

*bZIP19* and *bZIP23* coding sequences CDS were first cloned in the pENTR/D-TOPO vector, and then transferred to pDEST15 vector (Invitrogen) by LR reaction following the manufacturer's instructions. The GST-bZIP19 and GST-bZIP23 fusion proteins were expressed in *Escherichia coli* Rosetta 2(DE3) pLysS (Novagen, Darmstadt, Germany). Transformed cells were grown in a phosphate-buffered rich medium (Terrific broth) at 37°C containing appropriate antibiotics until the OD<sub>660</sub> reached 0.7-0.8. After induction with 1 mM IPTG (isopropyl-b-D-thiogalactoside) for 16 h at 22 °C, bacteria were harvested by centrifugation (6000 ×g, 10 min, 4 °C) and suspended in 1X PBS buffer containing lysozyme from chicken egg white (Sigma) and complete protease inhibitor cocktail (Roche). The resulting cell suspension was sonicated and centrifuged at 15,000 ×g, for 15 min at 4°C to remove intact cells and debris. The proteins extract was mixed with buffered glutathione sepharose beads (GE Healthcare, Freiburg, Germany), and incubated at 4°C for 3 h. The resin was centrifuged (500 ×g, 10 min, 4°C) and washed five times with 1X PBS buffer.

*bZIP19* and *bZIP23* were then cleaved from GST using 25 unit/ml of thrombin at room temperature for 16h. All fractions were subjected to SDS-PAGE, and proteins concentrations were determined. For protein quantification, absorbance measurements were recorded on a nanodrop spectrophotometer (Model No.1000, Thermo Scientific Inc., Wilmington, Delaware, USA) at 280 nm, and in parallel on a VICTOR2™ microplate reader (MULTILABEL



COUNTER, life sciences) at 660 nm using the Pierce 660 nm Protein Assay (Pierce/Thermo Scientific, Rockford; (Antharavally *et al.*, 2009))

### **Electrophoretic Mobility Shift Assay (EMSA)**

EMSA was performed using purified proteins and DNA probes labeled with Biotin-TEG at the 3' end. Biotin-TEG 3' end-labeled single-stranded DNA oligonucleotides were incubated at 95 °C for 10 min and then annealed to generate double-stranded DNA probes by slow cooling. The sequences of the oligonucleotide probes were synthesized by Eurofins Genomics and are as following: 5'-ttaggttcac**gtgtcgacat**gaaaggagct-3' and 5'-catatccat**gtgtcgaaa**acccgattttt-3' The binding of the purified proteins ( $\approx$  150 ng) to the Biotin-TEG labelled probes (20 fmol) was carried out using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, USA) in 20  $\mu$ L reaction mixture containing 1X binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), 2.5% glycerol, 5 mM MgCl<sub>2</sub>, 2  $\mu$ g of poly (dI-dC) and 0.05% NP-40. After incubation at 24° C for 30 min, the protein–probe mixture was separated in a 4% polyacrylamide native gel at 100 V for 50 min then transferred to a Biodyne B Nylon membrane (Thermo Scientific) by capillary action in 20X SSC buffer overnight. After ultraviolet crosslinking (254 nm) for 90 s at 120 mJ.cm<sup>-2</sup>. The migration of Biotin-TEG labelled probes was detected using horseradish peroxidase-conjugated streptavidin in the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's protocol, and then exposed to X-ray film.

### **Phospholipid Extraction**

Lipids were extracted from 18-days-old *Arabidopsis thaliana* shoots (Col-0) grown in the presence or absence of Zn, following the Folch's method (Folch *et al.*, 1957). The total phosphorus (P) contained in lipids was measured using a spectrophotometer with an absorbance at 830 nm. Lipid separation and quantification was performed using Thin Layer Chromatography (TLC). The lipid composition was detected and quantified using a GAMAG TLC SCANNER 3 (Muttenez, Switzerland), operating in the reflectance mode. The plates were scanned at 715 nm after dipping in a solution of Blue Spray (Sigma, France) and heating for 3 min at 55 °C. The WinCat software program was used to scan bands, the different classes of phospholipids (Fouret *et al.*, 2015) were identified by comparing their retention factor (Rf) to authentic standards and the quantities of each phospholipid were evaluated against the corresponding calibration curve (Fouret *et al.*, 2015).

## Statistical analysis

Statistical analysis of quantitative data was performed using the GraphPad prism 5.01 software program for Windows (GraphPad 156 Software, CA, USA, <http://www.graphpad.com>). For all the t-test analyses the difference was considered statistically significant when the test yielded a *P*-value < 0.05.

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## Contributions

H.R designed the research and supervised this project. M.K., N.B., D.S. and A.M performed most experiments. W.B., A.S., G.K. and M.G.M.A contributed identifying the new binding site for bZIP23. R.C.A. prepared and contributed plant material. MK. and G.F. helped conducting the PLs experiment and data analysis. H.R, R.C.A., M.G.M.A and W.B. analyzed the data and wrote the manuscript.

## Competing interests

The authors declare that no competing interests exist.



## **Chapitre IV.**

### **General Conclusion and Perspective**

## Chapitre IV. General Conclusion and Perspective

### Conclusion générale et perspective

Le travail de ma thèse avait pour objectif d'identifier les gènes impliqués dans la coordination de l'homéostasie du Pi et celle du Zn chez les plantes. Au moment de commencer ma thèse, mon équipe de recherche avait déjà identifié le gène *PHO1;H3* qui s'est caractérisé par son niveau d'expression remarquablement élevé en condition de carence en Zn chez *Arabidopsis thaliana* (Khan et al., 2014). L'ensemble des résultats obtenus par Khan et al (2014) ont permis d'émettre l'hypothèse que *PHO1;H3* pourrait jouer un rôle de régulateur négatif dans le chargement du Pi dans le xylème en condition de carence en Zn, rôle jusque-là inconnu chez les plantes. Lorsque j'ai commencé mon travail de thèse, nous avons élaboré un plan de recherche pour déterminer les protéines régulatrices de l'expression de *PHO1;H3* en condition de carence en Zn. Vu la complexité des interactions entre ces voies de signalisation (Zn et Pi), et le nombre de gènes impliqués, les approches de biologie classique nous semblaient insuffisantes pour décrire et comprendre les mécanismes moléculaires impliqués dans la régulation du *PHO1;H3*. En collaboration avec Dr Gabriel Krouk (CNRS- Montpellier), nous avons développé un programme nommé TransDetect permettant de prédire des facteurs de transcription (TFs) régulant l'expression de *PHO1;H3*. Ensuite, nous avons validé l'implication de quatre de ces TFs (MYB15, MYB84, BHLH35, ICE1) qui contrôlent l'expression de *PHO1;H3* en condition de carence en Zn. C'est ainsi que ces TFs interviennent dans la régulation de l'accumulation du Pi dans les feuilles d'*Arabidopsis*. Le détail du travail a été présenté dans la chapitre II et publié dans la revue Plant Physiology (2017).

D'après le phénotype observé chez le mutant *pho1h3*, nous avons conclu que ce gène ne peut pas être le seul responsable du contrôle de l'accumulation du Pi en condition de carence de Zn. C'est pour cela que nous avons employé une deuxième approche dite GWAS pour découvrir des nouveaux gènes impliqués dans cette interaction Pi-Zn chez *A. thaliana*. Cette partie du travail a été réalisée en collaboration avec Dr Wolfgang BUSH (GMI-Vienne Autriche). Notre objectif était atteint et nous avons identifié une nouvelle voie, contrôlant l'accumulation du Pi en condition de carence en Zn, dans laquelle le gène *LPCAT1*, joue un rôle central. Dans l'ensemble, nous pensons qu'il y a plusieurs nouveaux aspects dans ce travail. Premièrement, nous avons fait une première analyse de l'accumulation du Pi chez les plantes (230 génotypes d'*Arabidopsis thaliana*) en utilisant l'approche de GWAS, aussi bien dans les conditions de contrôle (présence de Zn, +Zn), qu'en condition de carence en Zn (-Zn). Nous

avons montré que ce trait (accumulation du Pi en  $-Zn$  ou  $+Zn$ ) est largement contrôlé par des facteurs génétiques, et nous avons identifié puis validé le gène *LPCAT1* comme déterminant clé de l'accumulation du Pi chez *Arabidopsis* en condition de carence en Zn. En outre, nous avons montré que la variation d'expression de ce locus contribue significativement à la variation naturelle de l'accumulation de Pi dans la condition  $-Zn$ .

Deuxièmement, nous avons pu démontrer que *LPCAT1* est régulé au niveau transcriptionnel par un régulateur majeur de la signalisation  $-Zn$ , c'est le facteur de transcription bZIP23, fournissant ainsi un lien moléculaire entre le signal de la carence en Zn et la régulation de l'expression de *LPCAT1*. L'analyse *in silico* couplée à une analyse EMSA nous a permis de montrer que le TF bZIP23 peut se lier au promoteur du *LPCAT1* au niveau d'un site (motif) de fixation qui lui est spécifique. Ce nouveau motif montre un polymorphisme (SNP) entre différentes accessions ayant une capacité contrastée d'accumuler le Pi en condition de carence en Zn. En utilisant des approches de génétique inverse, nous avons montré que la mutation dans *bZIP23* ou *LPCAT1* modifie le ratio Lyso-PC / PC, suggérant que le phospholipide pourrait être impliqué dans la signalisation de la carence en Zn, ce qui est en accord avec d'autres organismes comme les cellules de la levure et des mammifères.

Enfin, l'analyse d'expression du transporteur de phosphate *PHT1;1* effectuée chez le mutant *lpcat1* cultivé en condition de carence en Zn, révèle une induction de l'expression du *PHT1;1* qui est plus importante que celle observée chez les plantes sauvages (Col-0). Ceci démontre le rôle spécifique de ce transporteur dans l'accumulation du Pi chez les plantes en condition de carence en Zn.

En conclusion, ce travail a permis de révéler un lien important entre le métabolisme des phospholipides et l'interaction de l'homéostasie Pi-Zn *via* le gène *LPCAT1*. Il ouvre des nouvelles voies de recherches en vue de mieux comprendre le rôle de Lyso-PC et PC dans le contrôle de l'interaction entre l'homéostasie des macro- et micronutriments. D'une manière plus générale, notre découverte offre une nouvelle perspective sur la façon d'améliorer le contenu en Pi dans les plantes, car nos résultats suggèrent que la modulation de la voie de signalisation de la carence en Zn pourrait être une approche simple et bonne pour cela. Enfin, des nouveaux gènes régulant la teneur en Pi dans la plante en condition de carence en Zn ont été clonés, et nous sommes en train de les caractériser. De nouveaux mécanismes sont donc en cours de validation.

En perspective du travail de thèse, et encouragés par les résultats obtenus dans la recherche des bases génétiques de l'accumulation du Pi en condition de carence en Zn chez *Arabidopsis thaliana*, nous avons choisi d'utiliser l'approche de GWAS pour identifier des marqueurs génétiques et des gènes impliqués dans la régulation de l'accumulation du Pi chez une plante d'intérêt agronomique, le blé dur (*Triticum turgidum durum*). Cet effort ambitionne d'aller au-delà de l'accumulation des connaissances fondamentales à la proposition de ces marqueurs moléculaires pour sélectionner de meilleures variétés de blé dur qui soient moins consommatrices d'engrais phosphatés *via* la modification de la voie de signalisation de carence en Zn. Ce projet a commencé en collaboration avec Pr. Jacques David (AGAP., SupAgro Montpellier).

Le choix du blé dur est basé sur le fait que c'est la céréale emblématique de la Région Languedoc-Roussillon. Les céréales occupent environ 120 000 ha cultivés par 5 500 exploitations réparties sur la région. Elles se placent en deuxième position dans le palmarès des cultures, derrière la vigne. Parmi ces cultures de tradition méditerranéennes, le blé dur présente des enjeux sociétaux et économiques majeurs. Transformé en semoule, il est principalement consommé, autour du bassin méditerranéen, sous la forme de pâtes alimentaires et de couscous. Ainsi, la zone méditerranéenne dans son ensemble consomme 62 % du blé dur mondial et est la principale zone importatrice de la planète. Un plan de relance pour soutenir cette culture dans notre région et pour améliorer la productivité des systèmes de culture de blé dur est devenu une nécessité.

La production du blé dur nécessite une utilisation intensive des engrais phosphatés ce qui pèse défavorablement sur le coût de sa production. Sachant que le phosphore (P) est une ressource naturelle non renouvelable et qu'il est estimé que sa réserve mondiale sera épuisée dans les prochaines décennies, il est primordial d'anticiper le future manque de cet élément, indispensable à la croissance des plantes, en créant des variétés productrices avec un minimum d'apport en engrais phosphaté, et se caractérisant donc par une meilleure efficacité d'utilisation du Pi disponible.

En collaboration avec Pr. Jacques David nous avons initié ce projet, et nous avons utilisé une collection de 180 lignées de blé dur issues d'une population originale créée par le laboratoire AGAP (David *et al.*, 2014). Cette population, dite EPO pour « Evolutionary Pre-breeding pOpulation », a une base génétique large et possède les caractéristiques idéales pour des approches GWAS (peu de structuration génétique, faible déséquilibre de liaison). Ces

lignées ont été génotypées dans le cadre du Méta-Programme INRA Selgen à très haut débit grâce à une puce Axiom à ADN de 420 000 marqueurs, mise au point pour le blé tendre dans le cadre du projet Investissement d'Avenir BreedWheat, piloté par l'INRA de Clermont Ferrand (Paux et al, in prep). Pour 172 de ces lignées, un séquençage supplémentaire du transcriptome (RNA seq) de jeunes plantules a permis l'identification de nouveaux polymorphismes nucléotidiques, offrant la possibilité de « génotypage par séquençage » (David *et al.*, 2014).

Toutes ces ressources sont mises à disposition pour aller au-delà de mon travail de thèse. Ce projet est pour l'instant unique au niveau international et permet de lancer des études GWAS ambitieuses sur le blé dur. A titre de comparaison, l'étude la plus récente publiée sur le blé dur rapporte 183 lignées élites génotypées sur 957 marqueurs (Cane *et al.*, 2014). En rassemblant les données de la puce Axiom avec le séquençage du transcriptome des lignées, la diversité génétique de l'EPO peut finalement être décrite avec un total d'environ 170 000 marqueurs de bonne qualité, répartis sur l'ensemble des 14 paires de chromosomes. Les lignées EPO présentent un niveau élevé de diversité tant génétique que morphologique et apparaissent très adaptées aux programmes de GWAS, ce qui est bien adapté à la suite de notre projet. Ces données permettent d'envisager des études similaires aux meilleures études actuelles sur le blé tendre (Cormier *et al.*, 2014). Ces accessions sont (et seront), par ailleurs, décrites pour un nombre croissant de caractères par AGAP (Roumet, Communications personnelles), ce qui permettra, à terme, de comprendre l'interaction entre Pi et Zn (ou autres caractères d'intérêts agronomiques). Par ailleurs, il est important de noter que l'équipe AGAP continuera à accumuler des données de génotypage sur ce matériel au gré des différentes opportunités offertes (Plan Relance Blé Dur, Meta Programme INRA, collaborations internationales). Enfin, mise en regard avec des données phénotypiques de valeur, ces données génomiques permettent également de réfléchir pour lancer des projets pilotes de prédiction et de sélection génomique pour accélérer le progrès génétique sur la base de marqueurs.

En ce qui concerne la perspective de mon travail de thèse, nous avons déjà commencé des premières expériences. Nous avons cultivé les 180 lignées EPO sur un milieu hydroponique pendant 28 jours. Nous avons considéré deux conditions de culture: sur un milieu complet (présence du Zn) et une condition qui cause une sur-accumulation du Pi dans la plante (absence du Zn). Nous avons, ensuite, collecté les feuilles et les racines de chacune de ces lignées. Nous avons mesuré l'accumulation de Pi dans ces échantillons (Table IV. 1.) L'expérience a été répétée trois fois. En utilisant ces données, nous avons réalisé des analyses d'association entre

les données phénotypiques (contenu en Pi) et données génotypiques. Nous avons identifié la présence d'associations entre les deux groupes de données (accumulation du Pi en conditions de +Zn et -Zn), et identifié les gènes candidats. Les résultats sont très encourageants et constitueront sans doute la base d'une publication très originale. L'application de ces résultats aura un impact direct aux champs en générant des plantes avec une utilisation efficace du Pi.

## General Conclusion and Perspective

The aim of my thesis was to study the physiological and molecular basis of the interaction between Pi and Zn homeostasis in plants, and to identify the interconnections between Pi and Zn signaling pathways. At the time of starting my work, my research team had identified the *PHO1;H3* gene which is characterized by the induction of its expression by Zn deficiency (Khan et al., 2014). The set of results obtained by Khan et al., (2014) lead us to hypothesize that *PHO1;H3* plays a negative regulatory role in the loading of Pi into the xylem under Zn deficiency. When I started my thesis, we developed a research plan to determine the proteins (transcription factors, TFs) involved in the regulation the expression of *PHO1;H3* in Zn deficiency condition. Given the fact that multiple TFs are involved in the regulation of the expression of a given gene, classical biology approaches have proved insufficient. In collaboration with Dr. Gabriel Krouk we have developed a program (TransDetect) to predict TFs regulating the expression *PHO1;H3*. We identified then validated the involvement of the 4 TFs (MYB15, MYB84, BHLH35, ICE1) activated under Zn deficiency conditions to control the expression of *PHO1;H3*, and consequently modulate the accumulation of Pi in *Arabidopsis* leaves. The details of the work were presented in Chapter II and published in the Plant Physiology journal (2017).

Based on the phenotype observed in the *pho1;h3* mutant, we concluded that this gene cannot be the only gene responsible for controlling Pi accumulation under Zn deficiency. For this reason we used a second powerful approach, namely GWAS, to discover more genes and establish pathways involved in the regulation of Pi accumulation in -Zn in *Arabidopsis thaliana*. This work was done in collaboration with Dr. Wolfgang BUSH (GMI-Vienna Austria). Our goal was achieved and we have identified a new pathway, controlling the accumulation of Pi under Zn deficiency, in which the *LPCAT1* gene plays a central role. Overall, my work has several notable aspects. First, my work was the first analysis of Pi accumulation in 223 genotypes of *A. thaliana* using the GWAS approach, not only in control

conditions (presence of Zn), but also in response to Zn deficiency (-Zn). I have shown that this trait (Pi accumulation in -Zn) is largely controlled by genetic factors, and I have identified the *LPCAT1* gene as a key determinant of Pi accumulation specifically under -Zn. We then showed that the variation in the transcriptional regulation of this locus contributes significantly to the natural variation of the accumulation of Pi in -Zn.

Secondly, we have demonstrate that *LPCAT1* is transcriptionally regulated by a major regulator of -Zn signaling, the transcription factor bZIP23, providing a plausible mechanistic link between the Zn deficiency signal and the regulation of *LPCAT1* expression. In silico analysis coupled with an EMSA analysis allowed us to show that TF bZIP23 can bind to the *LPCAT1* promoter at a specific binding site (motif). This new motif shows a polymorphism (SNP) between different accessions having a contrasting ability to accumulate Pi in Zn deficiency condition. Using reverse genetic approaches, we show that the mutation in bZIP23 or *LPCAT1* modifies the phospholipid ratio Lyso-PhosphatidylCholine / PhosphatidylCholine (Lyso-PC / PC), suggesting that phospholipid may be involved in Zn deficiency signaling, which is in agreement with other organisms like yeast and mammalian cells.

Finally, the expression analysis of the phosphate transporter *PHT1;1* carried out in the *lpcat1* mutant cultured under Zn deficiency, reveals an induction of the expression of *PHT1;1* which is greater than that observed in wild type (Col) plants. This demonstrates the specific role of this transporter in the accumulation of Pi in plants under Zn deficiency.

In conclusion, my work has revealed an important link between phospholipid metabolism and the interaction of Pi-Zn homeostasis via *LPCAT1*, and opens avenues for research to better understand the role of Lyso-PC and PC in control of macro-and micronutrient homeostasis interaction. More broadly, our discovery offers a new perspective on how to improve Pi content in plants, as our results suggest that modulation of the Zn deficiency signaling pathway could be a simple and good approach for this. Finally, new genes regulating the P content in the plant under Zn deficiency have been cloned, and we are characterizing them. New mechanisms are therefore being validated.

In perspective of my thesis, the encouraging results we have obtained in the research of the genetic bases of the accumulation of Pi under conditions of Zn deficiency in *Arabidopsis thaliana*, prompted us to use this powerful "Genome Wide Association Studies" (GWAS) approach to identify genetic markers and genes involved in phosphate nutrition of durum wheat (*Triticum turgidum* subsp. *Durum*), to select better varieties of durum wheat that are less consuming phosphate fertilizer by modulating the Zn deficiency signaling pathway. This project started in collaboration with Pr. Jacques David (AGAP, SupAgro Montpellier).

The production of durum wheat requires intensive use of phosphate fertilizers which adversely affect the cost of its production. Knowing that phosphorus (P) is a non-renewable natural resource and that it is estimated that the world P reserve will be exhausted in the coming decades, it is essential to anticipate the future scarcity of this essential element for the growth of plants by creating varieties capable of producing with less input of phosphate fertilizer, and therefore characterized by a better efficiency of use of available Pi.

We initiated this project, and we used a collection of 180 durum wheat lines from an original population created by the AGAP laboratory (Dr Jacques David). This population, known as EPO for Evolutionary Pre-breeding population, has a broad genetic base and has the ideal characteristics for GWAS approaches (little genetic structuring, low linkage disequilibrium). These lines were genotyped in the framework of the very high throughput in INRA Selgen Meta-Program thanks to 420,000 markers in Axiom DNA chip developed for soft wheat as part of the Future Investment Breed Wheat project piloted by the INRA in Clermont Ferrand (Paux et al, in prep). For 172 of these lines, additional sequencing of the transcriptome (RNA seq) of young seedlings allowed the identification of new nucleotide polymorphisms, offering the possibility of "genotyping by sequencing" (David et al, 2014).

At first, we cultivated the 180 EPO lines on a hydroponic medium for 28 days. We considered two growing conditions: on a complete medium (presence of zinc) and a condition that causes an over-accumulation of phosphate in the plant (absence of zinc). We then collected the leaves and roots of each of these lines (Table IV. 1.), the experiment is repeated three times. We performed association analyzes between phenotypic data (phosphate content) and genotypic data using the same association models described above. We identified the presence of associations between the two groups of data and identified the candidate genes. My results are very encouraging. If these results are confirmed, there is no doubt that they will form the basis of a very original publication. The application of these results will have a direct impact on the fields by generating plants with efficient use of phosphate.



## **Chapter V.**

## **References**

## Chapitre V. References.

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# **Annexe 1**

## **Supplementary Tables**



**Table II.1.** AGI number occurrence in TransDetect analysis for At1g14040, Our analysis identified a total of 165 TFs organized in pairs.

<b>No. of</b>	<b>AGI</b>	<b>occurrence</b>			
<b>AGI</b>		<b>in model</b>			
1	At4g37790	64	25	At4g37260	4
2	At2g46510	49	26	At5g62610	4
3	At1g31050	44	27	At5g49520	4
4	At5g04760	37	28	At1g12610	4
5	At5g57150	25	29	At2g36890	4
6	At4g31800	20	30	At2g33860	4
7	At3g49690	17	31	At5g67300	3
8	At3g23250	16	32	At5g65790	3
9	At4g24060	15	33	At5g52660	3
10	At3g50060	10	34	At5g47390	3
11	At5g60890	8	35	At5g47370	3
12	At4g34410	8	36	At3g61850	3
13	At1g43160	8	37	At3g55730	3
14	At2g23320	7	38	At3g54810	3
15	At3g11020	7	39	At4g38620	3
16	At1g03040	6	40	At4g29100	3
17	At4g16780	5	41	At1g19850	3
18	At3g26744	5	42	At1g19210	3
19	At1g74500	5	43	At1g68150	3
20	At1g72360	5	44	At1g53170	3
21	At1g05805	5	45	At1g74930	3
22	At1g30650	5	46	At1g79180	3
23	At1g22810	5	47	At1g70000	3
24	At4g17500	4	48	At2g46590	3
			49	At1g61660	3
			50	At2g37260	3

51	At2g16720	3	81	At2g44840	2
52	At5g25810	2	82	At2g24260	2
53	At5g25830	2	83	At2g34830	2
54	At5g61890	2	84	At2g38470	2
55	At5g48560	2	85	At4g17490	1
56	At5g47220	2	86	At4g17785	1
57	At5g46830	2	87	At1g25330	1
58	At5g41410	2	88	At5g04150	1
59	At5g37260	2	89	At4g36930	1
60	At5g37020	2	90	At4g36900	1
61	At5g10510	2	91	At5g16770	1
62	At5g07580	2	92	At5g26930	1
63	At4g32710	2	93	At5g67190	1
64	At1g25550	2	94	At5g66320	1
65	At1g30330	2	95	At5g65310	1
66	At1g66470	2	96	At5g62430	1
67	At3g24050	2	97	At5g59780	1
69	At3g25890	2	98	At5g59340	1
70	At3g16770	2	99	At5g58010	1
71	At3g01530	2	100	At5g57390	1
72	At3g01220	2	101	At5g56270	1
73	At1g74080	2	102	At5g52830	1
74	At1g69810	2	103	At5g51990	1
75	At1g68670	2	104	At5g49240	1
76	At1g05710	2	106	At5g42630	1
77	At2g36080	2	107	At5g16600	1
78	At1g22640	2	108	At5g15130	1
79	At1g13260	2	109	At5g05410	1
80	At1g80840	2	110	At5g02840	1

111	At3g61890	1	140	At1g15720	1
112	At3g61150	1	141	At1g79840	1
113	At3g57800	1	142	At1g80590	1
114	At3g46590	1	143	At2g41710	1
115	At4g34610	1	144	At1g79700	1
116	At4g32890	1	145	At2g46400	1
117	At4g05100	1	146	At2g33710	1
118	At1g51700	1	147	At3g48920	1
119	At3g24120	1	148	At2g01060	1
120	At3g04670	1	149	At4g30080	1
121	At3g01560	1	150	At4g00760	1
122	At1g71450	1	151	At1g22190	1
123	At1g21340	1	152	At3g24310	1
124	At2g28510	1	153	At1g49560	1
125	At1g09250	1	154	At1g64620	1
126	At1g27050	1	155	At1g36060	1
127	At2g47520	1	156	At2g46410	1
128	At1g22985	1	157	At2g24570	1
129	At5g53290	1	158	At1g77200	1
130	At5g51190	1	159	At1g09530	1
131	At4g25480	1	160	At1g51190	1
132	At4g25490	1	161	At2g28350	1
133	At4g25470	1	162	At2g45050	1
134	At1g28370	1	163	At2g38250	1
135	At4g28110	1	164	At1g69600	1
136	At4g21340	1	165	At2g03470	1
137	At4g00940	1			
138	At4g00480	1			
139	At1g29860	1			

**Table II.2.** List of primers used in this work, The PHO1;H3 transcript abundance were.

Gene		Sequence
At5g57150	F	ATGGATAAAGCCTCAATAATCAA
	R	TTCGAGAACTTCGATGAGA
At3g23250	F	TCTTTCGAAACTTTTGGTGC
	R	TGAAAACGCAGCCTCTAA
At3g49690	F	AACAACAACAAATCCAAACTTC
	R	GGGTTTGTTTTCTCCAGTTC
At1g14040	F	CATCACATTCAAACGAAGAACCAAC
	R	TCATCGTCTAGTCGCCGGAA
At3g26744	F	GTTTTCTTAACCAAATCCATGCTCC
	R	AACAAAACCACCATTAGCAGGAC

**Table III.1.** Shoots inorganic phosphate (Pi) concentration (umole/gFW) in the 223 *Arabidopsis thaliana* accessions grown under two conditions presence or absence of zinc for 18 days.

Acc. Code	Acc. Name	Mean -Zn	Mean +Zn	latitude	longitude	Country of Origin
86	CUR-8	8.64659755	5.25508313	45	1.75	FRA
96	LAC-5	7.43131535	5.18422346	47.7	6.81667	FRA
149	LDV-58	7.76731344	5.74034752	48.5167	-4.06667	FRA
204	MIB60	11.5761743	5.38178506	47.3833	5.31667	FRA
224	MIB86	7.75006884	6.17411688	47.3833	5.31667	FRA
236	Mogl1	8.99304991	5.90436303	48.6667	-4.06667	FRA
262	PAR-8	8.08726839	4.88237958	46.65	-0.25	FRA
266	RAN	11.404434	6.5466901	48.65	-2	FRA
394	Vou-5	7.66027737	5.47858735	46.65	0.166667	FRA
461	EM-183	8.08767877	4.48577323	51.3	0.5	UK
936	FOR-5	10.8626461	4.91050851	42.3853	-71.1173	USA
1829	Mdn-1	10.8875332	NA	42.051	-86.509	USA
1874	MNF-Pot_80	11.579257	NA	43.595	-86.2657	USA
2057	Map-42	6.82099616	NA	42.166	-86.412	USA
2171	Paw-26	13.9574629	5.01024095	42.148	-86.431	USA
2187	Pent-1	5.864071	6.06395077	43.7623	-86.3929	USA
2290	Ste-3	8.6746105	7.53257036	42.03	-86.514	USA
2320	Wilcox-4	7.47760338	5.00225154	43.48	-89.46	USA
5723	Chr-1	6.86665722	3.8123823	51.3	1	UK
5729	Coc-1	6.94604421	4.54707471	54.7	-3.4	UK
5731	Crl-1	6.92491999	3.46953803	54.9	-2.9	UK
5736	Ema-1	6.51548937	3.33911055	51.3	0.5	UK
5742	Frd-1	5.82633996	4.8938863	51.3	1.1	UK
5745	Hil-1	7.02451115	4.0940101	51	-1.5	UK
5751	Kyl-1	6.14449459	4.20778915	57.3	-5.7	UK
5752	Lan-1	6.62222955	4.59453618	55.7	-3.8	UK
5837	Bor-1	6.29851268	4.76933645	49.4013	16.2326	CZE
6008	Duk	8.23211564	4.89075132	49.1	16.2	CZE
6016	Eds-1	6.41118936	3.70526311	62.9	18.4	SWE
6040	Kni-1	10.4149321	6.03090205	55.66	13.4	SWE
6074	Ör-1	11.681465	7.65502492	56.4573	16.1408	SWE
6243	Tottarp-2	9.08451812	6.01837824	56.27373	13.90045	SWE
6730	CIBC5	5.01353138	3.47933397	51.4083	-0.6383	UK
6897	Ag-0	7.79247819	5.05319258	45	1.3	FRA
6898	An-1	5.21396827	5.14882101	51.2167	4.4	BEL
6899	Bay-0	8.45146923	5.9151698	49	11	GER
6903	Bor-4	6.36791746	4.23453355	49.4013	16.2326	CZE

6904	Br-0	11.4305206	5.22447937	49.2	16.6166	CZE
6906	C24	7.0837264	4.89707248	40.2077	-8.42639	POR
6907	CIBC17	8.73921409	4.82734503	51.4083	-0.6383	UK
6909	Col-0	9.58737146	5.6599176	38.3	-92.3	USA
6910	Ct-1	8.94041885	6.59289613	37.3	15	ITA
6911	Cvi-0	9.32169956	4.99698102	15.1111	-23.6167	CPV
6913	Eden-2	10.9673625	7.40516224	62.877	18.177	SWE
6915	Ei-2	9.86253485	8.03031705	50.3	6.3	GER
6916	Est-1	5.17253557	6.09899038	58.3	25.3	RUS
6919	Ga-0	8.26519977	6.30294801	50.3	8	GER
6920	Got-22	7.55785073	4.54889874	51.5338	9.9355	GER
6922	Gu-0	6.54082842	3.82384992	50.3	8	GER
6923	HR-10	12.7713905	4.70594058	51.4083	-0.6383	UK
6926	Kin-0	9.97890104	5.02727376	44.46	-85.37	USA
6928	Kno-18	13.8286886	6.60872765	41.2816	-86.621	USA
6929	Kondara	5.3083583	3.27163651	38.48	68.49	TJK
6930	Kz-1	10.3564261	6.23598193	49.5	73.1	KAZ
6931	Kz-9	6.88800237	4.03782745	49.5	73.1	KAZ
6932	Ler-1	8.11462831	4.45751914	47.984	10.8719	GER
6936	Lz-0	7.80398263	5.26091263	46	3.3	FRA
6937	Mrk-0	12.1105055	8.48509746	49	9.3	GER
6938	MS-0	9.71747766	4.6156442	55.7522	37.6322	RUS
6939	Mt-0	10.1152381	6.06375862	32.34	22.46	LIB
6940	Mz-0	10.6000923	6.54488658	50.3	8.3	GER
6942	Nd-1	9.71348287	6.59486837	50	10	SUI
6943	NFA-10	6.9774983	4.85218904	51.4083	-0.6383	UK
6944	NFA-8	6.90546824	5.30922124	51.4083	-0.6383	UK
6945	Nok-3	6.44536357	5.19165847	52.24	4.45	NED
6946	Oy-0	10.6405948	6.51350185	60.23	6.13	NOR
6951	Pu2-23	7.99963372	5.49007188	49.42	16.36	CZE
6956	Pu2-7	9.2903414	5.34278869	49.42	16.36	CZE
6958	Ra-0	10.3043515	6.00053953	46	3.3	FRA
6959	Ren-1	12.2775708	6.63044708	48.5	-1.41	FRA
6960	Ren-11	9.48846364	7.48851151	48.5	-1.41	FRA
6961	Se-0	9.49237294	4.31802019	38.3333	-3.53333	ESP
6962	Sha	10.6938603	5.26163142	38.35	68.48	TJK
6963	Sorbo	8.08678387	4.33001527	38.35	68.48	TJK
6966	Sq-1	7.79012403	4.0390524	51.4083	-0.6383	UK
6967	Sq-8	8.56181834	6.72044323	51.4083	-0.6383	UK
6968	Tamm-2	6.79538731	5.24596714	60	23.5	FIN
6969	Tamm-27	7.73607615	5.12715017	60	23.5	FIN
6970	Ts-1	13.1144623	5.56964337	41.7194	2.93056	ESP
6971	Ts-5	10.0609691	7.11985858	41.7194	2.93056	ESP
6972	Tsu-1	8.32982981	5.43823785	34.43	136.31	JPN

6973	Ull2-3	8.14437663	7.39001593	56.0648	13.9707	SWE
6975	Uod-1	9.37415073	7.04818159	48.3	14.45	AUT
6976	Uod-7	8.71672711	6.85897666	48.3	14.45	AUT
6977	Van-0	8.55928918	6.02030445	49.3	-123	CAN
6979	Wei-0	8.48112113	7.59626824	47.25	8.26	SUI
6980	Ws-0	9.1737271	5.78577051	52.3	30	RUS
6981	Ws-2	10.3270506	9.9152386	52.3	30	RUS
6982	Wt-5	7.15007928	4.91101091	52.3	9.3	GER
6983	Yo-0	5.96810753	5.26245501	37.45	-119.35	USA
6984	Zdr-1	7.45261824	5.74378459	49.3853	16.2544	CZE
6985	Zdr-6	8.58088634	4.73729987	49.3853	16.2544	CZE
6990	Amel-1	6.8771875	3.81444828	53.448	5.73	NED
6992	Ang-0	4.95846887	3.58327915	50.3	5.3	BEL
6994	Ann-1	8.05568681	7.27824714	45.9	6.13028	FRA
7000	Aa-0	7.45449365	5.44208358	50.9167	9.57073	GER
7002	Baa-1	6.38979334	4.90406371	51.3333	6.1	NED
7004	Bs-2	6.2491472	4.66258752	47.5	7.5	SUI
7014	Ba-1	8.19115425	6.72158574	56.5459	-4.79821	UK
7015	Bla-1	8.33328875	5.51590748	41.6833	2.8	ESP
7026	Boot-1	7.10650939	6.3181962	54.4	-3.2667	UK
7028	Bch-1	8.36530647	8.09426424	49.5166	9.3166	GER
7031	Bsch-0	10.6700491	5.36815976	50.0167	8.6667	GER
7062	Ca-0	7.86743385	5.46362389	50.2981	8.26607	GER
7071	Chat-1	7.52505437	5.02194756	48.0717	1.33867	FRA
7075	Cit-0	7.94456459	6.57112367	43.3779	2.54038	FRA
7081	Co	7.23575172	3.66671335	40.2077	-8.42639	POR
7092	Com-1	6.16927493	3.09975715	49.416	2.823	FRA
7094	Da-0	10.2828062	4.3727976	49.8724	8.65081	GER
7098	Di-1	8.49317791	5.57176022	47	5	FRA
7102	Do-0	6.94714988	5.3464203	50.7224	8.2372	GER
7123	Ep-0	7.08839749	4.74554266	50.1721	8.38912	GER
7126	Es-0	7.3086231	5.66681966	60.1997	24.5682	FIN
7143	Gel-1	10.806962	6.18564115	51.0167	5.86667	NED
7147	Gie-0	6.94272167	4.48968966	50.584	8.67825	GER
7163	Ha-0	8.810963	5.17170467	52.3721	9.73569	GER
7164	Hau-0	6.78773492	4.96178032	55.675	12.5686	DEN
7166	Hey-1	7.53823426	6.46165965	51.25	5.9	NED
7172	Hl-3	7.97718679	5.86811565	52.1444	9.37827	GER
7176	Ls-1	6.32547827	4.89073939	50.5	7.5	GER
7178	Jm-1	9.15181808	4.9431708	49	15	CZE
7181	Je-0	9.79957806	3.76138002	50.927	11.587	GER
7192	Kil-0	8.82973131	6.10179792	55.6395	-5.66364	UK
7199	Kl-5	7.94581796	6.35087359	50.95	6.9666	GER
7201	Kr-0	10.9325058	4.67409943	51.3317	6.55934	GER

7205	Krot-2	9.15339614	6.10789842	49.631	11.5722	GER
7210	La-1	6.39671585	4.17808474	52.7333	15.2333	POL
7224	Li-3	7.73202841	4.79712939	50.3833	8.0666	GER
7231	Li-7	11.3629418	5.16733544	50.3833	8.0666	GER
7242	Lo-2	7.71339415	5.1200583	47.6166	7.6666	GER
7244	Mnz-0	7.93389353	6.9680808	50.001	8.26664	GER
7246	Ma-2	8.97716679	6.35097014	50.8167	8.7667	GER
7255	Mh-0	5.53070338	4.42362685	50.95	7.5	POL
7262	Nw-4	7.10410148	5.16409173	50.5	8.5	GER
7268	Np-0	7.71923568	5.19371526	52.6969	10.981	GER
7275	No-0	9.15447005	6.30889668	51.0581	13.2995	GER
7276	Ob-0	8.81959046	5.258508	50.2	8.5833	GER
7280	Old-1	10.736754	6.76394888	53.1667	8.2	GER
7282	Or-0	11.6649428	7.90523407	50.3827	8.01161	GER
7287	Ove-0	8.74076797	6.73815278	53.3422	8.42255	GER
7291	Pa-2	8.51188007	6.41892483	38.07	13.22	ITA
7297	Pf-0	10.4422539	6.32589599	48.5479	9.11033	GER
7299	Pi-2	7.95599904	6.34546893	47.04	10.51	AUT
7300	Pla-0	7.86176851	6.62896758	41.5	2.25	ESP
7306	Pog-0	10.3986032	6.10254231	49.2655	-123.206	CAN
7307	Pn-0	8.4622621	6.21962935	48.0653	-2.96591	FRA
7309	Po-1	9.00937887	7.28573088	50.7167	7.1	GER
7310	Pr-0	9.96809687	6.295954	50.1448	8.60706	GER
7316	Rhen-1	10.2791464	7.93981653	51.9667	5.56667	NED
7317	Ri-0	13.4529111	8.39003951	49.1632	-123.137	CAN
7320	Rou-0	6.38843653	5.65314018	49.4424	1.09849	FRA
7330	Sapporo-0	12.9693974	4.07552032	43.0553	141.346	JPN
7331	Sh-0	8.09315825	5.60304143	51.6832	10.2144	GER
7337	Si-0	5.50945003	4.24129871	50.8738	8.02341	GER
7351	Ty-0	10.0820986	8.0797601	56.4278	-5.23439	UK
7352	Te-0	6.72406063	5.41406315	60.0585	23.2982	FIN
7353	Tha-1	7.44017133	4.87171226	52.08	4.3	NED
7355	Tiv-1	12.0919328	8.96958852	41.96	12.8	ITA
7372	Tscha-1	6.57656138	4.69144192	47.0748	9.9042	AUT
7378	Uk-1	7.84291207	5.5734361	48.0333	7.7667	GER
7382	Utrecht	9.09608246	6.14615929	52.0918	5.1145	NED
7384	Ven-1	6.76723582	4.43952612	52.0333	5.55	NED
7404	Wc-1	7.06670489	4.48399246	52.6	10.0667	GER
7418	Zu-1	6.00441328	5.04307228	47.3667	8.55	SUI
7424	Jl-3	8.00692939	5.36277154	49.2	16.6166	CZE
7477	WAR	9.7850282	6.20008779	41.7302	-71.2825	USA
7514	RRS-7	10.103458	5.48557556	41.5609	-86.4251	USA
7515	RRS-10	5.20044803	4.81000765	41.5609	-86.4251	USA
7516	Var2-1	8.98153843	6.27854584	55.58	14.334	SWE



7519	Ömö2-3	8.15910492	5.65584318	56.14	15.78	SWE
7520	Lp2-2	7.35285572	5.11514318	49.38	16.81	CZE
7521	Lp2-6	7.90965761	6.15949456	49.38	16.81	CZE
7522	Mr-0	8.91990865	NA	44.15	9.65	ITA
7523	Pna-17	6.65759779	6.4260076	42.0945	-86.3253	USA
7524	Rmx-A02	5.70837813	4.55451799	42.036	-86.511	USA
7525	Rmx-A180	5.77096421	4.5488159	42.036	-86.511	USA
8214	Gy-0	7.28273627	4.59866272	49	2	FRA
8233	Dem-4	6.12109584	5.2413461	41.1876	-87.1923	USA
8236	Hsm	8.98147249	6.08792068	49.33	15.76	CZE
8240	Kulturen-1	9.51570991	6.68254886	55.705	13.196	SWE
8241	Liarum	8.42538982	6.22311818	55.9473	13.821	SWE
8243	PHW-2	14.4064503	8.17721855	43.7703	11.2547	ITA
8249	Vimmerby	9.07253309	6.97770416	57.7	15.8	SWE
8256	Bå1-2	6.49725867	4.26147396	56.4	12.9	SWE
8258	Bå4-1	8.85567024	6.31292833	56.4	12.9	SWE
8259	Bå5-1	9.40633163	5.68010601	56.4	12.9	SWE
8265	Blh-1	7.3813787	5.24282434	48	19	CZE
8270	Bs-1	8.74079588	8.48549562	47.5	7.5	SUI
8271	Bu-0	8.50834206	5.96804161	50.5	9.5	GER
8284	DraII-1	6.65544009	6.05309133	49.4112	16.2815	CZE
8290	En-1	8.80558568	7.06918082	50	8.5	GER
8296	Gd-1	8.97976785	6.11582923	53.5	10.5	GER
8297	Ge-0	8.4191512	5.34914061	46.5	6.08	SUI
8300	Gr-1	8.7371314	5.46060603	47	15.5	AUT
8306	Hi-0	7.42694983	5.47223389	56.1	13.74	SWE
8310	Hs-0	7.40180791	4.97734746	52.24	9.44	GER
8311	In-0	9.35674955	4.33806629	47.5	11.5	AUT
8312	Is-0	6.64786149	6.1463373	50.5	7.5	GER
8313	Jm-0	6.73628692	5.20154234	49	15	CZE
8314	Ka-0	6.05799316	3.96156601	47	14	AUT
8323	Lc-0	7.36556916	4.2893489	57	-4	UK
8325	Lip-0	12.4407196	7.87742081	50	19.3	POL
8329	Lm-2	11.5857516	5.57581886	48	0.5	FRA
8334	Lu-1	5.72598618	3.87445077	55.71	13.2	SWE
8337	Mir-0	9,534232005	6.050979748	44	12.37	ITA
8343	Na-1	6,43769515	4.158879498	47.5	1.5	FRA
8348	Nw-0	8,937974761	3.704357078	50.5	8.5	DEN
8354	Per-1	11,60148566	4.801792938	58	56.31	RUS
8365	Rak-2	9,375249107	6.179136936	49	16	CZE
8366	Rd-0	7,887709461	5.563545291	50.5	8.5	DEN
8369	Rev-1	11,50935801	8.475449025	55.69	13.45	SWE
8374	Rsch-4	15,15199849	8.351177146	56.3	34	RUS

8378	Sap-0	13,60100831	5.192483961	49.49	14.24	CZE
8387	St-0	8,830210708	6.189296788	59	18	SWE
8388	Stw-0	7,732442753	5.831787745	52	36	RUS
8395	Tu-0	6,694591949	5.460787705	45	7.5	ITA
8420	Kelsterbach-4	5,868723788	3.79638344	50.067	8.533	GER
8430	Lisse	8,531056663	4.768693743	52.25	4.567	NED
9104	Lag1-6	8,538205216	5.55174139	41.83	46.283	GEO
9165	Truk-5	9,676452071	4.91598767	50.463	30.541	UKR
9302	Edinburgh-5	6,663746329	3.551481478	55.968	-3.218	UK
9308	Ullapool-3	14,15935467	7.088564421	57.9	-5.15	UK
100000	Wil-1-Dean-Lab	6,152062949	4.066447268	54,6833	25,3167	LTU

**Table IV.1.** Shoots inorganic phosphate (Pi) accumulation (umole/gFW) in the 180 wheat plant *Triticum aestivum* accessions growth hydroponically for 3 weeks under two conditions presence (+Zn) or absence (–Zn) of Zinc.

Acc. No	Acc. Code	Mean +Zn	Mean –Zn
1	EL4X_3	23,0658554	25,2888285
2	GQ4X_4	21,0585771	30,1559889
3	GQ4X_5	24,4862348	33,6011495
4	EL4X_6	16,5497477	27,5206196
5	EL4X_9	19,3671582	31,4325507
6	GQ4X_10	22,8373445	26,2456827
7	EL4X_13	20,1346003	24,4305825
8	EL4X_16	19,4879806	27,4425363
9	EL4X_27	18,6267521	31,7944481
10	EL4X_28	14,4596541	29,1401931
11	EL4X_29	17,4483007	27,2893098
12	EL4X_31	15,3643079	22,6522989
13	EL4X_35	14,5453328	28,5832796
14	EL4X_38	14,2927745	20,4931057
15	EL4X_40	16,8744604	20,6675849
16	EL4X_41	18,8205114	30,2933179
17	EL4X_49	17,6504725	24,6428168
18	EL4X_59	16,0055145	26,6171468
19	EL4X_65	17,9811429	29,6490229
20	GQ4X_67	22,0704551	31,1997941
21	EL4X_68	15,9478801	25,2284445
22	EL4X_69	17,0670622	20,7576094
23	EL4X_70	20,8965232	30,4072006
24	EL4X_72	20,3321204	27,7793879
25	EL4X_73	21,8854397	28,2769941
26	EL4X_74	17,7077665	29,2600651
27	GQ4X_75	17,6888887	28,0672107
28	GQ4X_76	19,425473	28,6137354
29	EL4X_77	17,6495748	27,0448964
30	GQ4X_82	19,0832322	26,8300486
31	GQ4X_83	20,1507756	27,2566749
32	EL4X_85	19,9084742	25,5730195
33	EL4X_89	16,7511186	21,0836597
34	GQ4X_91	21,328153	25,2329654
35	EL4X_92	19,6375077	27,7578352
36	EL4X_94	16,8047125	24,9682732
37	GQ4X_95	14,6480712	27,8278652
38	EL4X_96	20,104913	25,0013427

39	EL4X_99	18,526497	26,9600765
40	EL4X_100	17,6175136	26,689932
41	EL4X_101	16,4847842	24,755466
42	EL4X_104	17,8019254	23,1847697
43	GQ4X_112	19,8835133	28,1088026
44	EL4X_114	17,4082407	25,687032
45	GQ4X_116	19,2289377	22,3935017
46	EL4X_117	17,0526331	24,0275037
47	EL4X_118	17,1405231	24,8399685
48	GQ4X_119	18,9811082	26,0166988
49	EL4X_120	21,6577154	28,7210791
50	EL4X_122	21,6778852	24,4202426
51	EL4X_124	18,4558286	28,3785227
52	GQ4X_126	19,6127691	25,7904235
53	EL4X_130	23,1976805	31,113535
54	EL4X_131	18,0591372	25,009335
55	GQ4X_138	15,6994348	25,3891531
56	GQ4X_139	14,9729879	21,5024208
57	GQ4X_140	17,7865567	30,7544637
58	EL4X_143	17,4349613	29,4834862
59	EL4X_145	19,2535723	28,3767603
60	EL4X_146	20,5419183	34,6022683
61	EL4X_148	12,8933373	22,0349655
62	GQ4X_149	16,932188	20,348861
63	EL4X_153	23,8960593	25,7347675
64	EL4X_165	15,8986458	27,382723
65	GQ4X_166	17,5238347	29,0762604
66	GQ4X_175	14,0814601	22,6793478
67	EL4X_176	15,9005099	24,1090134
68	EL4X_179	15,5762969	20,9689613
69	EL4X_182	17,7732184	20,0041614
70	EL4X_184	16,5837677	22,5199006
71	EL4X_185	16,969513	23,7261921
72	EL4X_187	23,6212789	27,5278132
73	EL4X_188	18,4187806	24,3932235
74	GQ4X_189	19,564679	30,3506282
75	EL4X_192	18,8638485	25,7361468
76	EL4X_194	18,425312	21,2305728
77	EL4X_195	15,1063767	21,9820617
78	EL4X_197	16,9581045	19,1633349
79	EL4X_198	23,4400442	23,8857178
80	EL4X_199	17,0436659	25,2117322
81	EL4X_201	11,1999928	25,397514
82	EL4X_202	14,0234921	28,5417815

83	EL4X_207	19,6415257	25,1972608
84	EL4X_212	17,3935701	23,0154442
85	EL4X_222	21,128303	28,0288512
86	EL4X_227	18,4432895	26,5447397
87	EL4X_229	18,6095735	24,3626879
88	EL4X_230	18,3027913	20,3575806
89	EL4X_233	15,1128209	26,5399353
90	EL4X_235	19,3107763	26,7061738
91	EL4X_237	18,3072218	26,083928
92	EL4X_239	20,1965996	27,7178456
93	EL4X_240	19,6448107	27,3027706
94	EL4X_241	32,1979417	34,0062366
95	EL4X_243	21,1285896	20,6464147
96	EL4X_244	22,6264463	29,7653487
97	EL4X_250	19,6429466	27,0925824
98	EL4X_251	27,3840201	28,9368808
99	EL4X_252	22,9760901	31,2305368
100	EL4X_261	23,2432728	23,3347501
101	EL4X_262	21,0885816	27,3729597
102	EL4X_265	18,5723031	22,2809466
103	EL4X_268	20,7020622	28,2210438
104	EL4X_278	18,8906022	25,0974036
105	EL4X_279	19,2708367	22,7664282
106	EL4X_288	24,9893748	35,0581629
107	EL4X_292	23,3737295	26,9032626
108	EL4X_295	20,6377466	23,4055799
109	EL4X_298	19,3096029	20,5400212
110	EL4X_303	28,992223	31,9188455
111	EL4X_305	20,1557999	26,3022055
112	EL4X_309	24,3426592	29,3979468
113	EL4X_311	21,15752	25,6168344
114	EL4X_315	25,2159174	30,4248417
115	EL4X_316	22,6248963	24,3924679
116	EL4X_329	20,1714784	22,95854
117	EL4X_331	20,2061879	24,8688895
118	EL4X_335	27,8003899	29,605021
119	EL4X_336	26,9573515	28,3195208
120	EL4X_341	25,7705699	30,4968495
121	EL4X_344	22,28369	20,0308263
122	EL4X_345	28,5334313	34,2453352
123	EL4X_346	23,2406754	19,1797679
124	EL4X_348	23,3218641	22,8796264
125	EL4X_350	24,2659522	26,4170271
126	EL4X_352	23,2641763	27,2464383

127	EL4X_353	21,4907034	23,4450716
128	EL4X_356	26,5102262	21,6073335
129	EL4X_366	22,8908947	19,8729954
130	EL4X_367	21,3645087	26,2757418
131	EL4X_368	22,8837447	26,3689848
132	EL4X_370	22,618659	22,7486251
133	EL4X_371	22,0751135	30,275902
134	EL4X_372	26,058387	32,0058874
135	EL4X_373	24,831217	34,0469831
136	EL4X_376	25,0105705	27,1433015
137	EL4X_377	21,4882267	23,975377
138	EL4X_383	25,0667985	25,0280459
139	EL4X_385	23,4837469	25,938614
140	EL4X_387	21,3967519	20,0871687
141	EL4X_388	23,1037536	25,1003639
142	EL4X_394	20,7597739	19,2283693
143	EL4X_395	21,2209322	22,7072584
144	EL4X_399	26,7416036	25,0949702
145	EL4X_401	21,9885923	34,2459434
146	EL4X_402	21,0938158	25,9929468
147	EL4X_407	23,9052804	22,3215237
148	EL4X_410	22,475198	21,6742135
149	EL4X_412	31,7667456	31,9259043
150	EL4X_415	22,4705444	32,7766358
151	EL4X_416	20,3697391	24,6454467
152	EL4X_423	22,4870606	26,4572196
153	EL4X_426	23,7015227	25,2082986
154	EL4X_427	31,1615768	36,8628346
155	EL4X_428	25,9176886	28,6145844
156	EL4X_435	26,7830088	27,5274014
157	EL4X_436	26,9205167	27,5014933
158	EL4X_441	31,5580429	34,7943609
159	EL4X_444	26,4388787	25,998769
160	EL4X_447	24,7675548	29,7293314
161	EL4X_451	23,2055845	26,8236482
162	EL4X_453	21,1106077	27,4980169
163	EL4X_459	24,7660877	24,164377
164	EL4X_460	21,4163589	19,206218
165	EL4X_461	25,3721581	30,7612694
166	EL4X_462	22,9479757	21,3904505
167	EL4X_464	25,8249976	29,158056
168	EL4X_465	22,3690605	20,2007496
169	EL4X_469	22,4270636	24,6845503
170	EL4X_470	27,5883019	35,5653562

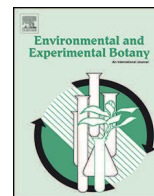
171	EL4X_474	26,2394731	31,4346698
172	EL4X_476	23,7150646	29,4455465
173	EL4X_480	27,1125865	29,5483828
174	EL4X_482	28,7266276	26,5159899
175	EL4X_483	26,5784166	29,0670232
176	EL4X_485	26,6659697	25,4221088
177	EL4X_486	31,5663464	28,585355
178	EL4X_488	22,5327468	24,1995643
179	EL4X_489	23,9377129	24,1739576
180	EL4X_504	24,8199226	26,6398948

## **Annexe 2**

### **Molecular mechanisms of phosphate and zinc signalling crosstalk in plants: Phosphate and zinc loading into root xylem in Arabidopsis**

**Mushtak Kisko, Nadia Bouain, Aida Rouached, Sikander Pal Choudhary & Hatem Rouached**





# Molecular mechanisms of phosphate and zinc signalling crosstalk in plants: Phosphate and zinc loading into root xylem in Arabidopsis



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## ABSTRACT

Inorganic phosphate (Pi) and zinc (Zn) are an essential macro- and micronutrients for plant survival. Control of Pi and Zn content in tissues is of major importance for normal plant growth and development. Zn deficiency typically leads to Pi over-accumulation in shoots (and vice versa), signifying the presence of complex interactions that link the homeostatic regulation of these two nutrients. Despite their primary importance, the molecular bases of these interactions remains poorly understood. Recent research has placed the co-regulation of these two elements at a limiting step in Pi and Zn distribution within plants, e.g. the loading of Pi and Zn into root xylem. In *Arabidopsis thaliana*, this process mainly involves members of the Phosphate 1 (PHO1 and PHO1;H1) family (for Pi) and the heavy metal ATPases protein (HMA2 and HMA4) family (for Zn). This review examines recent progress in determining the molecular mechanisms that regulate the loading of Pi and Zn into root xylem, by individually describing these specific genes. The first molecular evidence for their signalling crosstalk at this particular step of their transport in plants is also presented, with an emerging role for PHO1;H3. This recent progress is important for biotechnological and agronomic strategies aimed at enhancing Pi and Zn transfer to the aerial part of plants.

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## 1. Introduction

Pi and Zn are essential nutrients for all the living organisms. The macronutrient Pi is an essential structural component of RNA and DNA, as well as phospholipids. Pi is involved in many key biological processes in the cell, including numerous enzymatic reactions (Westheimer, 1987; Poirier and Bucher, 2002; Rouached et al., 2010). Several signal transduction cascades also rely on Pi via the modulation of enzyme activity by protein phosphorylation, which can be adversely altered in situations where Pi is present in too low a concentration (Poirier and Bucher, 2002). The micronutrient Zn is required for proper cell functioning (Berg and Shi, 1996; Salgueiro et al., 2000; Sinclair and Kramer, 2012), as it is a highly effective cofactor for hundreds of enzymes, the structural Zn-finger domains that mediate DNA-binding of transcription factors, and protein–protein interactions (Coleman, 1998; Shahzad et al., 2014). Due to the central roles of Pi and Zn in numerous aspects of

plant metabolism, it is not surprising that plants are profoundly affected by Pi or Zn starvation, and that their deficiencies provoke a coordinated series of morphological, physiological and biochemical adaptations (Mikulska et al., 1998; Poirier and Bucher, 2002; Misson et al., 2005; Rouached et al., 2010; Jain et al., 2013). It is therefore of great importance for cells to tightly control Pi and Zn homeostasis, which likely interact via a complex process (Cakmak and Marschner, 1986; Khan et al., 2014).

In plants, Pi and Zn are taken up at the root–soil interface, predominantly as free ions (Guerinot, 2000; Shahzad et al., 2014; Nussaume et al., 2011; Milner et al., 2013). In recent years, significant progress has been made in our knowledge of the regulation of Pi and Zn acquisition in plants, and this phenomenon has been documented in many research publications and elegantly summarised in multiple reviews (Sinclair and Kramer, 2012; Nussaume et al., 2011). *Arabidopsis* genome contains nine PHT1 family members and most of them are controlled by the endogenous Pi status of the plant (Poirier and Bucher, 2002; Nussaume et al., 2011). Some PHT1 genes are preferentially expressed in roots, and function as a high-affinity Pi uptake transporter (Muchhal et al., 1996; Misson et al., 2005; Remy et al., 2012; Bayle et al., 2011; Nussaume et al., 2011). Shin et al. (2004) provided genetic evidences proven that *PHT1;1* and *PHT1;4* play crucial role in Pi

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transport in roots during growth under both low- and high-Pi environments because the double mutant *pht1;1 pht1;4* shows a 75% reduction in Pi uptake capacity relative to the wild type (Shin et al., 2004). Research results are summarised on this subject in many reviews (Poirier and Bucher, 2002; Nussaume et al., 2011). For Zn, many Zn uptake transporters have been identified and belong to the Zrt/IRT-like protein (ZIP) family of Zn transporters. In *Arabidopsis thaliana*, the ZIP family contains 15 members (Maser et al., 2001), including the *AtIRT1* which localises preferentially to the plasma membrane of root epidermal cells (Vert et al., 2002). It has been reported that *irt1* mutant accumulates less Zn as compared to wild type, revealing its implication in Zn uptake (Henriques et al., 2002). For ample information on the regulation of Zn uptake in *Arabidopsis* readers are referred to Sinclair and Kramer (2012). After their acquisition at the root periphery, each element can be fixed into the root *via* transport into vacuoles. Alternatively, their symplastic journey, thought to be mediated by the plasmodesmata, ends with their loading into root xylem. For Pi, *PHO1* and its closest homologue *PHO1;H1* have been identified as key genes in the long-distance transfer of Pi from the root to the shoot (Poirier et al., 1991; Hamburger et al., 2002; Stefanovic et al., 2007; Stefanovic et al., 2011). For Zn, two members of the *Arabidopsis* P<sub>1B</sub>-ATPase subfamily: *HMA2* and its most closely related sequence in the *HMA* cluster, *HMA4* play crucial role in Zn loading into xylem (Hussain et al., 2004; Verret et al., 2004; Hanikenne et al., 2008; Siemianowski et al., 2011; Wong et al., 2009). The most recent reports on their biological functions and the molecular mechanisms of their regulation in *A. thaliana* will be reviewed below.

Research efforts over the past 10 years have contributed the first studies on Pi and Zn deficiency signalling pathways (Chiou and Lin, 2011; Assuncao et al., 2013). However, it is clear that these results are just part of a very complex process.

The Pi long-distance signalling network includes the MYB transcription factor *PHR1*, the ubiquitin E2 conjugase *PHO2*, and the miRNA A399 (Pant et al., 2008). In response to Pi deficiency, miRNA399 is transcriptionally regulated by *PHR1*, and then translocated from shoot to root by the phloem, where it targets the *PHO2* transcript (Bari et al., 2006; Lin et al., 2008; Pant et al., 2008). The repression of *PHO2* expression causes an increase in the expression of root Pi-uptake transporters (*PHT1;8* and *PHT1;9*), and therefore an increase in Pi acquisition by the roots as well as its translocation to the shoot (Bari et al., 2006; Lin et al., 2008). The Zn deficiency appears to be first sensed in shoots; the signal is then transmitted to the roots, where these cation transporters function (Assuncao et al., 2010, 2013). This suggests the presence of long-distance Zn deficiency signalling molecules (which are yet to be identified). A recent working model of Zn deficiency signalling (Assuncao et al., 2013) proposes that the *Arabidopsis* transcription factors *bZIP19* and *bZIP23* play important roles in the response to Zn deficiency by regulating downstream genes, including ZIP members (i.e. the Zrt/Irt-like proteins, candidates that mediate root Zn uptake and transport) (Guerinot, 2000; Assuncao et al., 2010).

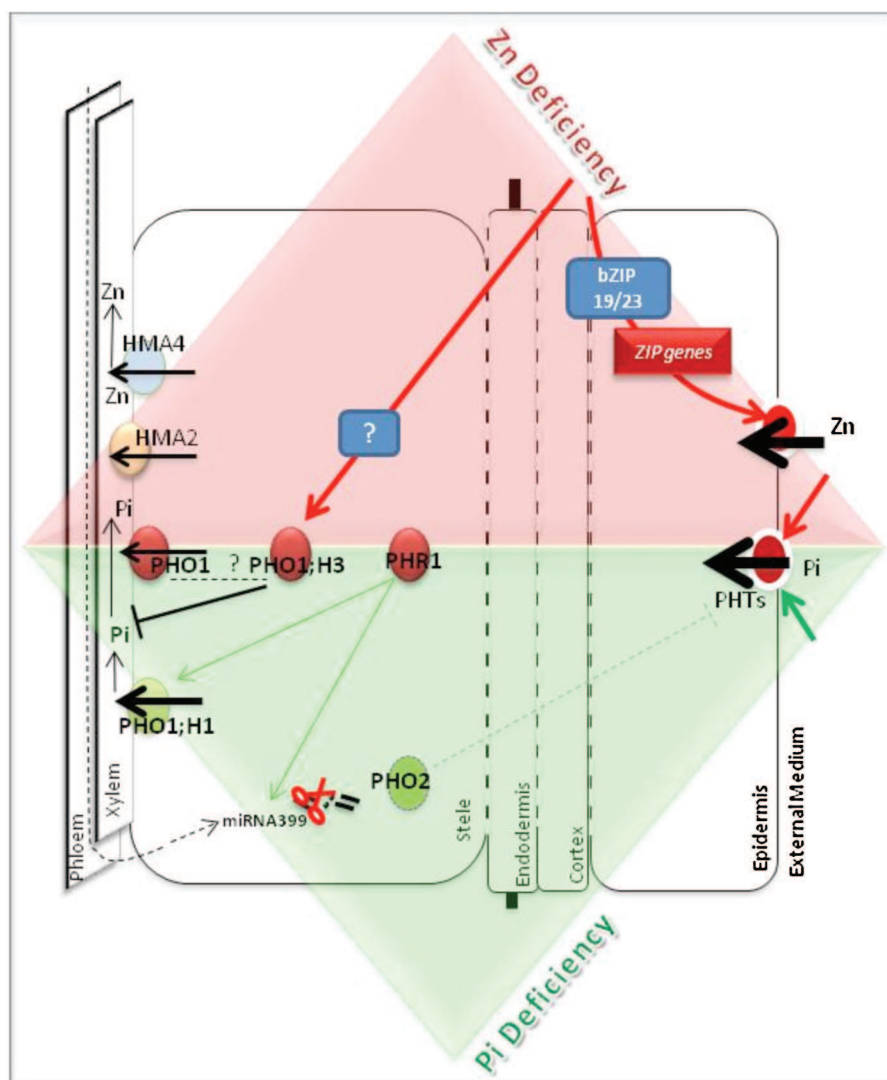
Interactions between Pi and Zn in plants have been reported in numerous plant species (Reed, 1946; Verma and Minhas, 1987; Webb and Loneragan, 1988; Tagwira et al., 1993; Loneragan et al., 1982; Gianquinto et al., 2000; Huang et al., 2000; Zhu et al., 2001; Shi et al., 2008). Such interaction is integrative as a plant loses its capacity to regulate Pi transport under Zn deficiency, despite the presence of an adequate Pi supply. Pi–Zn interaction is specific. Such specificity has been demonstrated by the fact that in barley only Zn deficiency could induce Pi uptake and not nitrogen, sulfur, nor manganese deficiency (Huang et al., 2000). Similarly, cotton or tomato plants do not show an over-accumulation of Pi under iron or copper deficiency (Cakmak and Marschner, 1986; Liu et al., 1998). This interaction is of agronomic importance and can account for the shortcomings of current models that are typically

focused on improving the assimilation of the individual elements. Earlier aforementioned studies have provided physiological evidence for the importance of Zn deficiency in Pi translocation to the shoots. More recently, Khan et al. (2014) have provided direct molecular evidence for the crosstalk between Pi and Zn nutrition in *A. thaliana*, by identifying genes involved in this crosstalk. Results from mining the transcriptomics data support the existence of genetic programmes that regulate Pi–Zn nutrition interaction in plants, as well as providing new research channels to elucidate this phenomenon (Misson et al., 2005; van de Mortel et al., 2006).

In this review, a molecular evidence for the Pi–Zn homeostasis and interaction in *A. thaliana* with an emerging role for *PHO1;H3* will be discussed. Given importance of genes involved in the regulation of Pi loading into roots xylem under Zn deficiency, a large part of this review is dedicated for detailing the current understanding on the molecular mechanisms that regulate this process. In preceding context roles of the Phosphate 1 (*PHO1* and *PHO1;H1*) family (for Pi) and the heavy metal ATPases protein (*HMA2* and *HMA4*) family (for Zn) will be reviewed. In addition, to further probe the regulation of these genes, results from data mining based on meta-analysis tools using the available sets of *Arabidopsis* microarray data will be presented.

## 2. Converging signalling pathways that regulate Pi and Zn loading into the root xylem: an emerging role for *PHO1;H3*

The existence of complex interactions that link the homeostatic regulations of Pi and Zn has long been recognised (Reed, 1946; Verma and Minhas, 1987; Webb and Loneragan, 1988; Tagwira et al., 1993; Loneragan et al., 1982; Cakmak and Marschner, 1986; Gianquinto et al., 2000; Huang et al., 2000; Zhu et al., 2001; Shi et al., 2008). In particular, Zn deficiency is associated with over-accumulation of Pi in the shoots of both dicotyledons and monocotyledons (Huang et al., 2000; Misson et al., 2005; Khan et al., 2014), although the genes underlying mechanisms of this process remain to be identified. Very recently, Khan et al. (2014) identified genes that are necessary for the increase in Pi over-accumulation in response to Zn deficiency in *Arabidopsis*. These genes include *PHR1*, *PHO1* and its homologue *PHO1;H3*. *PHR1* was already known as a major regulator of Pi deficiency signalling through its involvement in the so-called *PHR1-miRNA399-PHO2* regulatory pathway (Bari et al., 2006). However, this regulatory pathway is not involved in the over-accumulation of Pi in the shoot in response to Zn deficiency (Khan et al., 2014), and therefore a Zn-responsive signalling pathway involving *PHR1* remains to be elucidated. *PHO1* is most likely one of the final targets of the Zn-deficiency signalling pathway. Since its expression level does not change in response to Zn deficiency, it is likely that its activity is regulated through a protein–protein interaction, considering that a similar mechanism involving *PHO1* and *PHO2* has already been reported (Liu et al., 2012). Finally, *PHO1;H3* is involved in the control of Pi accumulation in response to Zn deficiency, and thus appears to be involved in the regulation of Pi transport (Khan et al., 2014), although no biological function has been identified so far. Nevertheless, it was recently reported that it is specifically and strongly induced by Zn deficiency, and that its expression pattern is similar to *PHO1*: both are expressed in cells of the root vascular cylinder and are localised to the Golgi when expressed transiently in tobacco cells (Khan et al., 2014). When grown in Zn-free medium, *pho1;h3* mutant plants displayed higher Pi contents in the shoots than wild-type plants. However, this was not observed in a *pho1 pho1;h3* double mutant, suggesting that *PHO1;H3* restricts root-to-shoot Pi transfer that requires *PHO1* function for Pi homeostasis in response to Zn deficiency (Khan et al., 2014). This makes *PHO1;H3* an interesting entry point to study Pi–Zn crosstalk in the root xylem. Future research to



**Fig. 1.** Pi and Zn transport in plant roots. Pi and Zn are respectively conveyed into the symplast by PHT1 and ZIP transporters in the root epidermis. Transport into the xylem mainly (but not exclusively) involves *PHO1* and *PHO1;H3* for Pi, and *HMA2* and *HMA4* for Zn. The presence of *PHO1;H1* under Pi deficiency is detectable through the action of the transcription factor *PHR1*. In low Pi conditions the action of *miRNA399* leads to the reduction of *PHO2* transcript (and consequently its protein level), which causes an increase in the uptake and translocation of Pi by *PHTs* and *PHO1*, respectively. Zn deficiency leads to an increase in Zn uptake and distribution in the plant. This phenomenon involves the transcription factor *bZIP19/23*, which likely targets many ZIP transporters. Zn deficiency also leads to the upregulation of *PHO1;H3*, which negatively regulates Pi loading into the root xylem. The transcription factor that regulates *PHO1;H3* is unknown.

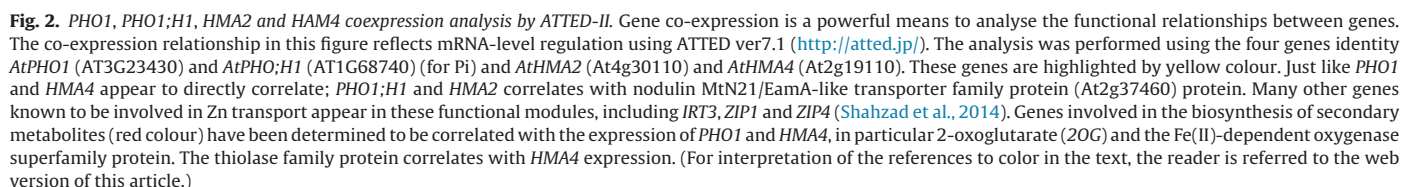
examine these questions should be aimed at uncovering the biological function of *PHO1;H3* in the regulation of Pi transport under Zn limitation, and identifying new proteins (particularly transcription factors) that regulate the expression of *PHO1;H3* (Fig. 1).

### 3. Pi and Zn deficiency signalling pathways interaction: elements from genes coexpression analysis

The aforementioned research work (Khan et al., 2014) has placed the co-regulation of Pi and Zn at a limiting step in Pi and Zn transport within plant: loading into Arabidopsis root xylem. This step involves key genes, namely *PHO1* and *PHO1;H1* (for Pi) and *HMA2* and *HMA4* (for Zn). Early reports clearly indicate that our current understanding of the transcriptional regulatory pathways that control the expression of *PHO1*, *PHO1;H1*, *HMA2* and *HMA4* is quite restricted, despite their essential role in the regulation of Pi and Zn transfer to the upper parts of plants. Fortunately, multiple sets of Arabidopsis microarray data have been assembled in various available databases, providing a unique opportunity to further probe the regulation of these genes in response to multiple

stresses. These databases, as well as data mining and analysis tool boxes such as TAIR (Rhee et al., 2003), NASCArrays (Craigon et al., 2004), the Stanford Microarray Database (Ball et al., 2005) and GENEVESTIGATOR (Zimmermann et al., 2004) could yield powerful clues for deciphering novel crosstalk networks. Furthermore, these data sources can be combined with several meta-analysis tools using a guilt-by-association principle to help visualise correlated gene expression, such as ATTED-II (Obayashi et al., 2007), and CressExpress (Srinivasasainagendra et al., 2008). The functional annotations in the gene's co-expression neighbourhood can then be used to hypothesise a biologically relevant relationship. In the context of this review, we have used the genes co-expression analysis tool available through ATTED-II, and considered that *PHO1*, *PHO1;H1*, *HMA2* and *HMA4* gene identities as input. The coexpressed rank was calculated from 1388 GeneChip data. Result obtained from NetworkDrawer Drawing gene networks in multiple query genes (Fig. 2). Interestingly, we have found that *PHO1*, *PHO1;H1*, *HMA2* and *HMA4* genes constitute a set of co-expressed genes. This observation nicely illustrates the existence of co-expression clusters that correspond to functional modules involved





Obviously, detailed analysis of the topology or node-to-node relationships within the network will contribute to progress in our understanding of the organisation and complexity of this transcriptional regulation. Several such approaches that were elegantly explained by [Aoki et al. \(2007\)](#) include co-expression analysis to identify new genes and functional modules combined with other omics data. For example, [Persson et al. \(2005\)](#) and [Ma et al. \(2007\)](#) successfully identified subclusters in Arabidopsis gene networks related to primary and secondary cell wall formation, and related to biochemical pathways and cold stress, respectively. Ideally, our first-hand results on *PHO1*, *PHO1;H1*, *HMA2* and *HMA4* co-expression, combined with information about cis-regulatory elements, could be used to determine the signals that are relayed to the transcription machinery. This could also preface further work that reconsiders the nature of the signal (s) involved in co-regulating the expression of these genes, and thus part of Pi-Zn signalling crosstalk.

As aforementioned, under Zn deficiency plant over-accumulates Pi in shoots (and *vice versa*). In *A. thaliana*, the loading of Pi and Zn into root xylem involves PHO1 and PHO1;H1 (for Pi) and HMA2 and HMA4 (for Zn). Interestingly, our co-expression analysis revealed that the expression of these genes correlate directly (for *PHO1* and *HMA4*) or indirectly (for *PHO1;H1* and *HMA2*). This result confirms the presence of a crosstalk between Pi and Zn regulatory networks in plants, which involves these specific genes. The current understanding on the molecular mechanisms that regulate the expression of *PHO1*, *PHO1;H1*, *HMA2* and *HMA4* is presented below. Future research work will be needed to answer how the Pi and Zn signals crosstalk are relayed to the transcription machinery of these genes.

It is believed that Pi loading into the root xylem in *A. thaliana* is mainly achieved by PHO1 (Poirier et al., 1991; Hamburger et al., 2002) and PHO1;H1, as the mutation of both genes results in a very low Pi level in the shoot, which severely compromises the plant life cycle (Stefanovic et al., 2007). This extreme phenotype can be alleviated by supplying the double mutants with high Pi (Stefanovic et al., 2007).

**Arabidopsis PHO1:** In pioneering work carried out by [Poirier et al. \(1991\)](#), an *AtPHO1* mutant was identified in a genetic screen (using EMS-mutagenesis approaches) for *Arabidopsis* mutants affected in Pi transport. The isolated *pho1* null mutants display several features associated with Pi deficiency, including severe reduction in shoot growth and accumulation of anthocyanins. Compared to the wild-type plant, *pho1* mutants have a normal Pi uptake rate, although there is a strong reduction (90%) in Pi transfer from the roots to the shoot; this results in very low Pi levels in shoots, e.g. 20-fold less free Pi in leaves than in control plants ([Poirier et al., 1991](#)).

The *pho1* mutation was mapped using a positional cloning strategy in the gene AT3G23430 (Hamburger et al., 2002). Examining the activity of the *PHO1* promoter fused to a reporter gene (GUS) revealed that the *PHO1* promoter is predominantly expressed in the root vascular system, which is consistent with its implied role in Pi loading into the xylem (Hamburger et al., 2002). This hypothesis further supported by micrografting experiments, which confirmed that the loss of *PHO1* expression in the roots is responsible for the reduced growth capacity and low shoot Pi content phenotypes (Stefanovic et al., 2007). At the protein level, the subcellular *PHO1*-GFP expression pattern was associated with similar punctate structures that colocalised with the Golgi/trans-Golgi network and uncharacterised vesicles (Arpat et al., 2012). Additionally, the *PHO1*-GFP construct could be partially relocated to the plasma membrane in leaves infiltrated with a high-phosphate solution (Arpat et al., 2012).

Experimental evidence showing that *PHO1* specifically exports Pi from the cell has been provided through the use of transgenic plants expressing *PHO1* under the control of an inducible promoter (Arpat et al., 2012). This study used ectopic expression of Arabidopsis *PHO1* in tobacco leaves (*Nicotiana tabacum*), Arabidopsis leaves, or mesophyll protoplasts to demonstrate that *PHO1* mediates Pi export into the apoplasts, establishing *PHO1* as a Pi exporter. Although the functional study of *PHO1* in Pi loading to the xylem has been investigated, several early attempts with heterologous systems have failed to reveal a Pi transport activity associated with the *PHO1* protein. Recently, a crucial role for *PHO1* in Pi efflux was demonstrated by Stefanovic et al. (2011), where it was demonstrated that *PHO1* overexpression strongly increases Pi concentration in leaf xylem exudates. This leads to an increase in shoot Pi content and a severe reduction in shoot growth, as compared to the wild-type plant. *In vivo*  $^{31}\text{P}$  NMR experiments demonstrated that *PHO1* overexpression causes a rapid loss of the vacuolar Pi pool, reflecting a dramatic efflux of Pi out of cells into the infiltration medium. Together, these results strongly indicate that *PHO1* is a Pi exporter (Stefanovic et al., 2011).

The *PHO1* gene is slightly up-regulated at the transcriptional level by Pi deprivation stress (Hamburger et al., 2002). This regulation involves members of the AtWRKY family. The most prominent feature of these proteins is the WRKY domain, which contains about 60 amino acids with a conserved WRKYGQK sequence followed by a C2H2- or C2HC-type of zinc finger motif (Eulgem et al., 2000). Indeed, Chen et al. (2009) revealed that the regulation of *PHO1* expression in response to low-Pi stress involves WRKY6 and WRKY42. WRKY6 can repress the expression of *PHO1* in a Pi-dependent manner by binding to two W-boxes present in its promoter. The accumulation of the WRKY6 protein is diminished in the low Pi condition, which is likely caused by 26S proteasome-mediated proteolysis (Chen et al., 2009). Consequently, *PHO1* expression is released by reducing WRKY6 binding to its promoter. In contrast to wild-type seedlings and the *wrky6-1* mutant, WRKY6 overexpression lines and the *pho1* mutant accumulate low Pi content in shoots (Chen et al., 2009).

It is worth noting that a unique mode of post-transcriptional regulation has been observed for the *PHO1* gene in rice (*Oryza sativa*). This has an unexpected implication of cis-natural antisense transcripts (cis-NAT) in promoting *PHO1* translation, ultimately affecting Pi homeostasis and plant fitness (Jabnourne et al., 2013). The rice genome has three *PHO1* homologues, all of which have a cis-natural antisense transcript located at the 5' end. Secco et al. (2010) have shown that *OsPHO1;2* plays a key role in the transfer of Pi from roots to shoots in rice, indicating that this gene could be regulated by its cis-NAT, which is expressed in the same tissues. Remarkably, the overexpression of cis-NAT during Pi deficiency was observed to stimulate the translation of the *OsPHO1;2* sense mRNA,

leading to an increase in *OsPHO1;2* protein level (Jabnourne et al., 2013).

At the protein level, AtPHO1 interacts with the ubiquitin-conjugating E2 enzyme (also known as PHO2) to regulate Pi loading into the root xylem (Liu et al., 2012). The *pho2* mutant was characterised by an up to 4-fold increase in the amount of Pi in leaves (as compared to the wild-type), whereas the Pi level in roots was unchanged (Delhaize and Randall, 1995). Interestingly, the *pho2* suppressors are caused by missense mutations in *PHO1*. Lin et al. (2008) showed that the protein level of *PHO1* is increased in *pho2*, whereas this accumulation is alleviated in both *pho2* suppressors. *PHO1* and *PHO2* proteins partially colocalise and physically interact in the endomembrane of tobacco leaves (Liu et al., 2012). Protein studies have revealed that *PHO2* modulates the degradation of *PHO1* (involving multivesicular body-mediated vacuolar proteolysis) to maintain Pi root-to-shoot transfer in plants (Liu et al., 2012).

*PHO1* does not show any structural homology with any other Pi or ion transporters. The *PHO1* protein family harbours an SPX tripartite domain in the N-terminal hydrophilic portion, and an EXS domain in the conserved C-terminal hydrophobic portion. The SPX and EXS domains that have been identified in yeast (*Saccharomyces cerevisiae*) proteins are involved in either phosphate transport, sensing, or sorting of proteins to the endomembrane (Hamburger et al., 2002; Wang et al., 2004). Rouached et al. (2011) provided evidence for a novel *PHO1* role in regulating shoot growth and the Pi deficiency-signalling pathway, by characterising Arabidopsis transgenic lines whose *PHO1* transcript and protein levels were reduced through gene silencing (Rouached et al., 2011). Similar to *pho1* null mutants, *PHO1* under expressor lines showed a decrease in root-to-shoot Pi transport, resulting in very low levels of Pi accumulation in the shoots. However, in contrast to *pho1* null mutants, *PHO1* under expressor lines did not display shoot Pi-starvation phenotypes. Despite their low shoot Pi content, they did exhibit normal growth, similar to Pi-sufficient wild-type plants (Rouached et al., 2011). These results suggest that the function of *PHO1* in regulating shoot growth may involve a *PHO1*-dependent Pi-signalling pathway. This hypothesis has been further supported by transcriptomic studies that reveal that *PHO1* under expressor lines exhibit only a very small number of genes induced by Pi deficiency, in addition to a reduction in their fold change as compared to Pi-deficient wild-type plants (Rouached et al., 2011). These data argue in favour of a role for *PHO1* in regulating shoot growth via a *PHO1*-dependent long distance Pi-signalling pathway.

*Arabidopsis PHO1;H1*: Intriguingly, the Arabidopsis genome contains 10 additional genes that show homology to *PHO1* (Wang et al., 2004). Phylogenetic analysis indicates that the *PHO1* family is subdivided into at least three clusters. However, the functional complementation of the *pho1* mutant by expression of the *PHO1* homologous genes under the control of the *PHO1* promoter revealed that only *PHO1* and its closest homologue *PHO1;H1* (At1g68740) could rescue the phenotype of the *pho1* mutant, in terms of shoot growth and shoot Pi content (Stefanovic et al., 2007). Like *PHO1*, the *PHO1;H1* promoter is active in the vascular cylinder of roots. However, the *pho1pho1;h1* double mutant displayed a strong reduction in growth and in the capacity to transfer Pi from the root to the shoot, as compared to *pho1*. *PHO1;H1* expression was very low in Pi-sufficient plants, although it was strongly induced under Pi-deficient conditions; this appears to be largely controlled by the transcription factor PHR1 (Rubio et al., 2001; Stefanovic et al., 2007).

Together, these data reveal that *PHO1* and *PHO1;H1* are the key contributors for Pi loading into the root xylem, and that they are regulated by Pi deficiency through distinct signal transduction pathways. Under Pi deficiency, the increases of *PHO1;H1* expression is largely controlled by the transcription factor PHR1, whereas

the increase of *PHO1* expression is independent of *PHR1* (Stefanovic et al., 2007).

#### 4.2. HMA2 and HMA4:

The *Arabidopsis* genes *AtHMA2* (At4g30110) and *AtHMA4* (At2g19110) exhibit a high primary sequence similarity, which may have evolved as a result of gene duplication. Both proteins are found in the Zn/Co/Cd/Pb subclass of the  $P_{1B}$ -ATPases, and have well-documented roles in Zn loading into xylem (Hussain et al., 2004; Verret et al., 2004). In *Arabidopsis*, none of the individual *hma* mutants exhibit an observable or distinctive morphological phenotype when grown in soil, as compared to the wild-type (Hussain et al., 2004). The mutation of both genes results in a drastic Zn reduction in the shoot. This severely compromises the plant life cycle through visible morphological alterations, a stunted phenotype, and the formation of sterile flowers that lack pollen (decreasing plant fertility). This severe phenotype can be alleviated by supplying double mutants with high Zn.

*Arabidopsis HMA2*: Expression of the GUS reporter under the *HMA2* promoter was observed in vascular bundles, and appeared to be expressed in components of both the xylem and the phloem (Hussain et al., 2004). No decrease in the Zn content is observed in the single mutant *hma2*. Like *HMA4*, the *HMA2* gene encodes for a metal transporter located in the plasma membrane, with a very similar structure. Both proteins differ in the length of their C-terminal extensions (e.g. 244 aa for *HMA2*), and show no sequence homology to each other (Mills et al., 2003). Functional analysis of *HMA2* in *A. thaliana* suggests that the N-terminal domain of *HMA2* is essential for its function *in planta* (Eren et al., 2006). The *A. thaliana hma2hma4* double mutant shows a 2-fold Zn increase in root pericycle cells, which causes a 2-fold decrease in shoots. This *hma2hma4* double mutant Zn-deficiency phenotype can be restored with the expression of full-length *AtHMA2* (Wong et al., 2009). Nevertheless, mutated versions (either by removal of the entire N-terminal domain or by mutation of the Cys residues within the conserved sequence Cys17-Cys-X-X-Glu) show a failure to complement the *hma2 hma4* mutant (Eren et al., 2006).

*Arabidopsis HMA4*: *AtHMA4* was originally identified as a gene with increased expression in the Zn hyperaccumulator *A. halleri* as compared to its non-hyperaccumulator relative *A. thaliana* (Hanikenne et al., 2008). The high *HMA4* expression level in the shoots of *A. halleri* can be explained by a triplication of the gene and changes to cis regulatory elements that drive *HMA4* expression. This high *HMA4* expression level appears to be one of the underlying genetic determinants for the hyperaccumulation phenotype in some plant species (Hanikenne et al., 2008).

Driving *AtHMA4* expression with the *A. halleri* endogenous promoter in *A. thaliana* induced Zn uptake, resulting in increased shoot Zn levels. Zn root-to-shoot translocation was also facilitated, leading to Zn toxicity symptoms (Hanikenne et al., 2008). In *A. halleri*, RNAi was used to knock down *HMA4* (from 45% to 10%), resulting in a 12–35% decrease in shoot Zn concentration and a 49–134% increase in root Zn concentration (as compared to wild-type plants) (Hanikenne et al., 2008). The Zn content in the aerial parts of *hma4* mutant plants is decreased at a Zn concentration of 3  $\mu$ M, although it is not significantly affected at higher concentrations (100  $\mu$ M). Overexpression of *AtHMA4* in *A. thaliana* resulted in a 2-fold increase in Zn content in leaves, whereas no significant change was observed in root Zn content (Verret et al., 2004).

In *Arabidopsis* roots, *HMA4* expression levels appeared to be enhanced by Zn (Hanikenne et al., 2008), but it is still unclear whether the transcript level is modulated directly or indirectly by Zn. The specific mRNA activity of *HMA4* in *A. halleri* and *A. thaliana*

was observed in root pericycle and xylem parenchyma (Hanikenne et al., 2008). When the  $\beta$ -glucuronidase (GUS) reporter gene was placed under the control of the *HMA4* promoter in *A. thaliana*, expression was predominantly observed in root stellar cells located at the periphery of the xylem (Verret et al., 2004). This expression pattern is consistent with the involvement of *AtHMA4* in Zn loading into the xylem (Hussain et al., 2004). A possible role for *AtHMA4* in Zn efflux from the cytoplasm is also supported by its ability to restore growth to the *zntA* mutant in *S. cerevisiae*, which is defective in its endogenous Zn efflux pump at high Zn concentrations (Rensing et al., 1997; Mills et al., 2003, 2005; Verret et al., 2004).

The *HMA4* metal transporters are located at the plasma membrane. Based on *in silico* predictions, their hydropathy profile should contain three major domains: the hydrophilic N- and C-terminals, and a central membrane-spanning domain (Mills et al., 2005). The central domain is predicted to have six to eight transmembrane segments responsible for metal transport. The C-terminal extension of *AtHMA4* is characterised by its length (~470 amino acids), which does not show any sequence homology to other HMA (Mills et al., 2005, 2010). *AtHMA4* contains a long histidine stretch in the C-terminal region that is substantially longer than found in most other HMA sequences. The presence of histidine residues in this last region may suggest a binding role for Zn (Mills et al., 2003), although whether *AtHMA4* binds this metal remains to be elucidated. The presence of the haloacid dehydrogenase-like hydrolase domain, which contains the phosphorylated aspartate residue in the DKTGT motif and the GDGVNDAP motif in the putative hinge domain (Scarborough, 2000; Xu et al., 2002; Mills et al., 2003), indicates that it likely functions as an ATP-driven pump. *AtHMA4* also contains a number of motifs that are characteristic of heavy metal pumps, including the conserved CPx motif (cysteine, proline and either cysteine, histidine or serine) (Solioz and Vulpe, 1996). The functional significance of the C-terminal domain of *AtHMA4* has been demonstrated in plants and in yeast (Verret et al., 2005; Mills et al., 2010; Siemianowski et al., 2011). The functional complementation of the *Arabidopsis hma2 hma4* double mutant with an *AtHMA4* protein lacking the C-terminal region could only partially restore the rosette diameter in two out of five lines, while bolt production was not rescued (Mills et al., 2010).

Together, *HMA2* and *HMA4* play key roles in Zn loading into the root xylem, even though the signalling pathways that regulate their expression and activity remain poorly understood.

## 5. Conclusions and perspectives

In plants, Pi and Zn loading into root xylem are highly regulated and complex processes. Deeper investigations into the Pi–Zn nutrition interaction at the molecular level will be necessary to untangle their interconnected signalling networks, but also to improve Pi or Zn nutrition in higher plants. For example, understanding that how Zn deficiency induces Pi translocation to the shoot as well as its accumulation can be exploited to improve the Pi nutritional stress response in major crop plants. This does not mean that plants should be grown in Zn deficient conditions to improve Pi nutrition. Rather, key genes and mechanisms in the coordination of Pi and Zn transport and signalling must be identified and characterised to uncover specific mutants or genetic variants that could be used in breeding programmes. Zn deficiency also strongly limits growth, and a similar course of action could be applied to improve plant Zn nutrition for the benefit of human health, by exploring knowledge of Pi homeostatic regulation. These research directions will certainly have important consequences for both basic and applied research in agronomy, with additional benefits to agronomists and producers.



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## **Annexe 3**

### **Phosphorus transport in Arabidopsis and wheat: emerging strategies to improve P pool in seeds**

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Review

# Phosphorus Transport in Arabidopsis and Wheat: Emerging Strategies to Improve P Pool in Seeds

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**Abstract:** Phosphorus (P) is an essential macronutrient for plants to complete their life cycle. P taken up from the soil by the roots is transported to the rest of the plant and ultimately stored in seeds. This stored P is used during germination to sustain the nutritional demands of the growing seedling in the absence of a developed root system. Nevertheless, P deficiency, an increasing global issue, greatly decreases the vigour of afflicted seeds. To combat P deficiency, current crop production methods rely on heavy P fertilizer application, an unsustainable practice in light of a speculated decrease in worldwide P stocks. Therefore, the overall goal in optimizing P usage for agricultural purposes is both to decrease our dependency on P fertilizers and enhance the P-use efficiency in plants. Achieving this goal requires a robust understanding of how plants regulate inorganic phosphate (Pi) transport, during vegetative growth as well as the reproductive stages of development. In this short review, we present the current knowledge on Pi transport in the model plant *Arabidopsis thaliana* and apply the information towards the economically important cereal crop wheat. We highlight the importance of developing our knowledge on the regulation of these plants' P transport systems and P accumulation in seeds due to its involvement in maintaining their vigour and nutritional quality. We additionally discuss further discoveries in the subjects this review discusses substantiate this importance in their practical applications for practical food security and geopolitical applications.

**Keywords:** phosphate; seeds; Arabidopsis; wheat

## 1. Introduction

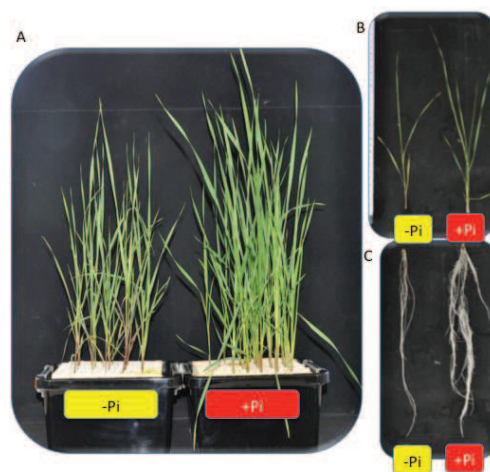
Phosphorus (P) is an essential macronutrient for plant growth and production [1,2]. P deficiency is a generally widespread stressor occurring in natural and agricultural environments. Consequently, global agricultural crop production has been severely affected [2,3]. It is estimated that crop yield on 30–40% of the world's total arable land is limited by inorganic phosphate (Pi) bioavailability [4,5]. Many causes could explain the low availability of Pi to plants, such as the Pi ( $\text{HPO}_4^{2-}$ ) interaction with soil cations such as zinc ( $\text{Zn}^{2+}$ ) or iron ( $\text{Fe}^{2+}$ ), which form an insoluble complex [6–8]. In addition, and most importantly, global Pi reserves are rapidly decreasing due to an increase in its demand [2,9–11]. Analysis of data collected over 14 years revealed that the usage of global P fertilizer considerably increased at a rate of about 357,000 t/annum (i.e., an annual increase of 2.4%) [12,13]. Experts agree that the world is facing serious P crisis [14,15] and that the global P reserve is not distributed uniformly [5]. Taken together, these issues constitute compelling evidence justifying a direct link

between Pi availability and the overwhelming world food security in coming few years. Developing a better understanding of how plants regulate Pi uptake for transport to different organs and seeds will help design new strategies to increase crop yield and simultaneously reduce P input.

Approximately 75% of Pi taken up by plant roots for use by vegetative tissues and vital storage in developing seeds is stored in the form of phytic acid (PA) [3]. Although P fertilizer supply has increased the yield of cereal grains such as wheat, a recent analysis showed that about 37% of wheat areas worldwide have experienced yield stagnation [16], highlighting the importance of precise management of P fertilizer application to achieve high wheat yield and quality. Achieving this objective necessitates a better understanding of how crops regulate P homeostasis. During the last decades, our knowledge on the molecular regulation of P transport and P redistribution in different plant organs during vegetative phase has progressed mainly in model plants such as *Arabidopsis thaliana* (for review, [17]). However, limited progress was achieved in crops such as wheat, a major dietary source of calories and protein for humans. In this short review, we present the recent progress of our understanding on Pi transport in wheat through information obtained for *A. thaliana*. We next highlight the importance of the knowledge generated on the molecular mechanisms which regulate the Pi transport and its accumulation in grains.

## 2. Phosphate Uptake and Transport in Wheat and Arabidopsis

In plants, Pi deficiency affects growth that manifests itself at a phenotypic level by affecting the aboveground and underground biomass. The effects of Pi availability on wheat growth is presented in Figure 1.



**Figure 1.** Changes in wheat growth under different phosphate conditions. (A) Wheat were grown hydroponically for three weeks in presence (+P) or absence (−P) of inorganic phosphate ( $\text{KH}_2\text{PO}_4$ ). (B) shoot and (C) root of wheat grown either in presence (0.5 mM) or in absence of inorganic phosphate for three weeks.

Pi is acquired by root systems, which undergo a significant architectural change in response to Pi deficiency (for review, [18]) that is associated with increasing Pi uptake capacity through an upregulation of regulators and transporters involved in P-homeostasis [19]. To improve P use efficiency in crop plants, it is therefore prerequisite to understand P distribution within the plant.

Numerous Pi transporters have been identified in wheat [20–22]. Gradual elucidation of these components and their roles have been effectively achieved through experiments either increasing P remobilization from senescing tissue or reducing the partition of P to developing grains [23]. However, initial identification of these transporters were generated from data collected for those of the model plant *Arabidopsis* [24]. In this dicot model plant, Pi transporters generally belong to a gene family referred to as phosphate transporter (PHT) [25]. The PHT family is divided into five

groups (*PHT1*, *PHT2*, *PHT3*, *PHT4*, and *PHT5*) differentiated primarily by their sub-cellular localization and functional properties. Plasma membrane-bound proteins belonging to the *PHT1* subfamily are primarily responsible for Pi uptake in Arabidopsis [25]. In contrast, *PHT2* proteins are localized in chloroplasts, while *PHT3*/MPT proteins are mostly mitochondrial membrane transporters and *PHT4* proteins are Golgi-apparatus located transporters. A vacuolar Pi transporter was identified in Arabidopsis and designated as *PHT5*/VPT/SPX-MFS proteins [26].

Pi is also transported outside the root cells for distribution between different plant organs. The Phosphate 1 (*PHO1*) gene family contains 11 Pi exporter proteins mostly involved in the translocation of Pi from roots to shoots [27,28]. Among these molecular players, the mutation of both *PHT1;1* and *PHT1;4* or *PHO1* and *PHO1;H1* causes the most severe phenotype that is characterized by a decreased Pi accumulation in Arabidopsis [19,28], demonstrating the importance of these Pi transporters and exporters in Pi uptake and Pi translocation to shoots. For more information, readers are referred to these reviews [25,29].

The identification of Arabidopsis proteins involved in Pi distribution accelerated the discovery of wheat Pi transporters by exploring regions of the wheat genome with similar sequence to the genomic sequences of these Arabidopsis Pi transporters [20]. Validation and characterization of identified wheat Pi transporter candidates have been attained through evaluation of their genetic function either in Arabidopsis or by means of complementation with yeast mutants defective in Pi transport [30,31]. A cumulative list of members of the crop origin Pi-transporters is summarized in Table 1.

**Table 1.** Inventory for the list of genes and regulators those are involved in sensing, uptake and signaling during Pi limiting conditions in model plant Arabidopsis and *Triticum aestivum*.

Components of Phosphate Transport	<i>Arabidopsis thaliana</i>	<i>Triticum aestivum</i>
Sensing	<i>AtSIZ1</i> ([32])	NA*
	<i>AtSPX1</i> , <i>AtSPX2</i> , <i>AtSPX3</i> & <i>AtSPX4</i> ([33,34])	<i>TaSPX1</i> ([35])
	<i>AtIPK1</i> ([36])	<i>TaIPK1</i> ([37])
Uptake & Transport	<i>AtPHT1;1–AtPHT1;9</i> ([38])	<i>TaPHT1.1–TaPHT1.13</i> ([20–22,39])
	<i>AtPHT2;1</i> ([40])	<i>TaPHT2.1</i> ([41])
	<i>AtPHT3;1–AtPHT3.3</i> ([42,43])	<i>TaPHT3.1–TaPHT3.3</i> ([20,44])
	<i>AtPHT4;1–AtPHT4;6</i> ([45])	<i>TaPHT4.1–TaPHT4.6</i> ([20])
	<i>AtPHT5;1–AtPHT5;3</i> ([46])	NA
	<i>AtPHO1</i> ([27])	<i>TaPHO1</i> ([47])
Signaling	<i>AtPHR1</i> ([48])	<i>TaPHR1</i> ([49])
	<i>AtPHL1</i> ([50])	NA
	<i>AtPHF1</i> ([51])	NA
	<i>AtPHO1</i> ([52])	NA
	<i>AtPHO2</i> ([53])	<i>TaPHO2</i> ([47])
	<i>AtZAT6</i> ([54])	NA
	<i>AtARP6</i> ([55])	NA
	<i>AtH2A.Z</i> ([55])	NA
	<i>AtWRKY6</i> ([56])	NA
	<i>AtWRKY42</i> ([57])	NA
	<i>AtWRKY45</i> ([58])	NA
	<i>AtWRKY75</i> ([59])	NA
	<i>AtNLA</i> ([60])	NA
	<i>AtIPS1</i> ([61])	<i>TaIPS1</i> ([44])
	<i>AtmiR399</i> ([62])	<i>Tae-miR399-A1</i> ([47])
	<i>AtMiR827</i> ([63])	NA
	<i>AtALIX</i> ([64])	NA

\* NA: Not applicable.

The wheat genome contains several TaPHT members that could be divided into four subfamilies, PHT1 (*TaPHT1.1–1.13*), PHT2 (*TaPHT2.1*), PHT3 (*TaPHT3.1–3.3*), and PHT4 (*TaPHT4.1–4.6*). Their transcripts demonstrate enhanced expression in Pi limited roots and shoots ([20]). The complexity in discerning a total number of TaPHT1 members within the wheat genome has been recently discussed ([21]) while experiments characterizing the role of specific members involved in P uptake within two wheat genotypes (*KN9204* and *SJZ8*) have been performed. Differential expression pattern of high-affinity *TaPHTs* was observed in different wheat varieties under varying Pi regimes ([39]). Interestingly, the expression of *TaPHT1.1*, *1.2*, *1.9*, and *1.10* at flowering positively correlated with P uptake after stem elongation in different wheat varieties under field conditions supplemented with different P rates ([21]). Under Pi deprivation, Pi uptake increases and involves a high-affinity PHT1 member *TaPHT2* ([65]). Down-regulation of *TaPHT2.1* was able to induce a pronounced decrease in Pi accumulation in both sufficient and Pi-deficient wheat, suggesting its association with other PHTs involved in Pi uptake and translocation within plants ([41]). This reinforces the impact the intracellular Pi transport mechanism has upon regulating the plant Pi uptake efficiency. Still, in contrast to the data known about Arabidopsis, little is known about the regulation of wheat Pi transporters at the protein levels ([66]). It is worth to note that apart from its role in Pi uptake, *TaPHT2.1* was functionally characterized as an important P signalling component involved in Pi translocation from cytosol to chloroplast in Pi-stressed leaves ([41]). Whether any Pi transporter in wheat could play an additional transceptor role remains an open question. Membrane proteins that fulfil a dual nutrient transport function, such as the *PHO1* ([67]) or nitrogen transporter *NRT1* ([68]), serve as extant examples. Other than *PHT2* members and some reports showing differential expression of *TaPHT3* and four transcript abundance under Pi-depleted roots and shoots ([20,44]), there is no detail on their precise biological role in wheat tissues/cell. In addition, recent expression profiles of wheat *PHT1* sub-family genes during hydroponic and field-grown plant tissues were correlated with the presence of cis-acting promoter elements ([22]). These studies showed growing interests in the crop Pi-transporters among researchers.

### 3. Phosphate Sensing and Signalling in Arabidopsis and Wheat

How plants sense and signal Pi deficiency has been a long-standing question. In Arabidopsis, decades of research eventually identified the Pi starvation signalling pathway, defined as *SPX1-PHR1-miR399-PHO2-PHT1/PHO1* (for review: [29,69]). Key genes encoding for SPX proteins was initially reported to be upregulated in response to P deficiency via transcriptome analysis ([70]). *SPX* genes perform diverse functions in plant tolerance to Pi starvation ([33]) and play an important role in sensing P concentration in the cytosol through its ability to bind the inositol polyphosphate signalling molecule ([71]). *SPX1* interacts with the transcription factor Phosphate Response 1 (*PHR1*) in presence of Pi and dissociates under Pi deficiency ([71]). *PHR1* regulates many Pi-related genes ([50]), such as the *miRNA399* that eventually targets Phosphate 2 (*PHO2*) transcripts. The reduction of *PHO2* protein abundance leads to the accumulation of *PHT1* and *PHO1* proteins ([72]) and consequently an increase of the plant capacity to uptake Pi and translocate Pi to shoots ([73]). Noteworthy, the proper functioning of this signalling pathway requires the contribution of many others genes such the *SUMO E3 ligase SIZ1* ([32]), *PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1)*, ([51]) and *NITROGEN LIMITATION ADAPTATION (NLA)*, ([60]). *SIZ1* is involved in the regulation of *PHR1* by sumoylation ([32]). *PHF1* is required for the trafficking of Pi transporters to the plasma membrane [51]. *NLA* is proposed to function at the plasma membrane to direct the degradation of *PHT1s* ([60]), a fine-tuning process required for Pi uptake capacity of plants.

In wheat, transcript profiles of genes involved in Pi starvation response with organ-specific Pi allocation patterns were studied in roots and shoots of Chinese 80–55 (P-efficient cultivar) and Machete (less-efficient cultivar) under Pi deficiency [44]. This report revealed the distinct modes for allocation of Pi and organic P compounds between the source and sink tissues that modulate the adaptation under varying Pi condition. The P-allocation patterns in the multiple plant organs correlated with

the transcript expression patterns, suggestive of molecular signatures for improved phosphorus use efficiency (PUE) during limited Pi supply. Few genes involved in Pi starvation signalling responses have been reported for hexaploid wheat [35], such as an ortholog of the Arabidopsis transcription factor PHR1 characterized for its function in regulating Pi-signalling and plant growth in wheat [49]. Under both Pi-sufficient and deficient conditions, over-expression of the *TaPHR1-A1* homolog moderately up-regulated the expression levels of *TaPHR1* throughout the plant, resulting in a moderate increase of leaf Pi concentration and thus avoiding resultant toxicity ([49]). Pi uptake was positively favoured by *TaPHR1-A1* over-expression by increasing root tip number, lateral root length, and *TaPHTs* expression (*TaPHT1.2* in roots and *TaPHT1.6* in shoots). Utilizing bimolecular fluorescence complementation assays, it has been confirmed that wheat PHR1 forms a homodimer and confers transcriptional activation of a putative downstream target Pi-transporter *TaPHT1.2* [49].

The presence of Arabidopsis PHO2 orthologs in hexaploid wheat has also been proposed [35]. Detailed analysis of respective mutant lines for three *TaPHO2* genes from homologous group1 (*A1*, *B1*, and *D1*) showed remarkably different effects on P uptake, distribution, and plant growth [47]. The overall expression of *TaPHO2* in wheat was severely reduced in a *tapho2-d1* mutant, leading to high total shoot P under limited Pi conditions, but also showed inhibited growth and yield [47]. This resembled the phenotype observed in a *pho2* mutant of both monocots (e.g., rice) and dicots (e.g., Arabidopsis) [74,75]. Interestingly, *tapho2-a1* knockout mutant plants showed reduced *TaPHO2* expression that leads to only a moderate increase of total P and Pi levels in leaf under both sufficient and deficient P conditions [47]. Unlike the *tapho2-d1* mutant, *tapho2-a1* mutants demonstrated a moderate increase in P levels and accumulation alongside improved plant growth and grain yield [47]. In light of these interesting data, the involvement of *TaPHO2-D1* in Pi homeostasis to maintain plant growth rather than a simple Pi starvation signalling pathway has been proposed [47]. The Pi starvation signalling pathway *PHR1-IPS1-miR399-UBC24/PHO2-PHT1/PHO1* seems to be conserved and functional in numerous plant species. Manipulating the components of this pathway could be an important strategy for improving Pi nutrition in crops.

These genes and molecular mechanisms involved in Pi stress response are specifically induced during Pi deficiency and not under any other modes of stress known to alter Pi homeostasis. These observations indicate the existence of additional unknown genes and pathways regulating the Pi content in plants [8,52]. For instance, it is now well established that Pi content in plants is altered when plants are challenged by zinc limitation (–Zn) [6,76–79]. Intriguingly, under single –Zn stress, an excess of Pi supply causes loss of wheat biomass in comparison with plants grown under –P-Zn simultaneous stress [78]. Nevertheless, despite its fundamental importance, very little is known about the regulatory network established during Zn deficiency to control Pi homeostasis [52]. Studying Zn/Pi homeostasis interactions will lead us to uncover new genes and pathways controlling plant Pi homeostasis. This knowledge will be an additional resource for the improving Pi usage through perturbing Zn deficiency signalling pathways.

These experiments have all begun to clarify the components and systems that regulate P/Pi sensing and signalling within plants. The emergence of genome editing tools holds promise for further studies perturbing specific Pi-related genes/pathways, or genes involved in modulating the Pi content, such as genes involved in Zn deficiency signalling, to ultimately improve Pi nutrition in crops [80].

#### 4. Phosphate in Seeds

The accumulation of nutrients in the seed is important for seed vigour and germination [81]. The amount of micronutrients in seeds has declined systematically since the beginning of the green revolution in the 1960s [82,83]. In contrast, phytic acid (PA), the organic form of P in seeds, has increased following global changes (e.g., elevated atmospheric CO<sub>2</sub>) in various plant species including wheat [84]. Since PA is considered an anti-nutrient, increasing Pi content in grain while decreasing PA has become a trait of great interest [85].



In general, while Pi uptake and its intracellular and long-distance transport in plants has been extensively studied, the Pi transport in seeds has received little attention [86]. Current knowledge on the role of seed-specific PHTs is largely lacking [87]. In the seeds, nutrients reach the embryo via various pathways and at different developmental stages. Transfer of nutrients from the maternal seed coat to the filial endosperm and embryo is required for seed production and quality. In the case of Pi, the transfer of this element from the seed coat to the embryo requires Pi exporters. Recently, the *PHO1* gene was shown to be expressed in the chalazal seed coat in Arabidopsis, suggesting a role in the transfer of P from the seed coat to the embryo in developing seeds [88]. Consistently, Pi transfer from the seed coat to the embryo is perturbed in the *pho1* mutant. This observation and experimental data state the ignition point for a deep investigation of Pi transport in seeds, which may help to start understanding the mechanism regulating P accumulation.

In wheat, the mature grain may contain up to 90% of the total shoot P, with 20–90% of this being translocated from other tissues (for details, see [89]). PA accounts for up to 1–2% of the total weight [90,91]. P and PA concentrations in the grain increases as P application increases [92]. Increased PA concentration greatly decreases the bioavailability of nutritional minerals in wheat grain, such as Zn [92]. Therefore, reduction of PA in cereal grains is considered an important trait that is generated either through breeding or biotechnological approaches. Reducing PA in grains may provide a dual gain with less grain P loss and more micronutrient retention [93,94]. Generation of low PA crops may be achieved by targeting PA biosynthesis genes or transport [29,95–98]. As an alternative strategy to achieve low PA grains, roles for other families of transporters are also emerging. For instance, knockdown of the rice Pi transporter *OsPHT1.8* resulted in lowered PA accumulation in the embryo and mature grains [94]. Subsequently, they showed that rice *PHT1.8* performs a novel biological function during crosstalk between Pi and auxin signalling. This was one of the recent reports that provided clues for the link between auxin and -Pi responses [99]. Sulphate transporters have also implicated in grain PA and P content regulation. Map-based cloning and complementation tools resulted in the identification of rice sulphate transporters referred as *OsSULTR3;3*, which are involved in compositional changes of Pi and PA in developing grains [100]. Subsequently, another sulphate transporter family gene named the *SULTR-like phosphorus distribution transporter (SPDT)* has demonstrated involvement in the intervacular transfer of P, especially at the nodes by unloading P from xylem toward the phloem [101]. Therefore, such studies have suggested that node-localized transporters could affect the preferential accumulation of P in grains [101].

Nevertheless, the relative dearth of information has led to few varieties of wheat being studied in regards to grain total P and PA. It is still unclear whether transportation of P to the grain occurs directly from phloem or via xylem through recycling from roots, and to what extent translocation of P between plant organs is altered at different P-regimes [102]. In wheat, only two significant transgenic studies that include over-expression of *TaPHR1-A1* and knockout of *TaPHO2-A1* were able to achieve enhanced P uptake and grain yield under low Pi condition [47,49]. It is reasonable to speculate that manipulating these Pi-related genes, among others yet to be discovered, will allow intentional modulation of Pi loading in grains [20,88].

Although the current knowledge of *PHT* expression and that of other regulators in seeds is in its early stage across plant species, some rice transporters demonstrate function in seed filling with Pi. A recent study investigating suppression of rice *OsPHT1.8* suggested its role in P redistribution and allocation of Pi in both embryo and endosperm seed tissue [94]. Hence, it will be important to implement such functional strategies for addressing transport and accumulation of Pi into grains [20].

## 5. Conclusions

It has been reported that plants use only 20%–30% of the Pi fertilizers applied to soil [103]. The significant remaining Pi is lost and can leach into aquatic ecosystems, instigating ecological issues such as eutrophication. Thus, it is clear that the excessive use of Pi fertilizers is not only an unsustainable and costly practice, but also ecologically unfriendly. Therefore, research on Pi nutrition



in plants should lead to changes in agricultural practices that would be both economically and environmentally beneficial.

How societally pertinent crop plants such as wheat maintain P homeostasis and respond to changes of Pi concentration remain poorly understood. Improving wheat Pi nutrition will require a full understanding of the physiology and molecular regulation of P remobilization from vegetative tissues to grains. In the future, it will be interesting to identify the complete list of genes that are involved in Pi transport between different wheat grain tissues, Pi acquisition, and Pi mobilization in embryo development. Although some uptake Pi transporters were discovered through classical molecular approaches, their regulatory mechanisms at the transcriptional and posttranscriptional levels remain obscure. This is particularly challenging in wheat because of the current unavailability of the complete genomic sequence. For the ones identified, the availability of sequenced mutant populations [104] alongside current genome editing tools like Clustered regularly interspaced short palindromic repeats-Cas9 technology will constitute an invaluable resource for their functional validation. In addition, a proper combination of omics approaches (such as RNA-seq), empowered with system biology tools, will help to construct regulatory pathways regulating Pi accumulation in wheat during its different developmental stages. Gaining this knowledge is vital to create crop varieties with improved P-use efficiency and modulate the Pi accumulation in grain.

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