

REVIEW PAPER

# Phosphate and zinc transport and signalling in plants: toward a better understanding of their homeostasis interaction

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## Abstract

**Inorganic phosphate (Pi) and zinc (Zn) are two essential nutrients for plant growth. In soils, these two minerals are either present in low amounts or are poorly available to plants. Consequently, worldwide agriculture has become dependent on external sources of Pi and Zn fertilizers to increase crop yields. However, this strategy is neither economically nor ecologically sustainable in the long term, particularly for Pi, which is a non-renewable resource. To date, research has emphasized the analysis of mineral nutrition considering each nutrient individually, and showed that Pi and Zn homeostasis is highly regulated in a complex process. Interestingly, numerous observations point to an unexpected interconnection between the homeostasis of the two nutrients. Nevertheless, despite their fundamental importance, the molecular bases and biological significance of these interactions remain largely unknown. Such interconnections can account for shortcomings of current agronomic models that typically focus on improving the assimilation of individual elements. Here, current knowledge on the regulation of the transport and signalling of Pi and Zn individually is reviewed, and then insights are provided on the recent progress made towards a better understanding of the Zn–Pi homeostasis interaction in plants.**

**Key words:** Cross-talk, homeostasis, phosphate, signalling, transport, zinc.

## Introduction

Plants require an adequate supply of inorganic phosphate (Pi) and zinc (Zn) for their survival, while also serving as the major entry point for these two elements into the food chain. For this reason, the effects of phosphorus (P) and Zn deficiencies on crop yield have become a worldwide concern in recent years, in terms of food availability and malnutrition. Specifically, three issues present a major concern. First, Pi sustainability, as the world is facing ‘a potential P crisis’ because of the rapid depletion of current Pi reserves (Abelson, 1999). The second problem is low Zn content in the food that is a major public health issue (Shahzad *et al.*,

2014), since it causes Zn malnutrition which is associated with retarded growth, skeletal abnormalities, delayed wound healing, increased abortion risk, and diarrhoea (Salgueiro *et al.*, 2000a, b). Thirdly, at the end of the plant life cycle, Pi is mainly stored in the grain as phytic acid (PA), which is composed of six Pi molecules linked to a myo-inositol backbone. The abundance of PA in crop seeds used as feed and food constitutes a long-standing issue. High PA content in cereal seeds is one of the major P sources that contribute to eutrophication (Bali and Satyanarayana, 2001). PA also has antinutritional properties since it acts as a chelating factor

for several essential cations such as Zn, reducing its bioavailability in the human digestive system, and thus leading to Zn deficiency (Maga *et al.*, 1982; Raboy, 2009; Veum *et al.*, 2009). To counter these alarming situations, substantial efforts have been made to improve Pi and Zn nutrition in plants, based on determining how plants respond to Pi and Zn deficiency at the physiological and molecular levels. This knowledge has been documented in many research publications and elegantly summarized in multiple reviews (Poirier and Bucher, 2002; Rouached *et al.*, 2010; Sinclair and Kramer, 2012; Shahzad *et al.*, 2014). Both Pi and Zn are taken up at the root–soil interface, predominantly as free ions. Under Pi or Zn deficiency, root architecture changes and increases its capacity for nutrient import (Nussaume *et al.*, 2011; Jain *et al.*, 2013). Once within the root symplast, each element can be stored in the root through transport into vacuoles. Alternatively, they can undergo symplastic transport towards and into the vascular cylinder, for subsequent transfer to the shoot. Export from cells is required to load Pi or Zn into the dead xylem, and thus for their translocation from the root to the shoot via the xylem (Hamburger *et al.*, 2002; Hanikenne *et al.*, 2008; Rouached *et al.*, 2010; Sinclair and Kramer, 2012). Numerous genes controlling these crucial steps of Pi or Zn transport in plants have been identified, and characterized at the molecular level, including the high affinity phosphate transporter (PHT1) (Nussaume *et al.*, 2011), phosphate exporter (PHO1) (Rouached *et al.*, 2010), transporters belonging to ZIP (ZRT, IRT-like protein) and members of P<sub>1B</sub>-ATPases (HMA2 and HMA4) (Sinclair and Kramer, 2012). Despite accumulating information on the identity of these genes, developing crop plants with improved P-use efficiency and Zn content by breeding or engineering remains a target, yet not achieved. A complex yet neglected interaction between Pi and Zn nutrition *in planta* is a part of the explanation of the shortcomings of current agronomic models targeted at improving the nutrition of each element individually.

The interconnection between the homeostasis of these two nutrients has been observed in many crop species and can be summarized as follows: Zn-deficient plants overaccumulate Pi in the shoot and, conversely, Pi-deficient plants overaccumulate Zn in the shoot (Reed, 1946; Bingham and Martin, 1956; Loneragan *et al.*, 1982; Cakmak and Marchsner, 1986; Norvell and Welch, 1993; Huang *et al.*, 2000; Zhu *et al.*, 2001; Misson *et al.*, 2005; Khan *et al.*, 2014). In >90% of land plants, symbiotic associations are formed with mycorrhizal fungi (Schachtman *et al.*, 1998). Mycorrhizae plays an important role in the acquisition of P for the plant (Smith and Read, 1997), and they appeared also to facilitate Zn transport in the soil–fungi–plant continuum (Chen *et al.*, 2003). Nevertheless, the molecular basis of the Pi–Zn interaction in plants remains poorly understood in both mycorrhizal (Cakmak and Marchsner, 1986; Huang *et al.*, 2000; Zhu *et al.*, 2001; Watts-Williams *et al.*, 2013) and non-mycorrhizal genera (Khan *et al.*, 2014). To gain insight into this phenomenon, in this review we focus mainly on *Arabidopsis* (a non-mycorrhizal genus) for which more physiological and ‘omics’ data on the regulation of Zn and Pi homeostasis are available. The published transcriptomics data (Misson *et al.*, 2005; Van

de Mortel *et al.*, 2006; Bustos *et al.*, 2010; Rouached *et al.*, 2010) and characterization of some *Arabidopsis* mutants (Khan *et al.*, 2014) have highlighted the presence of a genetic programme that co-regulates the homeostasis of the P and Zn elements as an integral part of the adaptive responses to these stresses. These important phenomena have consequences in terms of comprehending the regulation of homeostasis for each of these individual elements. Therefore, future work that aims to elucidate the regulation of Pi or Zn homeostasis should first consider the possibility of cross-talk between the regulatory pathways of the two elements. The aim of this article is to present a condensed summary of the most relevant findings on the regulation of Pi and Zn transport and signaling, and to review the growing body of evidence supporting the Pi and Zn homeostasis interaction in plants.

## Phosphate in plants

### *Pi: an essential macronutrient for plant growth*

Pi is an essential nutrient that ensures various basic biological functions. It is a component of RNA and DNA, as well phospholipids. Pi plays an essential role in the energy metabolism of the cell: the Pi–Pi bond present in molecules such as ATP is energy rich, and is essential to numerous enzymatic reactions. Pi also participates in several signal transduction cascades via the modulation of enzyme activity by protein phosphorylation (Poirier and Bucher, 2002). Due to the central role of Pi in numerous aspects of plant metabolism, it is not surprising that their metabolism is profoundly affected by Pi starvation, and that Pi deficiency is associated with a coordinated series of morphological, transcriptomic, and metabolic adaptations (Poirier and Bucher, 2002; Misson *et al.*, 2005; Lan *et al.*, 2012).

### *The Pi transport system in plants*

Pi is relatively inaccessible to plant roots because of its low solubility and high capacity for adsorption to soil particles. Plants must therefore use a complex series of Pi transporters that are involved in Pi acquisition from the soil, and its subsequent distribution to all tissues and subcellular organelles. Pi acquisition at the root periphery in plants is coupled to proton entry (Pi:H<sup>+</sup> symporter) and mediated by members of the *PHT1* gene family (Schachtman *et al.*, 1998). The *Arabidopsis* and rice genomes contain nine and 13 *PHT1* family members, respectively (Paszukowski *et al.*, 2002; Poirier and Bucher, 2002). The *PHT1* genes are preferentially expressed in roots, and the encoded proteins function as high-affinity Pi uptake transporters (Mucchhal *et al.*, 1996; Misson *et al.*, 2005; Bayle *et al.*, 2011; Nussaume *et al.*, 2011; Remy *et al.*, 2012). The expression of *PHT1* genes is, however, not restricted to roots and can be detected in other vegetative and reproductive tissues, implicating their role beyond Pi uptake at the root surface (Mudge *et al.*, 2002; Karthikeyan *et al.*, 2002; Nagarajan *et al.*, 2011).

Regulation at the transcriptional as well as post-transcriptional levels has been documented for the *PHT1* genes At

the transcriptional level, *PHT1* gene expression is influenced by the internal Pi concentration of cells, with Pi deficiency leading to the accumulation of a steady-state level of mRNA for several *PHT1* genes (Muchhal *et al.*, 1996; Muchhal and Raghothama, 1999; Misson *et al.*, 2005). The transcript levels of *PHT1* family members are also influenced by active photosynthesis, exogenous sugars, or even phytohormone application (Martin *et al.*, 2000; Franco-Zorrilla *et al.*, 2005; Karthikeyan *et al.*, 2007; Hammond and White, 2008; Lejay *et al.*, 2008; Rubio *et al.*, 2009; Hammond and White, 2011). A number of genes encoding transcription factors are differentially regulated by phosphate starvation, including *WRKY75* (Devaiah *et al.*, 2007a), *MYB62* (Devaiah *et al.*, 2009), *ZAT6* (Devaiah *et al.*, 2007b), and *PHR1* (Bustos *et al.*, 2010). Molecular studies have now revealed that PHR1 recognizes an imperfect palindromic motif (GNATATNC) found in the promoter sequence of some *PHT1* genes (Rubio *et al.*, 2001; Franco-Zorrilla *et al.*, 2004). Bustos *et al.* (2010) demonstrated that mutation of this motif in the promoter of two Pi starvation-responsive genes impaired their responsiveness to Pi starvation, thus revealing the importance of the PHR1 binding sequence as an integrating *cis*-regulatory motif (Bustos *et al.*, 2010).

A post-translational regulatory level has also been proposed for *PHT1*. It was recently shown that the C-terminal phosphorylation of PHT1 proteins is required for their access to the plasma membrane (Bayle *et al.*, 2011), with the assistance of phosphate transporter traffic facilitator1 (PHF1) (Gonzalez *et al.*, 2005). The presence of PHT1 at the plasma membrane is a highly regulated process. When Pi is present in the medium, PHT1 is degraded through the protein ubiquitination machinery. Under Pi deficiency, the abundance of ubiquitin E2 conjugase *PHO2* transcripts and the *NITROGEN LIMITATION ADAPTATION* (*NLA*) gene is reduced through the action of two microRNAs (miR399 and miR827, respectively), leading to an increase in PHT1 (Lin *et al.*, 2013).

Once within the root system, Pi is transferred from roots to shoots via its loading into the root xylem vessels by the PHO1 protein. PHO1 is preferentially expressed in the root vascular cylinder (Poirier *et al.*, 1991; Hamburger *et al.*, 2002; Stefanovic *et al.*, 2007; Arpat *et al.*, 2012), and the protein is primarily localized to the Golgi and *trans*-Golgi network (Hamburger *et al.*, 2002; Arpat *et al.*, 2012). PHO1 could be partially relocated to the plasma membrane in the presence of a high Pi concentration (Arpat *et al.*, 2012). Ectopic expression of *Arabidopsis* PHO1 in tobacco leaves, or in *Arabidopsis* leaves or mesophyll protoplasts, has shown that PHO1 mediates Pi export into the apoplasts, thus establishing PHO1 as a Pi exporter (Arpat *et al.*, 2012). The *Arabidopsis* *PHO1* gene family is composed of 11 members, namely *PHO1*, and 10 homologues named *PHO1;H1* to *PHO1;H10* (Wang *et al.*, 2004). Interestingly, of the 11 genes comprising the *PHO1* family, only *PHO1* and its closest homologue *PHO1;H1* could rescue the phenotype of the *pho1* mutant, in terms of shoot growth and shoot Pi content (Stefanovic *et al.*, 2007). Like *PHT1*, *PHO1* is regulated at the transcriptional and post-transcriptional levels.

In *Arabidopsis*, Pi deficiency leads to a strong increase in the activity of the *PHO1;H1* promoter, and a less intense increase in the *PHO1* promoter. The increase in *PHO1;H1* transcript level under Pi deficiency is under the control of the PHR1 transcription factor, and sequences recognized by PHR1 are found in the promoter of that gene (Stefanovic *et al.*, 2007). In contrast, stimulation of *PHO1* transcription by phosphate deficiency occurs independently of PHR1 (Stefanovic *et al.*, 2007). In agreement with this distinct regulation of *PHO1*, the WRKY6 transcription factor was found to bind to the W box of the *PHO1* promoter, where its transcription regulates *PHO1* transcription activity (Chen *et al.*, 2009). At the post-transcriptional level, PHO1 has been shown to be a target for PHO2-mediated degradation (Liu *et al.*, 2012). Study of the expression of the *OsPHO1;2* homologue of *Arabidopsis* *PHO1* in rice has revealed a unique mode of post-transcriptional regulation (Jabnourne *et al.*, 2013). A *cis*-natural antisense transcript was found associated with the sense *OsPHO1;2* gene, and was expressed in the same tissues as *OsPHO1;2*. Overexpression of the *cis*-natural antisense transcript under Pi deficiency was shown to stimulate the translation of the sense *OsPHO1;2* mRNA, leading to an increase in *OsPHO1;2* protein level (Jabnourne *et al.*, 2013).

Following Pi uptake into the root and its loading into the root xylem vessel, Pi must be reabsorbed into the leaf cells. Furthermore, Pi is highly mobile between tissues, with a flux between leaves and roots, leaves and seeds, and old source leaves and young sink leaves. This Pi distribution characteristically involves the movement of Pi in and out of the phloem. Although our current view of the long-distance movement of Pi between organs is incomplete, it is likely to involve members of the *PHO1* and *PHT1* gene families. In addition to its distribution between cells, Pi must also be transported into all subcellular compartments of the cell, including plastids and mitochondria. These processes involve genes belonging to the *PHT2*, *PHT3*, *PHT4*, and *pPT* gene families (Poirier and Bucher, 2002; Nussaume *et al.*, 2011). Briefly, *Arabidopsis* *PHT2* is predominantly expressed in green tissue and found associated with chloroplasts (Versaw and Harrison, 2002). Expression of *PHT2* in yeast revealed that it is a low-affinity H<sup>+</sup>/Pi symporter, with a potential role in Pi transfer from the cytosol to the chloroplast (Daram *et al.*, 1999; Versaw and Harrison, 2002; Rausch *et al.*, 2004; Guo *et al.*, 2013). Members of the *PHT3* family probably function as mitochondrial Pi transporters (Kiiskinen *et al.*, 1997; Millar and Heazlewood, 2003). Members of the *PHT4* family are localized in either the chloroplasts or Golgi (Roth *et al.*, 2004; Guo *et al.*, 2008). *PHT4;6* has been implicated in the transfer of Pi out of the Golgi (Cubero *et al.*, 2009). The *pPT* family mediates the exchange of Pi with other metabolites across the plastid membrane and is composed of four main groups: (i) TPT, a chloroplast triose-phosphate/Pi translocator precursor (Flugge, 1999); (ii) PPT, a plastidic phosphoenolpyruvate/Pi translocator (Fischer *et al.*, 1997); (iii) AGPT, a third group of plastidic Pi antiporters (Kammerer *et al.*, 1998); and (iv) XPT, a plastidic Pi translocator (Eicks *et al.*, 2002).

### *Pi sensing and signalling pathways*

Some key pieces in the Pi signalling puzzle have now been identified, revealing a complex regulatory network that plants activate in response to Pi deficiency stress. Progress in the field of Pi homeostasis regulation in plants has been summarized in several recent reviews (Doerner, 2008; Rouached *et al.*, 2010; Chiou and Lin, 2011). The most documented mechanism is the Pi long-distance signalling network, which includes PHR1, the ubiquitin E2 conjugase PHO2, and miR399 (Pant *et al.*, 2008). It has been demonstrated that miR399 is transcriptionally regulated by the MYB transcription factor PHR1, in response to Pi deficiency (Bari *et al.*, 2006). Furthermore, miR399 mediates long-distance Pi signalling from the shoot to the root via the phloem, and targets the transcription of PHO2 (Lin *et al.*, 2008; Pant *et al.*, 2008). The repression of PHO2 transcription causes an increase in the expression of root Pi uptake transporters, and hence an increase in the acquisition of Pi by the roots and its translocation to the shoot (Bari *et al.*, 2006; Lin *et al.*, 2008). The presence of a conserved 23 bp region complementary to miR399—with a mismatch at its expected cleavage site in the transcript of the non-coding gene *IPSI* (INDUCED BY PHOSPHATE STARVATION1)—appears to contribute to the regulation of the PHO2–miR399 pathway via a mechanism known as ‘target mimicry’ (Franco-Zorrilla *et al.*, 2007). This finding highlights the involvement of non-coding RNAs in Pi signalling. Many other Pi-responsive miRNAs have been identified (Fujii *et al.*, 2005; Hsieh *et al.*, 2009; Pant *et al.*, 2009). Although some targets have been proposed for these miRNAs, it is necessary to confirm them in order to appreciate fully the role of these miRNAs in Pi signalling. A recent report has provided new insights into the role of miR82, which is induced by Pi deprivation and targets the *NITROGEN LIMITATION ADAPTATION (NLA)* gene (Hsieh *et al.*, 2009) during Pi homeostasis regulation (Lin *et al.*, 2013). This demonstrates that Pi deficiency signalling networks are complex and appear to involve cross-talk between two miRNA regulatory pathways (Lin *et al.*, 2013). In *Arabidopsis*, the signalling network for Pi is also coordinated with other minerals, such as sulphate (Rouached *et al.*, 2011a, b), iron (Ward *et al.*, 2008), and Zn (Jain *et al.*, 2013; Khan *et al.*, 2014).

In recent years, the importance of proteins having an SPX domain in Pi homeostasis and signalling has been reported. In *Arabidopsis*, expression of all four *Arabidopsis* genes, named *AtSPX1–AtSPX4*, is regulated by Pi starvation. Whereas *AtSPX1*, *AtSPX2*, and *AtSPX3* are induced by Pi starvation, expression of *AtSPX4* is suppressed by Pi starvation (Duan *et al.*, 2008). Furthermore, expression of all the members was found to be under the control of PHR1 (Rubio *et al.*, 2001; Miura *et al.*, 2005).

A number of signalling players and networks have begun to surface for the regulation of the Pi deficiency response, revealing coordination between the Pi, phytohormone, and phytoassimilate signalling pathways. The cross-regulation between Pi starvation responses and phytohormones is perhaps best illustrated by the case of Pi and cytokinins. It has been shown that cytokinin supply can repress the expression of Pi uptake

transporters and many other Pi starvation-induced genes (Martin *et al.*, 2000; Brenner *et al.*, 2005). Such an interaction involves the cytokinin receptors CYTOKININ RESPONSE 1/WOODEN LEG/ARABIDOPSIS HISTIDINE KINASE 4 (CRE1/WOL/AHK4) (Martin *et al.*, 2000; Karthikeyan *et al.*, 2002; Franco-Zorrilla *et al.*, 2005; Hou *et al.*, 2005; X. Wang *et al.*, 2006). The interaction between the Pi and gibberellin (GA) signal transduction pathways has also been proposed based on results showing that the overexpression of the transcription factor gene *MYB62*, that is induced in response to Pi deficiency, resulted in a reduction in the expression not only of several Pi starvation-induced genes but also of some GA biosynthetic genes, and that the observed GA deficiency phenotype of the *MYB62* overexpressor lines could be partially rescued by external GA application (Devaiah *et al.*, 2009). Finally, auxin and ethylene are known to modulate the developmental adaptations of roots under Pi limitation (Rubio *et al.*, 2009). The Pi signalling pathway also interacts with those regulating the carbon status of the plants (Wissuwa *et al.*, 2005). Lejay *et al.* (2003) reported that some high-affinity Pi transporters are regulated diurnally and induced by sucrose supply, and that two other members of the same family are induced by sucrose supply and are proposed to be upstream of hexokinase (HXK) sugar-sensing pathways (Lejay *et al.*, 2008). Taken together, these observations provide strong evidence for the existence of an interconnection between hormones, sucrose, and Pi starvation signalling in plants.

## Zinc in plants

*Zn: an essential micronutrient for plant growth*

Zn is the only metal represented in all six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Coleman, 1998), and it also plays a structural role in regulatory proteins (Berg and Shi, 1996). In plants, Zn deficiency is described as causing reduction of biomass, interveinal necrosis, deformed and chlorotic leaves, and reduction in yield. In most crops, the typical leaf Zn concentration required for adequate growth is approximately between 15 mg kg<sup>-1</sup> and 20 mg kg<sup>-1</sup> dry weight (Marschner, 1995). Despite its essentiality, high concentrations of Zn in the growth medium can cause toxicity in plants. At the cellular level, elevated Zn concentration causes oxidative stress, a decrease in accumulation of ATP, disintegration of cell organelles, and development of vacuoles (Sresty and Madhava Rao, 1999; Xu *et al.*, 2013).

Diverse techniques with different thresholds in their spatial resolution or sensitivity have been used to investigate Zn<sup>2+</sup> cellular distribution in plant cells and tissues. These include electron-spectroscopic or energy-dispersive X-ray analysis imaging (Lanquar *et al.*, 2005), elemental mapping using synchrotron radiation-induced X-ray fluorescence and particle-induced X-ray emission (Kim *et al.*, 2006; Punshon *et al.*, 2009; Schnell Ramos *et al.*, 2013), or inductively coupled plasma atomic emission spectroscopy (ICP-AES) after subcellular

fractionation (Seigneurin-Berny *et al.*, 2006; Lanquar *et al.*, 2010). However, these approaches provide only static information on the total Zn concentration. Interestingly, the recent development of tools to image/monitor the dynamics of the cytosolic Zn, namely the combination of genetically encoded Förster resonance energy transfer (FRET) sensors and Root Chip technology (Lanquar *et al.*, 2014), offers a great opportunity to measure the intracellular free Zn concentrations in plants cells. These tools will certainly help to study Zn<sup>2+</sup> homeostasis, transport, and signalling in plant cells.

### Zn transport system in plants

Soil is the source of Zn for plants; thus an efficient Zn uptake by plants is crucial. However, Zn uptake is not closely related to the Zn concentration in the soil solution (Ernst and Nelissen, 2000). The metal uptake by plants depends upon the solubility of metals in soils which varies considerably depending on the soil composition, soil pH, and soil P (Tagwira *et al.*, 1992, 1993; Sauvé *et al.*, 2000; Loosemore *et al.*, 2004; Houben *et al.*, 2013). The relationship between Zn availability and soil pH and P status will be detailed below. The precise type(s) of root cells and mechanisms underlying Zn uptake are not completely known. Transport across the plasma membrane is thought to be mediated by a transport system and intracellular high-affinity binding sites. Transporters belonging to the ZIP (ZRT, IRT-like protein) family are thought to be the primary Zn transporters involved in Zn uptake. The ZIP family comprises 15 members in *A. thaliana* (Maser *et al.*, 2001). AtIRT1, a member of the ZIP family from *A. thaliana*, localizes to the plasma membrane especially of root epidermal cells (Vert *et al.*, 2002; Barberon *et al.*, 2011), and *A. thaliana* mutant for IRT1 accumulates less Zn as compared with the wild type, indicating its physiological role in Zn uptake (Henriques *et al.*, 2002). Some other transporters of the ZIP family are also thought to be involved in Zn uptake based on results obtained during transcriptomic studies and in heterologous systems. Functional heterologous expression of six *A. thaliana* ZIP genes, ZIP1, ZIP2, ZIP3, ZIP7, ZIP11, and ZIP12, complemented the *Saccharomyces cerevisiae* Zn-uptake deficient mutant, *zrt1 zrt2* (Milner *et al.*, 2013). At the transcriptional level, ZIP1, ZIP3, and ZIP4 are induced in Zn-starved *A. thaliana* roots (Grotz *et al.*, 1998), while ZIP1, ZIP3, ZIP4, ZIP9, and ZIP10 are constitutively overexpressed in the Zn hyperaccumulator species, *A. halleri*, as compared with *A. thaliana* (Talke *et al.*, 2006). It is worth mentioning that a passive Zn<sup>2+</sup> influx can occur through the depolarization-activated non-selective cation channel (DA-NSCC) type as reported in the Zn-hyperaccumulating *Thlaspi caerulescens* (Piñeros and Kochian 2003). The transport of Zn<sup>2+</sup> can also be mediated by the voltage-independent NSCCs (VI-NSCCs) which depend on the insensitivity/permeability of these channels to Zn<sup>2+</sup> ion (Demidchik and Maathuis, 2007).

In addition to these transporters, Zn movement in roots is also thought to be mediated by metal chelators: phytosiderophores (PSs), phytochelatin (PCs), and metallothioneins (MTs) (Palmgren *et al.*, 2008). For the most part, they

keep the free metal concentrations very low, and interact with and donate metals to apometalloproteins or transport proteins that mediate the sequestration or efflux of metal ions (Callahan *et al.*, 2006). PSs, hexadenate metal chelators, are compounds of the mugineic acid (MA) family capable of forming complexes with iron [stable Fe(III) chelates] (Tagaki *et al.*, 1984) and probably with Zn (Von Wirén *et al.*, 2000). The graminaceous monocot plants (Strategy II-plants: secrete PSs) and most other plants (Strategy I-plants: do not secrete PSs) can be distinguished based on these two distinct strategies that they use for uptake of sparingly soluble iron from the soil. Strategy I-plants use the reduction strategy which is characterized by plasma membrane-localized ferric reductases coupled with Fe(II) transporters. Strategy II-plants use the chelation strategy through the secretion of PSs and the absorption of the Fe(III)–PS complex via a specific uptake systems located at the root surface (Romheld and Marchner, 1986). Interestingly, the release of PSs is induced under Zn-deficient conditions in cereals (Tolay *et al.*, 2001; Suzuki *et al.*, 2008; Arnold *et al.*, 2010); nevertheless, whether this observation can be explained by an induced physiological deficiency for Fe remains an open question (Pedler *et al.*, 2000). It has been suggested that Strategy II-plants may take up Zn as Zn–PS complexes (von Wiren *et al.*, 1996; Erenoglu *et al.*, 2000; Tolay *et al.*, 2001). von Wiren *et al.* (1996) have demonstrated that Zn enters Strategy II-plants via a non-PS-mediated route.

Nicotianamine (NA) is the precursor of MA-family PSs, and has been proposed to form stable complexes with many heavy metals including Zn and to play a role in their circulation in the xylem and phloem (Stephan and Scholz, 1993). Interestingly, endogenous or exogenous expression of *NA synthase* (*NAS*) genes from various species under the control of either constitutive or tissue-specific promoters resulted in an increase in PS excretion, Zn uptake, and translocation, and Zn accumulation in polished rice grains (Masuda *et al.*, 2009, 2012, 2013a, b; Johnson *et al.*, 2011; Lee *et al.*, 2011). Taken together, these data clearly illustrate the importance of NA in uptake and translocation of Zn in plants. NA is present in both Strategy I- and Strategy II-plants but does not give rise to MA in Strategy I-plants. It has been reported that Zn deficiency stimulates the synthesis of MA forms, resulting in more PS secretion in barley and rice (Suzuki *et al.*, 2006, 2008). Under this condition (–Zn), barley takes up more Zn–MA complex than Zn<sup>2+</sup> ions (Suzuki *et al.*, 2006). The Zn–MA chelates may possibly be taken up into roots by YSL transporters. In line with this idea, it has been shown that the maize *ys1* mutant absorbs less Zn(II)–MA than wild-type plants (von Wiren *et al.*, 1996), thus revealing a physiological relevance of ZmYS1 in Zn–MA chelate uptake into roots. PCs are oligomers of glutathione, produced by the enzyme phytochelatin synthase. A role for PCs in Zn sequestration has been described in different mutants of yeast and *A. thaliana* (Tennstedt *et al.*, 2009). MTs are small cysteine-rich proteins involved in metal homeostasis. Very recently, Schiller *et al.* (2014) reported that MT proteins regulate the concentrations of Zn and other metal ions, in particular during germination and grain filling in barley. *Arabidopsis* transgenic

lines overexpressing a barley *MT2a* gene exhibited more sensitivity to Zn excess (Schiller *et al.*, 2014). It has been shown using *in vitro* assays that AtMT2 could chelate Zn (Robinson *et al.*, 1996). Functional heterologous expression of *AtMT2* could partly complement Zn hypersensitivity in mutants of *Synechococcus*. A study in rice also reported the involvement of *OsMT1a* in Zn homeostasis (Yang *et al.*, 2009). *OsMT1a* was found to be predominantly expressed in the roots and was induced by Zn treatment. Overexpression of *OsMT1a* caused an increase in Zn accumulation in the *OsMT1a*-overexpressing rice lines (Yang *et al.*, 2009). Taken together, these data prove the involvement of the MTs in regulating Zn homeostasis in plants. A novel class of proteins 'plant defensins type I (PDF1s)' were described for their involvement in cellular Zn tolerance (Mirouze *et al.*, 2006). PDF1s are small cysteine-rich peptides (Lay and Anderson, 2005), and are constitutively highly overexpressed in the Zn hyper-accumulators; *A. halleri* and *T. caerulescens* (Van de Mortel *et al.*, 2006; Shahzad *et al.*, 2013). Functional heterologous expression of PDF1s from *A. halleri* in yeast as well as in *A. thaliana* increased their Zn tolerance (Mirouze *et al.*, 2006; Shahzad *et al.*, 2013). The mechanism by which PDF1s play a role in Zn tolerance is still unknown. However, it seems likely that Zn might be chelated by the cysteines present in PDF1s. Bioinformatics analysis indeed revealed that an atom of Zn could be chelated by PDF1 (L. Marquès, personal communication).

Specific molecular mechanisms for Zn loading/unloading into/from the xylem have also been proposed. The plasma membrane transporters, AtHMA2 and AtHMA4, members of P<sub>1B</sub>-ATPases, have been well documented for their role in Zn loading into the xylem (Hussain *et al.*, 2004; Verret *et al.*, 2004). Moreover, using *A. thaliana hma2 hma4* double mutants or overexpressors of *HMA4* in *A. thaliana* has suggested the role of these gene in Zn interorgan distribution (Hussain *et al.*, 2004; Verret *et al.*, 2004; Hanikenne *et al.*, 2008). In the roots of *A. thaliana* and *A. halleri*, specific mRNA activity of *HMA4* was observed in the root pericycle and xylem parenchyma (Hanikenne *et al.*, 2008). It is noteworthy that the localization of *HMA2* and *HMA4* promoter activity in the vascular tissues of shoots and leaves indicates their possible involvement in xylem unloading as well (Hussain *et al.*, 2004; Hanikenne *et al.*, 2008). In addition, the localization of *HMA4* expression in the xylem parenchyma and the leaf cambium suggests a possible role for *HMA4* in Zn distribution within the leaf (Hanikenne *et al.*, 2008). Once loaded into the dead xylem, free Zn may move upward to shoots along the xylem sap. However, ligands of Zn with citrate or NA have also been proposed to be involved in long-distance transport of Zn in the dead xylem (Suzuki and Ishioka, 2008; Rellán-Álvarez *et al.*, 2010; Pineau *et al.*, 2012).

Proper functioning of cellular machinery requires a certain amount of Zn in the cytosol to serve the needs of cell organelles. Therefore, the Zn in excess of the nutritional needs is sequestered to avoid cytotoxic effects which can be remobilized when required. Vacuoles are assumed to be major sites of Zn sequestration and thereby detoxification (Martinoia *et al.*, 2007). In *A. thaliana*, metal tolerance protein 1 (MTP1)

and metal tolerance protein 3 (MTP3), members of the cation diffusion facilitator (CDF) family, heavy metal associated 3 (HMA3), a member of the P(1B-2) subgroup of the P-type ATPase family, and Zn-induced facilitator1 (ZIF1), a member of a vacuolar membrane major facilitator superfamily, have been shown to be implicated in vacuolar Zn sequestration (Drager *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; Arrivault *et al.*, 2006; Kawachi *et al.*, 2009; Morel *et al.*, 2009; Shahzad *et al.*, 2010; Haydon *et al.*, 2012). Under Zn-deficient conditions, the Zn stored in the vacuole has to be remobilized to the cytosol to serve the needs of the cell. We still have very poor knowledge of the transporters that could be involved in this process. It is thought that possible candidates should belong to either ZIP family transporters, yellow stripe1 (YSL1) family, or natural resistance-associated macrophage proteins (NRAMPs). A transporter involved in iron remobilization, AtNRAMP4, might also be involved in Zn remobilization. AtNRAMP4 is a tonoplast member of the NRAMP gene family (Lanquar *et al.*, 2005). Heterologous expression of *AtNRAMP4* complemented the growth phenotype of a *zrt1 zrt2 S. cerevisiae* mutant, suggesting its role in Zn transport across the membranes (Lanquar *et al.*, 2004). Transcript analysis revealed that *AtNRAMP4* was induced in the roots under excess Zn conditions (Van de Mortel *et al.*, 2006). The previous findings have established the role of AtNRAMP4 in the remobilization of iron from the vacuole. Whether NRAMP4 or some other member of the NRAMP family could also play a role in Zn remobilization still remains to be carefully examined. All these findings support the critical role of vacuoles that needs to be considered while developing Zn-biofortified cereals (Shahzad *et al.*, 2014).

The *Arabidopsis ZIF1* gene and the two other ZINC-INDUCED FACILITATOR-LIKE genes (*AtZIFL1* and *AtZIFL2*) form a distinct membrane protein family involved in regulating Zn homeostasis (Haydon and Cobbett, 2007). The expression of the *ZIF1* gene is up-regulated by Zn excess, and its mutation affects Zn distribution (Haydon and Cobbett, 2007; Haydon *et al.*, 2012). *ZIFL* genes are thought to accomplish a role in Zn translocation to the seeds. In *Arabidopsis*, *ZIF1* and *ZIFL1* are listed as candidates for seed Zn concentrations through a study of quantitative trait loci (QTLs) (Waters and Grusak, 2008). In barley, transcriptomic data analyses revealed that a *ZIF1-like* gene is expressed in the aleurone layer of seeds and its expression is induced in the embryo upon foliar Zn application (Tauris *et al.*, 2009). For further information on the *ZIFL* gene family (68 family members) in plants, readers are referred to Ricachenevsky *et al.* (2011). The remobilization of metal reserves such as Zn from leaves and its continuous uptake during seed filling constitutes the major source of metal supply to the seed (Curie *et al.*, 2001; Waters *et al.*, 2008; Inou *et al.*, 2009). Reverse genetics have revealed a role for the YSL members in the remobilization of Zn from senescing leaves, in the formation of pollen, and in the Zn loading of seeds. In *Arabidopsis*, expression of *YSL1* and *YSL3* has been observed in leaves during senescence (Waters *et al.*, 2006). Both genes are also expressed in pollen grains (Le Jean *et al.*, 2005; Waters *et al.*, 2006). Genetic evidence for the involvement of *YSL1* and *YSL3* in

pollen development was revealed through the characterization of the double *ys1/ys3* loss-of-function mutant (Waters *et al.*, 2006). All together, these data support the involvement of YSL1 and YSL3 in seed formation.

In conclusion, it has become clear that Zn transport and homeostasis in plants involve a large number of membrane proteins and chelators. It is necessary to undertake the task of determining the subcellular localization of the proteins involved in Zn transport, which would pave the way to understanding the physiological significance of their co-expression, and bring us one step closer to answering questions regarding their functional redundancy and/or interplay. Future challenges will be the purification of these large membrane proteins and obtaining their crystal structures. Such progress is necessary to obtain a better understanding of the molecular mechanisms regulating Zn transport in plants.

### *Zn sensing and signalling pathway*

How plants sense and transmit the signal of Zn deficiency remains poorly understood. Nevertheless, using available data on the identified transcription factors and target genes, together with their effects on Zn transport and accumulation in plants, a putative working model on Zn deficiency signalling has been proposed (Assuncao *et al.*, 2013). The two members of the bZIP transcription factors in *Arabidopsis*, bZIP19 and bZIP23, have been identified to play an important role in the response to Zn deficiency through the regulation of downstream genes (Assuncao *et al.*, 2010a). These target genes include members of the ZIP family of cation transporters (Guerinot, 2000). As mentioned above, ZIP members are likely candidates mediating root Zn uptake and transport, and hence are important players in the Zn homeostasis network. Because the Zn deficiency seems to be first sensed in shoots and the signal is then transmitted to the roots where these cation transporters are functioning (Assuncao *et al.*, 2013), suggests the presence of a long-distance Zn deficiency signalling molecule, yet to be identified. Within the set of bZIP19/23 target genes there are also the NAS genes *NAS2* and *NAS4*, encoding enzymes that catalyse the synthesis of NA (Assuncao *et al.*, 2010a, b). Both categories of supposed target genes (*ZIP* and *NAS*) of the bZIP19 and bZIP23 transcription factors are induced in response to Zn deficiency and contain a palindromic 10 bp motif, RTGTCGACAY, in their promoter region. An experimental demonstration of the importance of this *cis*-regulatory motif associated with genes that are induced by Zn starvation is awaited. Further research would focus on and should lead to the identification and characterization of additional transcription factors controlling the expression of key Zn-responsive genes identified by microarray approaches (Van de Mortel *et al.*, 2006). This could be achieved through direct genetics approaches. An *A. thaliana* transgenic line expressing a reporter gene (GFP or luciferase) under the control of the Zn-responsive promoter could be mutagenized using ethyl methanesulphonate (EMS), and used to screen for (i) mutant(s) impaired in the (reporter gene) response to Zn deprivation and (ii) mutant(s) showing unusual constitutive expression of the reporter gene

under control conditions (in the absence of Zn deprivation). Then, positional cloning or sequencing approaches can be used to identify one of the genes harbouring the mutations responsible for the altered response(s). To characterize these transcription factors functionally and gain more insights into how these regulatory proteins modulate the Zn signalling pathways, the TRANSPLANTA collection of *Arabidopsis* lines can be used (Coego *et al.*, 2014). This collection is a powerful resource; each line expressing one of 949 transcription factors under the control of a  $\beta$ -oestradiol-inducible promoter. Such an approach would allow the problem of severe developmental defects caused by constitutive overexpression of transcription factors to be overcome, and would permit a controlled conditional overexpression. In conclusion, discovering and characterizing transcription factors governing the expression of Zn-responsive genes will provide new insights into Zn homeostasis signalling pathways.

### **Interaction between zinc and phosphorus homeostasis.**

P interacts with many micronutrients such as manganese (Mn), iron (Fe), copper (Cu), and Zn (Murphy *et al.*, 1981; Broadley *et al.*, 2010; Perez-Novo *et al.*, 2011; Pedas *et al.*, 2011). Here, we will focus on the interplay between P and Zn at the physiological and molecular levels.

The Pi and Zn interaction occurs in the soil, the natural medium for plant growth. Numerous studies performed in various crop species indicate the existence of a negative relationship between Pi and Zn accumulation in plants (Reed, 1946; Bingham and Martin, 1956; Loneragan *et al.*, 1982; Cakmak and Marchsner, 1986; Norvell and Welch, 1993; Huang *et al.*, 2000; Zhu *et al.*, 2001). Lately such a negative relationship was also reported in *A. thaliana* (Misson *et al.*, 2005; Khan *et al.*, 2014). These studies have revealed the effect of Zn starvation on the overaccumulation of Pi nutrition in plants, which can lead to the appearance of Pi toxicity symptoms in high external Pi concentrations (Loneragan *et al.*, 1982). However, to date, the effect of Pi starvation on Zn nutrition has not been studied extensively, and very little information is available, such as a higher accumulation of Zn being observed during long-term Pi deprivation (Misson *et al.*, 2005). As already mentioned, the molecular bases of the Pi–Zn interactions remain poorly understood. In an attempt to better understand this interconnection, we will present below the available data on Pi–Zn interaction in soil, in yeast, and in plants. The evidence for the genetic basis of the Pi–Zn co-regulation that mediates the adaptation of a plant to the available Pi and Zn will be reviewed.

#### *Pi–Zn interaction in soil: influences of soil pH and root exudates*

The bioavailability of Pi and Zn in soil is critical for plant growth. Both these elements must be available, continuously, and in balanced proportions to support the metabolic demands of plants. Nevertheless, the bioavailability of Pi

and Zn concentrations in soil is low. For Pi, the low mobility can be attributed to its strong reaction in both solution and solid phases of the soil (Tagwira *et al.*, 1992, 1993; A.S. Wang *et al.*, 2006). Regarding Zn, its reduced bioavailability is often induced owing to its interactions with P, which become stronger in acidic soils (Tagwira *et al.*, 1992, 1993). Variations in soil pH affect Zn availability. At high pH, Zn availability in soil is low; while at low pH, Zn becomes bioavailable but the plant's capacity to absorb Zn becomes a limiting factor. To cope with low availability of Zn in alkaline soils, the plant excretes organic acids such as citrate, which lowers the soil pH, thus ultimately increasing the Zn availability. For instance, if a Zn deficiency occurs due to high pH and P levels in soil during the growing season, a foliar application of Zn is highly recommended (Tagwira *et al.*, 1992, 1993; A.S. Wang *et al.*, 2006). In wheat plants grown in calcareous soil with high pH, where Zn is not readily available, addition of Zn gives a higher Zn concentration in grains despite the decrease in Zn uptake caused by a simultaneous Pi supply (Lu *et al.*, 2011). The presence of P, especially at high concentrations, was found to boost Zn adsorption (Perez-Novo *et al.*, 2011). It is noteworthy that the choice of the Pi fertilizers can either raise or lower soil pH (Butcher *et al.*, 1989; Kashem and Singh, 2002) and consequently increase or decrease (Clark and McBride, 1984; Thakur *et al.*, 2006) the mobility of Zn. Interestingly, low Zn availability in soil can be linked to the type of Pi fertilizer that can decrease (monoammonium phosphate) or increase the pH (diammonium phosphate), presenting a typical reaction of Pi-induced Zn deficiency (Levi-Minzi and Petruzzelli, 1984). Application of superphosphate at different rates appears to have no effects on soil pH, whereas the application of Pi fertilizer contained in mixtures has been related to a decrease in soil pH (Hamon *et al.*, 1998; Kashem and Singh, 2002). From these considerations, it is clear that the type of Pi fertilizer is an important parameter to be taken into account in the fertilization models to increase the bioavailability of Pi and Zn and to regulate plant growth capacity. At the rhizosphere, the capacity of plants to modulate the development of the root architecture including root elongation and the frequency of lateral root formation also influences the uptake rate of Pi and Zn. The plant roots play an important role in producing exudate such as organic acids that chelate metal ions as a part of the mechanisms improving ion uptake (Curie *et al.*, 2001). For example, in white lupin, Pi deficiency provokes the formation of proteoid roots known to exude large quantities of citric acid (Gardner *et al.*, 1983; Tang *et al.*, 2013). Citrate exuded into the rhizosphere enhances Pi availability to the plant by solubilizing P in the soil that is bound to metal ions such as Zn (Gardner *et al.*, 1983; Fox *et al.*, 1990; Tang *et al.*, 2013), and this may explain the high productivity of white lupin on soils that have low plant-available P. Nevertheless, it remains unclear whether Zn transport occurs in chelated forms with organic acids such as citrate from the rhizosphere into plant roots, a phenomenon known for the absorption of the iron-MA complexes via YSL1 (Curie *et al.*, 2001). Therefore, additional work will be required to identify the chemical profile of these secreted compounds under Pi or Zn deficiency, and the

understanding of their biosynthetic pathways and their transport system to explore their possible roles in plant response to Pi and Zn homeostasis cross-talk.

The molecular mechanisms underlying how plants integrate multiple nutritional stimuli into root developmental programmes remains poorly understood (Gruber *et al.*, 2013; Kellermeier *et al.*, 2014). Pi deficiency has a marked effect on primary root growth, lateral root formation, and root hair production, elongation, and density (Bates and Lynch, 1996). Close inspection revealed that the Pi limitation causes a reduction in cell elongation as well as cell cycle activity in the root meristem, and consequently reduces the growth capacity of primary roots (Poirier and Bucher, 2002). Zn-deficient seedlings showed a reduction in primary root length and an increase in first- and higher order lateral root number (Jain *et al.*, 2013). In contrast to Pi starvation, Zn deficiency does not affect meristematic cells in the primary root (Jain *et al.*, 2013). It will be interesting to assess how plants regulate root growth in response to combined Pi and Zn deficiencies in comparison with Pi or Zn single stress.

#### *Involvement of phytic acid in the P and Zn transport and signaling*

As already mentioned in the Introduction, up to 90% of the total seed P is stored in the grain as PA, accounting for at least 1% of seed dry weight (Bohn *et al.*, 2008). PA is mainly found in the protein bodies of the embryo and aleurone layers (Steadman *et al.*, 2001), where other minerals are also preferentially deposited at a high concentration. The negatively charged Pi in PA strongly binds to metal cations such as Zn, to form a mixed salt called 'phytin' or 'phytate' (Bohn *et al.*, 2008). In cereals, numerous low PA plants have been generated thorough mutagenesis, such as wheat (Guttieri *et al.*, 2004), rice (Liu *et al.*, 2007), maize (Raboy *et al.*, 2000; Pilu *et al.*, 2003; Shi *et al.*, 2003), and barley (Rasmussen and Hatzack 1998). Silencing of a multidrug resistance-associated protein (MRP) transporter in sorghum causes a decrease in the PA concentration in seeds up to 86%, and consequently an increase in Zn absorption when analysed in Caco-2 cell lines (Kruger *et al.*, 2013). Future work would provide more insights into the effects of low PA cereals on Zn bioavailability in animal systems. The combined effect of supplying P and Zn to wheat in field conditions has been investigated and revealed that with added P the PA concentration in the grain was increased (Lu *et al.*, 2011). Moreover Zn-PA complexes were found in the vacuolar compartments of chalazal endosperm which is known to play a role in loading of minerals into the embryo at different stage of its development. For instance, in *A. thaliana*, Zn-PA crystals were shown to disappear from the endosperm early, between the globular and heart stages during bent-cotyledon stages (Otegui *et al.*, 2002). Beyond seeds, another study has shown that PA synthesis can occur in cell suspension culture of *Catharanthus roseus* and accumulates in vacuoles (Mitsuhashi *et al.*, 2005). This observation suggests the presence of a mechanism for PA transport from the cytosol, where it is synthesized into other organelles; however, so far, no plant protein has yet

been identified that mediates subcellular sequestration of PA. The same study also showed that plants supplemented simultaneously with high P and Zn could accumulate PA in non-vacuolar compartments, presumably the cytosol, probably because of the insolubility of phytate–Zn chelates. The precise mechanisms involved in PA–Zn subcellular location and its intracellular transport are not clearly understood and require further attention.

In a number of Zn-tolerant species such as *A. halleri* and *A. lyrata*, PA is implicated in binding and storage of Zn in roots (Rauser, 1999; Haydon and Cobbett, 2007). Sarret *et al.* (2002) reported a positive correlation between Zn and P concentrations in roots, and suggested that Zn is coordinated to P as Zn–PA in roots of both species and in leaves of *A. lyrata*. These data further support the presence of co-regulation of Pi–Zn nutrition in vegetative cells of intact plants, which may influence both P and Zn uptake and transport within a plant body. However, while the role of PA in regulation of Pi homeostasis and signalling has been demonstrated in a low PA *Arabidopsis* mutant (INOSITOL-PENTAKISPHOSPHATE 2-KINASE 1) *ipk1* (Stevenson-Paulik *et al.*, 2005), no available evidence on its implication in the regulation of Zn transport and/or signalling has been recorded. Therefore, further work should reconsider the role of PA in co-regulating Pi and Zn transport and signalling cross-talk.

#### *Insights on the Pi–Zn interaction in plants from transcriptomics*

Microarray technology has been successfully used to investigate the global transcriptional changes in roots and/or shoots in Pi-starved *Arabidopsis* plants (Hammond *et al.*, 2003; Wu *et al.*, 2003; Misson *et al.*, 2005; Muller *et al.*, 2007; Rouached *et al.*, 2011b; Bustos *et al.*, 2010; Woo *et al.*, 2012). In contrast, limited information is available on the effects of Zn deficiency on the global gene expression in *Arabidopsis*. Indeed, in the literature, only transcriptomic data in roots of *A. thaliana* Zn-deficient plants have been reported (Wintz *et al.*, 2003; Van de Mortel *et al.*, 2006). Therefore, obtaining transcriptomic data from shoots of Zn-deficient plants and/or plants exposed to Pi and Zn combined stresses is necessary to bring us closer to answering questions regarding the functional categories of genes involved in Zn and Pi cross-talk.

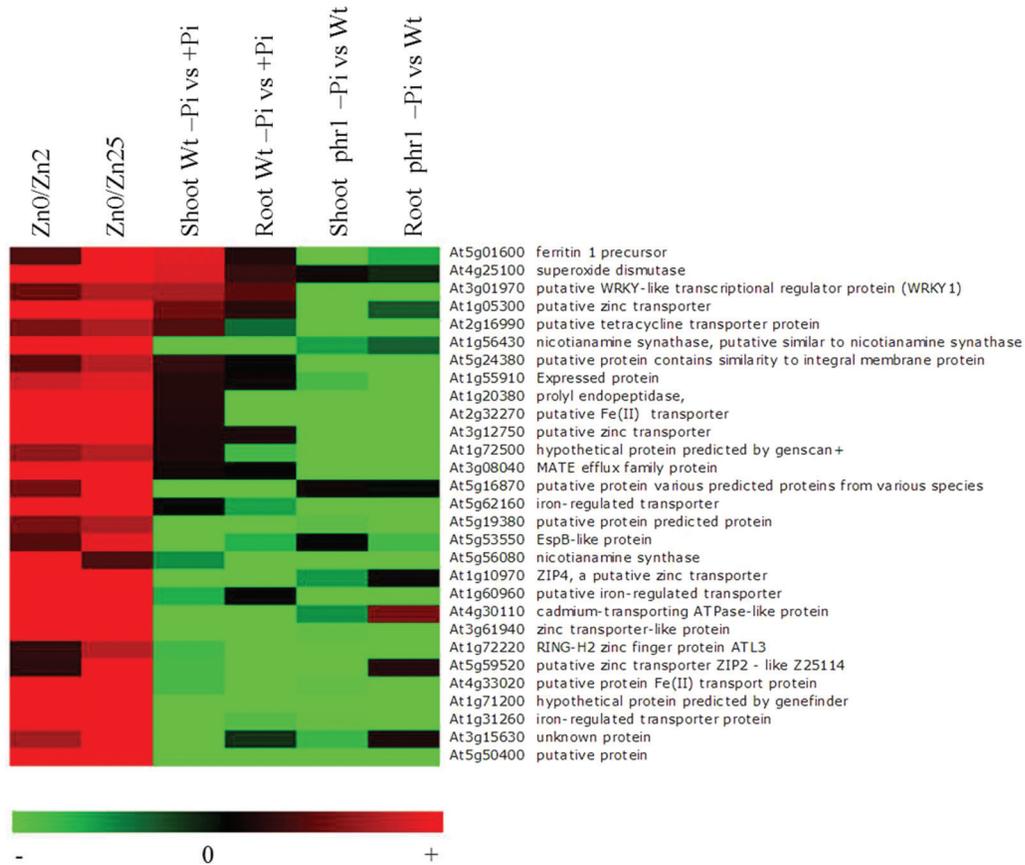
In the frame of this review, we considered transcriptomic data published by Bustos *et al.*, (2010) for Pi and by van de Mortel *et al.* (2006) for Zn deficiency in *A. thaliana*, and the expression levels of both Pi- and Zn-related genes were compared. On the one hand, analysis revealed that many Pi-related genes were induced in Zn-deficient *Arabidopsis* roots (van de Mortel *et al.*, 2006), consisting of those known to be involved in several physiological and biochemical processes tightly connected to Pi homeostasis, including signal transduction and sugar metabolism such as inositol-3-phosphate synthase isozyme 1 (At4g39800) and trehalose-6-phosphate phosphatase (At2g22190), respectively. On the other hand, large number of genes involved in heavy metal homeostasis were found to be up-regulated in *Arabidopsis* Pi-starved plants, including those encoding a member of the

MATE (multidrug and toxic compound extrusion) efflux family, FRD3 (AT3G08040) (Pfam profile: PF01554), the metal-associated domain-containing protein (Pfam profile PF00403), ABC-like transporter or ABC transporter such as NRAMP4 (AT3G13100), many Zn-finger proteins (e.g At5g54040, At3g52800, At3g19580, At5g18550, At1g76410, At5g04340, At2g19810, and At5g59820), and C2H2-type Zn finger proteins (ZFPs). These genes are known to be involved in the maintenance of metal homeostasis, and their induction may reflect the molecular response to Zn overload in plants induced by Pi deficiency (Misson *et al.*, 2005). The involvement of some of these genes in the regulation of Pi homeostasis in *Arabidopsis* has been demonstrated, for instance a gene from the subclass of C2H2 ZFP transcription factors (ZAT6) regulates Pi homeostasis through the control of root architecture (Devaiah *et al.*, 2007b).

Expression levels of Zn deficiency response genes identified by van de Mortel *et al.* (2006) in the microarray data obtained from the *Arabidopsis* wild type deficient for Pi and the *phr1* mutant were further analysed (Bustos *et al.*, 2010) (Fig. 1). This expression data analysis showed that the Pi and Zn deficiencies often act in the opposite directions on these sets of genes. Many genes induced by Zn deficiency were repressed by Pi deficiency (Fig. 1). Interestingly most of these genes were further repressed in the *phr1* mutant background, which further support the implication of the PHR1 transcription factor in the regulation of Pi-related genes under Zn deficiency. This observation is consistent with recent reports by Khan *et al.* (2014) which revealed the involvement of PHR1 in Pi–Zn homeostasis cross-talk in *Arabidopsis*. Our analysis also revealed the presence of gene clusters that are induced by both Zn and Pi deficiency (Fig. 1), including the putative Zn transporter (At1g05300), transcriptional regulator protein WRKY1-like (At3g01970), superoxide dismutase (At4g25100), and ferritin 1 precursor (At5g01600). Taken together, this expression analysis confirms the presence of a cross-talk between Pi and Zn regulatory networks in plants. Additional research efforts will be needed to determine how the Pi and Zn signals are relayed to the transcription machinery and to provide new insights into Pi–Zn signalling cross-talk.

#### *Pi–Zn interaction at the cellular level; lessons from studies in yeast*

The interaction between Pi and Zn occurs in yeast and has been revealed through studying the effect of Zn availability on the expression of the Pi transporter PHO84 and its activity. The *PHO84* gene encodes a cell surface high affinity transporter for Pi, and it is rapidly activated with relatively small changes in intracellular Pi (Pettersson *et al.*, 1999; Ogawa *et al.*, 2000; Wykoff and O'Shea, 2001; Springer *et al.*, 2003; Wykoff *et al.*, 2007). Mutation in the transcription factor *pho80* causes a constitutive expression of PHO84 (Pettersson *et al.*, 1999). Interestingly, it was observed that either *pho80* mutation or Zn deficiency stress causes a common effect on PHO84 expression. The relationships between Zn nutrition and Pi transport in yeast involving PHO84 is supported by



**Fig. 1.** Effects of varying concentrations of zinc and phosphate on the expression levels of Zn starvation response genes in *Arabidopsis*. Genes induced in roots of Zn-deficient *Arabidopsis* plants identified by Van de Mortel et al. (2006) are presented. The expression profile of these genes in response to Pi starvation in shoots and roots of the *Arabidopsis* wild type and the *phr1* mutant are listed (Bustos et al., 2010). The red colour represents an increase in expression relative to the wild type; the green colour represents a decrease in expression relative to the wild type. Zinc concentrations in the medium were 25  $\mu$ M (Zn25), 2  $\mu$ M (Zn2) or 0  $\mu$ M (Zn0).

additional lines of evidence; for instance, yeast cells lacking *PHO84* ( $\Delta$ *pho84*) are resistant to Zn toxicity (Jensen et al., 2003). In this context, *PHO84* not only controls cellular Pi but can also influence the uptake or homeostasis of Zn (Jensen et al., 2003). It is clear that to complement heterologous studies in yeast, future work should aim to explore the role of genes involved in the maintenance of Pi homeostasis signalling in the regulation of Zn transport/accumulation in plants. Additional regulatory mechanisms to those found in yeast might exist in plants, considering their complex morphology and biochemistry. More recently, Khan et al. (2014) provided evidence of the implication of *PHO* genes in the Pi-Zn interaction in *Arabidopsis* (detailed below).

#### *Pi-Zn interaction in plants: effect of Zn deficiency on Pi transport system and its accumulation in shoots*

The connection between the effects of Zn availability on Pi uptake and accumulation and vice versa has been widely described in various studies (Reed, 1946; Loneragan et al., 1982; Cakmak and Marschner, 1986; Norvell and Welch, 1993; Huang et al., 2000; Zhu et al., 2000; Khan et al., 2014). The Pi concentration decreases with the application of Zn (Robson and Pitman, 1983; Verma and Minhas, 1987), while application of Pi causes a decrease in Zn concentration in crop

plants (e.g. wheat and maize) (Robson and Pitman, 1983). Experiments performed in well-controlled mineral solutions have shown that the physiological interaction between Pi and Zn must occur to explain such antagonistic effects (Cakmak and Marschner, 1987). Very recently, Bouain et al. (2014) showed that the increase in Pi concentration in the growth medium decreases Zn accumulation in lettuce roots. These data suggest that Pi nutrition may directly influence the Zn uptake mechanisms in plants.

The expression of *PHT1* genes encoding high-affinity Pi transporters in roots is tightly controlled at the transcription level by the Pi availability (Muchhal and Raghothama, 1999). Interestingly, in barley, Zn deficiency causes an up-regulation in the expression of the *PHT1* Pi transporter and its activity despite an adequate supply of Pi (Huang et al., 2000). In *A. thaliana*, Jain et al. (2013) and Khan et al. (2014) have reported that Zn deficiency could induce the expression of *PHT1;1* in shoots with concurrent down-regulation in roots. This clearly indicates that the expression of specific *PHT1;1* is controlled by both the Pi and Zn status of the plants, probably through a coordination between Zn and Pi signalling pathways. Thus, under Zn deficiency, the plants lose the capacity to down-regulate the expression of genes encoding high-affinity Pi transporters in roots. Interestingly, this Zn-Pi relationship appears to be specific, since the expression of Pi

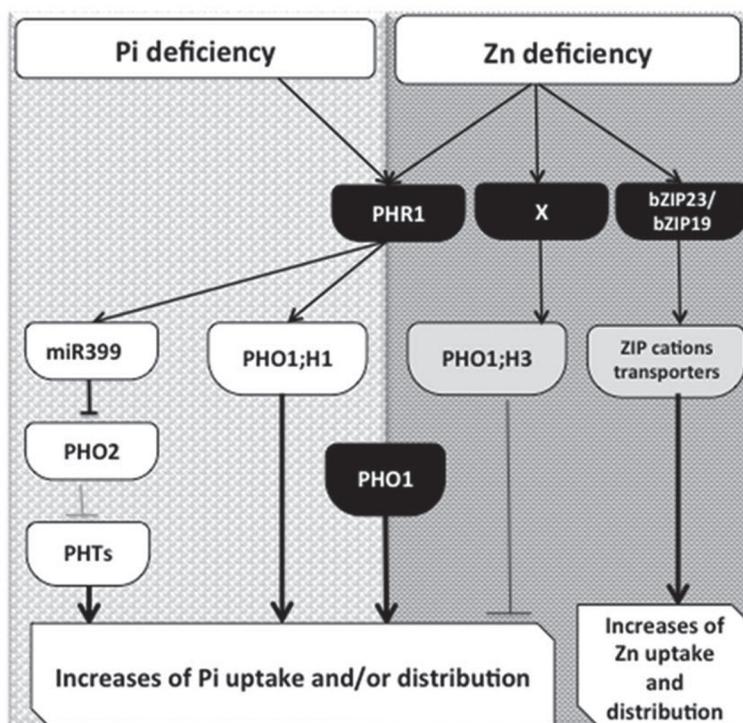
transporters is not induced by other macro- or micronutrients tested in barley roots (Huang *et al.*, 2000), in cotton (Cakmak and Marschner, 1986), or in tomato roots (Liu *et al.*, 1998). However, the intricate and complex regulation of Zn–Pi homeostasis is yet to be investigated to underpin the signalling pathways, which are activated by Zn deficiency to regulate Pi homeostasis. Furthermore, as the PHT1s are regulated post-transcriptionally in response to Pi starvation, how the activity of these transporters is regulated at the post-translational level under Zn deficiency remains to be elucidated.

The accumulation of Pi in the shoots of plants grown under Zn deprivation has been frequently observed, and the potential role of the shoot-derived signal as a determinant of this important process has been suggested (Cakmak and Marschner, 1986); however, no genes have been identified so far acting in the phenomenon. Very recently, Khan *et al.* (2014) demonstrated that *A. thaliana* Zn-deficient plants accumulate Pi in the shoots, and identified PHO1 and its homologue PHO1;H3, responsible for Pi export into the xylem, as key players in this process. Interestingly, when grown in Zn-free medium, the *pho1;h3* mutant plants display a higher Pi content in the shoots than wild-type plants (Khan *et al.*, 2014). Thus PHO1;H3 appears to act as a negative regulator of the Pi transfer to the shoot under Zn deficiency, even though this process requires PHO1. It is noteworthy that the expression patterns of PHO1;H3 and PHO1 show co-localization in the endomembrane system of the plant cells. Additionally, the same authors showed that the transcription factor PHR1 is also involved in the Pi–Zn cross-talk, and excluded the role of PHO2 in this Pi–Zn interaction,

since the *pho2* mutant exhibited the same phenotype as wild-type plants, overaccumulating Pi in their shoots when grown under Zn deficiency. Based on the evidence mainly from studies in *A. thaliana*, it is becoming possible to propose an integrative model for the regulation of genes involved in intracellular and interorgan Pi and Zn homeostasis under the deficiency of either one. PHR1 acts positively to regulate the root Pi uptake and its translocation under Pi deficiency, including *PHT1* gene family members and the *PHO1;H1* gene. *PHR1*, *PHO1*, and *PHO1;H3* are the common genes that link Zn and Pi nutrition and homeostasis (Khan *et al.*, 2014). Regulation of *PHO1;H3* expression by Zn deficiency involves as yet unidentified transcription factor, and deserve further investigation. Figure 2 represents the illustration of a molecular basis for the Zn–Pi signalling cross-talk.

## Conclusion

Continuing research in the field has made it increasingly evident that maintaining Zn and Pi homeostasis in plants is interconnected and requires tightly controlled mechanisms. This phenomenon is likely to provide great advantages for plants to cope with the fluctuations in the availability of Pi and Zn in various environments and stress conditions. Recent studies have provided evidence in favour of a genetic basis for the interconnection of these two elements. Bouain *et al.* (2014) showed that the co-regulation of Pi–Zn homeostasis may vary in varieties of the same plant species, for example lettuce plant



**Fig. 2.** Schematic representation of the regulatory pathways and their interconnections involved in plant phosphate and Zn deficiency responses. Under Pi deficiency, the PHR1–miR399–PHO2–PHT pathway is activated. Under Zn deficiency, the signalling pathways that involve bZIP23 and bZIP19 and specific cation transporter genes are activated (Assuncao *et al.*, 2013). *PHO1;H3*, which is induced by Zn deficiency, and the PHR1 and PHO1 proteins contribute to the Zn–Pi interaction. Negative and positive regulatory effects are indicated by grey lines and arrowheads, respectively.

varieties, which showed a contrasting behaviour in terms of the co-regulation of Pi and Zn homeostasis. **Broadley *et al.*, (2010)** reported that Zn deficiency leads to a substantial increase in the shoot P concentrations of many *Brassica oleracea* L. genotypes, thus proving to be a sufficient natural genetic variation to study some of the interactions between Zn and P nutrition. The future challenge will be to develop a comprehensive understanding of the coordination mechanisms of the homeostasis of these two elements by discovering new signalling and regulatory networks and elucidating how Zn and Pi signalling pathways are integrated into functional networks and to help plants grow better. The combination of transcriptomics and metabolomics can be one of the effective tools used to clarify cross-talk between metabolic pathways of Pi and Zn and could help the assigned metabolites to play negative and/or positive regulatory roles in the co-regulation of Pi and Zn nutrition. Finally, the development of a comprehensive understanding of Pi–Zn signalling cross-talk will be of great scientific interest, and crucial for sustainable agriculture worldwide.

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