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Overexpression of the bacterial phytase US417 in *Arabidopsis* reduces the concentration of phytic acid and reveals its involvement in the regulation of sulfate and phosphate homeostasis and signaling

Running head: *PA is involved in regulating P and SO4 homeostasis*

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Abbreviations: Pi, inorganic phosphate; MS, Murashige and Skoog;P, phosphorus; PA, phytic acid.

Abstract

Phytic acid (PA) is the main phosphorus storage form in plant seeds. It is recognized as an anti-nutrient for humans and non-ruminant animals, as well as one of the major sources of phosphorus that contributes to eutrophication. Therefore, engineering plants with low PA content without affecting plant growth capacity has become a major focus in plant breeding. Nevertheless, lack of knowledge on the role of PA seed reserves in regulating plant growth and in maintaining ion homeostasis hinders such an agronomical application. In this context, we report here that the overexpression of the bacterial phytase PHY-US417 in *Arabidopsis* leads to a significant decrease in seed PA, without any effect on the seed germination potential. Interestingly, this over-expression also induced a higher remobilization of free iron during germination. Moreover, the PHY-overexpressor lines show an increase in inorganic phosphate and sulfate contents, and a higher biomass production after phosphate starvation. Finally, phosphate sensing was altered because of the changes in the expression of genes induced by phosphate starvation or involved in phosphate or sulfate transport. Together, these results show that the over-expression of *PHY-US417* reduces PA concentration, and provide the first evidence for the involvement of PA in the regulation of sulfate and phosphate homeostasis and signalling.

Keywords

Homeostasis, phosphorus, phytic acid, phytase, sulfate, signalling.

Introduction

Phosphorus (P) is an essential element for optimal plant growth and animal nutrition. Plants serve as the major entry point for this element into the food chain. P is absorbed by plant roots from the soil predominantly as inorganic phosphate (Pi), which is present in very low concentrations (Marschner, 1995; Poirier and Bucher, 2002, Lin et al. 2014). At the end of the plant life cycle, Pi is mainly stored in the seed as phytic acid (PA), which is a myo-inositol ester with six phosphate groups (Raboy, 2009). The abundance of PA in crop seeds used as food and feed is a long-standing issue. PA is considered as a key anti-nutrient element for humans and non-ruminant animals (Raboy, 2006; Raboy, 2009). Indeed, the PA-associated P is in general not bioavailable to the non-ruminant animals because they produce little or no phytase in their digestive tract (Nys et al. 1996; Wodzinski et al. 1996). Phytases are special acid phosphatases with the capacity to hydrolyze PA by cleaving the Pi off the inositol backbone (Lei et al., 2013; Yao et al., 2011). They can be classified into four families namely histidine acid phosphatases, β-propeller phytases, purple acid phosphatases and more recently protein tyrosine phosphatase-like phytases (for review see Lei et al. 2013). Thus, monogastric diets need to be supplemented with Pi to meet the P requirement for animal growth. PA has additional anti-nutritional properties since it acts as a chelating agent for several essential cations such as iron (Fe), preventing their absorption, and thus contributing to mineral deficiencies (Maga et al. 1982; Raboy, 2009; Veum et al. 2009). On the other hand, high PA content in cereal seeds is one of the major P sources that contribute to eutrophication, which is aggravated by the release of Pi from the manure (Bali and Satyanarayana, 2001). Therefore, reducing the PA levels in grain would contribute to improving animal and human diets through better micronutrient resorption, and decreasing the negative impact of P excess on the environment.

The regulation of Pi homeostasis, transport and signaling in plants are highly regulated by complex processes (Rouached et al. 2010; Lin et al. 2014). At the transport level, multiple Pi transporters implicated in acquiring Pi at the root surface, distributing Pi to various organelles and organs have been identified including members of the PHT1 and the PHO1 gene families (Nussaume et al. 2011; Stefanovic et al. 2011; Bouain et al. 2014). At the signaling level, some regulatory pathways describing plant response to Pi starvation have been proposed (Bari et al. 2006; Lin et al. 2008a; Rouached et al. 2010; Lin et al. 2014), including key regulators such as the *Arabidopsis* noncoding RNAs *AT4* and *IPS1*, which were first described as phosphate starvation induced genes (Shin et al. 2006). Mutants affected in the above cited genes cause an alteration of Pi inter-organ distribution (for review Lin et al. 2014; Lott and West, 2001). The transport of Pi in plant is also influenced by a complex interconnection between the Pi homeostasis and those of essential nutrients such as iron (Fe) (Ward et al., 2008; Bournier et al., 2013; Bouain et al., 2014).Therefore, it is clear that future work that aims to modulate PA content in seed by manipulation of the Pi transport and/or signaling should first consider the complexity of these regulatory pathways.

In crop plants, various genetic and biotechnological strategies have been employed to reduce the PA contents (Lucca et al. 2001; Brinch-Pedersen et al. 2003; Chiera et al. al. 2004; Drakakaki et al. 2005). The classical mutagenesis approach has been used to generate mutants having low phytic acid (*lpa*) content (Raboy, 2009). This strategy was shown to be effective in cereals including wheat (Guttieri et al. 2004), maize (Raboy et al. 2000; Pilu et al. 2003; Shi et al. 2005), and rice (Larson et al. 2000; Kim et al. 2008a, b). The maize mutants, namely *lpa1-1* and *lpa2-1*, show a reduction in PA content by 66% and 50% in mature seeds respectively (Raboy et al. 2000). Molecular studies led to the identification of the genes responsible for the *lpa2* and *lpa3* phenotype in maize. LPA2 and LPA3 have been shown to be an inositol phosphate kinase and a myo-inositol kinase, respectively (Shi et al. 2003;

2005). While the maize *lpa1* gene encodes a multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi et al. 2007). However, many other genes responsible for the *lpa* phenotype remain largely unknown. It is important to note that the reduction in PA levels in the *lpa* mutants is sometimes associated with the alteration of agronomic traits such as crop yield (Roby, 2009). For this reason, Shi et al., 2007 proposed a new alternative where through silencing of the MRP-ABC transporter in an embryo-specific manner, they successfully produced maize and soybean seeds with low-PA and high-P contents and which germinate normally without significant decreases in seed dry weights. However, such a strategy was not always successful since in rice the seed-specific silencing of a MRP (osMRP5) causes a reduction of PA levels but also a decrease in seed weight accompanied by reductions in seed germination and seedling emergence (Li et al., 2014).

Despite its primary importance, the biological role of PA in plants remains poorly understood either under standard growth conditions or under challenging environments. A different strategy is now actively pursued, which is the generation of transgenic crops by manipulating the PA biosynthetic pathway (Kuwano et al. 2006; Kuwano et al. 2009; Suzuki et al.2007, Zhao et al. 2013; Kim and Tai.- 2011). Such a strategy becomes feasible in the light of a better understanding of the PA biosynthetic pathway, which involves both inositol-lipid intermediates and a lipid-independent pathway (Stevenson-Paulik et al.2005; Raboy, 2009). Some genes of the PA biosynthetic pathway have been cloned such as the inositol 3-phosphate synthase (MIPS) and several inositol polyphosphate kinases (*IPK1*, *IPK2*, *IPK3*, *INK1*) (Raboy, 2009). In rice, *lpa* lines were generated by seed-specific down regulation of MIPS, the key enzyme which catalyzes the conversion of glucose 6-phosphate into inositol monophosphate (Ins(3)P), the early substrate in PA biosynthetic pathway (Ali et al. 2013a; Kuwano et al. 2009). The *IPK1*gene is required for the later steps of PA synthesis and its mutation in *Arabidopsis* has lowered the PA content in seed (Stevenson-Paulik et al. 2005;

Kim and Tai, 2011). Furthermore, molecular and physiological analyses of several *lpa* mutants revealed the relevance of *IPK*s in phosphorylating IP intermediates. Recently, the RNAi-mediated seed-specific silencing of *OsIPK1* resulted in a substantial reduction in seed PA levels without negatively affecting the growth and development of transgenic rice plants (Ali et al. 2013b). This finding strongly supports the possibility of uncoupling low PA content from plant growth capacity.

On another hand, multiple attempts to increase Pi availability via PA hydrolysis through heterologous expression of microbial phytases *in planta* have been successfully made in the last decade (Brinch-Pedersen et al. 2002, Lei et al. 2013). In most cases, a fungal phytase of *Aspergillus niger* or bacterial phytases from *Bacillus subtilis* strains were expressed in transgenic plants as intracellular or secreted forms (Richardson et al. 2001; Brinch-Pedersen et al. 2002; Yip et al. 2003; Lung et al. 2005; Bilyeu et al. 2008). So far, numerous transgenic plants heterologously expressing a phytase have been developed, but one ideal enzyme for all applications does not exist. Therefore, screening for phytases with suitable catalytic properties and stability is of primary interest. In this report, we explored the effects of a novel thermostable phytase PHY-US417 from *B. subtilis* (Farhat et al. 2008) on plant growth in *Arabidopsis*. Our results showed that the overexpression of an intracellular form of PHY-US417 leads to a significant decrease in the PA content, which is concomitant with a higher Fe remobilization during seedling establishment compared to control plants. Remarkably, PHY-US417 overexpression results in up to 50% and 45 % increase in shoot Pi and sulfate (SO_4) concentrations respectively, and promotes plant growth with a significant enhancement in root growth capacity after Pi starvation. The results presented here strongly support a role for PA in the regulation of the Pi and the SO4 homeostasis and signalling pathways.

Results

The purified bacterial phytase PHY-US417 promotes growth of *Arabidopsis* seedlings under P-limited conditions

Few reports described previously the contribution of extracellular phytases or externally applied phytases in promoting the plant-growth capacity under Pi stress conditions (Idriss et al. 2002, Lung et al. 2008, Richardson et al. 2001). In this study, the β-propeller phytase PHY-US417 of *Bacillus subtilis* US417 was chosen because of its interesting catalytic properties since the *in vitro* enzymatic activity exhibited a great pH stability with an optimum at pH 7.5, and was previously reported to be specifically active on PA (Farhat et al. 2008). We were interested here to investigate whether these enzymatic properties can be of a beneficial effect for plant growth under P-limited conditions. For this reason, wild type *Arabidopsis* seedlings were germinated on standard MS growth medium and then transferred to low-P medium containing PA and in the presence or the absence of the purified enzyme PHY-US417. Compared to standard conditions, seedlings grown on low-P medium are dwarfed and exhibit small dark green leaves, which are typical Pi deficiency features (Figure 1A). When PA (0.33mM) was supplied as the sole P-source in the growth media, the growth of seedlings is also inhibited but to a less extent in comparison with those grown on low-P medium (Figure 1A). In the presence of the purified phytase PHY-US417 together with PA as a sole P-source, the seedlings grow far better, and even more vigorously than those grown on a standard MS. Under these conditions, the purified phytase seems to stimulate significantly root growth (Figure 1B), and the seedlings display up to ~ 5 times more shoot and root fresh biomasses than those grown on low-P medium (Figure 1C). Like other β-propeller phytases, PHY-US417 is expected to hydrolyse PA by sequential removal of only 3 Pi groups to finally generate myo-inositol triphosphates (Greiner et al., 2007; Kerovuo et al., 2000), and the addition of PA at a concentration of 0.33 mM would result in the release of ≅ 1mM Pi, the

usual concentration used in a standard MS medium. Therefore, this finding indicates that under P-limited conditions, the bacterial phytase is capable to restore and promote the growth of *Arabidopsis* seedlings most likely by hydrolyzing the PA present in the medium leading to the increase of the Pi bio-availability.

Generation of transgenic *Arabidopsis* plants overexpressing the bacterial phytase PHY-US417

The capacity of the supplied phytase PHY-US417 to promote plant growth under P-limited conditions prompted us to investigate the effect of the overexpression of the *phy*-US417 gene in *Arabidopsis* plants. The full length ORF encoding the mature form of this phytase (presumably cytoplasmic according to protein subcellular localization prediction programs, such as WoLF PSORT software http://wolfpsort.org) was first cloned in the binary vector pCAMBIA1302 under the control of the 35S promoter and the NOS terminator. The vector also bears the *HPT* gene (conferring resistance to hygromycin) as a selectable marker for plant transformation. After *Agrobacterium*-mediated transformation of *Arabidopsis* plants, several transformants were selected on an hygromycin-containing medium (see Materials and Methods). PCR screenings on genomic DNA of a number of T1 transformants allowed the amplification of both *HPT* and *phy*-US417 transgenes (supplemental figure 1B, C). Homozygous T3 plants were then generated, where both transgenes were shown to be stably expressed (supplemental figure 1D) at seemingly variable levels that might be linked to the transgene integration sites. Two transgenic lines (L7 and L9) harboring single transgene inserts were finally chosen for further molecular and physiological analyses.

The transgenic lines overexpressing *phy*-US417 gene exhibit higher phytase activities and lower seed PA concentrations

To assess whether PHY-US417 is active *in planta*, two week-old seedlings from both lines L7 and L9 grown on MS medium, were harvested for phytase assays. These assays were performed under optimal conditions (pH 7.5 and 55°C) for PHY-US417 but not for endogenous *Arabidopsis* phytases. Our results showed that phytase activities were approximately 8 and 28 times higher in the extracts of lines L7 (1.31±0,81U/g FW) and L9 (4.79±0,64U/g FW), respectively, in comparison with the wild-type control (0,17±0.04 U/g FW). Together with the expression data of the *phy*-US417 gene (supplemental figure 1D), this finding demonstrates that *Arabidopsis* plants can produce an active form of this phytase. The effects of the *phy*-US417 gene overexpression on the PA accumulation were determined in the seeds of the two transgenic lines L7 and L9 and wild type plants (Col) grown under standard condition as described by Haug and Lantzsch, 1983. Our results showed that both transgenic lines contain ~ 40% less PA compared to the control (Figure 2), thus demonstrating that the phytase PHY-US417 activity in planta resulted in decreasing the PA contents.

The overexpression of *phy*-US417gene in *Arabidopsis* improves plant growth after P re-supply

In order to assess the effect of the constitutive overexpression of *phy*-US417 on the tolerance of the transgenic lines L7 and L9 to Pi starvation, plant growth and fresh and dry masses were evaluated in plants grown for 5 to 20 days under Pi deficiency and then transferred to standard MS medium containing 1mM KH_2PO_4 (+Pi) for 10 additional days (Figure 3). The *Arabidopsis ipk1-1* mutant known to accumulate low PA level (Stevenson-Paullik et al. 2005) was also included in this experiment. Both transgenic lines show a better recovery from P-starvation than wild-type plants (Figure 3A). Although to a less extent, the *ipk1-1* mutant has also a better capacity to recover from P starvation. After ten days of growth on MS medium, L7 and L9 lines exhibited higher growth rates (especially of roots) which were illustrated by

net increases (up to 50%) in fresh and dry masses compared to wild-type plants (Figure 3B, C). These data demonstrate that the overexpression of the bacterial phytase promotes *Arabidopsis* growth during recovery from Pi-starvation.

The rate of Fe remobilization is higher in low phytic acid lines compared to wild-type plants

PA is deposited in seeds within protein storage vacuoles as mixed salts (Raboy, 2009) that are made with different metal cations such as Fe. In *Arabidopsis*, it has been shown that during germination, the Fe pool is remobilized from mature embryos to feed the developing tissues of the seedling (Roschzttardtz et al. 2009). Here, the effect of lowering seed PA on the Fe remobilisation during germination was examined using an Fe histochemical detection assay as described previously (Roschzttardtz et al. 2009). The Fe staining of the wild type, *ipk1-1* and the two PHY-overexpressor lines reveal that in mature embryos the Fe pattern is identical in all genotypes (Figure 4A). However, two days after germination it is clear that the concentration of Fe in the vascular tissues of the mutant and transgenic lines is significantly lower than in the wild-type, indicating that during the two days of post-germinative growth, the rate of Fe remobilization was higher in the mutant lines, compared to the wild type (Figure 4B). These data strongly suggest that the decrease in PA levels, via the overexpression of the *phy*-US417 gene may enhance the Fe remobilization during seed germination in *Arabidopsis*.

The PHY-US417 overexpression alters phosphate and sulfate concentrations in *Arabidopsis.*

We have investigated the effects of lowering PA levels in the *PHY*-overexpressing lines on the concentrations of major inorganic anions including Pi. Plants were grown on medium with low Pi concentration for 15 days. Only subsets of these plants were transferred to standard medium containing 1mM KH_2PO_4 (+Pi) for 10 additional days. Measurement of anion

concentrations revealed that under P-starvation (-Pi), the transgenic lines accumulated ~40-50% more Pi in their shoots than the wild type (Figure 5). Once transferred to medium with added Pi, the differences in shoot Pi-accumulation between wild type plants and transgenic lines were attenuated. In the roots, no significant differences were recorded between tested lines, neither under P starvation nor during the recovery phase. Interestingly, among other inorganic anions, the SO_4 anions accumulated to higher levels in the shoots (up to 45% increase) of the transgenic plants during Pi starvation (-Pi) and the recovery phase (+Pi), compared to wild-type plants (Figure 5).There is a less significant difference regarding SO_4 concentrations in the roots of the lines tested. No significant changes were recorded for other inorganic (*i.e.* nitrate) and organic anions (*i.e.* malate) in shoots or roots of the transgenic lines, in comparison to wild-type plants (data not shown). Therefore, these data demonstrate that the overexpression of PHY-US417 in P-limited *Arabidopsis* plants, affects inter-organ distribution of two essential macronutrients, Pi and SO_4.

Expression of phosphate and sulfate transporters is up-regulated in the PHY-US417 transgenic lines.

To better understand the underlying molecular mechanisms by which the reduction of the PA levels in the PHY-overexpressors affects Pi and SO_4 inter-organ distribution, we performed expression analysis of a subset of transporters: *PHT1;1, PHT1;4*, *PHO1*, and *PHO1;H1* (for Pi) (Stefanovich et al. 2007; Misson et al. 2004; Shin et al. 2004) and *SULTR1;1* and *SULTR1;2* (for SO4) (Yoshimoto et al. 2002). This expression analysis included also the nitrate (NO3) uptake transporters, *NRT1;1* and *NRT2;1,* (Forde, 2000; Tsay et al. 2007) as well as *IRT1* gene which is involved in Fe uptake (Vert et al. 2002). Both transgenic lines (L7 and L9), the *ipk1-1* mutant and wild-type plants were grown under –Pi conditions for 15 days and then transferred to standard conditions (+Pi) for 10 days, and the mRNA accumulation levels of these root transporters were determined by quantitative RT-PCR as described by

Rouached et al. (2008). The transcript abundances of the *PHT1;1, PHT1;4*, and *PHO1;H1*, but not *PHO1*, show an up-regulation in both L7 and L9 transgenic lines compared to wild-type plants (Figure 6). Also, the transcript accumulation of *SULTR1;2*, but not *SULTR1;1*, increases in these lines. No significant changes in the expression levels of the two tested NO_3 transporters, and of the *IRT1* gene, were observed. This result clearly indicates that the overexpression of PHY-US417, and consequently the reduction of PA levels, may affect both the Pi and SO_4 transport systems in *Arabidopsis*. Of note, the *ipk1-1* mutant shows expression patterns similar to those observed with both transgenic lines, which strongly suggests that the PA is involved in the signalling cascades regulating the expression of a subset of genes involved in Pi and SO_4 transport in the plant.

The overexpression of the *phy*-US417 gene affects phosphate sensing and signalling in plants

Because the L7 and L9 transgenic lines appear to have altered Pi homeostasis (similar to the *ipk1-1* mutant), and show a greater root growth capacity after Pi-starvation, we were interested to see whether the sensitivity to extracellular Pi supply was also altered. Seeds from the PHY-US417 overexpressor lines, the *ipk1-1* mutant, and wild-type plants were germinated in a vertical position on low-P MS medium for 5 days and then transferred to Pi-containing MS medium (+1 mM Pi) for 5 additional days. Subsequently, light microscopy observations of root sections revealed that both transgenic lines and the *ipk1-1* mutant produce long and numerous root hairs, which were completely absent on the roots of wild type plants (Figure 7A). These data, which are in line with those reported for the *ipk1-1* mutant (Stevenson-Paullik et al. 2005), strongly suggest that the overexpression of this bacterial phytase reduces the capacity of *Arabidopsis* to sense extracellular Pi levels, since L7 and L9 transgenic lines are unable to form shorter root hairs at higher Pi concentrations. To strengthen our hypothesis,

we further analysed the transcript accumulation of the two Pi starvation induced non coding *RNAs*, *IPS1* and *AT4*, in the *phy*-US417 transgenic lines and *ipk1-1* mutant in comparison to wild type plants grown on standard media (1 mM Pi). Interestingly, our results showed that compared to wild type plants, these marker genes are both induced in the transgenic lines albeit to lower levels than in the *ipk1-1*mutant (Figure 7B). Therefore, this finding reinforces the idea that lowering PA levels, either in the *PHY-US417* overexpressing lines or in the *ipk1-1* mutant affects Pi signalling in *Arabidopsis*.

Discussion

A comprehensive understanding of the role of PA in plant biology is of a great scientific interest, with implications for biotechnological and agronomic strategies aimed at reducing PA levels in the grain. PA is known as the storage compound of phosphorus in seeds. Divergent effects of lowering the PA content on seed germination potential have been reported (Kuwano et al. 2009; Nunes et al. 2006; Campion et al. 2009; Doria et al. 2009; Nusrat et al. 2013, Zhao et al. 2013). In this study, we have generated *Arabidopsis* transgenic lines compromised in their ability to accumulate PA by the overexpression of an intracellular form of the *B.subtilis* phytase PHY-US417. Interestingly, the decrease in PA levels in PHY-US417 transgenic seeds did not alter the germination potential, and even resulted in a better seedling establishment. So far, only few studies have described normal seed germination behaviour in plants accumulating lower levels of PA. In rice, silencing of the *IPK1* gene can mediate a substantial reduction in seed PA levels without negatively affecting the growth and development of transgenic plants (Ali et al. 2013). In *Arabidopsis*, the *ipk1* mutation causes a severe decrease in PA level without altering the kinetics of seed germination or the cotyledon's appearance (Stevenson-Pauliket al. 2005). So far, the transgenic lines reported in

this study, together with the *ipk1-1* mutant, offer a good opportunity to assess the biological role of PA in seed germination in *Arabidopsis*.

PA is a natural cation chelator (Borg *et al*. 2009), because of its negatively charged Pi side groups. It is generally assumed that the appropriate release of essential ions during seed germination is crucial to determine seedling vigor. Interestingly, the PHY-US417 transgenic lines, as well as the *ipk1-1* mutant, show a stronger remobilisation of Fe during germination compared to wild type (Figure 4). Therefore, it is tempting to propose that reduction of PA level leads to an increase in the proportion of bioavailable Fe in the endosperm, hence improving seedling establishment. Although the site of Fe storage is now well described in *Arabidopsis* embryos, the chemical form of this vacuolar pool of Fe remains unknown (Roschzttardtz et al. 2009). Nevertheless, the co-localization of Fe and P atoms in globoids inside the storage vacuoles (Lanquar et al. 2005) has led these authors to propose that Fe may be bound to PA and thus that during germination, the remobilization of Fe may require specific biochemical processes to release Fe atoms from this type of ligand. Although not demonstrated in the present report, the increased remobilization of Fe in mutants affected in their PA content is a strong indication that in the globoids, Fe is associated to PA and that specific enzymes may be required to allow the correct solubilisation of Fe before its efflux from the vacuole (Lanquar et al. 2005). Taken together, these results suggest that the normal germination potential and the growth promotion observed in the transgenic lines described here may be due to the phytase activity of PHY-US417, which by PA hydrolysis can reduce the harmful effects of PA linked to its binding capacity to essential cations for plant metabolism, such as Fe (Raboy et al.2009). This finding represents the first experimental illustration for a link between PA and Fe remobilisation during seedling establishment.

The regulation of plant growth is a complex process and involves several phytohormones, such as auxin, which may interact with PA. It has been reported that auxin response mutants,

axr1 and *aux1*, affect plant development and particularly root growth (Pitts et al. 1998). Interestingly, a link between PA and auxin has been established, since PA was found in crystals of the auxin receptor TIR1, and discussed as potent functional cofactor; but its precise role remains to be confirmed (Tan et al. 2007). Similarly inositol pentakisphosphate (InsP5) was found to be a cofactor of jasmonate receptor COI (Sheard et al. 2010). Therefore, the high affinity of PA and InsP5 with two homologous plant hormone receptors, COI1 and TIR1, offers profound implications for the role of inositol polyphosphates in plant hormone signaling. In our study, whether the alteration in PA concentration in the transgenic lines expressing the *phy*-US417 gene interferes with auxin and or jasmonate biosynthesis or responses (which ultimately may improve plant growth), requires further investigation.

On the other hand, regulation of ion homeostasis plays a key role in determining plant growth and responses to various environmental stresses. Results presented here provide lines of evidence that the heterologous expression of an intracellular phytase can improve *Arabidopsis* growth capacity under nutritional stress, namely Pi deficiency, without any apparent negative traits. The low-PA transgenic lines, L7 and L9, exhibit better growth under Pi-starvation compared to the control, as they appear more vigorous with expanded roots and shoots after stress recovery, suggesting a potential role for PA in the response of plants to Pi availability. The majority of low-PA mutants (*i.e. Atipk1-1* mutant) described in the literature exhibited an increase in inorganic phosphate, which is in favour of improving the availability of Pi and other micronutrients, such as Fe that are chelated by PA. The current study further supports this general observation (Figure 4 and Figure 5). Of note, the PHY-US417 overexpressing lines show an increase of intracellular Pi and SO4 concentrations in their shoots, under Pi-starvation, indicating an apparent defect in the 'rheostat' that monitors the Pi and SO4 status of the leaves. Once transferred to standard conditions, the shoot Pi and SO4 concentrations levels remains higher in *ipk1-1*mutant and transgenic lines in comparison to wild type. The

observed increase in the expression of genes involved in Pi and SO4 transport is consistent with the accumulation of Pi and SO4 in the shoot of the PHY-overexpressors and *ipk1-1* mutant. An interaction between SO4 and Pi homeostasis has been reported (Rouached et al. 2011), but no metabolite has yet been identified in higher plants that affects, at gene expression and physiological levels, the response to both elements. Our finding introduces PA as an attractive candidate for controlling Pi and SO4 transport and homeostasis. PA is known to accumulate in large amounts in seeds but much less in other organs including leaves (Bentsink et al., 2003; Raboy, 2003). It has been also shown that PA synthesis can occur in vacuoles of cell suspension cultures (Mitsuhashi et al., 2005). In this work, we showed that the PA levels decrease in the seeds of PHY-overexpressors. Whether PA contents are also affected in other organs of these transgenic lines, remains to be determined. Although PA concentrations in mature roots/shoots were not detected by the method used, the results indicated that the modulation of PA levels may affect directly or indirectly the expression of P-related genes and root architectural traits. It is likely that such effects could be due not only to Pi mobilization but also to regulatory, and in particular hormonal networks.

In addition, it appears that low-PA transgenic lines L7 and L9 are affected in Pi sensing. Indeed, the microscopic analysis revealed that in contrast to wild-type plants, the roots of both transgenic lines develop root hairs even when Pi was supplied in the media. These observations are in line with the previous report by Stevenson-Paulik et al. (2005). Taken together, these results strongly suggest that low PA levels interfere with the regulation of Pi sensing. The Pi signalling is also altered in these lines, since the expression of *IPS1* and *AT4* genes is up-regulated in the roots of the transgenic lines grown under standard conditions whereas in wild type plants, both genes are only induced under Pi-starvation (Burleigh and Harrison, 1999; Liu et al. 1997). It is worth to note that *IPS1* and *AT4* genes are also known to interfere with the shoot-to-root Pi distribution (Shin et al. 2006). In this context, given their

similar phenotypes, it will be interesting to explore a possible genetic interaction between *Arabidopsis at4* or *ips1* and the low PA lines.

In conclusion, this report demonstrates that the bacterial phytase *PHY-US417* is effective in *Arabidopsis* and its expression leads to a significant decrease in the seed PA content, without any effect on the seed germination potential. Such a feature would encourage exploiting this bacterial phytase as a biotechnological means for sustainable and environmentally friendly agriculture. The data presented here strongly support an active role of PA in the regulation of Pi and SO_4 homeostasis including transport and signalling. The future challenge will be to develop a comprehensive understanding of the role of PA in the acquisition and assimilation of these two elements by discovering new regulatory networks. Therefore, this knowledge will be certainly valuable not only for an effective and a more targeted approach to engineering low-phytate crops, but also to fully appreciate the role of PA in the regulation of plant physiology and metabolism.

Materials and Methods

Plant material, growth conditions and P-stress treatments

Seeds of-*Arabidopsis thaliana* ecotype Columbia (Col-0) (used as a wild-type control for all experiments), transgenic lines expressing *phy*-US417 gene (see below) and the *ipk1-1* mutant (Stevenson-Pauliketal. 2005) were used in these experiments. Seeds were sterilized with 70% ethanol for 5 min and with 5%bleach for 15 min, rinsed five times with sterile water and plated on MS medium agar with 1 % sucrose and 0.8% (w/v) agar (Sigma, A1296) under a photoperiod of light/dark cycle of 8 h/16 h, light intensity of 250 μmol·m^{-2}·s^{-1}, and temperature of 22–24°C.

For growth analyses under low phosphate conditions, 7 day-old wild-type seedlings grown on MS agar medium (Murashige and Skoog, 1962) were transferred onto the following media: standard MS medium (with 1mM KH_2PO_4) or low-P MS medium (with 5 µM KH_2PO_4) in which Pi was replaced with 0.33 mM PA without or with added phytase PHY-US417 (6.5U) purified as previously described (Farhat et al. 2008).

For recovery tests, seedlings were first grown on low-P MS medium for 5, 10, 15 or 20 days and then transferred carefully to complete MS medium for 10 days. After photo documentation, the plant materials (shoots and roots) were collected for fresh and dry weight determinations, as well as quantitative real-time PCR and High-Performance Ion Chromatography (HPIC) analyses.

For light microscopy, seedlings were grown on vertical plates on low-P medium for 5 days and then transferred to MS medium for further 5 days. Then roots were regularly scanned using an Epson Perfection 1240U scanner.

Molecular cloning and generation of transgenic plants

The nucleotide sequence encoding the mature form of the phytase PHY-US417 (accession no. AM501550) of *Bacillus sutilis* (Farhat et al. 2008) was amplified by PCR with *Pfu*Turbo

DNA polymerase (Stratagene; La Jolla, CA, USA), using the PHYC-Bg (5'GCAGATCT**ATG**TCCGATCCTTATCATTTTACC3'), and PHYC-Sp (5'GCACTA-GTTTATTTTCCGCTTCTGTCGGTC3') primers, which harbour the *Bgl*II and *Spe*I restriction sites respectively. An ATG start codon was introduced into the forward primer PHYC-Bg, immediately upstream the coding sequence of the mature peptide. The amplified fragment was first subcloned into the pGEM-T easy cloning vector (Promega) and then introduced as *Bgl*II-*Spe*I fragment into the pCAMBIA1302 binary vector between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. This binary vector contains also the hygromycin resistance gene (*HPT*) as a selection marker. The resulting construct pC1302-phy US417 (Supplemental figure 1A) was used to transform *Arabidopsis thaliana* (ecotype Columbia, Col-0) plants by floral dipping using *Agrobacterium* strain GV3101 (Clough and Bent, 1998).

Transgenic plants were selected on MS agar medium supplemented with 15 µg/ml hygromycin. From twenty T1 independent transformants, six T2 lines showing a 3:1 segregation for hygromycin resistance (indicative of single transgene insertion) were grown up to T3 generation to identify plants homozygous for the transgene and from which two lines, L7 and L9, were chosen for further detailed molecular and physiological analyses.

Molecular characterization of transgenic lines

Genomic DNA was prepared from fresh leaves of independent transformants. The presence of *phy-US417* and *HPT* genes was verified by PCR screening. The forward and reverse primers for *HPT* gene were HPTIIF (5'ATGAAAAAGCCTGAACTCAC3') and HPTIIR (5' CTCT-ATTTCTTTGCCCTCG3'), and those for the *phy-US417*gene were PHYC-Bg and PHYC-Sp.

To perform expression analysis by RT-PCR, total RNA was first extracted from 200 mg of two week-old seedlings of wild type *Arabidopsis* and transgenic lines using Trizol reagent

(Invitrogen) according to the manufacturer's protocol.To remove contaminating DNA, RNAs were treated with DNaseI (Promega) at 37°C for 15 min. DNase-treated RNA samples (2 ug) were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reactions were performed at 37°C for 1 h using 2 μM oligo-dT18. Two microlitres of first strand cDNAs were employed as a template for PCR amplifications with a pair of *phy-US417*-specific primers, PHYC-Bg/PHYC-Sp or HPT-specific primers HPTIIF/HPTIIR. An *Arabidopsis* Actin gene fragment, used as an internal control, was amplified with the primers: ActF: 5' GGCGATGAAGCTCAATCCAAACG-3' and ActR: 5'-GGTCACGACCAGCAAGATCAAGACG-3'. For PCR reactions, samples were denatured for 5 min at 94 °C and then run for 30 cycles of 30 sec at 94 °C, 1 min at 56°C, and 30 sec at 72 °C with a final extension of 5 min at 72 °C. Finally, the RT-PCR products were separated by agarose gel electrophoresis.

Quantitative RT-PCR

Total RNA was isolated from frozen root tissues of *Arabidopsis* plants using the RNeasy Plant mini Kit (Qiagen) according to the manufacturer's protocol. RNA samples were quantified with Nano Drop spectrophotometer (Thermo Scientific) and cDNA was synthesized from 1 μg of total RNA using an oligo(dT) primer and M-MLV reverse transcriptase (Promega). Quantitative real-time RT-PCR was performed with the LightCycler® 480 Real-Time PCR System (Roche) using SYBR green dye technology. PCR reactions were done in a 10 μl containing 500 nM of each forward and reverse primers, 5 ul of the SYBER green I master and 1 μl of 1:3 cDNA dilution. Reactions were performed in lightCyber® 480 Multiwell Plates 384 (Roche) covered with optical film (Sarstedt). Specific primers used for quantitative real-time RT-PCR analysis are described in Pfaffl et al. (2002) and Rouached et al. (2008). All mRNA levels were calculated from threshold cycle values

(C_T, Livak and Schmittgen, 2001) and as relative to controls and normalized with respect to Ubiquitin 10 (UBQ10) transcript levels according to Gruber et al. (2001).

Phytase Activity assay

Phytase activity was estimated colorimetrically by monitoring the release of Pi from PA (Na-InsP6; P-8810; Sigma) as previously reported (Engelen *et al.* 1994). Briefly, two-week old seedlings (three replicates, 20 plants per replicate) were ground in a mortar and pestle at 4°C with three volumes (v/w) of MES/Ca buffer (15 mM MES buffer with 0.5 mM CaCl2, pH 7.5) containing 1 mM EDTA. Extracts were then centrifuged for 10 min at 12,000g and from which100 µg were reacted at 55°C for 30 min in a 100 mM NaOAc (pH 7.5) assay buffer containing 2 mM PA as a substrate. The reaction was terminated with the addition of an equal volume of 10% (w/v) trichloroacetic acid. The released Pi was quantified spectrophotometrically at 820 nm with molybdate-blue method (Murphy and Riley, 1962). Background readings due to Pi contamination (time 0) were subtracted before calculation. One unit (U) of phytase is the activity that releases 1 µmol of phosphate per minute under these assay conditions. The protein concentrations were determined according to Bradford method (1976) using bovine serum albumin as a standard.

Phytic acid measurement

The seed PA contents were determined by colorimetric method as described previously (Haug and Lantzsch, 1983). Four mg of seeds were extracted with 0.4 N HCl during 3.5 h with constant stirring at room temperature (RT). The extract was transferred into a glass tube with a stopper ammonium iron (III)-sulfate solution (0.02% NH_4Fe(III)-sulfate in 0.2 N HCl). Next, the mixture was heated at 95°C for 30 min and cooled on ice for 15 min, followed by an incubation at RT for 20 min. Then, the mix was centrifuged at 4000g at room temperature for 30 min and the supernatant was transferred to another glass tube to which 1% (w/v) 2,2'-

bipyridine in 1% (v/v) thioglycolic acid was added. After a defined time, absorbance was measured at 519 nm.

High-performance ion chromatography analysis

Anion content measurements were performed as described by Rouached et al. (2008). Briefly, after determining the mass of the fresh shoot and root materials, anions were extracted by incubating the tissue in Milli-Q water heated at 70°C for 60 min. The extract was centrifuged for 10 min at maximum speed, and the supernatants were passed through 0.2 µm filters. Anion content of the final clear supernatant was determined by HPIC (LC20 Dionex) using an IonPaq AS11 column and a sodium hydroxide linear gradient (Rouached et al. 2008). The identification and quantification of each anion were performed using the Chromeleon software (Dionex).

Iron histochemical detection

Embryos were dissected from dry seeds after 2h of imbibition in water. Seedlings were germinated on half-strength MS medium without sucrose and Fe and grown for 2 days at 21°C in long days (16h light). For Fe histochemical detection, mature embryos and 2-day-old seedling were stained using the Perls/DAB procedure described by Roschzttardtz et al.(2009). The presence of Fe is revealed as brown pigments.

Statistical analyses

Statistical analyses of the data were performed using analysis of variance (ANOVA) and the Tukey's test to compare mean values. Only differences with P values < 0.05 were considered as significant.

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Figures legends

Figure1. Effect of *in vitro* phytase supply on the growth of *Arabidopsis* wild type plants.

(A) Seedlings were first grown on MS medium for one week then transferred to standard MS medium (MS with 1 mM Pi, as a control), or to low-P medium (MS-P), in the absence or in the presence of 0.33 mM phytic acid (PA) and the phytase PHY-US417 (6.5U). (**B)** Photographs of the roots of seedlings grown under the above conditions. All photographs were taken 15 days after transfer. Representative data of three biological replicates are shown. (C) Fresh weights of shoots and roots from seedlings grown under conditions indicated in A). Measurements were recorded 15 days after transfer. Data are mean± SD of three individual replicates. Each replicate was obtained from the analysis of shoots and roots collected from a pool of six plants.

Figure 2. Measurement of seed phytate concentrations in wild type plants and in the two PHY-US417 transgenic lines L7 and L9. All plant genotypes including wild type Col-0, were grown in the green house and seeds were collected at the end of their life cycle. Seed PA concentrations were determined by colorimetric method as described previously (Haug and Lantzsch,1983) and expressed as μmol PA/g seed. Data are mean± SD of three individual replicates. Each replicate was obtained from the analysis of seeds collected from a pool of six plants.

Figure 3. Growth performance of *Arabidopsis* PHY-US417 overexpressing lines grown under Pi-limited conditions. Seedlings germinated on Pi deficient MS medium (-Pi) were transferred after 5, 10, 15 or 20 days, to standard MS plates (+Pi) and grown for additional 10 days.**(A)** Photographs of representative wild-type (Col), *ipk1-1* mutant and transgenic lines (L7, L9)

were taken 10 days after transfer to MS medium (+Pi), following their growth on low-P medium for 15 days. **(B), (C)** Fresh and dry weights of shoots and roots of WT, *ipk1-1* mutant and L7 and L9 lines which were grown on low-P medium for 5 to 20 days as indicated, and then transferred to MS medium for 10 days. Mean ± SD is shown for three replicates. Stars indicate a statistically significant difference ($P<0.05$).

Figure 4. Fe remobilization is faster in low phytate mutants during germination.
Fe was stained with Perls/DAB in mature embryos extracted from dry seeds (upper panels) or in 2-days old seedlings (of wild type, *ipk1-1*, and transgenic lines L7 and L9) after germination in liquid medium without Fe (lower panels).

Figure 5. Effect of the overexpression of PHY-US417 in *Arabidopsis* on organ phosphate and sulfate concentrations. Wild-type plants (Col-0), *ipk1-1*, as well as the PHY-US417 transgenic lines were grown on medium containing low Pi (-Pi) for 15 days, followed by additional 10 days in media containing 1 mM Pi (+Pi). Shoots and roots were harvested separately and anion concentrations expressed as $\mu mol.gFW^{-1}$, were quantified by HPIC. Individual measurements performed before (-Pi) and after (+Pi) transfer of seedlings to MS containing 1mM of Pi, were obtained from the analysis of shoots or roots collected separately from three biological repeats. Each repeat was obtained from the analysis of shoots and roots collected from a pool of six plants. Error bars indicate SD; (a) and (b) are values significantly different ($P < 0.05$) from WT plants.

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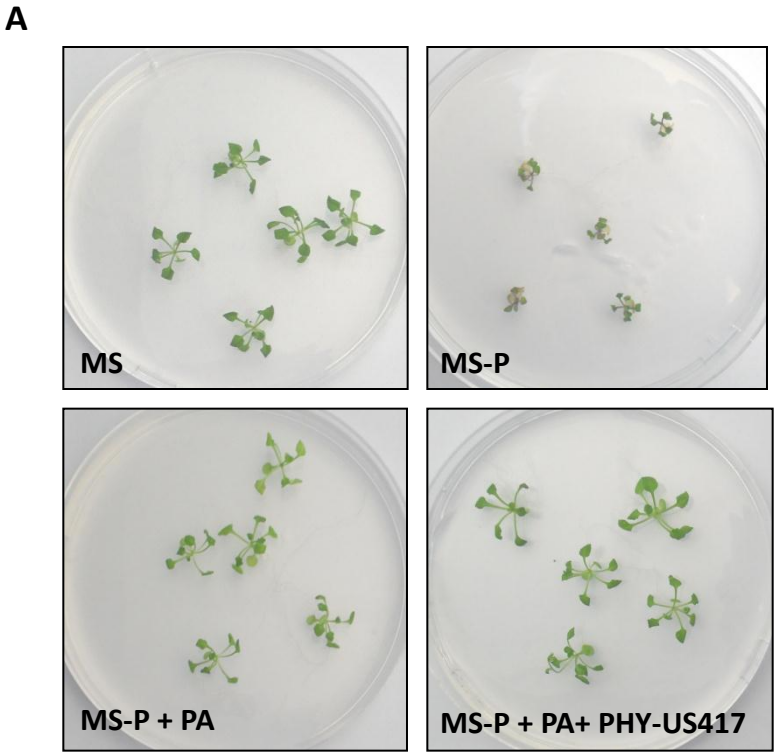
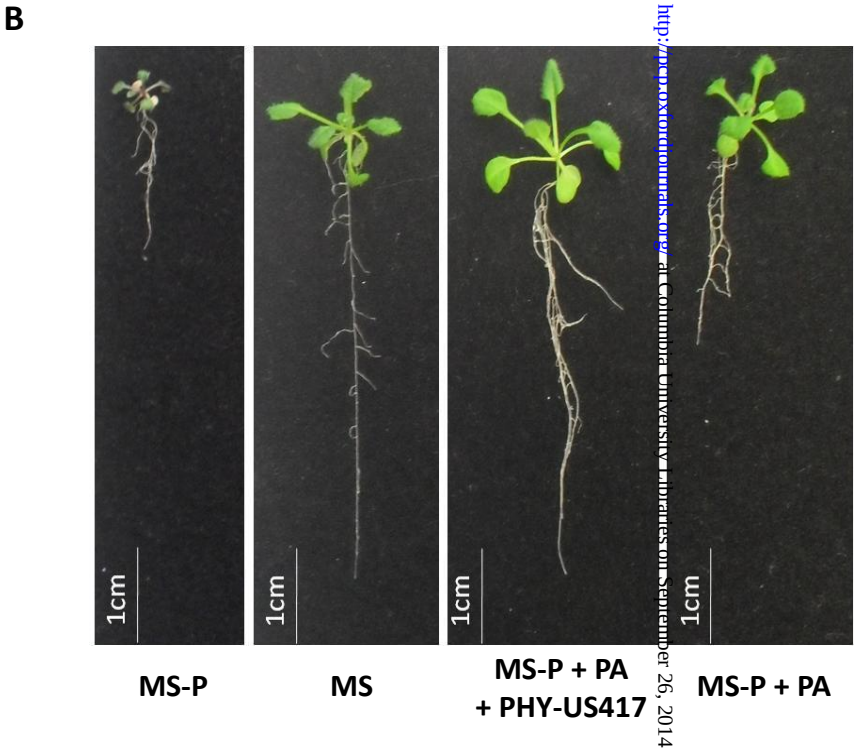
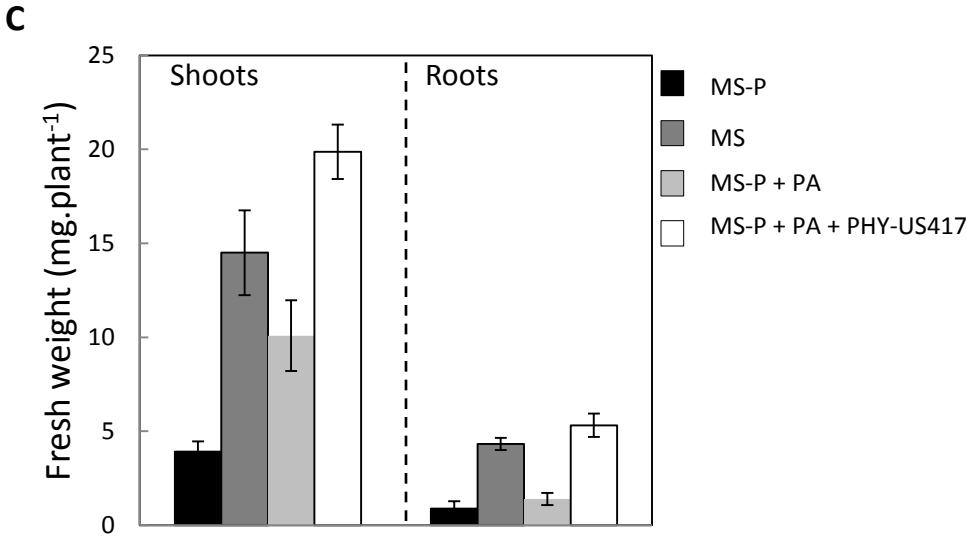
Figure 7. The overexpression of *PHY*-US417 gene alters Pi sensing and signalling in *Arabidopsis*.**(A)** Representative images of primary root and root hairs of 10 day-old Wild-type (Col), *ipk1-1*mutant and PHY-US417 overexpressor linesL7 and L9 grown for 5 days on MS +Pi after 5 days on low-P medium. Images were taken by light microscopy (Olympus Soft imaging system). The root segments chosen for examination of root hair formation are indicated in additional windows. **(B)** Expression levels of two phosphate starvation induced non-coding RNAs, *IPS1* and *AT4*, determined by quantitative RT-PCR from 10-old seedlings grown as described in A).Transcript levels were normalized to the level of the control ubiquitin-10 mRNA (*UBQ10*: At4g05320). Error bars correspond to SD of three biological repeats and each repeat was obtained from the analysis of roots collected from a pool of six plants.

Supplemental figure 1. Generation of transgenic *Arabidopsis* plants overexpressing *phy*-US417 gene. **(A)** Expression construct pC1302-phy US417, containing the CaMV 35S promoter (P35S), the *phy*-US417gene and the NOS polyadenylation signal (Nos-PA), within

T-DNA left (LB) and right (RB) borders. PCR screenings for HPT **(B)** and *phy-US417***(C)** genes on 14 independent transformants (lanes 1-14) together with wild type plant (WT). (-) indicates control for PCR where H_2O was used instead of DNA. Expected sizes of the PCR products are indicated. Lane M: λPstI marker. **(D)** RT-PCR analyses of *HPT* and *phy*-US417 expression in10 representative transformants (lanes 1-10) and in a line transformed with the pCAMBIA1302 empty vector (lane V). (-RT): without reverse transcriptase. A 380 bp *Actin* fragment was amplified by RT-PCR as internal control.







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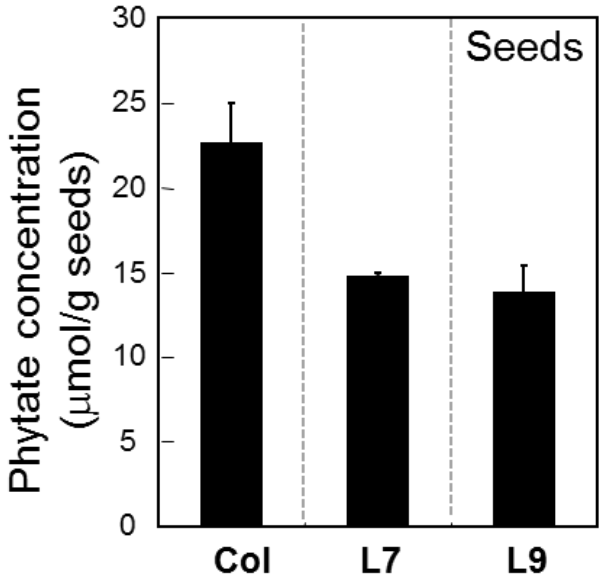

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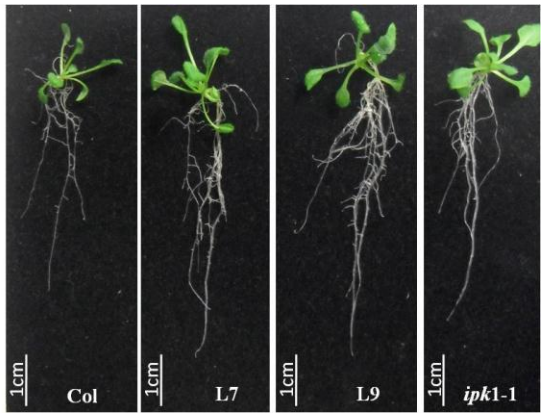

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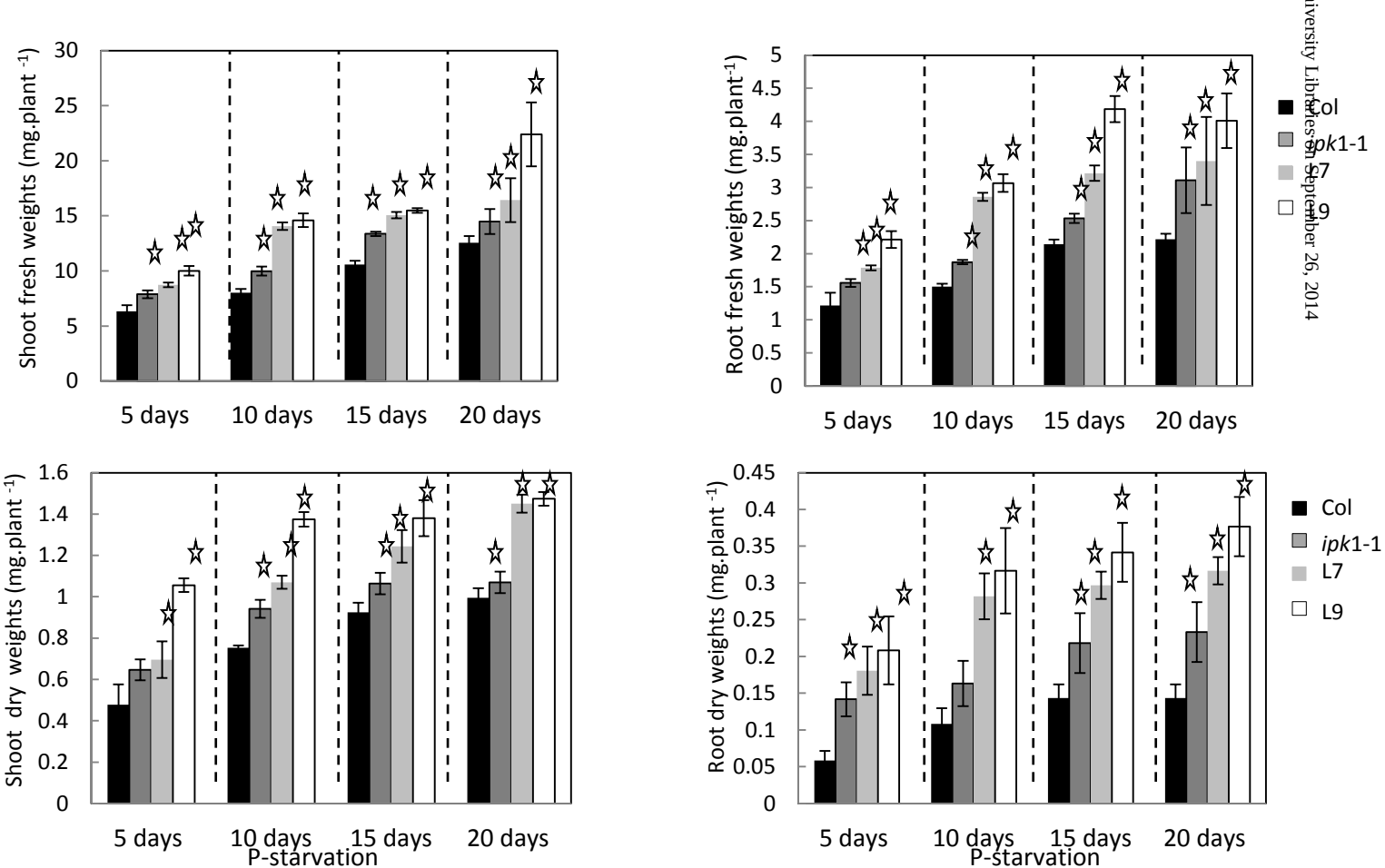

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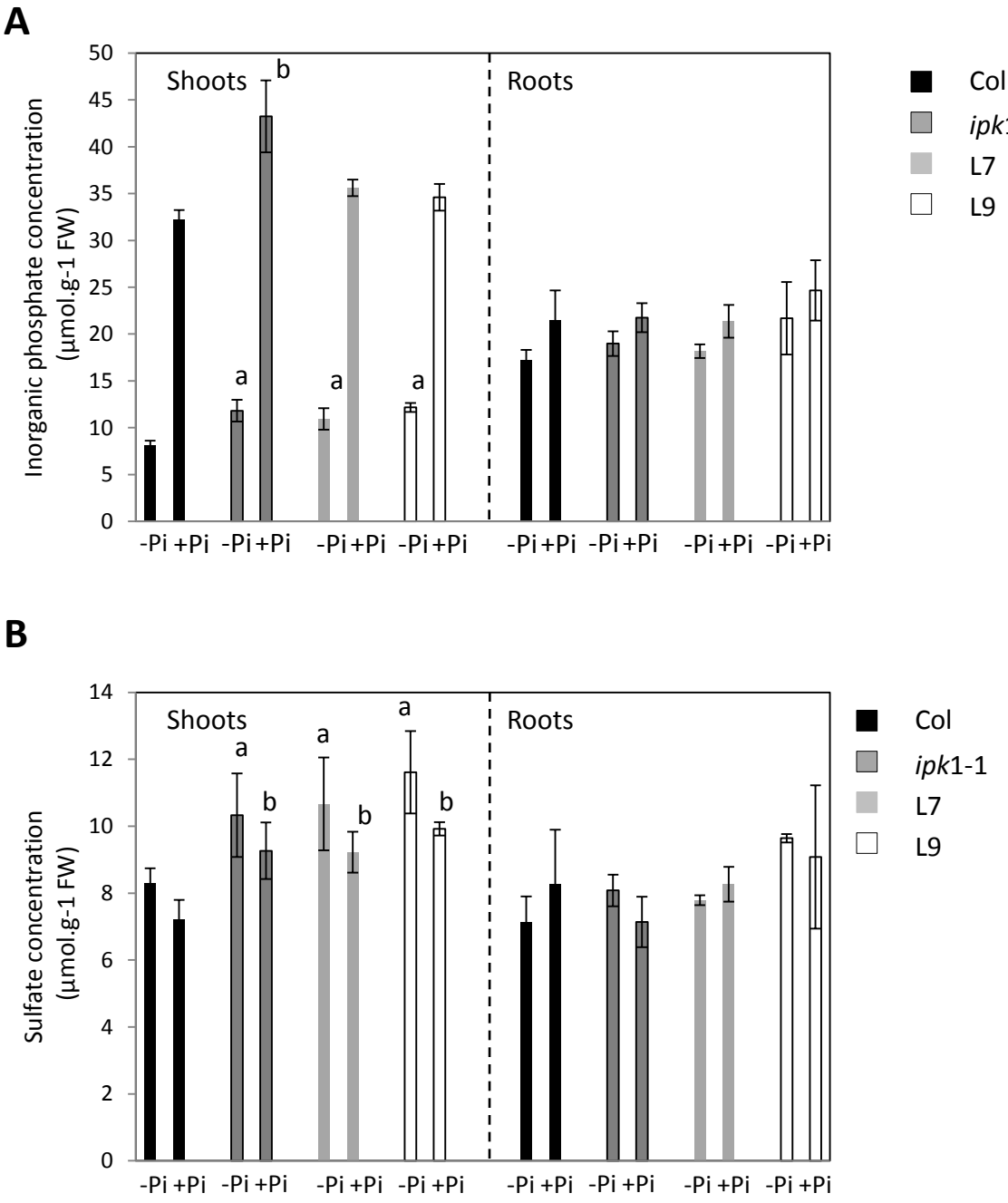

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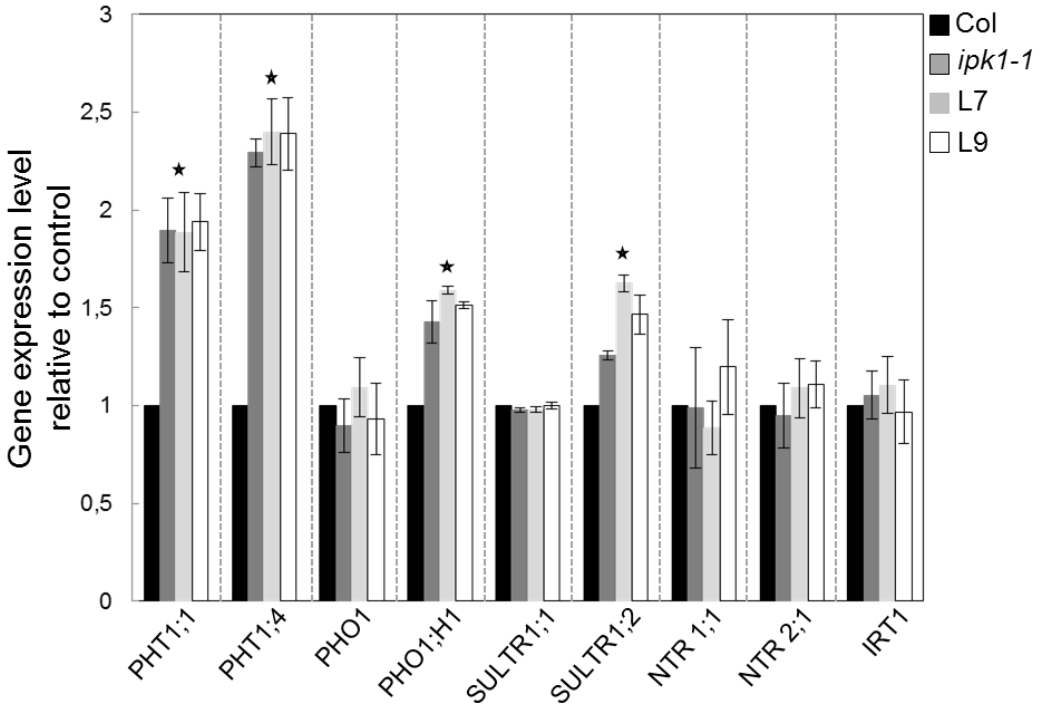

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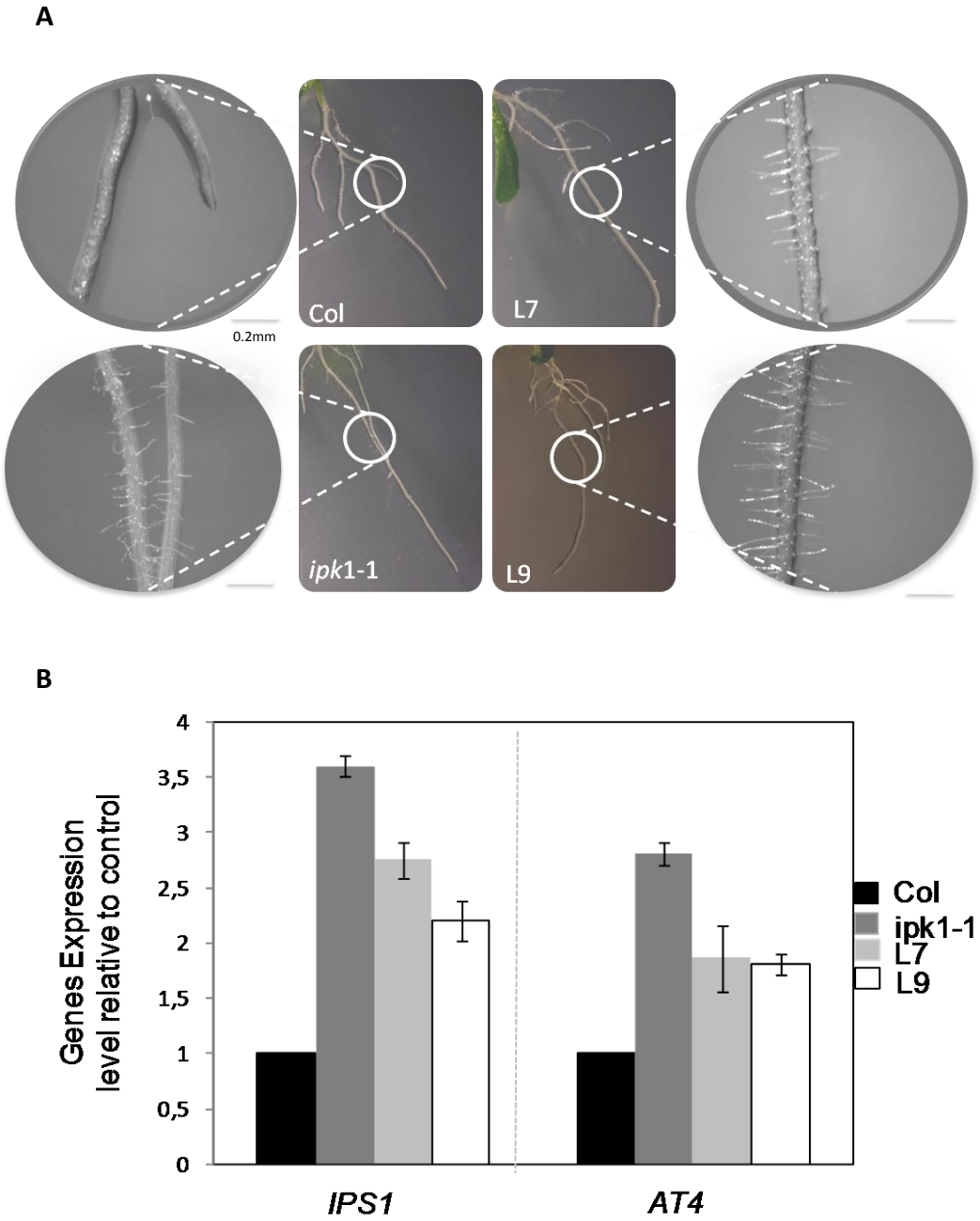

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