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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



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For Peer Review

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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Subject area: environmental and stress responses

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1 supplemental figure

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

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

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26

27

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

Abstract

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

47

Keywords

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

50

51 **Introduction**

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

75 The regulation of Pi homeostasis, transport and signaling in plants are highly regulated by
76 complex processes (Rouached et al. 2010; Lin et al. 2014). At the transport level, multiple Pi
77 transporters implicated in acquiring Pi at the root surface, distributing Pi to various organelles
78 and organs have been identified including members of the PHT1 and the PHO1 gene families
79 (Nussaume et al. 2011; Stefanovic et al. 2011; Bouain et al. 2014). At the signaling level,
80 some regulatory pathways describing plant response to Pi starvation have been proposed (Bari
81 et al. 2006; Lin et al. 2008a; Rouached et al. 2010; Lin et al. 2014), including key regulators
82 such as the *Arabidopsis* noncoding RNAs *AT4* and *IPSI*, which were first described as
83 phosphate starvation induced genes (Shin et al. 2006). Mutants affected in the above cited
84 genes cause an alteration of Pi inter-organ distribution (for review Lin et al. 2014; Lott and
85 West, 2001). The transport of Pi in plant is also influenced by a complex interconnection
86 between the Pi homeostasis and those of essential nutrients such as iron (Fe) (Ward et al.,
87 2008; Bournier et al., 2013; Bouain et al., 2014). Therefore, it is clear that future work that
88 aims to modulate PA content in seed by manipulation of the Pi transport and/or signaling
89 should first consider the complexity of these regulatory pathways.

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

100 2005). While the maize *lpa1* gene encodes a multidrug resistance-associated protein (MRP)
101 ATP-binding cassette (ABC) transporter (Shi et al. 2007). However, many other genes
102 responsible for the *lpa* phenotype remain largely unknown. It is important to note that the
103 reduction in PA levels in the *lpa* mutants is sometimes associated with the alteration of
104 agronomic traits such as crop yield (Roby, 2009). For this reason, Shi et al., 2007 proposed a
105 new alternative where through silencing of the MRP-ABC transporter in an embryo-specific
106 manner, they successfully produced maize and soybean seeds with low-PA and high-P
107 contents and which germinate normally without significant decreases in seed dry weights.
108 However, such a strategy was not always successful since in rice the seed-specific silencing of
109 a MRP (*osMRP5*) causes a reduction of PA levels but also a decrease in seed weight
110 accompanied by reductions in seed germination and seedling emergence (Li et al., 2014).

111 Despite its primary importance, the biological role of PA in plants remains poorly understood
112 either under standard growth conditions or under challenging environments. A different
113 strategy is now actively pursued, which is the generation of transgenic crops by manipulating
114 the PA biosynthetic pathway (Kuwano et al. 2006; Kuwano et al. 2009; Suzuki et al. 2007,
115 Zhao et al. 2013; Kim and Tai.- 2011). Such a strategy becomes feasible in the light of a
116 better understanding of the PA biosynthetic pathway, which involves both inositol-lipid
117 intermediates and a lipid-independent pathway (Stevenson-Paulik et al. 2005; Raboy, 2009).

118 Some genes of the PA biosynthetic pathway have been cloned such as the inositol 3-
119 phosphate synthase (MIPS) and several inositol polyphosphate kinases (*IPK1*, *IPK2*, *IPK3*,
120 *INK1*) (Raboy, 2009). In rice, *lpa* lines were generated by seed-specific down regulation of
121 MIPS, the key enzyme which catalyzes the conversion of glucose 6-phosphate into inositol
122 monophosphate (Ins(3)P), the early substrate in PA biosynthetic pathway (Ali et al. 2013a;
123 Kuwano et al. 2009). The *IPK1* gene is required for the later steps of PA synthesis and its
124 mutation in *Arabidopsis* has lowered the PA content in seed (Stevenson-Paulik et al. 2005;

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

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

148

149 Results

150 The purified bacterial phytase PHY-US417 promotes growth of *Arabidopsis* seedlings 151 under P-limited conditions

152 Few reports described previously the contribution of extracellular phytases or externally
153 applied phytases in promoting the plant-growth capacity under Pi stress conditions (Idriss et
154 al. 2002, Lung et al. 2008, Richardson et al. 2001). In this study, the β -propeller phytase
155 PHY-US417 of *Bacillus subtilis* US417 was chosen because of its interesting catalytic
156 properties since the *in vitro* enzymatic activity exhibited a great pH stability with an optimum
157 at pH 7.5, and was previously reported to be specifically active on PA (Farhat et al. 2008). We
158 were interested here to investigate whether these enzymatic properties can be of a beneficial
159 effect for plant growth under P-limited conditions. For this reason, wild type *Arabidopsis*
160 seedlings were germinated on standard MS growth medium and then transferred to low-P
161 medium containing PA and in the presence or the absence of the purified enzyme PHY-
162 US417. Compared to standard conditions, seedlings grown on low-P medium are dwarfed and
163 exhibit small dark green leaves, which are typical Pi deficiency features (Figure 1A). When
164 PA (0.33mM) was supplied as the sole P-source in the growth media, the growth of seedlings
165 is also inhibited but to a less extent in comparison with those grown on low-P medium (Figure
166 1A). In the presence of the purified phytase PHY-US417 together with PA as a sole P-source,
167 the seedlings grow far better, and even more vigorously than those grown on a standard MS.
168 Under these conditions, the purified phytase seems to stimulate significantly root growth
169 (Figure 1B), and the seedlings display up to ~ 5 times more shoot and root fresh biomasses
170 than those grown on low-P medium (Figure 1C). Like other β -propeller phytases, PHY-
171 US417 is expected to hydrolyse PA by sequential removal of only 3 Pi groups to finally
172 generate myo-inositol triphosphates (Greiner et al., 2007; Kerovuo et al., 2000), and the
173 addition of PA at a concentration of 0.33 mM would result in the release of \cong 1mM Pi, the

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

178

179 **Generation of transgenic *Arabidopsis* plants overexpressing the bacterial phytase PHY-** 180 **US417**

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

196

197 **The transgenic lines overexpressing *phy*-US417 gene exhibit higher phytase activities** 198 **and lower seed PA concentrations**

199 To assess whether PHY-US417 is active *in planta*, two week-old seedlings from both lines L7
200 and L9 grown on MS medium, were harvested for phytase assays. These assays were
201 performed under optimal conditions (pH 7.5 and 55°C) for PHY-US417 but not for
202 endogenous *Arabidopsis* phytases. Our results showed that phytase activities were
203 approximately 8 and 28 times higher in the extracts of lines L7 (1.31 ± 0.81 U/g FW) and L9
204 (4.79 ± 0.64 U/g FW), respectively, in comparison with the wild-type control (0.17 ± 0.04 U/g
205 FW). Together with the expression data of the *phy-US417* gene (supplemental figure 1D),
206 this finding demonstrates that *Arabidopsis* plants can produce an active form of this phytase.
207 The effects of the *phy-US417* gene overexpression on the PA accumulation were determined
208 in the seeds of the two transgenic lines L7 and L9 and wild type plants (Col) grown under
209 standard condition as described by Haug and Lantzsch, 1983. Our results showed that both
210 transgenic lines contain ~ 40% less PA compared to the control (Figure 2), thus demonstrating
211 that the phytase PHY-US417 activity in planta resulted in decreasing the PA contents.

212

213 **The overexpression of *phy-US417* gene in *Arabidopsis* improves plant growth after P re-** 214 **supply**

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

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

227

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

230 PA is deposited in seeds within protein storage vacuoles as mixed salts (Raboy, 2009) that are
231 made with different metal cations such as Fe. In *Arabidopsis*, it has been shown that during
232 germination, the Fe pool is remobilized from mature embryos to feed the developing tissues
233 of the seedling (Roschztardt et al. 2009). Here, the effect of lowering seed PA on the Fe
234 remobilisation during germination was examined using an Fe histochemical detection assay as
235 described previously (Roschztardt et al. 2009). The Fe staining of the wild type, *ipk1-1* and
236 the two PHY-overexpressor lines reveal that in mature embryos the Fe pattern is identical in
237 all genotypes (Figure 4A). However, two days after germination it is clear that the
238 concentration of Fe in the vascular tissues of the mutant and transgenic lines is significantly
239 lower than in the wild-type, indicating that during the two days of post-germinative growth,
240 the rate of Fe remobilization was higher in the mutant lines, compared to the wild type (Figure
241 4B). These data strongly suggest that the decrease in PA levels, via the overexpression of the
242 *phy-US417* gene may enhance the Fe remobilization during seed germination in *Arabidopsis*.

243

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

246 We have investigated the effects of lowering PA levels in the PHY-overexpressing lines on
247 the concentrations of major inorganic anions including Pi. Plants were grown on medium with
248 low Pi concentration for 15 days. Only subsets of these plants were transferred to standard
249 medium containing 1mM KH₂PO₄ (+Pi) for 10 additional days. Measurement of anion

250 concentrations revealed that under P-starvation (-Pi), the transgenic lines accumulated ~40-
251 50% more Pi in their shoots than the wild type (Figure 5). Once transferred to medium with
252 added Pi, the differences in shoot Pi-accumulation between wild type plants and transgenic
253 lines were attenuated. In the roots, no significant differences were recorded between tested
254 lines, neither under P starvation nor during the recovery phase. Interestingly, among other
255 inorganic anions, the SO₄ anions accumulated to higher levels in the shoots (up to 45%
256 increase) of the transgenic plants during Pi starvation (-Pi) and the recovery phase (+Pi),
257 compared to wild-type plants (Figure 5). There is a less significant difference regarding SO₄
258 concentrations in the roots of the lines tested. No significant changes were recorded for other
259 inorganic (i.e. nitrate) and organic anions (i.e. malate) in shoots or roots of the transgenic
260 lines, in comparison to wild-type plants (data not shown). Therefore, these data demonstrate
261 that the overexpression of PHY-US417 in P-limited *Arabidopsis* plants, affects inter-organ
262 distribution of two essential macronutrients, Pi and SO₄.

263
264 **Expression of phosphate and sulfate transporters is up-regulated in the PHY-US417**
265 **transgenic lines.**

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

276 Rouached et al. (2008). The transcript abundances of the *PHT1;1*, *PHT1;4*, and *PHO1;H1*,
277 but not *PHO1*, show an up-regulation in both L7 and L9 transgenic lines compared to wild-
278 type plants (Figure 6). Also, the transcript accumulation of *SULTRI;2*, but not *SULTRI;1*,
279 increases in these lines. No significant changes in the expression levels of the two tested NO₃
280 transporters, and of the *IRT1* gene, were observed. This result clearly indicates that the
281 overexpression of PHY-US417, and consequently the reduction of PA levels, may affect both
282 the Pi and SO₄ transport systems in *Arabidopsis*. Of note, the *ipk1-1* mutant shows expression
283 patterns similar to those observed with both transgenic lines, which strongly suggests that the
284 PA is involved in the signalling cascades regulating the expression of a subset of genes
285 involved in Pi and SO₄ transport in the plant.

286

287 **The overexpression of the *phy*-US417 gene affects phosphate sensing and signalling in** 288 **plants**

289 Because the L7 and L9 transgenic lines appear to have altered Pi homeostasis (similar to the
290 *ipk1-1* mutant), and show a greater root growth capacity after Pi-starvation, we were
291 interested to see whether the sensitivity to extracellular Pi supply was also altered. Seeds from
292 the PHY-US417 overexpressor lines, the *ipk1-1* mutant, and wild-type plants were germinated
293 in a vertical position on low-P MS medium for 5 days and then transferred to Pi-containing
294 MS medium (+1 mM Pi) for 5 additional days. Subsequently, light microscopy observations
295 of root sections revealed that both transgenic lines and the *ipk1-1* mutant produce long and
296 numerous root hairs, which were completely absent on the roots of wild type plants (Figure
297 7A). These data, which are in line with those reported for the *ipk1-1* mutant (Stevenson-
298 Paullik et al. 2005), strongly suggest that the overexpression of this bacterial phytase reduces
299 the capacity of *Arabidopsis* to sense extracellular Pi levels, since L7 and L9 transgenic lines
300 are unable to form shorter root hairs at higher Pi concentrations. To strengthen our hypothesis,

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

308

309 Discussion

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

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

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

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

350 *axr1* and *aux1*, affect plant development and particularly root growth (Pitts et al. 1998).
351 Interestingly, a link between PA and auxin has been established, since PA was found
352 crystals of the auxin receptor TIR1, and discussed as potent functional cofactor; but its precise
353 role remains to be confirmed (Tan et al. 2007). Similarly inositol pentakisphosphate (InsP5)
354 was found to be a cofactor of jasmonate receptor COI (Sheard et al. 2010). Therefore, the high
355 affinity of PA and InsP5 with two homologous plant hormone receptors, COI1 and TIR1,
356 offers profound implications for the role of inositol polyphosphates in plant hormone
357 signaling. In our study, whether the alteration in PA concentration in the transgenic lines
358 expressing the *phy-US417* gene interferes with auxin and or jasmonate biosynthesis or
359 responses (which ultimately may improve plant growth), requires further investigation.
360 On the other hand, regulation of ion homeostasis plays a key role in determining plant growth
361 and responses to various environmental stresses. Results presented here provide lines of
362 evidence that the heterologous expression of an intracellular phytase can improve *Arabidopsis*
363 growth capacity under nutritional stress, namely Pi deficiency, without any apparent negative
364 traits. The low-PA transgenic lines, L7 and L9, exhibit better growth under Pi-starvation
365 compared to the control, as they appear more vigorous with expanded roots and shoots after
366 stress recovery, suggesting a potential role for PA in the response of plants to Pi availability.
367 The majority of low-PA mutants (*i.e.* *Atipk1-1* mutant) described in the literature exhibited an
368 increase in inorganic phosphate, which is in favour of improving the availability of Pi and
369 other micronutrients, such as Fe that are chelated by PA. The current study further supports
370 this general observation (Figure 4 and Figure 5). Of note, the PHY-US417 overexpressing
371 lines show an increase of intracellular Pi and SO₄ concentrations in their shoots, under Pi-
372 starvation, indicating an apparent defect in the ‘rheostat’ that monitors the Pi and SO₄ status
373 of the leaves. Once transferred to standard conditions, the shoot Pi and SO₄ concentrations
374 levels remains higher in *ipk1-1* mutant and transgenic lines in comparison to wild type. The

375 **observed** increase in the expression of genes involved in Pi and SO₄ transport is consistent
376 with the accumulation of Pi and SO₄ in the shoot of the PHY-overexpressors and *ipk1-1*
377 mutant. An interaction between SO₄ and Pi homeostasis has been reported (Rouached et al.
378 2011), but no metabolite has yet been identified in higher plants that affects, at gene
379 expression and physiological levels, the response to both elements. **Our** finding **introduces** PA
380 as an attractive candidate for controlling Pi and SO₄ transport and homeostasis. **PA is known**
381 **to accumulate in large amounts in seeds but much less in other organs including leaves**
382 **(Bentsink et al., 2003; Raboy, 2003).** It has been also shown that PA synthesis can occur in
383 vacuoles of cell suspension cultures (Mitsuhashi et al., 2005). **In this work, we showed that**
384 **the PA levels decrease in the seeds of PHY-overexpressors. Whether PA contents are also**
385 **affected in other organs of these transgenic lines, remains to be determined. Although PA**
386 **concentrations in mature roots/shoots were not detected by the method used, the results**
387 **indicated that the modulation of PA levels may affect directly or indirectly the expression of**
388 **P-related genes and root architectural traits. It is likely that such effects could be due not only**
389 **to Pi mobilization but also to regulatory, and in particular hormonal networks.**

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

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

402 In conclusion, this report demonstrates that the bacterial phytase *PHY-US417* is effective in
403 *Arabidopsis* and its expression leads to a significant decrease in the seed PA content, without
404 any effect on the seed germination potential. Such a feature would encourage exploiting this
405 bacterial phytase **as a** biotechnological means for sustainable and environmentally friendly
406 agriculture. The data presented here strongly support an active role of PA in the regulation of
407 Pi and SO₄ homeostasis including transport and signalling. The future challenge will be to
408 develop a comprehensive understanding of the role of PA in **the acquisition and assimilation**
409 of these two elements by discovering new regulatory networks. Therefore, this knowledge
410 will be certainly valuable not only for an effective and a more targeted approach to
411 engineering low-phytate crops, but also to fully appreciate the role of PA in the regulation of
412 plant physiology and metabolism.

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417 **Materials and Methods**

418 *Plant material, growth conditions and P-stress treatments*

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

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

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

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

439 *Molecular cloning and generation of transgenic plants*

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

443 DNA polymerase (Stratagene; La Jolla, CA, USA), using the PHYC-Bg
444 (5'GCAGATCTATGTCCGATCCTTATCATTTTACC3'), and PHYC-Sp (5'GCACTA-
445 GTTTATTTTCCGCTTCTGTCCGGTC3') primers, which harbour the *Bg*III and *Spe*I
446 restriction sites respectively. An ATG start codon was introduced into the forward primer
447 PHYC-Bg, immediately upstream the coding sequence of the mature peptide. The amplified
448 fragment was first subcloned into the pGEM-T easy cloning vector (Promega) and then
449 introduced as *Bg*III-*Spe*I fragment into the pCAMBIA1302 binary vector between the
450 cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator.
451 This binary vector contains also the hygromycin resistance gene (*HPT*) as a selection marker.
452 The resulting construct pC1302-phy US417 (Supplemental figure 1A) was used to transform
453 *Arabidopsis thaliana* (ecotype Columbia, Col-0) plants by floral dipping using *Agrobacterium*
454 strain GV3101 (Clough and Bent, 1998).
455 Transgenic plants were selected on MS agar medium supplemented with 15 µg/ml
456 hygromycin. From twenty T1 independent transformants, six T2 lines showing a 3:1
457 segregation for hygromycin resistance (indicative of single transgene insertion) were grown
458 up to T3 generation to identify plants homozygous for the transgene and from which two
459 lines, L7 and L9, were chosen for further detailed molecular and physiological analyses.

460

461 *Molecular characterization of transgenic lines*

462 Genomic DNA was prepared from fresh leaves of independent transformants. The presence of
463 *phy-US417* and *HPT* genes was verified by PCR screening. The forward and reverse primers
464 for *HPT* gene were HPTIIF (5'ATGAAAAAGCCTGAACTCAC3') and HPTIIR (5' CTCT-
465 ATTTCTTTGCCCTCG3'), and those for the *phy-US417* gene were PHYC-Bg and PHYC-Sp.
466 To perform expression analysis by RT-PCR, total RNA was first extracted from 200 mg of
467 two week-old seedlings of wild type *Arabidopsis* and transgenic lines using Trizol reagent

468 (Invitrogen) according to the manufacturer's protocol. To remove contaminating DNA, RNAs
469 were treated with DNaseI (Promega) at 37°C for 15 min. DNase-treated RNA samples (2 µg)
470 were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse
471 transcription (RT) reactions were performed at 37°C for 1 h using 2 µM oligo-dT18. Two
472 microlitres of first strand cDNAs were employed as a template for PCR amplifications with a
473 pair of *phy-US417*-specific primers, PHYC-Bg/PHYC-Sp or HPT-specific primers
474 HPTIIF/HPTIIR. An *Arabidopsis* Actin gene fragment, used as an internal control, was
475 amplified with the primers: ActF: 5' GGCGATGAAGCTCAATCCAAACG-3' and ActR: 5'-
476 GGTCACGACCAGCAAGATCAAGACG-3'. For PCR reactions, samples were denatured
477 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
478 72 °C with a final extension of 5 min at 72 °C. Finally, the RT-PCR products were separated
479 by agarose gel electrophoresis.

480 *Quantitative RT-PCR*

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

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

494

495 *Phytase Activity assay*

496 Phytase activity was estimated colorimetrically by monitoring the release of Pi from PA (Na-
497 InsP6; P-8810; Sigma) as previously reported (Engelen *et al.* 1994). Briefly, two-week old
498 seedlings (three replicates, 20 plants per replicate) were ground in a mortar and pestle at 4°C
499 with three volumes (v/w) of MES/Ca buffer (15 mM MES buffer with 0.5 mM CaCl₂, pH
500 7.5) containing 1 mM EDTA. Extracts were then centrifuged for 10 min at 12,000g and from
501 which 100 µg were reacted at 55°C for 30 min in a 100 mM NaOAc (pH 7.5) assay buffer
502 containing 2 mM PA as a substrate. The reaction was terminated with the addition of an equal
503 volume of 10% (w/v) trichloroacetic acid. The released Pi was quantified
504 spectrophotometrically at 820 nm with molybdate-blue method (Murphy and Riley, 1962).
505 Background readings due to Pi contamination (time 0) were subtracted before calculation.
506 One unit (U) of phytase is the activity that releases 1 µmol of phosphate per minute under
507 these assay conditions. The protein concentrations were determined according to Bradford
508 method (1976) using bovine serum albumin as a standard.

509

510 *Phytic acid measurement*

511 The seed PA contents were determined by colorimetric method as described previously (Haug
512 and Lantzsich, 1983). Four mg of seeds were extracted with 0.4 N HCl during 3.5 h with
513 constant stirring at room temperature (RT). The extract was transferred into a glass tube with
514 a stopper ammonium iron (III)-sulfate solution (0.02% NH₄Fe(III)-sulfate in 0.2 N HCl).
515 Next, the mixture was heated at 95°C for 30 min and cooled on ice for 15 min, followed by an
516 incubation at RT for 20 min. Then, the mix was centrifuged at 4000g at room temperature for
517 30 min and the supernatant was transferred to another glass tube to which 1% (w/v) 2,2'-

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

520 *High-performance ion chromatography analysis*

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

529

530 *Iron histochemical detection*

531 Embryos were dissected from dry seeds after 2h of imbibition in water. Seedlings were
532 germinated on half-strength MS medium without sucrose and Fe and grown for 2 days at
533 21°C in long days (16h light). For Fe histochemical detection, mature embryos and 2-day-old
534 seedling were stained using the Perls/DAB procedure described by Roschztardt et al.(2009).
535 The presence of Fe is revealed as brown pigments.

536

537 *Statistical analyses*

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

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804

805 **Figures legends**

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

807 (A) Seedlings were first grown on MS medium for **one week** then transferred to standard MS
808 medium (MS **with 1 mM Pi**, as a control), or to low-**P** medium (MS-P), in the absence or in
809 the presence of **0.33 mM** phytic acid (PA) and the phytase PHY-US417 (6.5U). (B)
810 Photographs of the roots of seedlings grown under the above conditions. All photographs
811 were taken **15** days after transfer. Representative data of three biological replicates are shown.

812 (C) **Fresh weights of shoots and roots from seedlings grown under conditions indicated in A).**
813 **Measurements were recorded 15 days after transfer. Data are mean± SD of three individual**
814 **replicates. Each replicate was obtained from the analysis of shoots and roots collected from**
815 **a pool of six plants.**

816

817

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

825

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

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

835

836 **Figure 4.** Fe remobilization is faster in low phytate mutants during germination.

837 Fe was stained with Perls/DAB in mature embryos extracted from dry seeds (upper panels) or
838 in 2-days old seedlings (of wild type, *ipk1-1*, and transgenic lines L7 and L9) after
839 germination in liquid medium without Fe (lower panels).

840

841 **Figure 5.** Effect of the overexpression of PHY-US417 in *Arabidopsis* on organ phosphate and
842 sulfate concentrations. Wild-type plants (Col-0), *ipk1-1*, as well as the PHY-US417 transgenic
843 lines were grown on medium containing low Pi (-Pi) for 15 days, followed by additional 10
844 days in media containing 1 mM Pi (+Pi). Shoots and roots were harvested separately and
845 anion concentrations expressed as $\mu\text{mol.gFW}^{-1}$, were quantified by HPIC. Individual
846 measurements performed before (-Pi) and after (+Pi) transfer of seedlings to MS containing
847 1mM of Pi, were obtained from the analysis of shoots or roots collected separately from three
848 biological repeats. Each repeat was obtained from the analysis of shoots and roots collected
849 from a pool of six plants. Error bars indicate SD; (a) and (b) are values significantly different
850 ($P < 0.05$) from WT plants.

851

852 **Figure 6.** Effect of the overexpression of PHY-US417 in *Arabidopsis* on the expression of a
853 sub-set of phosphate, sulfate, and nitrate transporter encoding genes. Expression levels of
854 phosphate (*PHT1;1*, *PHT1;4*, *PHO1* and *PHO;H1*), sulfate (*SULTR1;1* and *SULTR1;2*), nitrate

855 (*NRT1;1* and *NRT2;1*) and iron (*IRT1*) transporter genes were analysed in the transgenic lines
856 (L7 and L9) and in the *ipk1-1* mutant in comparison to wild-type plants. Plants were grown on
857 Pi-limited conditions for 15 days and then transferred to MS medium containing 1 mM of Pi
858 for 10 additional days. Roots were harvested and the mRNA accumulation inferred from
859 quantitative PCR analysis. Individual measurements were obtained from the analysis of roots
860 collected from pools of five plants. Transcript levels were normalized to the level of the
861 control ubiquitin10 mRNA (*UBQ10*: At4g05320). Error bars correspond to SD of three
862 biological repeats and each repeat was obtained from the analysis of roots collected from a
863 pool of six plants.

864

865 **Figure 7.** The overexpression of *PHY-US417* gene alters Pi sensing and signalling in
866 *Arabidopsis*. (A) Representative images of primary root and root hairs of 10 day-old Wild-type
867 (Col), *ipk1-1* mutant and *PHY-US417* overexpressor lines L7 and L9 grown for 5 days on MS
868 +Pi after 5 days on low-P medium. Images were taken by light microscopy (Olympus Soft
869 imaging system). The root segments chosen for examination of root hair formation are
870 indicated in additional windows. (B) Expression levels of two phosphate starvation induced
871 non-coding RNAs, *IPSI* and *AT4*, determined by quantitative RT-PCR from 10-old seedlings
872 grown as described in A). Transcript levels were normalized to the level of the control
873 ubiquitin-10 mRNA (*UBQ10*: At4g05320). Error bars correspond to SD of three biological
874 repeats and each repeat was obtained from the analysis of roots collected from a pool of six
875 plants.

876

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

880 T-DNA left (LB) and right (RB) borders. PCR screenings for HPT **(B)** and *phy-US417***(C)**
881 genes on 14 independent transformants (lanes 1-14) together with wild type plant (WT).

882 **(-)** indicates control for PCR where H₂O was used instead of DNA. Expected sizes of the PCR
883 products are indicated. Lane M: λ PstI marker. **(D)** RT-PCR analyses of *HPT* and *phy-US417*
884 expression in 10 representative transformants (lanes 1-10) and in a line transformed with the
885 pCAMBIA1302 empty vector (lane V). (-RT): without reverse transcriptase. A 380 bp *Actin*
886 fragment was amplified by RT-PCR as internal control.

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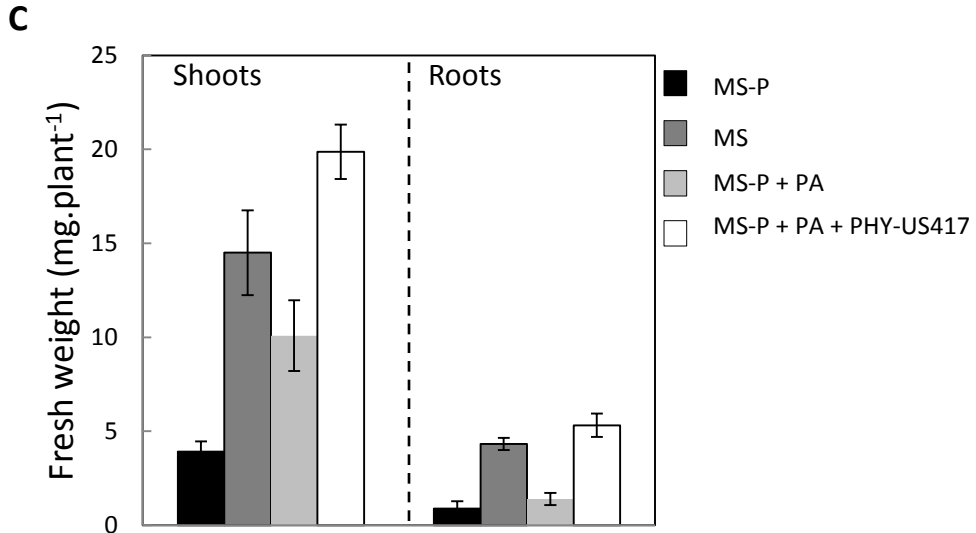
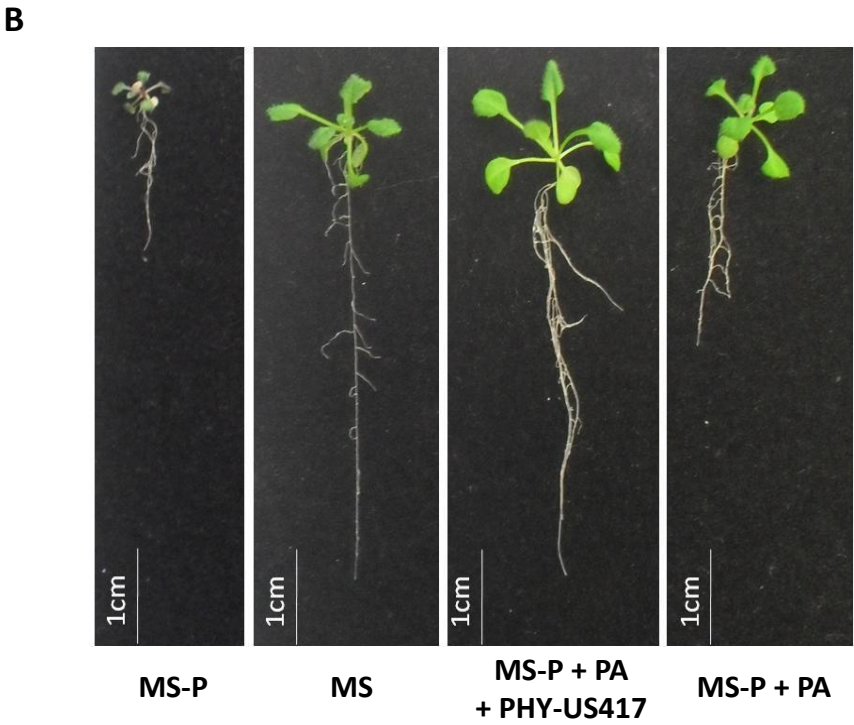
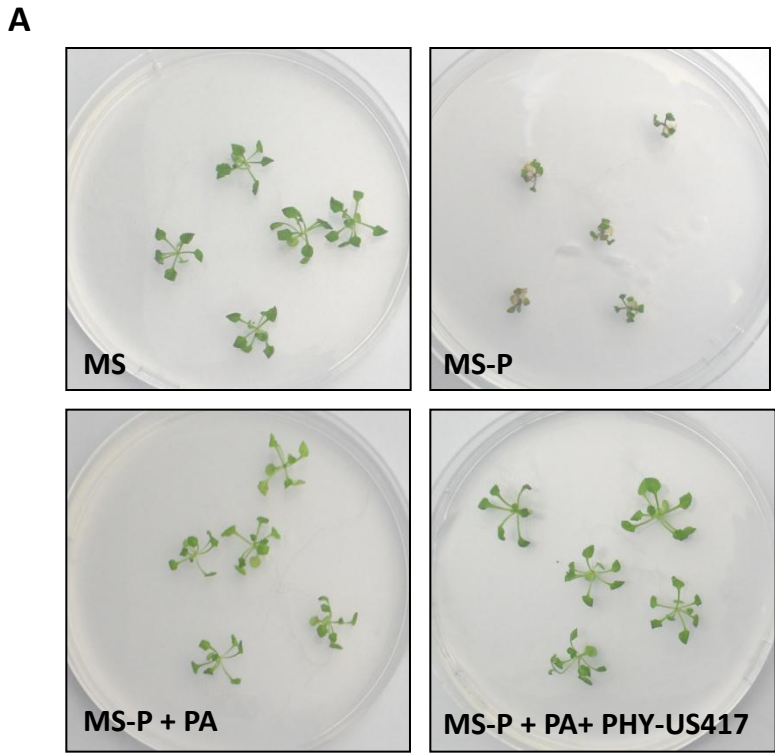


Figure 1. Effect of *in vitro* phytase supply on the growth of *Arabidopsis* wild type plants. **(A)** Seedlings were first grown on MS medium for one week then transferred to standard MS medium (MS with 1 mM Pi, as a control), or to low-P medium (MS-P), in the absence or in the presence of 0.33 mM phytic 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..

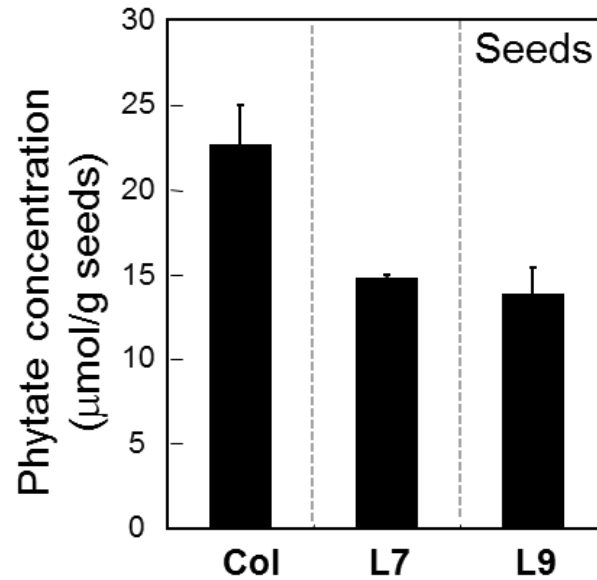


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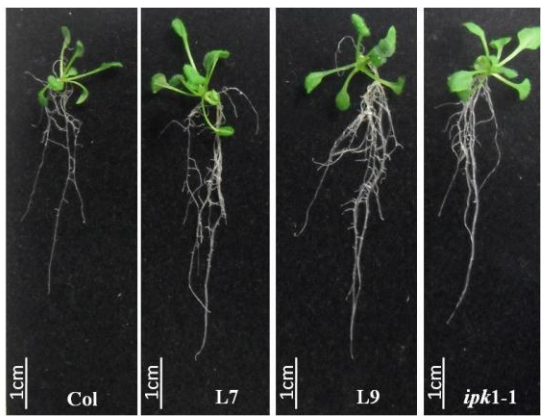


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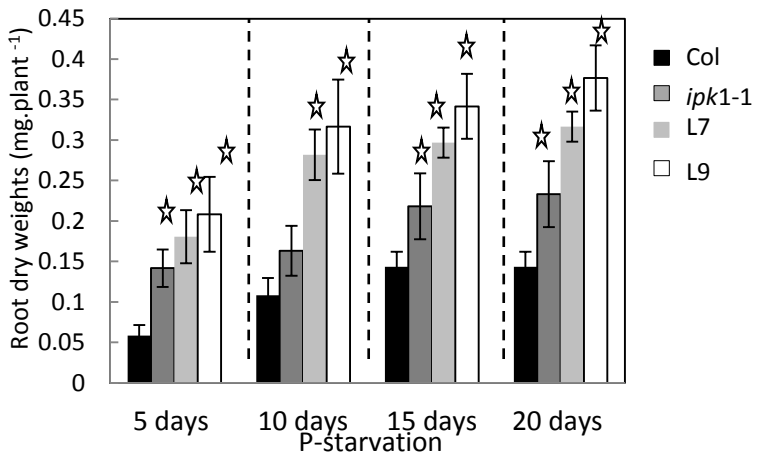
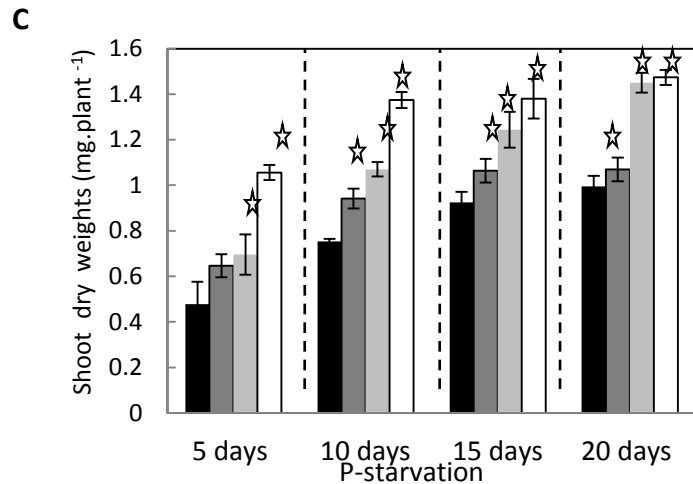
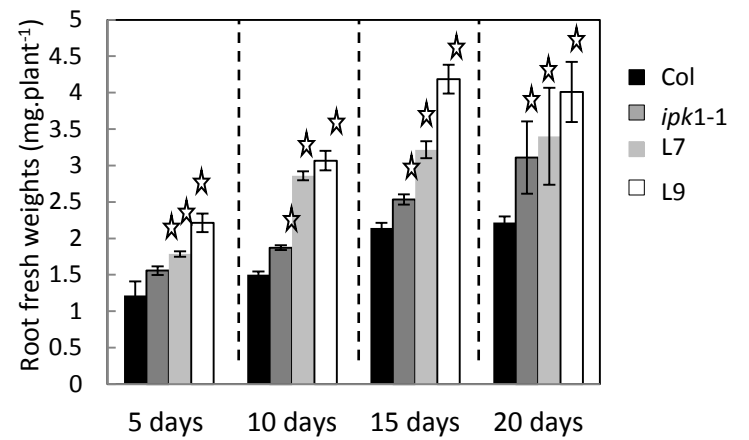
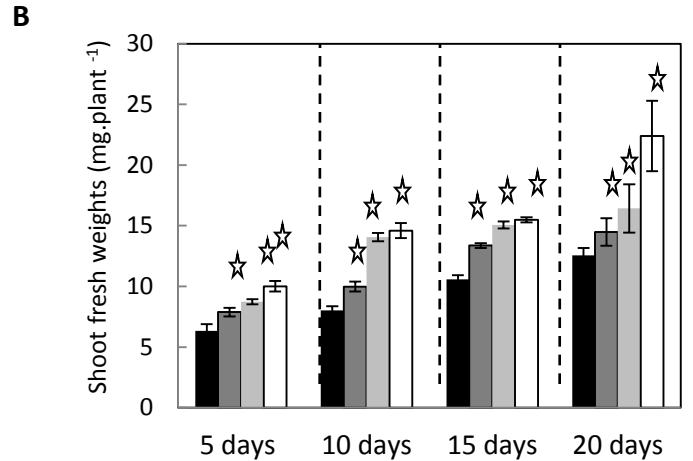




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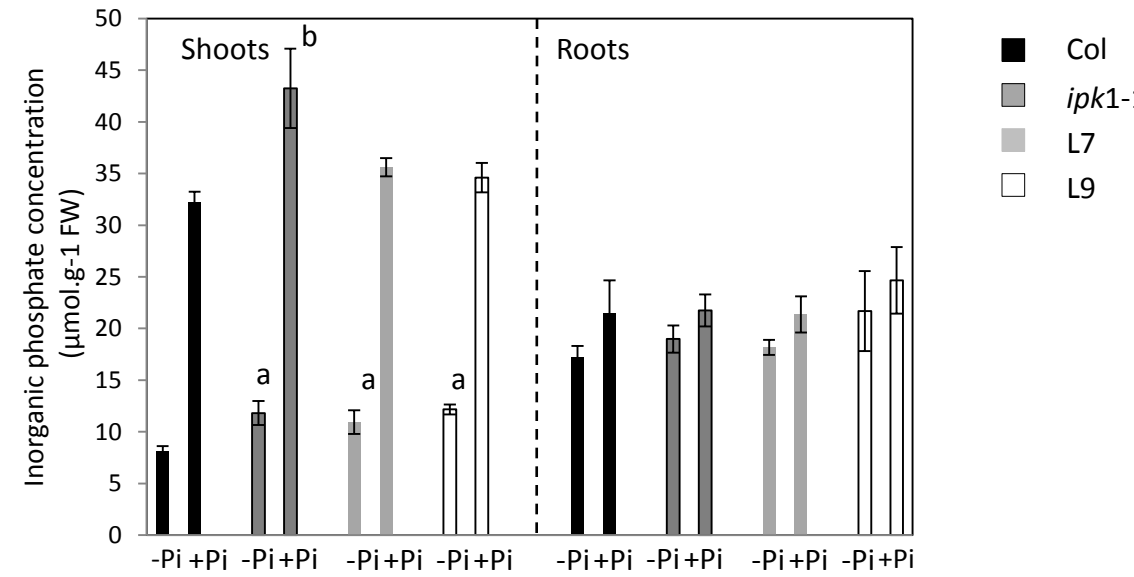
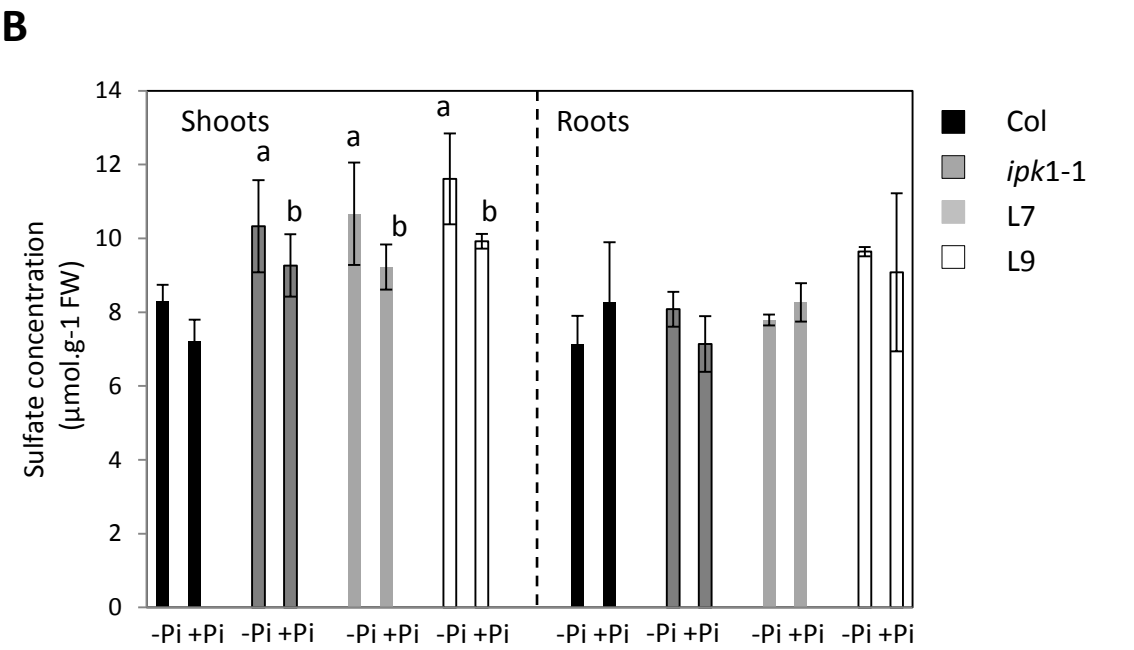


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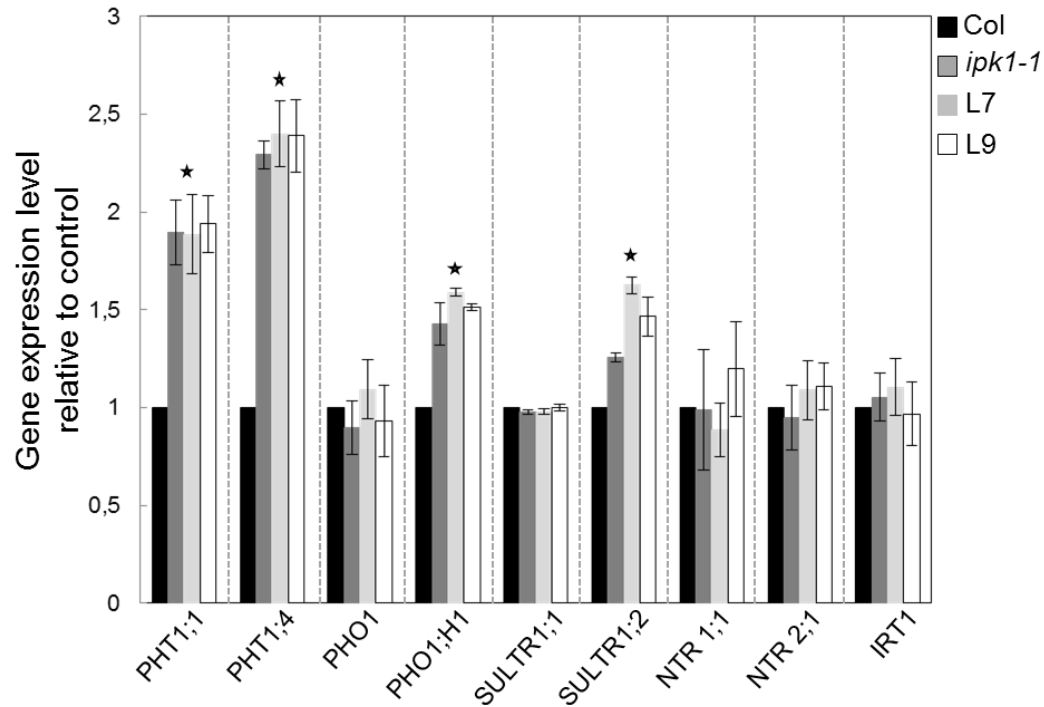


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A

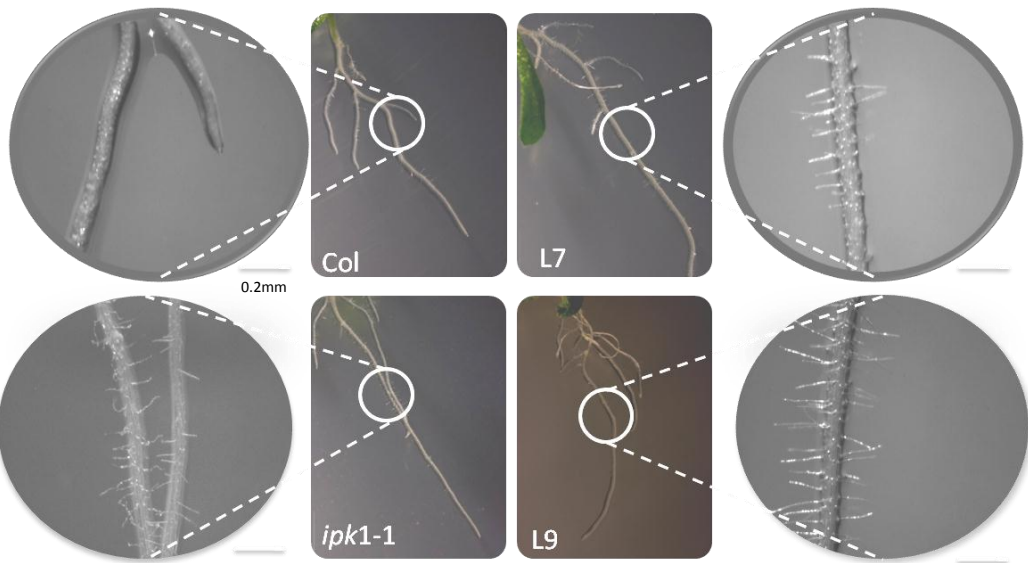


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B

