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1 **LPCAT1 controls phosphate homeostasis in a zinc-dependent**  
2 **manner.**

3  
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26

27 **Abstract**

28 All living organisms require a variety of essential elements for their basic biological functions.  
29 While the homeostasis of nutrients is highly intertwined, the molecular and genetic  
30 mechanisms of these dependencies remains poorly understood. Here, we report a discovery  
31 of a molecular pathway that control phosphate (Pi) accumulation plants in Zn deficiency. Using  
32 genome-wide association studies we first identified allelic variation of the *Lyso-*  
33 *PhosphatidylCholine (PC) AcylTransferase 1 (LPCAT1)* gene as the key determinant of shoot  
34 Pi accumulation under Zn deficiency. We then show that regulatory variation at the LPCAT1  
35 locus contributes significantly to this natural variation and we further demonstrate that the  
36 regulation of *LPCAT1* expression involves bZIP23 TF, for which we identified a new binding  
37 site sequence. Finally, we show that in Zn deficient conditions loss of function of *LPCAT1*  
38 increases the phospholipid *Lyso-PhosphatidylCholine/PhosphatidylCholine* ratio, the  
39 expression of the Pi transporter PHT1;1, and that this leads to shoot Pi accumulation.

## 40 Introduction

41

42 All living organisms require an adequate supply of nutrients for growth and survival. Nutrient  
43 deficiencies lead to decreased plant survival and lower nutritional value of foods, which has a  
44 profound impact on human health (Myers et al., 2014). In particular zinc (Zn) and iron (Fe)  
45 deficiencies affect up to 2 billion people worldwide (Hilty et al., 2010). According to the World  
46 Health Organization, about 800,000 child deaths per year are attributable to Zn deficiency  
47 alone (Akhtar, 2013). The widespread occurrence of deficiencies in micronutrients such as Zn  
48 and Fe in human populations is due to low dietary intake (Rouached, 2013; Myers et al., 2014;  
49 Shahzad et al., 2014). In the light of crop optimization for yield and nutritional quality, it is  
50 therefore an important goal to understand the genetic and molecular basis of plant nutrition. A  
51 complicating circumstance is that plant uptake, storage and use of these nutrients are partly  
52 dependent of each other (Rouached and Rhee, 2017). For instance, physiological Zn  
53 deficiency leads to over-accumulation of phosphorus (P) in the shoots (for review, Bouain et  
54 al., 2014; Kisko et al., 2015). Note worthy, when the Zn supply is low, increasing P supply  
55 causes a reduction of plant height, delayed development and severe leaf symptoms including  
56 chlorosis and necrosis (Ova et al., 2015). At high P supplies, Zn deficiency associated with  
57 elevated shoot P levels causes P toxicity (Marschner, 2012). Interestingly, this P-Zn interaction  
58 is also recognized in a wide variety of other biological systems, including rats (Wallwork et al.,  
59 1983), human cells (Sandström and Lönnerdal, 1989), and multiple fungal species (Freimoser  
60 et al., 2006). In *Saccharomyces cerevisiae* yeast, the Zn status acts as a major determinant of  
61 the ability to store P (Simm et al., 2007). Much like Zn nutrition, P homeostasis is of global  
62 relevance as current agricultural practices require large amounts of P. At the same time, world-  
63 wide P reserves are becoming increasingly scarce and a potential P crisis looms for  
64 agriculture at the end of this 21st century (Abelson, 1999; Neset and Cordell, 2012). How P  
65 and Zn homeostases are coordinated is therefore not only a fundamental biological question  
66 but has also serious implications for global agronomic and biotechnological applications.

67 P is a critical component of many metabolites and macromolecules, including nucleic acids  
68 and phospholipids (PLs) (Poirier and Bucher, 2002; Rouached et al., 2010). Of equal  
69 importance, Zn provides chemical, structural and regulatory functions in biological systems  
70 (Christianson, 1991), for instance as cofactor for hundreds of enzymes, or by binding to PLs to  
71 maintain membrane structure (Binder et al., 2001; Sinclair and Krämer, 2012). Plants have  
72 evolved the ability to adjust to large fluctuations in external P or Zn supply. P is taken up by  
73 the root system in the form of inorganic phosphate (Pi). In *Arabidopsis thaliana* (Arabidopsis),  
74 this uptake relies on members of the high affinity Pi transporter family (PHT1) (Nussaume et  
75 al., 2011), of which PHT1;1 is the major contributor (Ayadi et al., 2015). Upon P deficiency, the  
76 expression of some *PHT1* transporters increases as a result of the activation of the “PHR1-  
77 miR399-PHO2” signalling pathway (Bari et al., 2006; Lin et al., 2008; Pant et al., 2008),  
78 causing a strong increase in the acquisition of Pi and its subsequent translocation to the  
79 shoots (Lin et al., 2008; Pant et al., 2008). In contrast to our understanding of the molecular  
80 mechanisms involved in sensing and signalling of Pi abundance (Chiou and Lin, 2011; Zhang  
81 et al., 2014), little is known about how plants sense and signal Zn deficiency. A putative  
82 working model of Zn deficiency signalling was proposed by (Assunção et al., 2013), which is  
83 centred around two essential members of the bZIP transcription factor (TF) family in  
84 Arabidopsis, bZIP19 and bZIP23, without which plants are unable to respond to Zn starvation  
85 by inducing the expression of genes involved in Zn uptake and distribution such as the zinc  
86 transporter ZIP4 (Assunção et al., 2010). Beyond common set of genes targeted by these two  
87 TFs, each TF could regulate distinct genes (Inaba et al., 2015), but the identity of distinctive  
88 binding site recognized by each remains poorly unknown. Identifying such binding motif is  
89 necessary to better understand how plants regulate Zn homeostasis.

90  
91 The interaction between Zn and Pi homeostasis in plants is also obvious at the molecular level  
92 (for reviews, Bouain et al., 2014; Kisko et al., 2015). For instance, Zn deprivation causes an  
93 up-regulation of *PHT1;1* and consequently an over-accumulation of Pi in *Arabidopsis thaliana*  
94 (Jain et al., 2013; Khan et al., 2014). The expression of Pi uptake transporters is normally

95 tightly controlled in roots in response to the P status of the plant, but it is clear that this tight  
96 control is lost under Zn deficiency. Remarkably, although the involvement of PHOSPHATE  
97 RESPONSE1 transcription factor (PHR1) in the coordination of Pi-Zn homeostasis has been  
98 demonstrated (Khan et al., 2014), the Zn deficiency-induced Pi uptake transporter expression  
99 is independent of the aforementioned canonical “PHR1-miR399-PHO2” signalling pathway  
100 (Khan et al., 2014), indicative of room for new discoveries in Pi homeostasis under Zn  
101 deficiency in plants.

102

103 In this study we set out to identify the genes controlling such novel mechanisms to cause Pi  
104 accumulation in shoots of Zn-deficient Arabidopsis plants. Genome wide association (GWA)  
105 mapping was employed using a subset of 223 Arabidopsis accessions from the RegMap panel  
106 (Horton et al., 2012), which enabled us to demonstrate that there is heritable natural variation  
107 of Pi accumulation in responses to Zn deficiency and that one major locus governing this is the  
108 *LysoPhosphatidylCholine AcylTransferase 1 (LPCAT1)* gene. Under Zn deficiency, *lpcat1*  
109 mutants showed an alteration in the phospholipids *Lyso-*  
110 *PhosphatidylCholine/PhosphatidylCholine* (Lyso-PC/PC) ratio, and an up-regulation of the  
111 expression of the main high affinity Pi transporter gene *PHT1;1*. Finally, we demonstrate that  
112 *LPCAT1* acts downstream of one of the two key Zn starvation signalling TFs, bZIP23, for  
113 which we identified a new binding site sequence. Overall, this study uncovered a novel  
114 pathway, in which *LPCAT1* plays a key role in the coordination of Pi homeostasis and Zn  
115 deficiency response in plants through modulation of phospholipid metabolism and Pi  
116 transporter expression.

## 117 Results

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

120 To identify genes regulating shoot Pi concentration under Zn deficiency, genome wide  
121 association studies (GWAS) were conducted. To do so, a diverse set of 223 Arabidopsis

122 accessions, selected from the RegMap panel (Horton et al., 2012) was grown on agar medium  
123 supplemented with (+Zn) or without Zn (-Zn) for 18 days, before assessing their shoot Pi  
124 concentration (Supplementary file 1). As expected, Zn deficiency in shoots of Col-0 plants was  
125 associated with the induction of the expression of two Zn-deficiency marker genes, *ZIP4* and  
126 *ZIP12* (Jain et al., 2013) (Figure 1-figure supplement 1). Under the +Zn condition, shoot Pi  
127 concentration varied across the 223 accessions from 3 - 10  $\mu\text{mol}$  of Pi per gram of fresh  
128 weight (median  $\sim 5.45 \mu\text{mol}\cdot\text{gram}^{-1}$  fresh weight of Pi) (Figure 1-figure supplement 2A) while  
129 in -Zn, it increased to 4 - 16  $\mu\text{mol}$  of Pi per gram of fresh weight (median  $\sim 8.23 \mu\text{mol}\cdot\text{gram}^{-1}$   
130 fresh weight of Pi) (Figure 1-figure supplement 2B). The broad-sense heritability ( $H^2$ ) of the  
131 shoot Pi concentrations was 0.63 and 0.47 under +Zn and -Zn conditions, respectively. Using  
132 the genotype and the shoot Pi concentration as input, we performed a mixed model (AMM  
133 method (Seren et al., 2012)) GWAS that corrects for population structure (Korte et al., 2012)  
134 for both Zn conditions (Figure 1B, C, Figure 1-figure supplement 2C-D). Using a non-  
135 conservative 10% false discovery threshold (FDR), we identified 13 significant SNPs in 5  
136 distinct genomic loci to be associated with Pi concentration in the shoots, which was specific  
137 for the -Zn condition (Figure 1B). The most significantly associated SNP ( $P\text{-value} = 5.86 \times 10^{-8}$ ;  
138 FDR 1%) was located on Chromosome 1 (Supplementary file 2). A haplotype analysis centred  
139 on the 50-kb region surrounding the significantly associated SNP revealed one main haplotype  
140 (depicted in purple) that was associated with the marker SNP and high Pi concentration  
141 (Figure 1-figure supplement 3). The significantly associated SNP was located at the upstream  
142 and coding regions of two candidate genes, namely *At1g12640* and *At1g12650* (Figure 1D-E).  
143 *At1g12650* encodes an unknown protein likely to be involved in mRNA splicing via the  
144 spliceosome, and *At1g12640* encodes a member of the *Membrane Bound O-Acyl Transferase*  
145 (*MBOAT*) gene family known as *LysoPhosphatidylCholine AcylTransferase 1* (*LPCAT1*, (Wang  
146 et al., 2012)). *LPCAT1* is an evolutionarily conserved key enzyme that is involved in  
147 phospholipid metabolism and more precisely in the Lands cycle (Lands, 1960). In Arabidopsis  
148 *LPCAT1* has been shown to catalyze the conversion of lysophosphatidylcholine (Lyso-PC) to  
149 produce phosphatidylcholine (PC) (Zheng et al., 2012).

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

151 In order to determine the causal gene underlying the shoot Pi accumulation Quantitative Trait  
152 Locus (QTL) in  $-Zn$ , we used a reverse genetic approach. The first thing we studied was to  
153 test if any of these two genes is indeed involved in the  $-Zn$  specific variation in shoot Pi  
154 concentration. Therefore, wild-type Arabidopsis (Columbia-0, Col-0), T-DNA insertion mutant  
155 lines for *LPCAT1* (At1g12640) (Wang et al., 2012) and for *At1g12650* gene were grown for 18  
156 days on  $+Zn$  or  $-Zn$  media before assessing their shoot Pi concentration. In response to  $-Zn$ ,  
157 Col-0 plants showed a significant increase ( $\sim 29\%$  increase,  $P$ -value  $< 0.05$ ) in their shoot Pi  
158 concentration compared to  $+Zn$  conditions (Figure 2A), which is in line with a previous report  
159 (Khan et al., 2014). Importantly, while Pi accumulation in response to  $-Zn$  in *At1g12650*  
160 mutants was indistinguishable from Col-0, *lpcat1* mutants displayed a significant increase in  
161 shoot Pi concentration ( $\sim 36\%$  increase,  $P$ -value  $< 0.05$ ) (Figure 2A). We confirmed that this  
162 increase in shoot Pi concentration in the *lpcat1* mutants is specific to the  $-Zn$  treatment as no  
163 significant differences were observed in the  $+Zn$  condition compared to Col-0. These results  
164 showed that *LPCAT1*, and not *At1g12650*, is involved in regulating shoot Pi concentration in  
165 response to Zn deficiency in Arabidopsis. Our further efforts were therefore directed at  
166 understanding the transcriptional regulation of *LPCAT1* by  $-Zn$ , and then at resolving how  
167 allelic variation at the *LPCAT1* gene contributes to the variation in shoot Pi concentration.

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

169 To investigate the molecular causal links between Zn/*LPCAT1*/Pi we analyzed the *cis*-  
170 regulatory elements present within the 1500-bp region upstream of the *LPCAT1* start codon (in  
171 Col-0 background) using the search tool AthaMap (Bülow et al., 2010). We identified the  
172 presence of a single copy of the 10-bp Zinc Deficiency Response Element (ZDRE,  
173 RTGTCGACAY)(Assunção et al., 2010), located 377 bp upstream of the ATG (Figure 2B).  
174 This motif is a known binding site for the bZIP19 and bZIP23 transcription factors, the key  
175 transcriptional regulators of the  $-Zn$  response (Assunção et al., 2010). Given the presence of  
176 the ZDRE, we hypothesized that the expression of *LPCAT1* under  $-Zn$  could be controlled by



177 the bZIP19 or bZIP23 TFs. An electrophoretic mobility shift assay (EMSA) was performed,  
178 using a 30-bp promoter fragment containing the 10-bp potential ZDRE, which confirmed that  
179 both bZIP19 and bZIP23 could bind to this *cis*-regulatory element (Figure 2C), as had already  
180 been shown by (Assunção et al., 2010).

181 Further analysis of the regulatory regions of *LPCAT1* led us to identify a new motif  
182 GTGTCGAA (5' untranslated region of *LPCAT1*), very similar to that of the ZDRE motif  
183 (RTGTCGACAY) (Figure 2B). Due to the sequence similarity of this newly identified motif to  
184 that of ZDRE, we first tested the capacity of bZIP23 or bZIP19 to bind to this motifs.  
185 Interestingly, EMSA analysis revealed that bZIP23 could bind to the newly identified motif,  
186 while bZIP19 showed an extremely weak (if any) binding capacity to new motif (Figure 2C).  
187 These findings strongly support the Zn-dependency of *LPCAT1* expression. We therefore  
188 determined the transcript abundance of *LPCAT1* in shoots of Arabidopsis wild-type plants  
189 (Col-0) grown in -Zn for 6, 12 and 18 days. In response to -Zn, transcript accumulation of  
190 *LPCAT1* was changed, showing significant down-regulation compared +Zn conditions (Figure  
191 2D). This result shows that repression of *LPCAT1* upon low -Zn is associated with higher Pi  
192 levels and suggests that transcriptional regulation of *LPCAT1* is important for its involvement  
193 in Pi homeostasis. We next tested whether these bZIP TFs could be involved in regulating the  
194 expression of *LPCAT1* in -Zn. To test this, we determined the expression levels of *LPCAT1* in  
195 the *bzip19* and *bzip23* single and *bzip19/bzip23* double knock-out mutant lines and WT plants  
196 (Col-0) grown for 18 days in +Zn and -Zn conditions. The *LPCAT1* transcript was significantly  
197 up-regulated in the *bzip23* and *bzip19/bzip23* mutant lines, compared to Col-0 and *bzip19* in -  
198 Zn, which showed a significant down-regulation (Figure 2D). This indicates that bZIP23, but  
199 not bZIP19, is involved in negatively regulating the expression of *LPCAT1* under -Zn. We  
200 therefore hypothesized that *bZIP23* but not bZIP19 are necessary for the downregulation of  
201 *LPCAT1* in -Zn and subsequent Pi accumulation and there assessed the capacity of the  
202 mutants to accumulate Pi when grown with or without Zn for 18 days. While in +Zn, all plants  
203 showed similar shoot Pi content, we observed a significant decrease in shoot Pi content in the  
204 *bzip23* and *bzip19/bzip23* mutants compared to Col-0, confirming the regulatory role of bZIP23

205 and not bZIP19 (Figure 2E). Taken together, this suggests that bZIP23 represses *LPCAT1*  
206 upon  $-Zn$  and this repression leads to the over-accumulation of Pi in shoots in Arabidopsis  
207 grown under  $-Zn$  condition.

208  
209 **Allelic variation of *LPCAT1* determines natural variation of Pi content under zinc**  
210 **deficiency.**

211 We next wanted to test whether allelic variation of *LPCAT1* is causal for the observed  
212 differences in Pi accumulation under  $-Zn$ . For this, we selected two contrasting groups of  
213 accessions with either a high ratio (Br-0, Ts-1, PHW-2 and Sap-0) or a low ratio (Ang-0, CIBC-  
214 5, Col-0, EST-1, RRS-10) of Pi accumulated in shoots of  $-Zn$  plants compared to  $+Zn$  plants  
215 (Figure 3A). Interestingly, comparative sequence analysis of the regulatory regions of *LPCAT1*  
216 of these accessions using the sequence data from the 1001 genomes project (Alonso-Blanco  
217 et al., 2016) revealed that the common ZDRE motif (Figure 2B) didn't display any variation  
218 between these two groups of accession (Figure 3A), and that the newly identified *bzip23*  
219 specific motif (Figure 2B) showed clear variation between the two groups of accession with the  
220 accessions with low Pi ratio under  $-Zn$  exhibiting a Col-0 like GTGTCGAA motif and the high Pi  
221 accumulating accession displaying a GTGTCACA motif (Figure 3A, Figure 3-figure  
222 supplement 1, Supplementary file 3). We therefore tested the capacity of bZIP23 and bZIP19  
223 to bind to this latter version (GTGTCACA) of the ZDRE motif. EMSA analysis revealed that  
224 only bZIP23 could bind to this version of ZDRE motif (Figure 3B). Taken together, EMSA  
225 results (Figure 2C, Figure 3B) support the specificity of a new ZDRE motif for bZIP23.

226 We next assessed the effect of these motif sequence changes on the activity of the *LPCAT1*  
227 promoter using a quantitative *in planta* transactivation assay (Bossi et al., 2017). In this assay,  
228 we co-transformed tobacco leaves with an effector construct (35S::bZIP23 or 35S::YFP) with a  
229 reporter construct, containing either the *LPCAT1* Col-0 native promoter (with GTGTCGAA  
230 motif), the *LPCAT1* Col-0 mutated promoter (with GTGTCACA motif), or the promoter of the  
231 zinc transporter *ZIP4* promoter (as positive control) fused to a  $\beta$ -glucuronidase (*GUS*)-  
232 encoding reporter gene (Figure 3C). The comparison of the ability of 35S-bZIP23 or 35S-YFP

233 to activate these *LPCAT1* promoters was performed by quantifying the GUS activity. The  
234 average relative activity was calculated as the GUS activity of each promoter in the presence  
235 of 35S::bZIP23 divided by its GUS activity in the presence of 35S::C-YFP (Figure 3D). As  
236 expected, our results showed an induction of the positive control (*ZIP4* promoter by  
237 35S:bZIP23). Consistent with the hypothesis that the natural variation of the new ZDRE is  
238 relevant for *LPCAT1* regulation, bZIP23 acted as stronger repressor of the *LPCAT1* Col-0  
239 mutated promoter that contained the new ZDRE of Sap-0 compared to the Col-0 *LPCAT1*  
240 native promoter (Figure 3D). Moreover, *LPCAT1* was downregulated by a larger extent in  
241 accessions that accumulated more Pi upon -Zn (Figure 3E) and contained the new (non Col-  
242 0) ZDRE while the expression of *bZIP23* remained unchanged in all accessions and growth  
243 conditions tested.

244 To further test whether the difference in *LPCAT1* expression was due to the natural allelic  
245 variation in the regulatory regions and whether this was causal for the Pi accumulation, we  
246 focused on only two contrasting accessions, Sap-0 and Col-0, which displayed a significantly  
247 different capacity to accumulate shoot Pi in -Zn (Supplementary file 1). Noteworthy, the  
248 *LPCAT1* promoter and predicted amino acid coding sequences of Col-0 and Sap-0 displayed  
249 97.9% and 99.4% sequence identity respectively (data not shown). The *lpcat1* knock-out  
250 mutant (in Col-0 background) was then transformed with either an empty vector (control) or  
251 one of four constructs containing 1.5 kbp of the promoter (immediately upstream of the start  
252 codons) of either p*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup> respectively fused to either the coding region  
253 of *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup> (Figure 4A). Three independent, single locus insertion lines  
254 (based on segregation of the insertion in progeny of a hemizygous plant) were considered for  
255 the analysis. When expressed under the p*LPCAT1*<sup>Col-0</sup> promoter, *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup>  
256 complemented the *lpcat1-1* knock-out mutant phenotype and showed a similar Pi content to  
257 WT (Col-0) plants in both +Zn and -Zn conditions (Figure 4B). This indicates that the  
258 polymorphisms in the coding region are not responsible for the change in Pi content in -Zn  
259 conditions. In contrast, lines complemented with the p*LPCAT1*<sup>Sap-0</sup>:*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup>:  
260 *LPCAT1*<sup>Sap-0</sup> transgenic lines showed significantly higher Pi content compared to p*LPCAT1*<sup>Col-0</sup>

261 <sup>0</sup>:*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Col-0</sup>:*LPCAT1*<sup>Sap-0</sup> lines or WT (Col-0) in -Zn conditions (Figure 4B).  
262 This result demonstrates that regulatory variation in of the *LPCAT1* promoter determines Pi  
263 accumulation and favours the model that variation in the expression level of *LPCAT1* as the  
264 cause of the variation in Pi accumulation in -Zn. Therefore, we assessed *LPCAT1* mRNA  
265 accumulation in in WT (Col-0) and all transgenic lines grown in both +Zn and -Zn conditions.  
266 Our result showed that while *LPCAT1* is down-regulated in all tested lines by -Zn treatments,  
267 the lines complemented with the *LPCAT1* driven by p*LPCAT1*<sup>Sap-0</sup> accumulates significantly  
268 lower *LPCAT1* mRNA than that of those under the control of p*LPCAT1*<sup>Col-0</sup> and WT (Col-0)  
269 (Figure 4C). Taken together, our results indicate that the allelic variation between Col-0 and  
270 Sap-0 in the promoter of the *LPCAT1* gene causes the difference in *LPCAT1* expression, and  
271 confirm that this difference leads to the difference in Pi accumulation under -Zn. Importantly,  
272 the polymorphisms in the *bzip23* binding site in the promoter of *LPCAT1* suggest a potential  
273 cis-regulatory mechanism for this.

#### 274 ***LPCAT1* mutation impacts phospholipid concentrations in -Zn.**

275 While *LPCAT1* had not been implicated in any known process involving Zn, it is known to  
276 catalyse the conversion of lyso-phosphatidylcholine (Lyso-PC) to phosphatidylcholine (PC) in  
277 the remodelling pathway of PC biosynthesis (Figure 5A) (Lands, 1960; Chen et al., 2007;  
278 Wang et al., 2012). Consequently, we hypothesized that a mutation in *LPCAT1* or *bZIP23*  
279 would affect the Lyso-PC and PC under -Zn conditions. To test this, we measured the  
280 composition of these two phospholipid classes in the shoots of the Col-0 wild type and the  
281 *bzip23* and *lpcat1* mutants, in +Zn and -Zn conditions. In +Zn, no significant changes in the  
282 Lyso-PC and PC levels in the three different genotypes were observed (Figure 5B, C).  
283 However, under -Zn, *bzip23* showed a modest (but non-significant) decrease in the Lyso-  
284 PC/PC ratio while the mutation in *LPCAT1* resulted in a significant increase of Lyso-PC and a  
285 decrease of PC, resulting in an increase of the Lyso-PC/PC ratio (~1.2 fold, *P-value*< 0.05)  
286 compared to Col-0 plants (Figure 5D). These results demonstrate that the *LPCAT1* function is  
287 required to maintain the shoot Lyso-PC/PC ratio under -Zn. We next tested whether the

288 polymorphisms in the regulatory region of *LPCAT1* are responsible for the change in LPC/PC  
289 ratio that ultimately affects the Pi content in -Zn conditions. We determined the LPC, PC  
290 concentrations in the shoots of the plants expressing *LPCAT1* driven by the *LPCAT1*<sup>Col-0</sup>  
291 promoter (p*LPCAT1*<sup>Col-0</sup>::*LPCAT1*<sup>Col-0</sup>, p*LPCAT1*<sup>Col-0</sup>::*LPCAT1*<sup>Sap-0</sup>) or the *LPCAT1*<sup>Sap-0</sup>  
292 promoter (p*LPCAT1*<sup>Sap-0</sup>::*LPCAT1*<sup>Col-0</sup>, p*LPCAT1*<sup>Sap-0</sup>::*LPCAT1*<sup>Sap-0</sup>) in the *lpcat1* mutant  
293 background, grown in presence or absence of Zn for 18 days. WT plants of the Col-0 and Sap-  
294 0 accessions, and *lpcat1* transformed with the empty vector were included in this analysis. In  
295 the presence of Zn, no difference in PC or LPCA concentrations was observed between all  
296 plant lines. However, under -Zn conditions, Sap-0, p*LPCAT1*<sup>Sap-0</sup>::*LPCAT1*<sup>Col-0</sup>, p*LPCAT1*<sup>Sap-0</sup>  
297 0::*LPCAT1*<sup>Sap-0</sup> or *lpcat1* lines showed a significant decrease of PC and increase of LPC  
298 concentrations, leading to an increase of Lyso-PC/PC ratios (Figure 6). These results further  
299 support the association between the increase of LPC/PC ratios and the alterations in P content  
300 in the plant shoots under Zn deficiency (Figure 4b).

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

303 While the molecular function of *LPCAT1* is related Lyso-PC/PC homeostasis, it doesn't answer  
304 the question how it might cause Pi levels to increase under -Zn conditions. A first hint towards  
305 answering this question came from our GWAS data: The 3<sup>rd</sup> most significant associated peak  
306 (8% FDR) under -Zn conditions was located in a region of chromosome 5 containing members  
307 of the high affinity Pi transporters *PHT1* gene family, namely *PHT1;1*, *PHT1;2*, *PHT1;3* and  
308 *PHT1;6* (Chr5 : 17394363 – 174200000) (Figure 7 A-C, Supplementary file 2). Except for  
309 *PHT1;6*, the role of these genes in Pi uptake, transport and accumulation in Arabidopsis is well  
310 documented (Nussaume et al., 2011; Ayadi et al., 2015). To test, the activity of one of these  
311 genes might be related to the *LPCAT1* dependent Pi accumulation under -Zn, we assessed  
312 the expression of the *PHT1* transporter genes in the shoots of *lpcat1* mutant and WT (Col-0)  
313 plants grown in +Zn or -Zn for 18 days. In all genotypes, *PHT1;1* was the only member of the  
314 *PHT1* gene family to be significantly up-regulated in the -Zn condition (Figure 7D). Zn

315 deficiency induces transcription of *PHT1;1* already ~ 2.2 fold ( $P < 0.05$ ) in WT (Col-0) and this  
316 induction was further increased by 2-fold ( $P < 0.01$ ) in *lpcat1* mutants (Figure 7D), when  
317 compared to +Zn (Figure 7-figure supplement 1). The expression of the *PHT1;1* was thereafter  
318 tested for responsiveness to -Zn in roots of WT (Col-0) and the *lpcat1-1* mutant. While -Zn  
319 caused no significant change in expression of the *PHT1;1* in roots of WT, it increased its  
320 expression by ~2-fold in roots of *lpcat1* mutant (Figure 7-figure supplement 2). We next  
321 determined the effects loss of function for each phosphate transporter located under the  
322 second GWAS peak (*PHT1;1*, *PHT1;2* and *PHT1;3*) for the accumulation of Pi in -Zn in 18-  
323 day-old plants. The *pht1;1* mutant showed low Pi accumulation in presence of Zn compared to  
324 WT plants (Figure 7E) consistently with (Shin et al., 2004) that reported that the *pht1;1* mutant  
325 showed a reduction in Pi content of the shoots relative to wild type plants grown under control  
326 condition (+Pi+Zn). Importantly, no increase of Pi concentration was observed in the shoots of  
327 *pht1;1* grown in -Zn, which contrast with the Pi accumulation in *pht1;2* and *pht1;3* that was in a  
328 similar range to WT plants in presence or absence of Zn. These results show the involvement  
329 of *PHT1;1* in the overaccumulation of Pi in the shoot of *lpcat1* grown in -Zn, and further  
330 supports a second peak of the GWAS on the chromosomal region of *PHT1* genes.

331

## 332 Discussion

333

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

339

340 In *A. thaliana*, GWAS has been shown to be a powerful approach to detect loci involved in  
341 natural variation of complex traits including variation in the accumulation of non-essentials or  
342 toxic elements in plants, such as sodium (Baxter et al., 2010), cadmium (Chao et al., 2012) or  
343 arsenic (Chao et al., 2014). Here we used GWAS to identify genes involved in the regulation of

344 the essential macronutrient (P) concentration in its anionic form (Pi) in plants grown under  
345 control conditions (+Zn) and -Zn. In both conditions, our GWA analysis reveals that there is  
346 widespread natural variation in shoot Pi concentration, and supports the existence of genetic  
347 factors that affect this trait (Figure 1). The GWAS data support the -Zn specificity of this  
348 response, since no association was detected around the *LPCAT1* locus in our control  
349 condition (+Zn) (Figure 1). The presence of the Zinc Deficiency Response Element (ZDRE)  
350 (Assunção et al., 2010) in the promoter of *LPCAT1* and more particularly the newly identified  
351 binding motif specific for bZIP23 (Figure 2, 3) in the 5' untranslated leader of *LPCAT1* is a  
352 strong argument supporting the Zn-dependency of this response.

353 A ZDRE is present in the promoter regions of many genes targeted by bZIP19 and bZIP23  
354 (Assunção et al., 2010). In addition to their positive regulatory role by inducing several Zn  
355 deficiency related genes, publicly available microarray showed that bZIP19 and bZIP23 may  
356 have a negative regulatory role as many genes were induced in the *bzip19/bzip23* mutant  
357 background compared to WT plants grown in -Zn (Azevedo et al., 2016). A functional  
358 redundancy of these two TFs was proposed based on the oversensitivity of the *bzip19* and  
359 *bzip23* double mutant to -Zn, which was not observed with either *bzip19* or *bzip23* single  
360 mutants (Assunção et al., 2010). This redundancy may not be absolute, as recent  
361 physiological and genetic evidence indicates that bZIP19 and bZIP23 are not completely  
362 redundant and they not only regulate the same, but also separate sets of genes in Arabidopsis  
363 (Inaba et al., 2015). Our results support this finding by showing that only bZIP23 is involved in  
364 regulating *LPCAT1* in response to -Zn. bZIP23 is likely to do so through two *cis*-elements in  
365 the non-coding part of the *LPCAT1* gene. One being the aforementioned ZDRE, which can  
366 also be bound by the bZIP19 paralogue of bZIP23, the other a novel binding motif, with  
367 versions TGTCACA and TGTCGAA, which are specifically bound by bZIP23. Worth noting, in  
368 the accessions panel used in our work one other allele can be found, in the Alc-0 accession  
369 (TGTCAAA) that displayed the lowest Pi accumulation in our panel of accession  
370 (Supplementary file 3, Figure 3-figure supplement 1). Alc-0 is the only accession among all

371 Arabidopsis accessions for which sequence information is available in the 1001 genome  
372 database, with this version of the ZDRE motif.

373 The new ZDRE motif resides in the 5'-untranslated leader of *LPCAT1*. Binding of bZIP23 to  
374 this element therefore might physically block the transcription of the *LPCAT1* gene under Zn  
375 deficient conditions. This is further supported by the repressive role for bZIP23 on the  
376 expression of *LPCAT1* under Zn deficiency. Genomic sequence surveys screening for this  
377 new TF-binding site promise to further help identifying a complete list of genes potentially  
378 regulated by bZIP23 in order to fully understand the involvement of bZIP23 in the -Zn  
379 response in a genome-wide manner.

380 Based on our results we propose that the sequence change in the 5'UTR sequence impacts  
381 gene(s) expression, and consequently causes variation in the associated traits. It has been  
382 proposed that evolution of species complexity from lower organisms to higher organisms is  
383 accompanied by an increase in the regulatory complexity of 5'UTRs (Lui et al., 2012).  
384 Nevertheless, so far little is known about the role of the 5'UTR sequence in the regulation of  
385 gene expression at the transcriptional level. The role of 5'UTR in the regulation of gene  
386 expression is perhaps best studied in human. Several studies showed that point substitutions  
387 in the 5'UTR change the expression of several genes such as *Ankirin Repeat Domain 26*  
388 (Pippucci et al., 2011), *Solute Carrier Family 2 Member 4* (Malodobra-Mazur et al., 2016), and  
389 *Cyclin D1* (Berardi et al., 2003). In contrast, few examples exist on the role of 5'UTR as *cis*  
390 regulators in plants. In Arabidopsis, it has been shown that the regulation of *NRP1* gene  
391 expression involves WRKY DNA Binding Proteins, which act on a potential *cis*-acting  
392 regulatory element located within the 5'UTR of *NRP1* (Yu et al., 2001). More general, a large-  
393 scale study of DNA affinity purification sequencing, which is a high-throughput TF binding site  
394 discovery method, has revealed a global preference across TF families for enrichment at  
395 promoters and 5'UTRs (O'Malley et al., 2016). Taken together, our study has uncovered a new  
396 example for the role of the 5'UTR in gene expression regulation and evolution in plants.

397



398 Of a particular interest is that our study revealed that this novel bZIP23-interacting sequence  
399 motif is subject to natural variation in *A. thaliana* (Figure 3), and its alteration may be  
400 associated with changes in the binding capacity of bZIP23. There are several ways that  
401 genetic variants can mechanistically contribute to plant adaptation. Many reported examples  
402 with regards to nutrient accumulation involve a change in the coding sequence of a gene that  
403 then alters the amino acid sequence of the encoded protein, thus leading to the disruption of  
404 gene function and a phenotypic change (Baxter et al., 2010; Chao et al., 2012; Chao et al.,  
405 2014). Reports on the role of specific regulatory element polymorphisms in the regulation of  
406 complex traits such as nutrient homeostasis crosstalk are less common, also because it is  
407 difficult to identify these relevant sequence changes. In our study we demonstrated that allelic  
408 variation (SNPs) in the novel bZIP23 binding motif upstream of the *LPCAT1* gene is  
409 associated with variation in *LPCAT1* expression levels, which in turn results in variation in Pi  
410 accumulation in  $-Zn$  conditions. The *LPCAT1* natural variants such as found in this study offer  
411 new inspiration for agronomical and biotechnological applications to optimize Pi use efficiency  
412 in plants.

413 While mutation of *LPCAT1* results in altered Lyso-PC and PC concentrations; an altered Lyso-  
414 PC/PC ratio; the up-regulation of *LPCAT1* in *bzip23* mutant background has no significant  
415 effect on this ratio. The simplest explanation for this observation would be that in the *bzip23*  
416 mutant background a portion of the synthesized PC is fed into the Kennedy pathway leading to  
417 the biosynthesis of different molecules such diacylglycerol or phosphatidic acid, and therefore  
418 no changes in Lyso-PC/PC could be detected (Wang et al., 2012). Nevertheless, an attractive  
419 second explanation exists: *LPCAT1* could be subjected to a regulation at the protein level as a  
420 strategy to optimize phosphatidylcholine specie levels. In animals, it has been proposed that  
421 the *LPCAT1* primary protein sequence may contain additional motifs, structural features, or  
422 interact with second messengers or ligands that can, under certain circumstances, alter its  
423 protein half-life (Zou et al., 2011). The precise mechanism that regulates *LPCAT1* protein  
424 stability by the ubiquitin-proteasomal pathway was already shown (Zou et al., 2011). Further  
425 studies will be required to verify the presence of such regulation pathway for *LPCAT1* in

426 Arabidopsis, and if confirmed, such a mechanism would add another level of our  
427 understanding of LPCAT1 activity in plants.

428  
429 Mutation of *LPCAT1* results in increased *PHT1;1* expression levels (Figure 7D); and ultimately  
430 an over-accumulation of Pi under Zn deficiency. The induction of the expression of genes  
431 encoding P uptake transporters under Zn deficiency has been reported in crop plants such as  
432 barley (*Hordeum vulgare*) (Huang et al., 2000); and the model plant Arabidopsis (Jain et al.,  
433 2013; Khan et al., 2014; Pal et al., 2017). Our study showed an induction of *PHT1;1* in *lpcat1*  
434 plants grown under Zn deficiency. The increase in *PHT1;1* expression levels is likely to explain  
435 the increased shoot Pi concentration in *lpcat1* since it is known that CaMV 35S promoter  
436 driven overexpression of this Pi transporter significantly increases shoot Pi concentration  
437 (Mitsukawa et al., 1997; Shin et al., 2004; Catarecha et al., 2007). Moreover, our finding  
438 provides evidence supporting a role for a Lyso-PC/PC-derived signal in regulating Pi  
439 homeostasis under -Zn. Until recently our knowledge on PL-derived signals in plants was  
440 scarce; however, physiological and molecular studies have shown that some PL classes could  
441 serve as precursors for the generation of diverse signalling molecules (Spector and Yorek,  
442 1985; Testerink and Munnik, 2005). For instance, Lyso-PC was shown to act as a signal for  
443 the regulation of the expression of arbuscular mycorrhiza (AM)-specific Pi transporter genes in  
444 potato, tomato and recently in *Lotus japonicus* (Drissner et al., 2007; Vijayakumar et al., 2016).  
445 In addition to the involvement of individual PLs in specific physiological processes in plants  
446 (e.g ion transport), a broader importance of changes in Lyso-PC/PC ratio for the regulation of  
447 plants development and basic cell biology is emerging. For instance, in Arabidopsis alteration  
448 of the Lyso-PC/PC ratio shortens the time to flower (Nakamura et al., 2014). In human cells,  
449 the Lyso-PC/PC ratio was also associated with an impairment of cell function, signalling and  
450 metabolism (Mulder et al., 2003; Klavins et al., 2015). Our data now demonstrate a  
451 fundamental link between PL metabolism, particularly Lyso-PC/PC, and Pi accumulation in -  
452 Zn condition, and lays the foundation for exploring the role of Lyso-PC/PC-derived signal in

453 controlling ion homeostasis and response to environmental changes not only in plant cells but  
454 also in other organisms.

455  
456 Overall, our study shed light on molecular mechanism underlying an old observation made as  
457 early as 1970s, namely P-Zn interaction in plants (Warnock, 1970; Marschner and Schropp,  
458 1977; Loneragan et al., 1979). By combining GWAS and functional genomics approaches, we  
459 discovered a complete pathway involved in the regulation of shoot Pi accumulation in -Zn that  
460 can be defined as bZIP23-LPCAT1(Lyso-PC/PC)-PHT1;1. Beyond its fundamental  
461 importance, our study could have a direct impact on plants growth in field by improving plant  
462 growth while reducing P supply, and will help meeting one of challenges facing agriculture in  
463 the 21<sup>st</sup> century.

464

## 465 Materials and Methods

466

### 467 **Plant materials and growth conditions.**

468 A subset of 223 *Arabidopsis thaliana* accessions of the RegMap panel (Horton et al., 2012)  
469 was used for genome-wide association studies (Arabidopsis Biological Resource Center  
470 accession number CS77400). The names of accessions are provided in supplementary file 1.  
471 All lines were used side by side in the same growth chambers under the same conditions, 22  
472 °C under long days (16 h light and 8 h dark). Arabidopsis mutants used in this study are in the  
473 Columbia-0 genetic background. The *bzip19bzip23* mutant previously described by (Assunção  
474 et al., 2010) was used in this work. T-DNA insertion mutant lines for the At5g43350 (N666665,  
475 *pht1;1*), At5g43360 (N661080, *pht1;2*), At5g43370 (N448417, *pht1;3*), At1g12640 (N686743  
476 (*lpcat1-1*,(Wang et al., 2012)), N442842) and At1g12650 (N526222) genes were obtained from  
477 the European Arabidopsis Stock Centre (arabidopsis.info; University of Nottingham, UK).  
478 Plants were germinated and grown on vertically positioned agar-solidified media (A1296,  
479 Sigma). The complete nutrient medium contained: 9.5 mM KNO<sub>3</sub>, 10.3 mM NH<sub>4</sub>NO<sub>3</sub>, 1.5 mM  
480 MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 100 μM FeNaEDTA, 100 μM MnSO<sub>4</sub>, 30 μM ZnSO<sub>4</sub>, 100  
481 μM H<sub>3</sub>BO<sub>3</sub>, 5 μM KI, 1 μM Na<sub>2</sub>MoO<sub>4</sub>, 0.1 μM CuSO<sub>4</sub> and 0.1 μM CoCl<sub>2</sub> (adapted from

482 Murashige and Skoog, 1962). Zn-deficient medium was made by omitting ZnSO<sub>4</sub>. Seeds sown  
483 on plates were stratified at 4 °C for 3 days. Plates were then transferred to a growth chamber  
484 for 18 days set at the following conditions: 16/8h light/dark cycle, 250 μmol m<sup>-2</sup> s<sup>-1</sup> light, and  
485 24/20 °C (light/dark) .

486

#### 487 **Plasmid construction and plant transformation.**

488 The *LPCAT1* coding region driven by its native promoter (1.5 kbp fragment immediately  
489 upstream of the start codon including the 5' untranslated region (5'-UTR)) from Col-0 and Sap-  
490 0 accessions were amplified using PCR and the following primers p*LPCAT1*<sup>Col-0</sup>-forward 5'-  
491 cgctgcagggtgtcgaaaaccggtttt-3'; p*LPCAT1*<sup>Col-0</sup>-reverse 5'-cgggatcctgatcagagagtacaac  
492 aggagag-3'; p*LPCAT1*<sup>Sap-0</sup>-forward 5'-cgctgcagggtgtcacaaaccgggt-3' and p*LPCAT1*<sup>Sap-0</sup>-  
493 reverse 5'-cgggatccatgatcagatagttacaacaggagagg-3', and then cloned into the binary vector  
494 pCAMBIA1301 by restriction enzymes *Bam*HI and *Pst*I (site underlined). The *LPCAT1* coding  
495 regions were amplified using PCR and the following primers p*LPCAT1*<sup>Col-0</sup>-forward 5'-  
496 cgctgcagttattcttctttacgcggttttg-3'; p*LPCAT1*<sup>Sap-0</sup>-forward 5'-cgctgcagttattcttctttacgtggttttg-3'  
497 and p*LPCAT1*<sup>Col-0/ Sap-0</sup>-reverse 5'-cgctgcagatggatgatgagttcaatggctg-3'. *Pst*I was used for the  
498 fusion of p*LPCAT1*<sup>Col-0</sup> or p*LPCAT1*<sup>Sap-0</sup> promoters to either *LPCAT1*<sup>Col-0</sup> or *LPCAT1*<sup>Sap-0</sup>. The  
499 constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and then used for  
500 Arabidopsis transformation by the floral dip method (Clough and Bent, 1998). Transgenic  
501 plants were selected by antibiotic resistance, and only homozygote descendants of  
502 hemizygote T2 plants segregating 1:3 for antibiotic resistance: sensitivity were used for  
503 analysis.

504

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

506 All accessions were grown in the presence or absence of zinc for 18 days. Shoots were  
507 collected, weighed and ground into powder in liquid nitrogen. An aliquot (30 mg) was  
508 incubated at 70 °C in NanoPure water, for 1 hour. Inorganic phosphate (Pi) concentrations  
509 were determined using the molybdate assay as previously described by (Ames, 1966). The

510 shoot Pi concentrations across the analysed accessions was used as phenotype for GWA  
511 analysis. The GWA analysis was performed in the GWAPP web interface using the mixed  
512 model algorithm (AMM) that accounts for population structure (Seren et al., 2012) and using  
513 the SNP data from the RegMap panel (Atwell et al., 2010; Brachi et al., 2010; Horton et al.,  
514 2012). Only SNPs with minor allele counts greater or equal to 10 (at least 10 out of 223  
515 accessions contained the minor allele) were taken into account. To correct for multiple testing,  
516 the false discover rate was calculated using the Benjamini-Hochberg correction (Benjamini  
517 and Hochberg, 1995). An FDR threshold of 0.1 was used to detect significant associations.

518  
519

### **Haplotype analysis**

520 Haplotype analysis was performed as follows. SNPs from a 50 kb window around the  
521 significant marker SNP (chromosome 1, position 4306845) were extracted for the 221 natural  
522 accessions. SNP data was taken from the Regional Mapping Project SNP panel described in  
523 Horton et al. (2012). These SNPs were used as the input for fastPHASE version 1.4.0 (Scheet  
524 and Stephens, 2006). Results were then visualized using R.

525  
526

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

527 For expression analysis, the Plant RNeasy extraction kit (Qiagen) was used to extract total  
528 RNA free of residual genomic DNA from 100 mg frozen shoot material. Total RNA was  
529 quantified with a NanoDrop spectrophotometer (Thermo Scientific). Two  $\mu$ g of total RNA was  
530 used to synthesize cDNA. Reverse transcriptase PCR (RT-qPCR) was performed with a Light  
531 Cycler 480 Real-Time PCR System (Roche) using SYBR green dye technology (Roche) as  
532 described by (Khan et al., 2014). The primers used in this study are *LPCAT1*-forward 5'-  
533 ggtgtaagcttgacgaaac-3'; *LPCAT1*-reverse 5'-agagaaacaagaaccgga-3' and *UBQ10*-forward  
534 5'-aggatggcagaactcttgcct-3'; *UBQ10*-reverse. 5'-tcccagtcacgtcttaacg-3'. The primers used to  
535 quantify *ZIP4* are *ZIP4*-forward 5'-cggtaaacataagaaatcaggagc-3'; *ZIP4*-reverse 5'-  
536 taaatctcgagcgtgtgatg-3'; and for *ZIP12* are *ZIP12*-forward 5'-aacagatctcgcttgccg-3'; *ZIP12*-  
537 reverse 5'-aatgtgatcatcatcttggg-3'. Primers used to quantify the *PHT1* gene family member are  
538 designed according to (Khan et al., 2014). Quantification of mRNA abundance was performed

539 in a final volume of 20  $\mu$ L containing 10  $\mu$ L of the SYBR Green I master mix, 0,3  $\mu$ mol primers,  
540 and 5  $\mu$ L of a 1:25 cDNA dilution. PCR conditions were as 95°C for 5 min, and followed by 40  
541 cycles of 95°C for 10 s, 60°C for 10 s, 72 °C for 25 s. One final cycle was added in this  
542 program: 72 °C for 5 min. For every reaction , the cycle threshold (Ct) value was calculated  
543 from the amplification curves. For each gene, the relative amount of calculated mRNA was  
544 normalized to the calculated mRNA level of the *Ubiquitin10* control gene (*UBQ10*: At4g05320)  
545 and expressed as relative values against wild-type plants grown in the presence or absence of  
546 Zn in the medium. Quantification of the relative transcript levels was as described in(Rouached  
547 et al., 2008). The mRNA abundance of each gene was calculated following normalization  
548 against the CT values of *Ubiquitin10* mRNA, for instance  $\Delta Ct, LPCAT1 = Ct, LPCAT1 -$   
549  $(Ct, UBQ10)$ . Quantification of the relative transcript levels was performed as following, low Zn  
550 (-Zn) treatment was compared to control (Ct, +Zn), the relative mRNA accumulation of each  
551 gene was expressed as a  $\Delta\Delta Ct$  value calculated as follows:  $\Delta\Delta Ct = \Delta Ct, LPCAT1(-Zn) -$   
552  $\Delta Ct, LPCAT1(Ct)$ . The fold change in relative gene expression was determined as  $2^{-\Delta\Delta Ct}$ .

553

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

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

566 sepharose beads (GE Healthcare, Freiburg, Germany), and incubated at 4°C for 3 h. The resin  
567 was centrifuged (500 ×g, 10 min, 4°C) and washed five times with 1X PBS buffer.  
568 bZIP19 and bZIP23 were then cleaved from GST using 25 unit/ml of thrombin at room  
569 temperature for 16h. All fractions were subjected to SDS-PAGE, and proteins concentrations  
570 were determined. For protein quantification, absorbance measurements were recorded on a  
571 nanodrop spectrophotometer (Model No.1000, Thermo Scientific Inc., Wilmington, Delaware,  
572 USA) at 280 nm, and in parallel on a VICTOR2™ microplate reader (MULTILABEL  
573 COUNTER, life sciences) at 660 nm using the Pierce 660 nm Protein Assay (Pierce/Thermo  
574 Scientific, Rockford; (Antharavally et al., 2009)).

575

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

577 EMSA was performed using purified proteins and DNA probes labeled with Biotin-TEG at the  
578 3' end. Biotin-TEG 3' end-labeled single-stranded DNA oligonucleotides were incubated at  
579 95 °C for 10 min and then annealed to generate double-stranded DNA probes by slow cooling.  
580 The sequences of the oligonucleotide probes were synthesized by Eurofins Genomics and are  
581 as following: 5'-ttaggttcac**gtgtcgacat**gaaaggagct-3', 5'-catatccat**gtgtcgaa**aacccgattttt-3' and 5  
582 '-catatccat**gtgtcaca**aacccgggtttt-3. The binding of the purified proteins (≈ 150 ng) to the  
583 Biotin-TEG labelled probes (20 fmol) was carried out using the LightShift Chemiluminescent  
584 EMSA Kit (Thermo Scientific, Waltham, USA) in 20 µL reaction mixture containing 1X binding  
585 buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), 2.5% glycerol, 5 mM MgCl<sub>2</sub>, 2 µg of poly  
586 (dI-dC) and 0.05% NP-40. After incubation at 24°C for 30 min, the protein –probe mixture was  
587 separated in a 4% polyacrylamide native gel at 100 V for 50 min then transferred to a Biodyne  
588 B Nylon membrane (Thermo Scientific) by capillary action in 20X SSC buffer overnight. After  
589 ultraviolet crosslinking (254 nm) for 90 s at 120 mJ.cm<sup>-2</sup>. The migration of Biotin-TEG labelled  
590 probes was detected using horseradish peroxidase-conjugated streptavidin in the LightShift  
591 Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's protocol, and  
592 then exposed to X-ray film.

593

594 **Phospholipid Extraction**

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

606

607 ***In planta* transactivation assay.**

608 The *in planta* transactivation assay was performed in *N. benthamiana*. LPCAT Col-0 promoter  
609 and LPCAT Col-0 mutated promoter were cloned pLPCAT1Col-0-For 5'-  
610 ggggacaagtttgtaaaaaagcaggcttcggtgtcgaaaaccggtt-3'; pLPCAT1Col-0-Rev 5'-  
611 ggggaccactttgtacaagaaagctgggtctgatcagagttacaacaggagag -3' ;pLPCAT1Col-0-mutated-For  
612 5'-ggggacaagtttgtaaaaaagcaggcttcggtgtcacaaccggttt-3'. The LPCAT1 promoters were  
613 then fused to the  $\beta$ -GUS-encoding reporter gene using the Gateway system. These following  
614 primers were used to clone the promoter of the zinc transporter ZIP4-For 5'-  
615 ggggacaagtttgtaaaaaagcaggcttctggaaagtgaagtggattg-3'; ZIP4-REV 5'-  
616 ggggaccactttgtacaagaaagctgggtctgatcatcgacgaagaccatgggaacaagagt -3' (Lin et al., 2016).  
617 Each of these primer was then fused to  $\beta$ -GUS-encoding reporter gene. bZIP23 coding  
618 sequences CDS was placed under the CaMV. 35S promoter. The 35S::C-YFP construct was  
619 provided by Dr. Seung Y. Rhee (Bossi et al., 2017). Each construct was transformed into  
620 *Agrobacterium*. Positive clones per construct were grown overnight at 28 °C, and then washed  
621 four times in infiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES (pH 5.6) and 100  $\mu$ M



622 acetosyringone). The effector construct and reporter construct (OD600 of 0.8), were co-  
623 infiltrated at a ratio of 9 to 1 in fully expanded leaves (3<sup>rd</sup> or 4<sup>th</sup> leaves) of five to six week-old  
624 tobacco plants. Each plasmid combination was infiltrated in one leaf from different plants. We  
625 used C-YFP as negative controls. Three independent infiltrations per combination resulting in  
626 6 samples per construct. Three days after infiltration, leaves were collected, and the infiltrated  
627 areas in each leaf were excised and pooled into one sample. The GUS extraction and the  
628 GUS enzymatic activity measurements was performed as described by Bisso et al., (2017).  
629 The relative GUS enzymatic activity was determined by comparing the effect of bZIP23 TF and  
630 C-YFP protein on each promoter.

631  
632

### **Statistical analysis**

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

637

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## 651 Competing interests

652 The authors declare that no competing interests exist.

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## 894 Figure Legends

895 **Figure 1. Genome-wide association (GWA) analysis of Arabidopsis shoot Pi**  
896 **concentration.** 223 *Arabidopsis thaliana* accessions were grown supplemented with zinc (+Zn)  
897 or without zinc (-Zn) for 18 days under long day conditions, upon which shoot inorganic  
898 phosphate (Pi) concentrations were determined. (A) Mean shoot Pi concentration of  
899 *Arabidopsis* accessions in +Zn is plotted against the mean shoot Pi concentration of  
900 *Arabidopsis* accessions in -Zn. (B, C) Manhattan plots of GWA analysis of *Arabidopsis* shoot  
901 Pi concentration in -Zn (B) and +Zn (C). The five *Arabidopsis* chromosomes are indicated in  
902 different colours. Each dot represents the  $-\log_{10}(P)$  association score of one single nucleotide  
903 polymorphism (SNP). The dashed red line denotes an approximate false discovery rate 10%  
904 threshold. Boxes indicate the location of the *LPCAT1* (red) quantitative trait loci (QTL). (D)  
905 Gene models (upper panel) and SNP  $-\log_{10}(P)$  scores (lower panel) in the genomic region  
906 surrounding the GWA QTL at the *LPCAT1* (E); 5' and 3' indicate the different genomic DNA  
907 strands and orientation of the respective gene models. (E) Distribution of Pi concentrations in  
908 accessions with the phenotype A versus accessions with phenotype G. Asterisk indicates a  
909 significant difference between the two groups of accessions of  $P < 0.01$ .

910

911 **Figure 2. Loss of function mutation of *Lyso-PhosphatidylCholine AcylTransferase 1***  
912 **(*LPCAT1*), and not *At1g12650*, affects shoot Pi concentration in a Zn supply and *bZIP23***  
913 **dependent manner.** (A) Shoot Pi concentration of 18-days-old Col-0 wild-type plants, *lpcat1*  
914 and *At1g12650* mutants grown in +Zn or -Zn conditions. (B) Gene structure of *LPCAT1*. The  
915 grey box represents the promoter region, green boxes are 5' and 3' untranslated regions,  
916 black boxes represent exons, and black lines represent introns, the arrow head indicates the  
917 direction of transcription, ATG indicates the start codon. The Zinc Deficiency Response  
918 Element (ZDRE) binding site for *bZIP19* and *bZIP23*, and the newly identified binding site for  
919 *bZIP23* are indicated. (C) Differential binding of *bZIP19* and *bZIP23* to two promoter regions of  
920 *LPCAT1* gene. EMSA analysis on 30-bp promoter fragments from motif present in *LPCAT1*

921 promoter of contrasting accessions showed in (A). (D) Relative *LPCAT1* transcript abundance  
922 (-Zn/+Zn) in Col-0 wild-type plants grown on +Zn or -Zn agar medium for 6, 12 and 18 days.  
923 (E) Relative *LPCAT1* transcript abundance in Col-0 wild-type plants, *bzip19*, *bzip23*, and  
924 *bzip19/bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. The relative  
925 mRNA levels was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA  
926 level (*UBQ10*: At4g05320). (F) Shoot Pi concentration in Col-0 wild-type plants, *bzip19* and  
927 *bzip19/bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. Values are  
928 means of three to six biological replicates. Individual measurements were obtained from the  
929 analysis of shoots collected from a pool of 10 plants. Error bars indicate SD; One and two  
930 asterisks indicate a significant difference with WT plants (ANOVA and Tukey test) of  $P < 0.05$   
931 and  $P < 0.01$ , respectively.

932

933 **Figure 3. Identification of a new binding motif specific for bZIP23, and the variation of**  
934 **LPCAT1 gene expression between genotypes in -Zn condition.** (A) Sequence comparison  
935 for ZDRE and the new binding site motif for bZIP23 in the promoter of accession with high  
936 ratio of Pi accumulation in -Zn/+Zn (Col-0, Ang-0, CICB-5, Est-1, RRS-10) and low Pi  
937 accumulation ratio - Zn/+Zn (Sap-0, Ts-1, Br-0 and PHW-2). (B) Differential binding of bZIP19  
938 and bZIP23 to a specific ZDRE motif of *LPCAT1* (GTGTCACA). (C-D) *in planta* transactivation  
939 assay. (C) 35S:bZIP23 and 35S::C-YFP were used as effectors. p*LPCAT1*: the native Col-0  
940 *LPCAT1* promoter (with « GTGTCGAA » as new ZDRE), mp*LPCAT1*: a modified (point  
941 mutation) version of the Col-0 promoter to only contain the new ZDRE of Sap-0 («  
942 GTGTCACA ») ; pZIP4 ; promoter of the zinc transporter *ZIP4* gene. Each p*LPCAT1* (native,  
943 Col-0), mp*LPCAT1* (mutated version) or pZIP4 promoter was fused to a  $\beta$ -glucuronidase  
944 (*GUS*)-encoding reporter gene (reporter). (D) The effect of bZIP23 TF on the activity of each  
945 promoter p*LPCAT1*, mp*LPCAT1* or pZIP4 was determined by measuring GUS activity. The  
946 effect of C-YFP protein on the activity of each promoter p*LPCAT1*, mp*LPCAT1* or pZIP4 was  
947 used as a control to determine the basal level of GUS activity for each promoter. Comparing  
948 the effect of bZIP23 TF and C-YFP protein on each promoter enabled the determination of the



949 relative GUS activity. Error bars represent standard error from 3 independent experiments.  
950 The asterisks indicate that the relative GUS activity is statistically different from the YFP  
951 control (p-value <0.01, t-test). (E) Relative *bZIP23* and *LPCAT1* transcripts abundance in -Zn  
952 and +Zn conditions. Col-0, Ang-0, CICB-5, Est-1, RRS-10, Sap-0, Ts-1, Br-0 and PHW-2  
953 genotypes were grown on +Zn or -Zn agar medium. The relative mRNA level was quantified by  
954 RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320).  
955 Values are means of six biological replicates. Individual measurements were obtained from the  
956 analysis of shoots collected from a pool of 20 plants. Error bars indicate SD; one and two  
957 asterisk indicates a significant difference with Col-0 plants (ANOVA and Tukey test) of P <  
958 0.05 and P < 0.01 respectively.

959

960 **Figure 4. Natural allelic variation of *LPCAT1* locus causes phenotypic variation of Pi**  
961 **accumulation in Zn deficiency conditions.** (A) Schematic representation of the transgenic  
962 constructs used to complement the *lpcat1* null mutant (Col-0 background). (B) Shoot Pi  
963 concentration (-Zn / +Zn) of 18-days-old Col-0 wild-type plants, *lpcat1* mutant transformed  
964 with empty vector, or with constructs schematized in (A) grown in +Zn or -Zn conditions. (C)  
965 The ANOVA results are presented in the table. Significant codes: '\*\*\*\*' 0.001 and '\*' 0.05.  
966 Relative *LPCAT1* transcript abundance in wild-type plants (Col-0 background) and the  
967 transgenic lines generated using the construct schematized in (A) grown on +Zn or -Zn agar  
968 medium. The relative mRNA levels was quantified by RT-qPCR and normalized to the  
969 *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). Values are means of three to  
970 biological replicates. Individual measurements were obtained from the analysis of shoots  
971 collected from a pool of six plants. Error bars indicate SD; asterisks indicates a significant  
972 difference with Col-0 plants (ANOVA and Tukey test) of P < 0.05.

973

974 **Figure 5. Loss of function mutations of *LPCAT1* affect the lysoPC/PC ratio in -Zn**  
975 **conditions.** (A) Schematic representation of the biochemical function of *LPCAT1*, which  
976 catalyses the formation of phosphatidylcholine (PC) from lyso-PC and long-chain acyl-CoA.  
977 (B) Lyso-PC concentration (C) PC concentration (D) Lyso-PC/PC concentration ratios of Col-0

978 wild-type plants, *bzip23* and *lpcat1* mutant lines grown in +Zn or -Zn conditions for 18 days.  
979 Individual measurements were obtained from the analysis of shoots collected from a pool of  
980 five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant  
981 differences (ANOVA and Tukey test,  $P < 0.05$ ) between mutants and Col-0 are indicated with  
982 asterisks.

983

984 **Figure 6. Effect of the polymorphisms in the regulatory region of *LPCAT1* on the change**  
985 **in LPC/PC ratios in -Zn conditions.** (A) Lyso-PC concentration (B) PC concentration (C)  
986 Lyso-PC/PC concentration ratios of Sap-0, Col-0 wild-type plants, and *lpcat1* expressing  
987  $pLPCAT1^{Col-0}::LPCAT1^{Col-0}$ ,  $pLPCAT1^{Col-0}::LPCAT1^{Sap-0}$ ,  $pLPCAT1^{Sap-0}::LPCAT1^{Col-0}$ ,  
988  $pLPCAT1^{Sap-0}::LPCAT1^{Sap-0}$  constructs and *lpcat1* transformed with empty lines grown in +Zn  
989 or -Zn conditions for 18 days. Individual measurements were obtained from the analysis of  
990 shoots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates.  
991 Statistically significant differences (ANOVA and Tukey test,  $P < 0.05$ ) between mutants and  
992 Col-0 are indicated with asterisks.

993

994 **Figure 7. Loss of function mutations of *LPCAT1* show enhanced expression of *PHT1;1***  
995 **when compared to Col-0 wild-type plants.** (A, B) Genome-wide association (GWA) analysis  
996 of Arabidopsis shoot Pi concentration. 223 Arabidopsis thaliana accessions were grown on  
997 agar medium supplemented with zinc (+Zn) or without zinc (-Zn) for 18 days under long day  
998 conditions, upon which shoot inorganic phosphate (Pi) concentrations were determined.  
999 Manhattan plots of GWA analysis of Arabidopsis shoot Pi concentration in -Zn (A) and +Zn (B).  
1000 The five Arabidopsis chromosomes are indicated in different colours. Each dot represents the  
1001  $-\log_{10}(P)$  association score of one single nucleotide polymorphism (SNP). The dashed red  
1002 line denotes an approximate false discovery rate 10% threshold. Boxes indicate the location of  
1003 the PHT1 (blue) quantitative trait loci (QTL). (C) Gene models (upper panel) and SNP –  
1004  $\log_{10}(P)$  scores (lower panel) in the genomic region surrounding the GWA QTL at the or  
1005 PHT1 locus ; 5' and 3' indicate the different genomic DNA strands and orientation of the

1006 respective gene models. (D) Relative expression level of all members of the Arabidopsis *PHT1*  
1007 gene family in shoots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on -Zn  
1008 agar medium compared to their expression on +Zn. mRNA accumulation was quantified by  
1009 RT-qPCR, normalized to the mRNA level of the *UBIQUITIN10* reference gene (*UBQ10*:  
1010 At4g05320) and expressed as relative values against its *UBQ10* normalized mRNA level of  
1011 Col-0 grown in +Zn medium (control). (E) Shoot Pi concentration of 18-days-old Col-0 wild-  
1012 type plants, *pht1;1*, *pht1;2*, and *pht1;3* mutants grown in +Zn or -Zn conditions. Data are mean  
1013  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  
1014  $P < 0.05$  and  $P < 0.01$ ) are indicated by one or two asterisks.

1015  
1016

### Supplementary Figure

1017 **Figure 1-figure supplement 1. mRNA abundance of Zn-responsive genes *ZIP4* and *ZIP12***  
1018 **in roots of Col-0 plants grown in presence and absence of Zn.** Reverse transcriptase  
1019 qPCR analyses of transcript levels changes in response to Zn-deficiency of the genes *ZIP4*  
1020 (*At1g10970*) and *ZIP12* (*At5g62160*) in shoots of Arabidopsis (Col-0). Seedlings were grown  
1021 on vertical agar plate in presence or absence of Zn for 18 days. Transcript levels of these  
1022 genes are expressed relative to the average transcript abundance of the *UBIQUITIN10*  
1023 (*UBQ10*; *At4g05320*) that was used as an internal control. Every data point was obtained from  
1024 the analysis of shoots collected from a pool of six plants. Data presented are means of three  
1025 biological replicates  $\pm$  SE. Asterisks indicate statistically significant differences compared to  
1026 the +Zn condition for each gene analyzed ( $P < 0.05$ ).

1027

1028 **Figure 1-figure supplement 2. Genome-wide association (GWA) analysis of Arabidopsis**  
1029 **shoot Pi concentration.** 223 Arabidopsis thaliana accessions were grown supplemented with  
1030 zinc (+Zn) or without zinc (-Zn) for 18 days under long day conditions, upon which shoot  
1031 inorganic phosphate (Pi) concentrations were determined. (A, B) Histogram of the frequency  
1032 distribution of mean shoot Pi concentration of Arabidopsis accessions in +Zn (A) and -Zn (B).  
1033 (C, D) Manhattan plots of GWA analysis of Arabidopsis shoot Pi concentration in -Zn (C) and

1034 +Zn (D) generated using 1001 Genomes imputed SNPs. Each dot represents the  $-\log_{10}(P)$   
1035 association score of one single nucleotide polymorphism (SNP). Boxes indicate the location of  
1036 the *LPCAT1* (red) quantitative trait loci (QTL).

1037

1038 **Figure 1-figure supplement 3. Haplotype analysis of region around SNP C1P4306845.**

1039 (upper panel) Clusterplot represents the haplotype blocks according to fastphase2 for SNPs  
1040 25 kb up and downstream of the significantly associated SNP. Colours represent a specific  
1041 haplotype. X-axis: Accessions, Y-axis: SNPs. The horizontal blue line indicates the position of  
1042 the significant SNP. The red box indicates the *LPCAT* gene model. The blue arrow represents  
1043 the transcription direction of the gene model (*LPCAT* is on the - strand). (lower panel)  
1044 Normalized Pi content in -Zn condition of each accession (using the R scale function). Colour  
1045 indicates the genotype of the accession at SNP C1P4306845 (top GWAS hit), whereby red  
1046 colour indicates the minor SNP allele that is associated with high Pi content in -Zn.

1047

1048 **Figure 3-figure supplement 1. Shoot Pi concentrations in Arabidopsis accessions**

1049 **grouped by new ZDRE motif.** Shoot Pi concentrations of *Arabidopsis thaliana* accessions  
1050 grown on agar medium supplemented with zinc (+Zn) (A) or without zinc (-Zn) (B) for 18 days  
1051 under long day conditions. Data same as in Supplementary file 3. Grouping of accessions is  
1052 based on their ZDRE binding motif allele TGTCAAA, TGTCACA and TGTCGAA respectively.  
1053 Asterisk indicates a significant difference according to Student's T-test  $P < 0.01$ .

1054

1055 **Figure 7-figure supplement 1. Relative expression level of all members of the**

1056 ***Arabidopsis PHT1* gene family.** *Arabidopsis thaliana* Col-0, *lpcat1-1* and *lpcat1-2* mutant  
1057 lines were grown agar medium supplemented with zinc (+Zn) for 18 days under long day  
1058 conditions. mRNA level was quantified by RT-qPCR and normalized to the *Ubiquitin10*  
1059 reference mRNA level (*UBQ10*: At4g05320) and expressed as relative values against the  
1060 *UBQ10* normalized mRNA level in Col-0. Values are means of six biological replicates.  
1061 Individual measurements were obtained from the analysis of shoots collected from a pool of 20

1062 plants. Error bars indicate SD; one and two asterisk indicates a significant difference with Col-  
1063 0 plants (ANOVA and Tukey test) of  $P < 0.05$  and  $P < 0.01$  respectively.

1064

1065 **Figure 7-figure supplement 2. High Affinity of Phosphate Transporter (*PHT1;1*) gene**  
1066 **expression analysis.** Loss of function mutations of *LPCAT1* show enhanced expression of  
1067 *PHT1;1* when compared to Col-0 wild-type plants. Relative expression level of *PHT1;1* gene in  
1068 roots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on -Zn agar medium.  
1069 mRNA accumulation was quantified by RT-qPCR, normalized to the mRNA level of the  
1070 *UBIQUITIN10* reference gene (*UBQ10*: At4g05320) and expressed as relative values against  
1071 Col-0 grown in +Zn medium (control). Individual measurements were obtained from the  
1072 analysis of roots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological  
1073 replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.01$ ) are indicated  
1074 by two asterisks.

1075

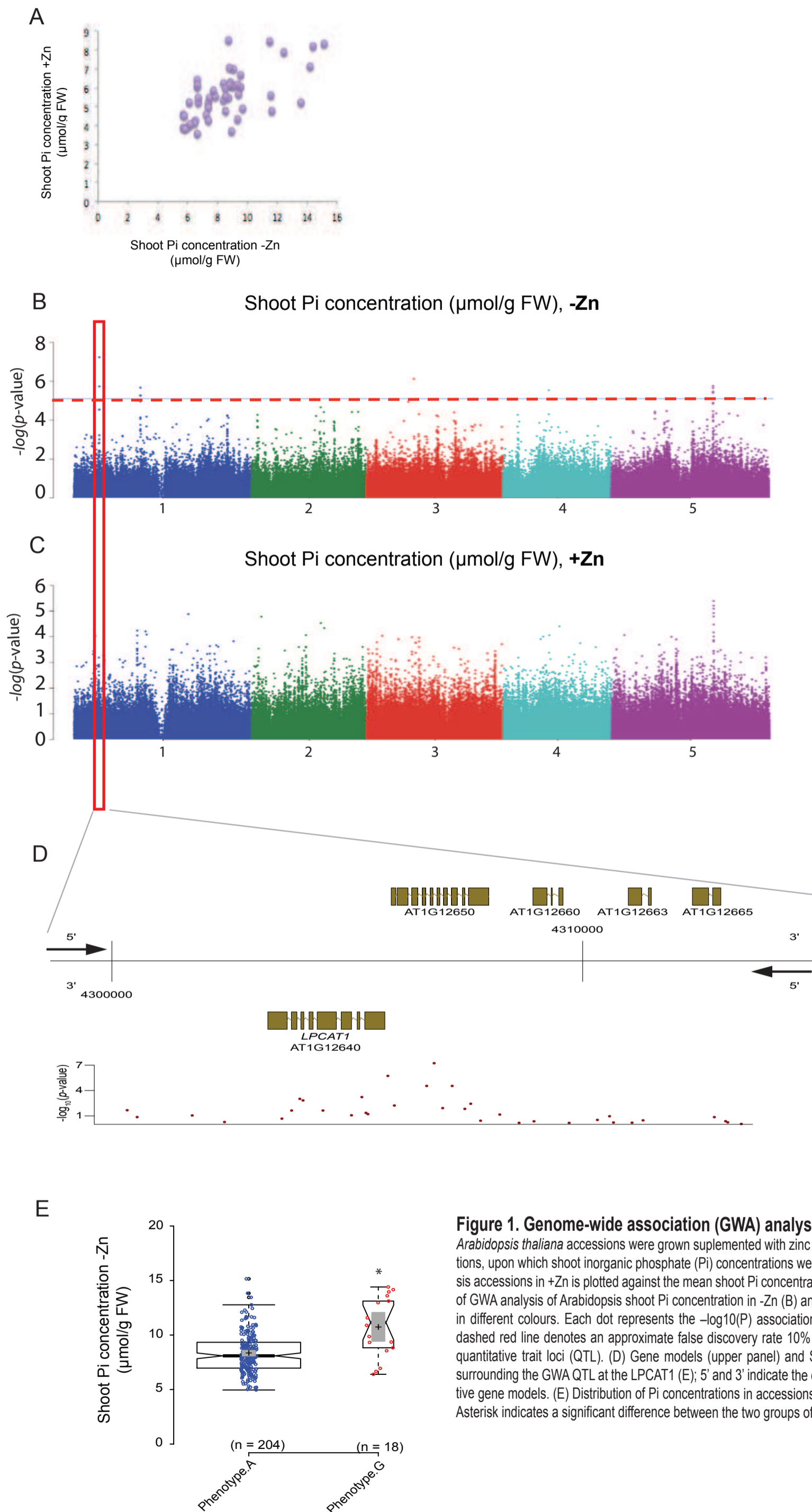
#### 1076 **Supplement Table**

1077 **Supplement file 1.** Shoots Pi concentration in the 223 *Arabidopsis thaliana* accessions grown  
1078 in presence or absence of zinc.

1079 **Supplement file 2.** Coordinates for the significant SNPs associated with Pi concentration in  
1080 shoots in Zn conditions.

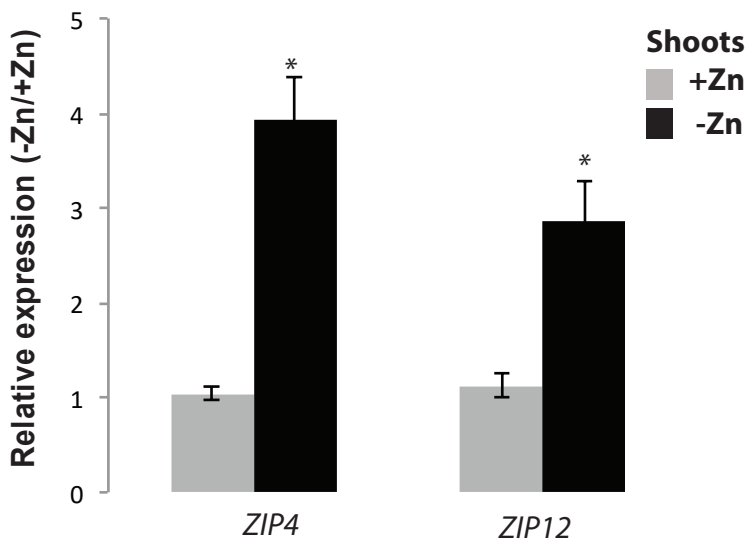
1081 **Supplement file 3.** List of new ZDRE motif in the *Arabidopsis thaliana* accessions and the  
1082 shoot Pi content in presence (+Zn) or absence of Zn (-Zn).

**Figure 1**



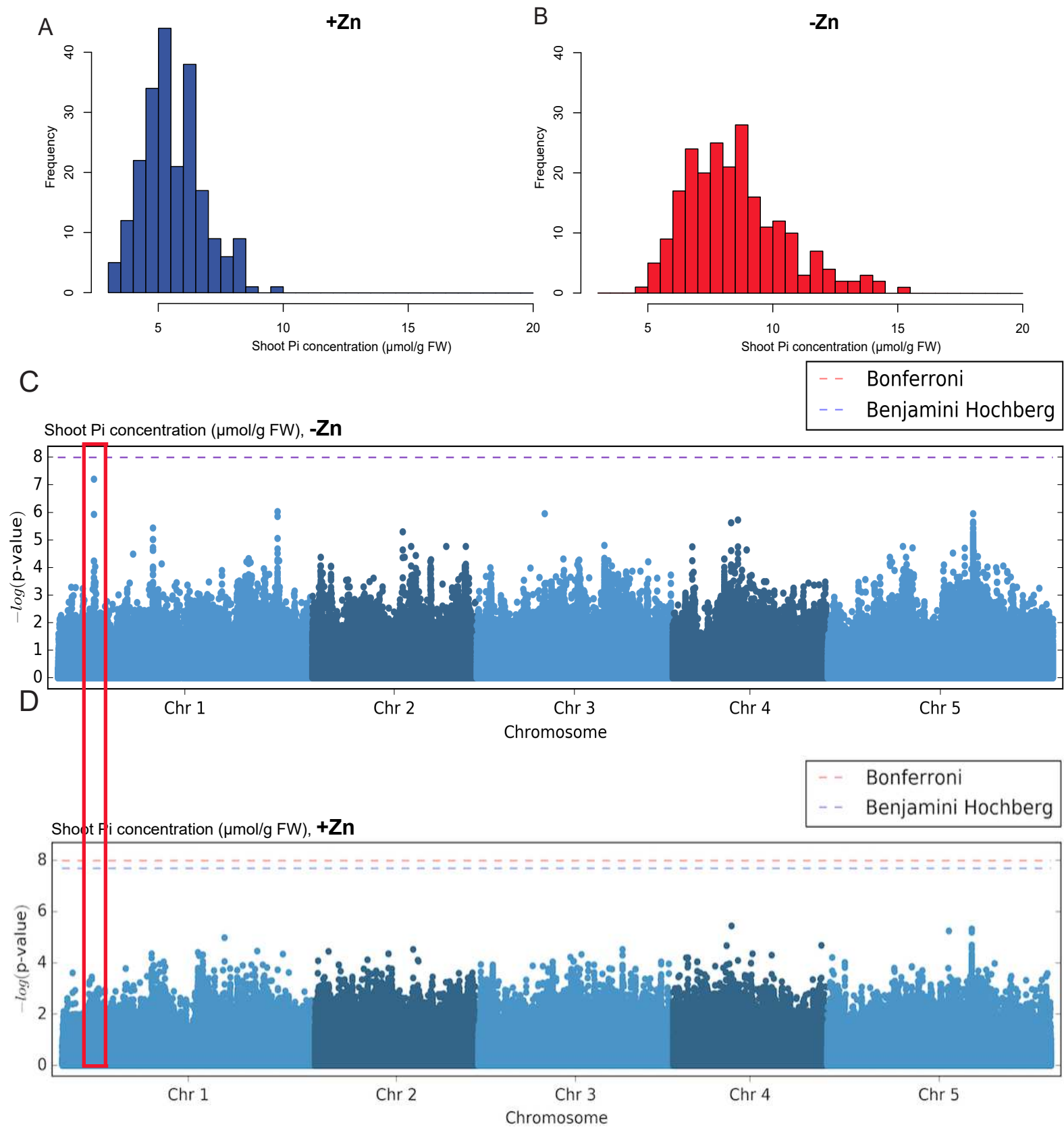
**Figure 1. Genome-wide association (GWA) analysis of Arabidopsis shoot Pi concentration.** 223 *Arabidopsis thaliana* accessions were grown supplemented with zinc (+Zn) or without zinc (-Zn) for 18 days under long day conditions, upon which shoot inorganic phosphate (Pi) concentrations were determined. (A) Mean shoot Pi concentration of Arabidopsis accessions in +Zn is plotted against the mean shoot Pi concentration of Arabidopsis accessions in -Zn. (B, C) Manhattan plots of GWA analysis of Arabidopsis shoot Pi concentration in -Zn (B) and +Zn (C). The five Arabidopsis chromosomes are indicated in different colours. Each dot represents the  $-\log_{10}(P)$  association score of one single nucleotide polymorphism (SNP). The dashed red line denotes an approximate false discovery rate 10% threshold. Boxes indicate the location of the LPCAT1 (red) quantitative trait loci (QTL). (D) Gene models (upper panel) and SNP  $-\log_{10}(P)$  scores (lower panel) in the genomic region surrounding the GWA QTL at the LPCAT1 (E); 5' and 3' indicate the different genomic DNA strands and orientation of the respective gene models. (E) Distribution of Pi concentrations in accessions with the phenotype A versus accessions with phenotype G. Asterisk indicates a significant difference between the two groups of accessions of  $P < 0.01$ .

**Figure 1-figure supplement 1**



**Figure 1-figure supplement 1. mRNA abundance of Zn-responsive genes ZIP4 and ZIP12 in roots of Col-0 plants grown in presence and absence of Zn.** Reverse transcriptase qPCR analyses of transcript levels changes in response to Zn-deficiency of the genes *ZIP4* (At1g10970) and *ZIP12* (At5g62160) in shoots of Arabidopsis (Col-0). Seedlings were grown on vertical agar plate in presence or absence of Zn for 18 days. Transcript levels of these genes are expressed relative to the average transcript abundance of the *UBIQUITIN10* (*UBQ10*; At4g05320) that was used as an internal control. Every data point was obtained from the analysis of shoots collected from a pool of six plants. Data presented are means of three biological replicates  $\pm$  SE. Asterisks indicate statistically significant differences compared to the +Zn condition for each gene analyzed ( $P < 0.01$ ).

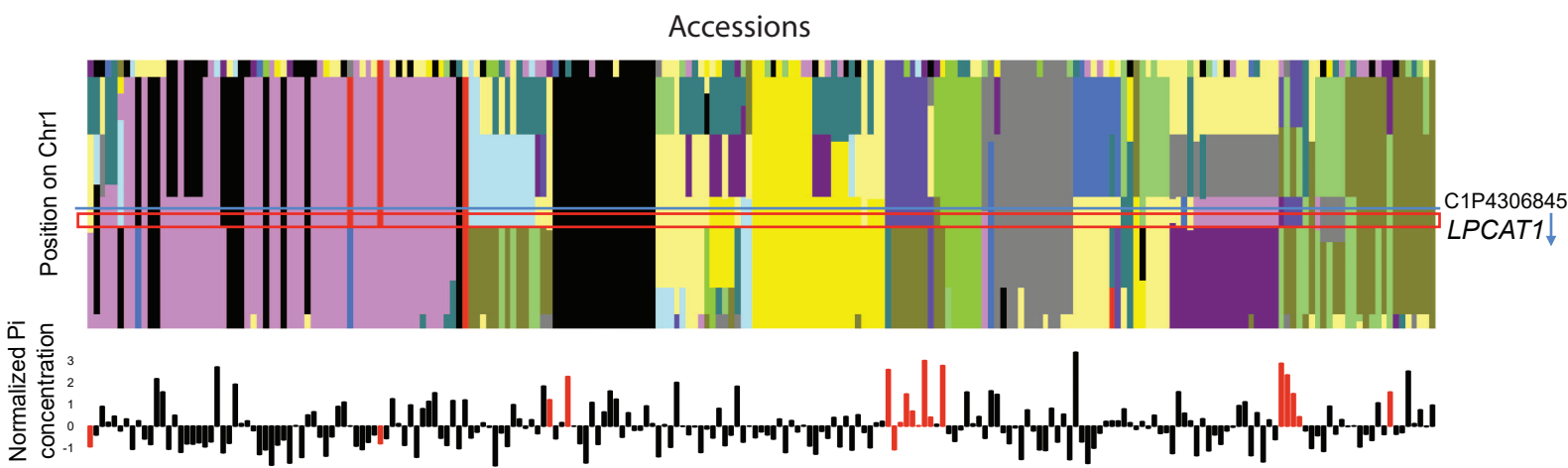
**Figure 1-figure supplement 2**



**Figure 1-figure supplement 2. Genome-wide association (GWA) analysis of Arabidopsis shoot Pi concentration.** 223 *Arabidopsis thaliana* accessions were grown supplemented with zinc (+Zn) or without zinc (-Zn) for 18 days under long day conditions, upon which shoot inorganic phosphate (Pi) concentrations were determined. (A, B) Histogram of the frequency distribution of mean shoot Pi concentration of *Arabidopsis* accessions in +Zn (A) and -Zn (B). (C, D) Manhattan plots of GWA analysis of *Arabidopsis* shoot Pi concentration in -Zn (C) and +Zn (D) generated using 1001 Genomes imputed SNPs. Each dot represents the  $-\log_{10}(P)$  association score of one single nucleotide polymorphism (SNP). Boxes indicate the location of the LPCAT1 (red) quantitative trait loci (QTL).

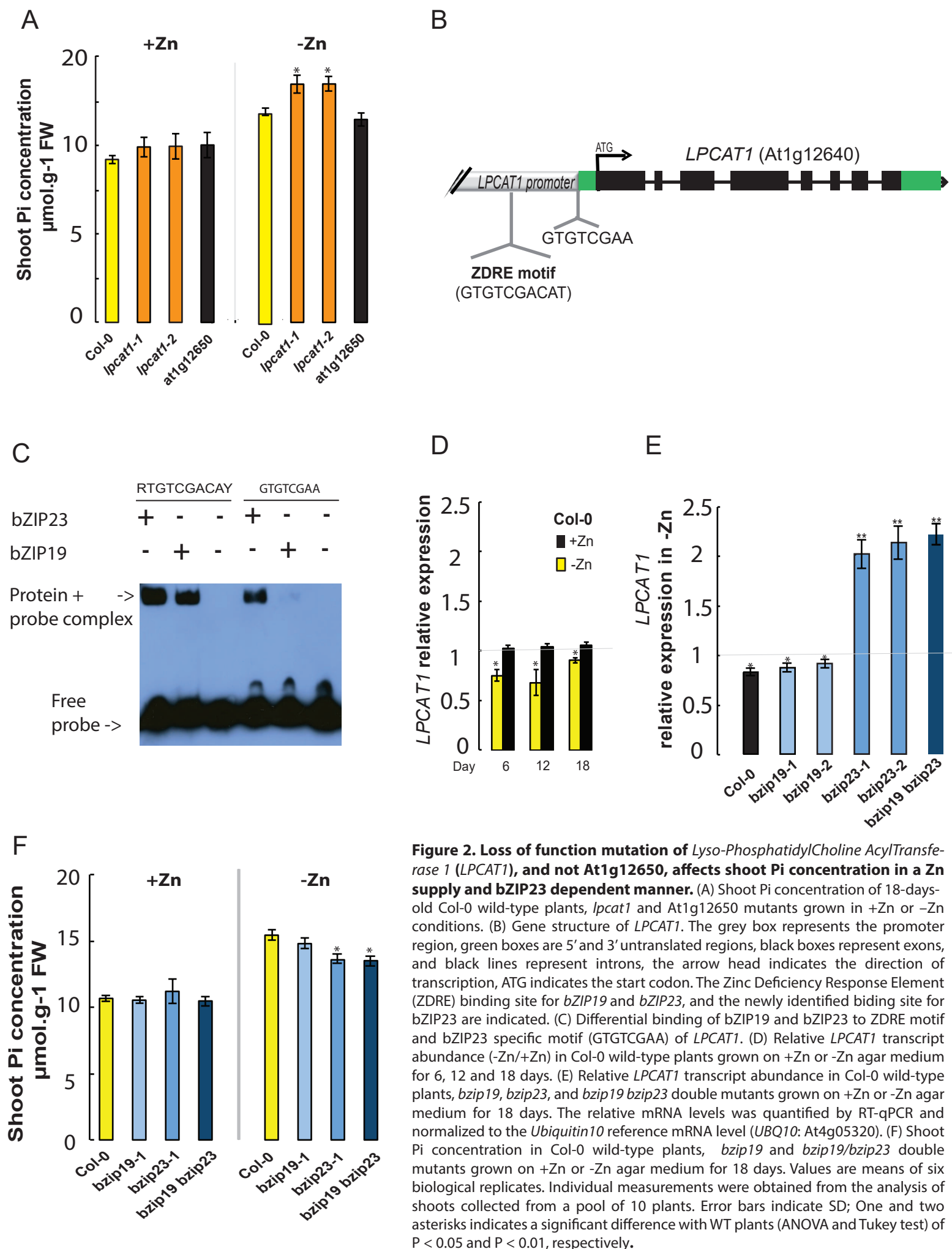


# Figure 1-figure supplement 3



**Figure 1-figure supplement 3. Haplotype analysis of region around SNP C1P4306845.** (upper panel) Clusterplot represents the haplotype blocks according to fastphase2 for SNPs 25 kb up and downstream of the significantly associated SNP. Colours represent a specific haplotype. X-axis: Accessions, Y-axis: SNPs. The horizontal blue line indicates the position of the significant SNP. The red box indicates the LPCAT gene model. The blue arrow represents the transcription direction of the gene model (LPCAT is on the - strand). (lower panel) Normalized Pi content in -Zn condition of each accession (using the R scale function). Colour indicates the genotype of the accession at SNP C1P4306845 (top GWAS hit), whereby red colour indicates the minor SNP allele that is associated with high Pi content in -Zn.

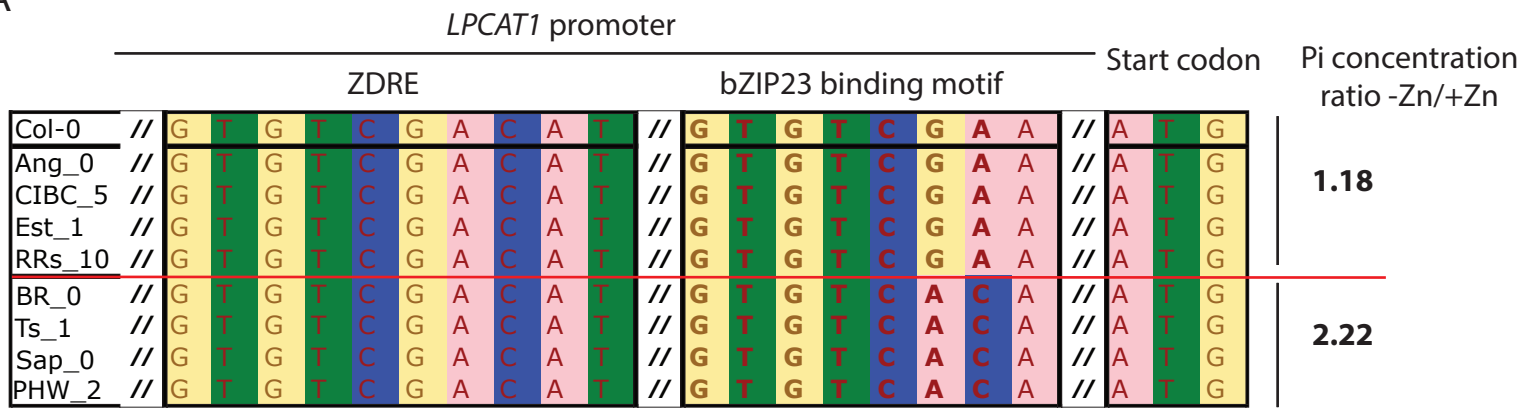
## Figure 2



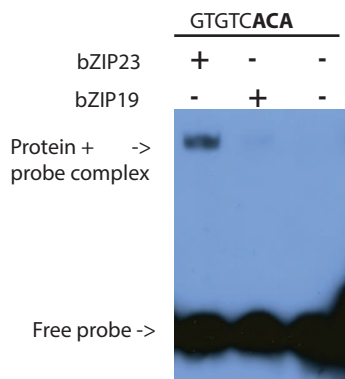
**Figure 2. Loss of function mutation of *Lyso-PhosphatidylCholine AcylTransferase 1 (LPCAT1)*, and not *At1g12650*, affects shoot Pi concentration in a Zn supply and bZIP23 dependent manner.** (A) Shoot Pi concentration of 18-days-old Col-0 wild-type plants, *lpcat1* and *At1g12650* mutants grown in +Zn or -Zn conditions. (B) Gene structure of *LPCAT1*. The grey box represents the promoter region, green boxes are 5' and 3' untranslated regions, black boxes represent exons, and black lines represent introns, the arrow head indicates the direction of transcription, ATG indicates the start codon. The Zinc Deficiency Response Element (ZDRE) binding site for *bZIP19* and *bZIP23*, and the newly identified binding site for *bZIP23* are indicated. (C) Differential binding of *bZIP19* and *bZIP23* to ZDRE motif and *bZIP23* specific motif (GTGTCGAA) of *LPCAT1*. (D) Relative *LPCAT1* transcript abundance (-Zn/+Zn) in Col-0 wild-type plants grown on +Zn or -Zn agar medium for 6, 12 and 18 days. (E) Relative *LPCAT1* transcript abundance in Col-0 wild-type plants, *bzip19*, *bzip23*, and *bzip19 bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. The relative mRNA levels was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). (F) Shoot Pi concentration in Col-0 wild-type plants, *bzip19* and *bzip19/bzip23* double mutants grown on +Zn or -Zn agar medium for 18 days. Values are means of six biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of 10 plants. Error bars indicate SD; One and two asterisks indicates a significant difference with WT plants (ANOVA and Tukey test) of  $P < 0.05$  and  $P < 0.01$ , respectively.

# Figure 3

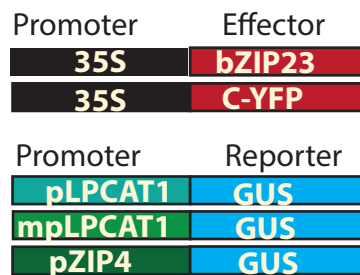
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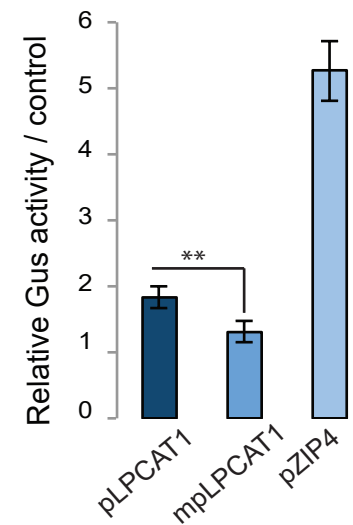
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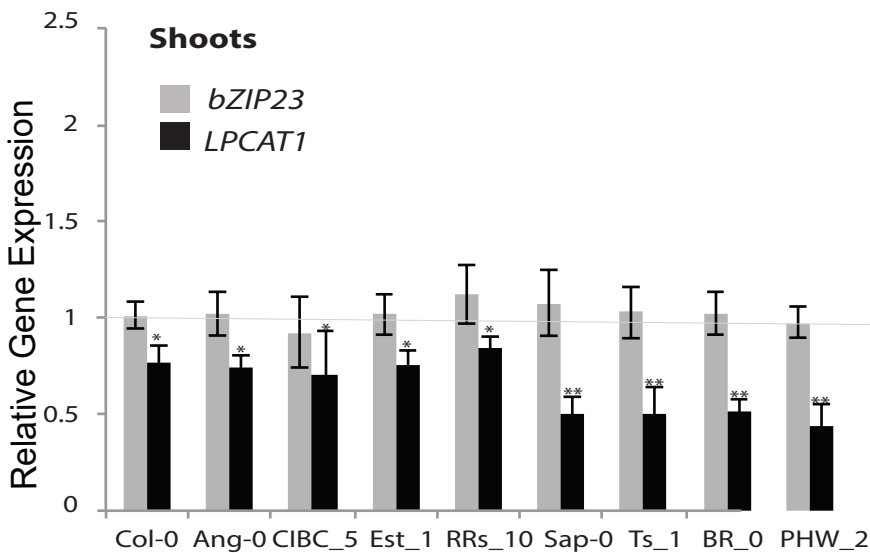
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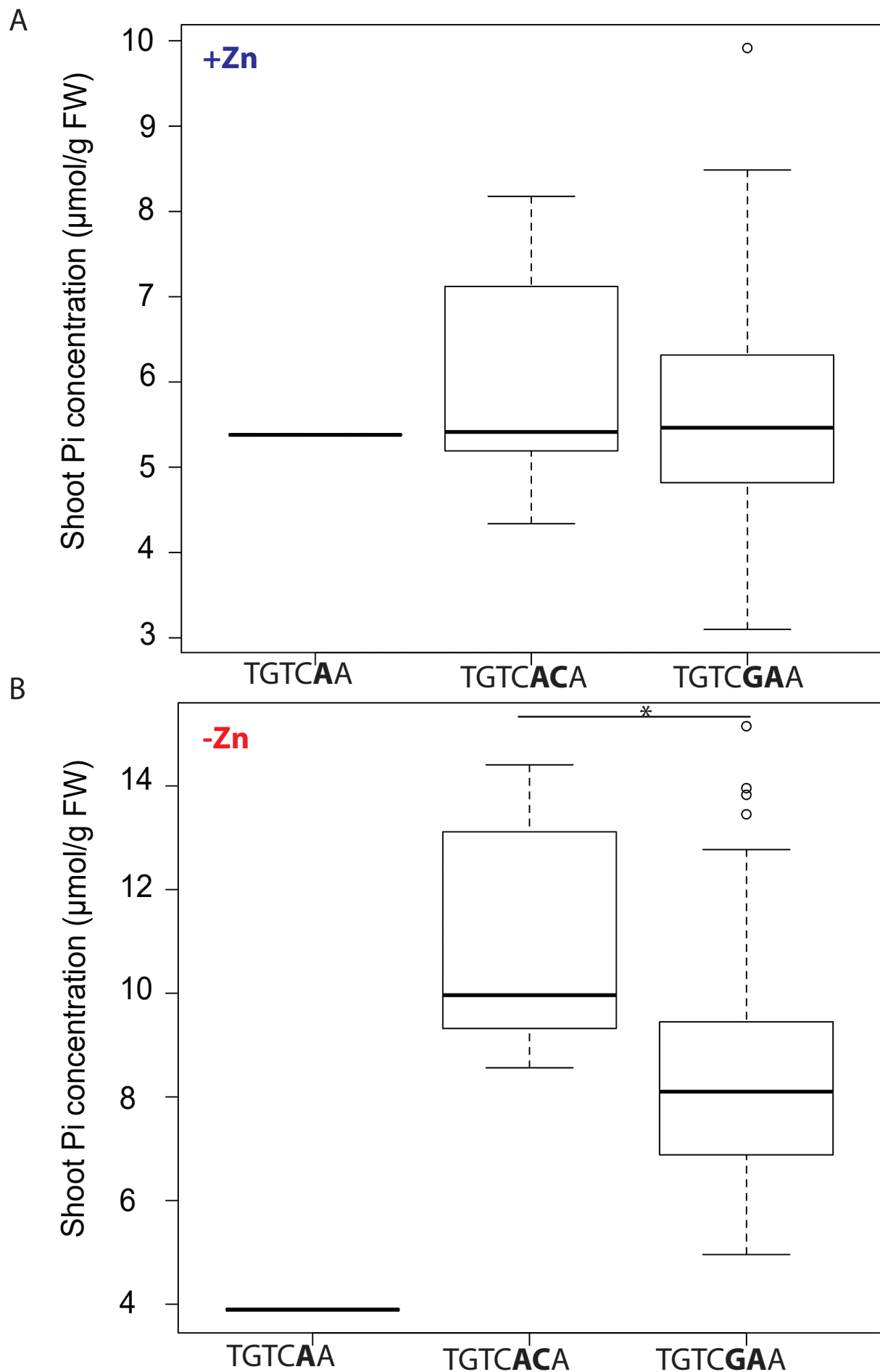


E



**Figure 3. Identification of a new binding motif specific for bZIP23, and the variation of LPCAT1 gene expression between genotypes in -Zn condition.** (A) Sequence comparison for ZDRE and the new binding site motif for bZIP23 in the promoter of accession with high ratio of Pi accumulation in -Zn/+Zn (Col-0, Ang-0, CIBC-5, Est-1, RRS-10) and low Pi accumulation ratio -Zn/+Zn (Sap-0, Ts-1, Br-0 and PHW-2). (B) Differential binding of bZIP19 and bZIP23 to a specific ZDRE motif of LPCAT1 (GTGTCACA). (C-D) in planta transactivation assay. (C) 35S:bZIP23 and 35S::C-YFP were used as effectors. pLPCAT1: the native Col-0 LPCAT1 promoter (with « GTGTCGAA » as new ZDRE), mpLPCAT1: a modified (point mutation) version of the Col-0 promoter to only contain the new ZDRE of Sap-0 (« GTGTCACA »); pZIP4; promoter of the zinc transporter ZIP4 gene. Each pLPCAT1 (native, Col-0), mpLPCAT1 (mutated version) or pZIP4 promoter was fused to a  $\beta$ -glucuronidase (GUS)-encoding reporter gene (reporter). (D) The effect of bZIP23 TF on the activity of each promoter pLPCAT1, mpLPCAT1 or pZIP4 was determined by measuring GUS activity. The effect of C-YFP protein on the activity of each promoter pLPCAT1, mpLPCAT1 or pZIP4 was used as a control to determine the basal level of GUS activity for each promoter. Comparing the effect of bZIP23 TF and C-YFP protein on each promoter enabled the determination of the relative GUS activity. Error bars represent standard error from 3 independent experiments. The asterisks indicate that the relative GUS activity is statistically different from the YFP control (p-value <0.01, t-test). (E) Relative bZIP23 and LPCAT1 transcripts abundance in -Zn and +Zn conditions. Col-0, Ang-0, CIBC-5, Est-1, RRS-10, Sap-0, Ts-1, Br-0 and PHW-2 genotypes were grown on +Zn or -Zn agar medium. The relative mRNA level was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). Values are means of six biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of 20 plants. Error bars indicate SD; one and two asterisk indicates a significant difference with Col-0 plants (ANOVA and Tukey test) of P < 0.05 and P < 0.01 respectively.

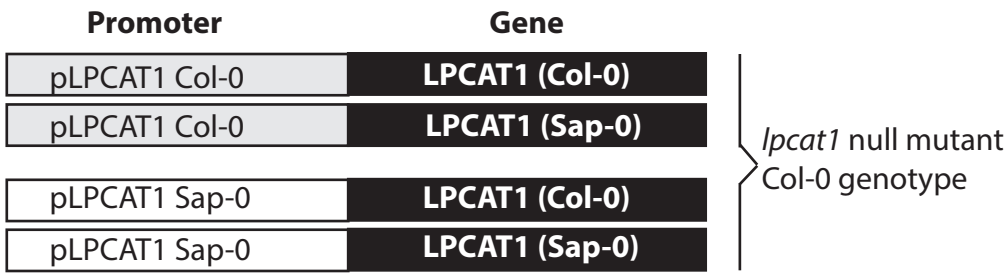
Figure 3- figure supplement 1



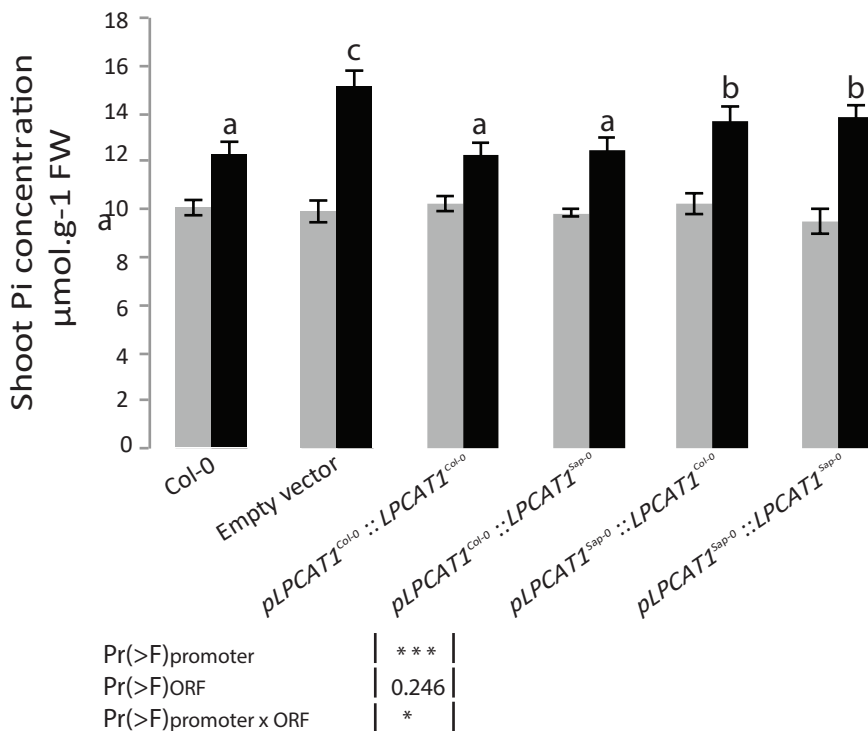
**Figure 3- figure supplement 1. Shoot Pi concentrations in Arabidopsis accessions grouped by new ZDRE motif.** Shoot Pi concentrations of *Arabidopsis thaliana* accessions grown on agar medium supplemented with zinc (+Zn) (A) or without zinc (-Zn) (B) for 18 days under long day conditions. Data same as in Table S3. Grouping of accessions is based on their ZDRE binding motif allele TGCAAA, TGTCACA and TGTCGAA respectively. Asterisk indicates a significant difference according to Student's T-test  $P < 0.01$ .

# Figure 4

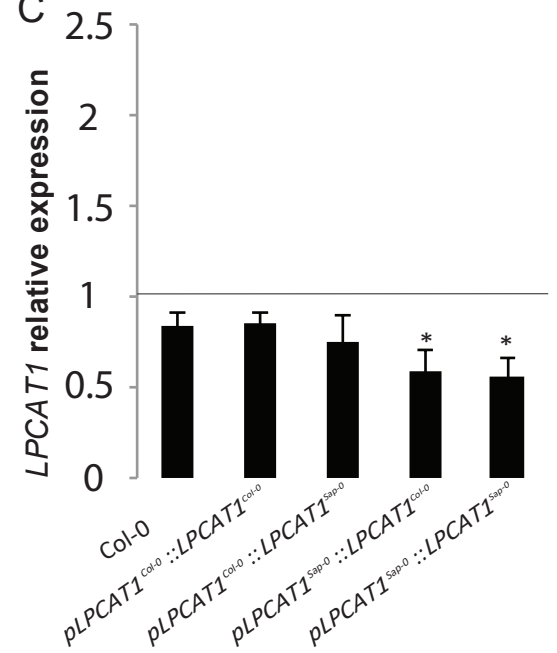
A



B

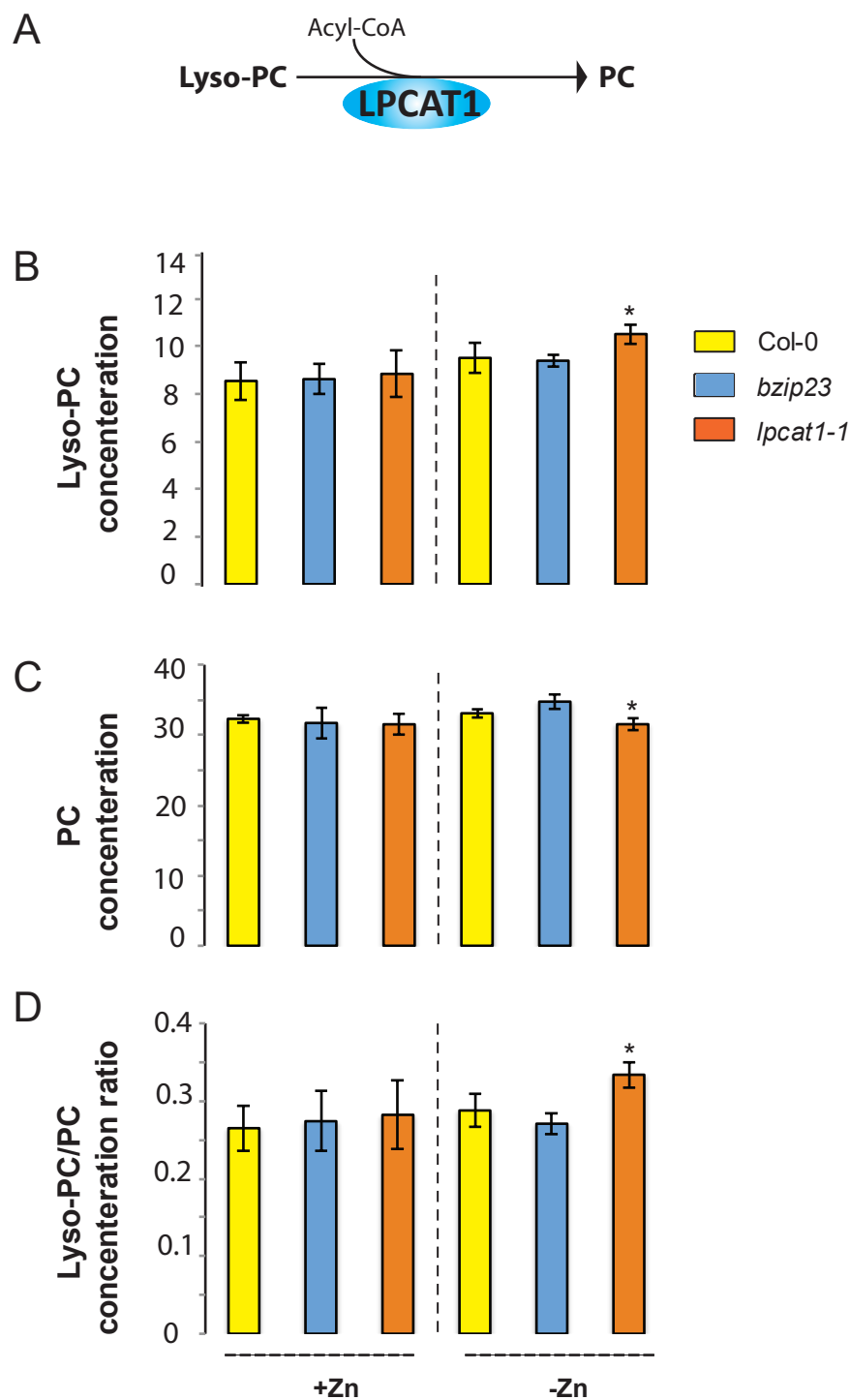


C



**Figure 4. Natural allelic variation of LPCAT1 locus causes phenotypic variation of Pi accumulation in Zn deficiency conditions.** (A) Schematic representation of the transgenic constructs used to complement the *lpcat1* null mutant (Col-0 background). (B) Shoot Pi concentration ratios (–Zn / +Zn) of 18-days-old Col-0 wild-type plants, *lpcat1* mutant transformed with empty vector, or with constructs schematized in (A) grown in +Zn or –Zn conditions. Two-way ANOVA was used to test the impact of Promoter (Col-0 v Sap-0), ORF(LPCAT1-Col-0 v LPCAT1-Sap-0) and their interaction on Pi concentration. The ANOVA results are presented in the table. Significant codes: ‘\*\*\*’ 0.001 and ‘\*’ 0.05. (C) Relative *LPCAT1* transcript abundance in wild-type plants (Col-0 background) and the transgenic lines generated using the construct schematized in (A) grown on +Zn or –Zn agar medium. The relative mRNA levels was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320). Values are means of six biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of six plants. Error bars indicate SD; letters indicates a significant difference between empty vector, Col-0 and transgenic lines (ANOVA and Tukey test) of  $P < 0.05$ .

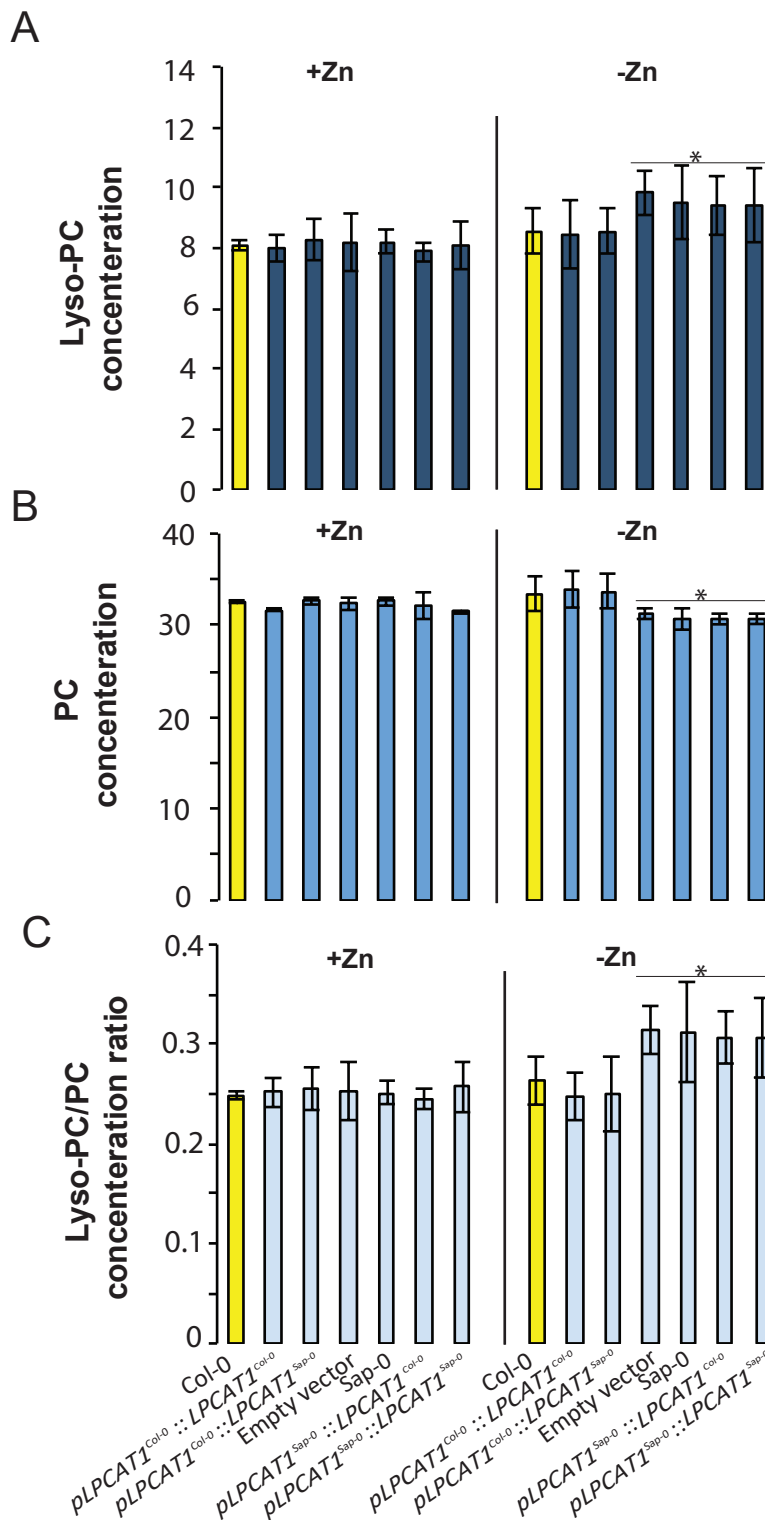
**Figure 5**



**Figure 5. Loss of function mutations of *LPCAT1* affect the lysoPC-PC ratio in -Zn conditions.**

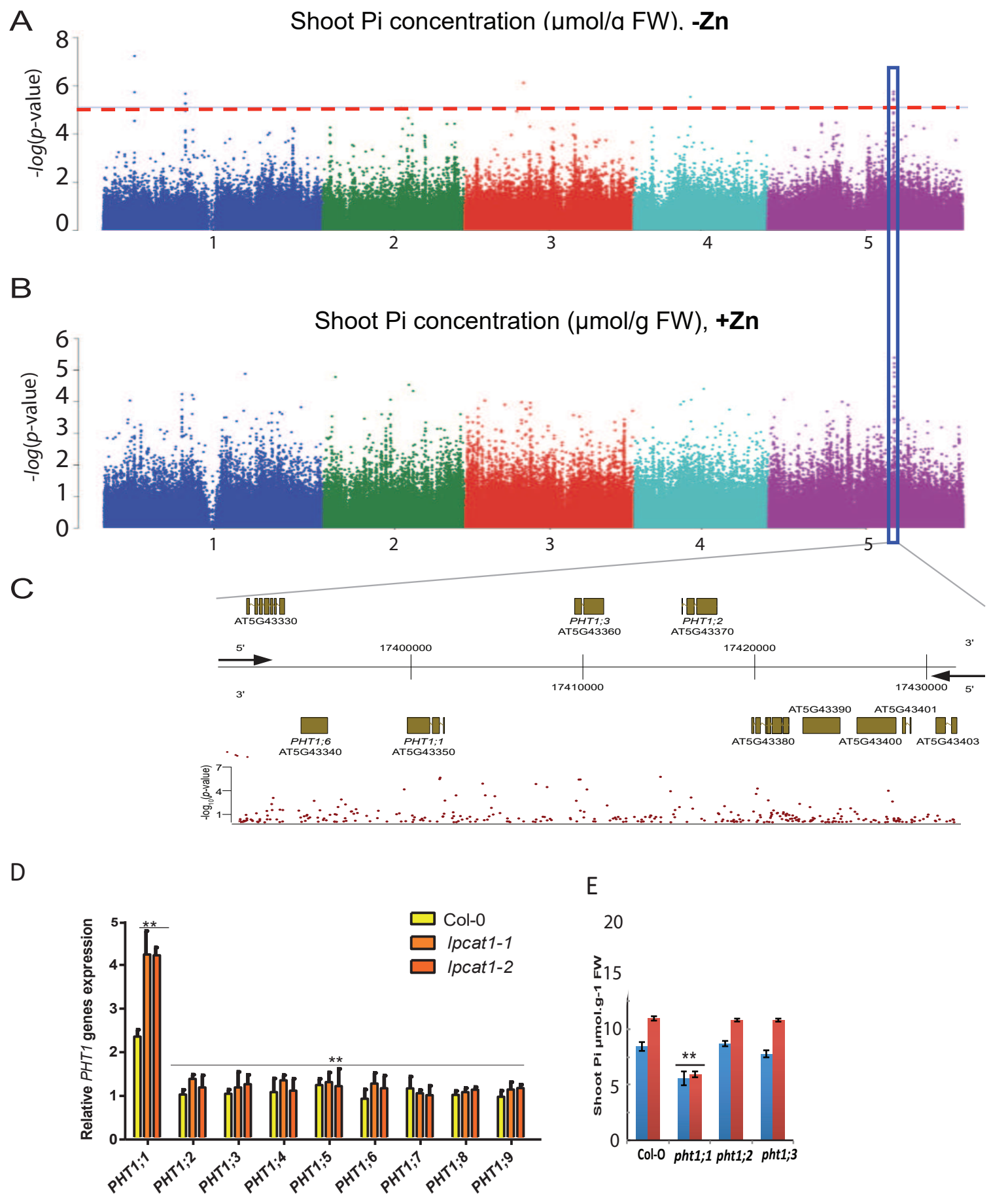
(A) Schematic representation of the biochemical function of *LPCAT1*, which catalyses the formation of phosphatidylcholine (PC) from lyso-PC and long-chain acyl-CoA. (B) Lyso-PC concentration (C) PC concentration (D) Lyso-PC/PC concentration ratios of Col-0 wild-type plants, *bzip23* and *lpcat1-1* mutant lines grown in +Zn or -Zn conditions for 18 days. Individual measurements were obtained from the analysis of shoots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.05$ ). between mutants and Col-0 are indicated with asterisks.

**Figure 6**



**Figure 6. Effect of the polymorphisms in the regulatory region of LPCAT1 on the change in LPC/PC ratios in -Zn conditions.** (A) Lyso-PC concentration (B) PC concentration (C) Lyso-PC/PC concentration ratios of *Sap-0*, *Col-0* wild-type plants, and *lpcat1* expressing *pLPCAT1<sup>Col-0</sup>::LPCAT1<sup>Col-0</sup>*, *pLPCAT1<sup>Col-0</sup>::LPCAT1<sup>Sap-0</sup>*, *pLPCAT1<sup>Sap-0</sup>::LPCAT1<sup>Col-0</sup>*, *pLPCAT1<sup>Sap-0</sup>::LPCAT1<sup>Sap-0</sup>* constructs and *lpcat1* transformed with empty lines grown in +Zn or -Zn conditions for 18 days. Individual measurements were obtained from the analysis of shoots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.05$ ) between mutants and *Col-0* are indicated with asterisks.

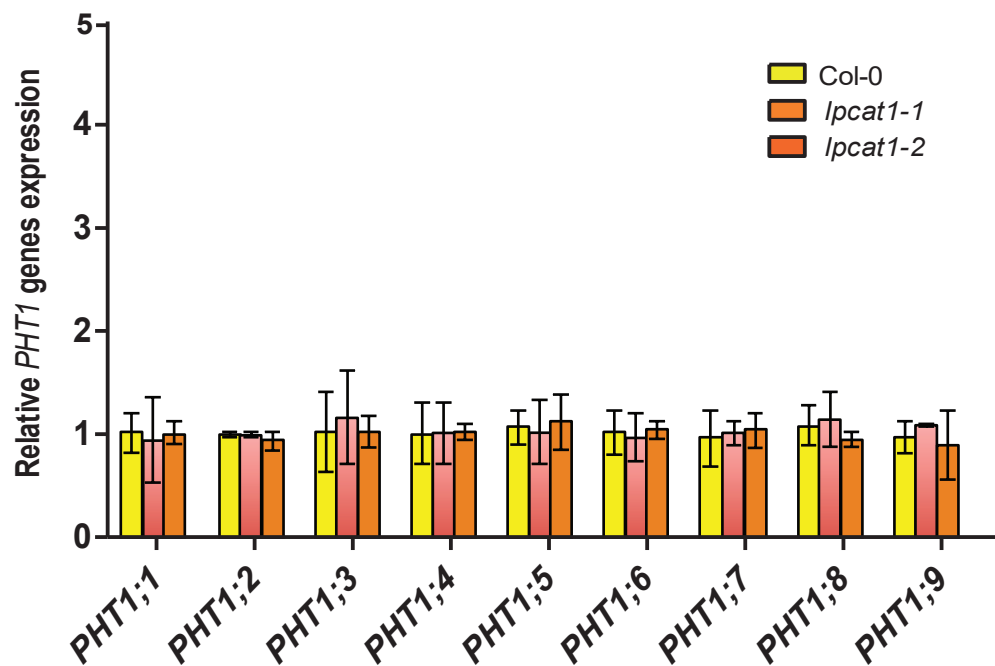
# Figure 7



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

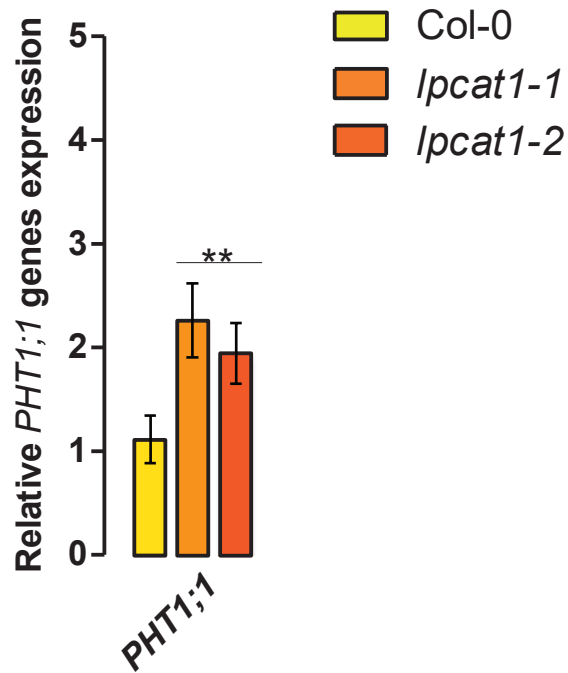


Figure 7-figure supplement 1



**Figure 7-figure supplement 1. Relative expression level of all members of the Arabidopsis PHT1 gene family.** *Arabidopsis thaliana* Col-0, *lpcat1-1* and *lpcat1-2* mutant lines were grown agar medium supplemented with zinc (+Zn) for 18 days under long day conditions. mRNA level was quantified by RT-qPCR and normalized to the *Ubiquitin10* reference mRNA level (*UBQ10*: At4g05320) and expressed as relative values against the *UBQ10* normalized mRNA level in Col-0. Values are means of six biological replicates. Individual measurements were obtained from the analysis of shoots collected from a pool of 20 plants. Error bars indicate SD; one and two asterisk indicates a significant difference with Col-0 plants (ANOVA and Tukey test) of  $P < 0.05$  and  $P < 0.01$  respectively.

Figure 7-figure supplement 2



**Figure 7-figure supplement 2. High Affinity of Phosphate Transporter (*PHT1;1*) gene expression analysis.**

Loss of function mutations of *LPCAT1* show enhanced expression of *PHT1;1* when compared to Col-0 wild-type plants. Relative expression level of *PHT1;1* gene in roots of 18-days-old Col-0 wild-type plants and *lpcat1* mutants grown on -Zn agar medium. mRNA accumulation was quantified by RT-qPCR, normalized to the mRNA level of the *UBIQUITIN10* reference gene (*UBQ10*: At4g05320) and expressed as relative values against Col-0 grown in +Zn medium (control). Individual measurements were obtained from the analysis of roots collected from a pool of five plants. Data are mean  $\pm$  SD of three biological replicates. Statistically significant differences (ANOVA and Tukey test,  $P < 0.01$ ) are indicated by two asterisks.