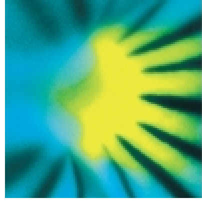

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Role of cis-zeatin in root responses to phosphate starvation

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Summary

- Phosphate (Pi) is an essential nutrient for all organisms. Root are underground organs, but the majority of the root biology studies have been done growing the root system in presence of light.
- Root illumination alters the Pi starvation response (PSR) at different levels. Thus, we have analyzed morphological, transcriptional and physiological responses to Pi starvation in dark-grown roots.
- We have identified new genes and pathways regulated by Pi starvation that were not described previously. We also show that Pi-starved plants increase the cis-zeatin (cZ)/trans-zeatin (tZ) ratio. Transcriptomic analyses show that tZ preferentially represses cell cycle and PSR genes while cZ induces genes involved in cell and root hair elongation and differentiation. In fact, cZ-treated seedlings show longer root system as well as longer root hairs than tZ-treated, increasing the total absorbing surface. Mutants with low cZ levels do not allocate free Pi in roots during Pi starvation.
- We propose that Pi-starved plants increase the cZ/tZ ratio to maintain basal CK responses and allocate Pi in the root system to sustain its growth. Therefore, cZ acts as a Pi-starvation response hormone that stimulates root and root hair elongation to enlarge root absorbing surface and to increase Pi levels in roots.

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Introduction

Phosphorous is one of the most important nutrient for plant growth and development. Plants have evolved complex regulatory mechanisms and crosstalk signaling pathways to improve the uptake inorganic phosphate (PO_4^{3-}; Pi), the absorbable form of P, from the rhizosphere, and the Pi usage to maintain intracellular its homeostasis (Baek *et al.*, 2017). In poor Pi environments, plants activate a general Pi-starvation response (PSR) aimed to mobilize and uptake Pi from rhizosphere and to reallocate endogenous Pi from storage-tissue. This PSR involves metabolic readjustment, morphological changes and the activation of specific transcriptional programs, mainly regulated by the PHR1- miR399-PHO2 module (Rubio *et al.*, 2001; Lopez-Bucio *et al.*, 2003; Bari *et al.*, 2006; Bustos *et al.*, 2010; Peret *et al.*, 2011; Secco & Whelan, 2014). Many of the morphological changes including the reduction of the main root, the increase in lateral roots (LR) density and root hairs, are aimed to enlarge the root surface area to search for phosphate in the soil.

The PSR is interconnected with several hormonal signaling pathways (Martin *et al.*, 2000; Rouached *et al.*, 2010; Baek *et al.*, 2017). Among them, cytokinins (CK) play an important role in controlling the balance of root/shoot ratio. Pi starvation reduces CK levels and the expression of the CK receptor CYTOKININ RESPONSE 1 (CRE1/AHK4) (Kuiper & SteingrÖVer, 1991; Franco-Zorrilla *et al.*, 2002). CK-signaling reduction leads to an increase in the root/shoot ratio by favoring root growth to the detriment of shoot. CK also down-regulated genes induced by Pi starvation (Martin *et al.*, 2000; Franco-Zorrilla *et al.*, 2002), likely by increasing intracellular Pi (Wang *et al.*, 2006). In addition, the auxin response factor *ARF16* negatively regulates Pi signaling and uptake in response to CK (Shen *et al.*, 2014). These data indicate that CK play an important role in the control of PSR

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and plant adaptation to low Pi levels. The CK receptors can perceive different CK-types with different affinities (Heyl *et al.*, 2012). The most abundant CK-type in Arabidopsis is the trans-zeatin (tZ), which is synthesized by the ADP/ATP IPTs. Another zeatin isomer present in higher plants is the cis-zeatin (cZ), although its function is less understood (Romanov *et al.*, 2006; Heyl *et al.*, 2012). cZ is synthesized by the tRNA-IPTs IPT2 and IPT9 enzymes using the tRNA degradation products (Miyawaki *et al.*, 2006). cZ-type have been involved in the regulation of plant growth but also in the response to different biotic and abiotic challenges (Großkinsky *et al.*, 2013; Schäfer *et al.*, 2015).

Usually, root biology studies, at least in the plant model Arabidopsis, are carried out in *in vitro* plates where the root system is exposed to light, condition that is not natural for this organ. The use of the D-Root device to grow roots in darkness proves that light has a negative effect on root growth and response to abiotic stresses, including nitrate deficiency or salinity (Silva-Navas *et al.*, 2015; Silva-Navas *et al.*, 2016). Here, we show that root illumination alters the Pi starvation response, enhancing the root and shoot growth arrest and reducing the root/shoot ratio as well as root hair elongation. A transcriptomic study in dark-grown roots (DGR) identifies several genes that respond to Pi deficiency but have not been previously reported. Hormone profiling revealed that Pi starvation reduces the level of tZ but significantly increases the amount of cZ and cis-Zeatin Riboside (cZR), which are partially dependent on *PHR1* function. Transcriptomic studies indicate that cZ modifies the expression of specific genes, positively regulating cell growth and root hair elongation while tZ seems to inhibit cell division genes. Furthermore, the molecular response to Pi starvation is less inhibited by higher cZ/tZ ratio. Likewise, both zeatins increase Pi levels in roots in detriment of Pi content in shoots and higher cZ/tZ ratio favors root growth and

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lateral root (LR) formation. Genetic analyses show that cZ is needed for root hair elongation and Pi allocation in the root during Pi starvation.

Methods

Plant material

Arabidopsis Columbia (Col-0) ecotype was used for all experiments, including mutants *ahk4-3/ahk3-4* (Franco-Zorrilla *et al.*, 2005), shy2-101 (Goh *et al.*, 2012) and *ipt2*, *ipt9* and *ipt2 ipt9* (Miyawaki et al., 2006). We used the reporter lines (TCSn (Liu & Muller, 2017), SKP2B::GUS (Manzano *et al.*, 2012) and CYCB1::CYCB1-GUS (Colon-Carmona *et al.*, 1999). Seedlings were sown on vertically oriented 12 cm square plates containing half-strength Murashige and Skoog (MS1/2) media, 0.05% MES, 1% sucrose and 1% agar (Difco). The pH was adjusted to 5.7 and after autoclaving, different concentrations of phosphate were added as indicated. Plants were grown with roots exposed to light-LGR: light grown roots- or using the D-ROOT device (Silva-Navas *et al.*, 2015) to maintain roots in darkness. The different Pi-media used contained the following Pi concentration: +Pi, MS1/2 with 625 µM of H_2KPO_4; -Pi, MS1/2 with 0 µM of H_2KPO_4 and 5Pi, MS1/2 with 5 µM of H_2KPO_4. Sterile H_2KPO_4 was added after the medium was autoclaved.

Arabidopsis seedling were grown in chambers under a 16-h light/8-h dark photoperiod at 20°C. Light fluency rate was around 100 µmol m-1 sec-1 at the bottom of the plate (see Fig. S1a).

Root growth assays and microscopic analysis

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Root meristem size was calculated based on the number of meristematic cortex cells and/or the distance from the quiescent center (QC) to the last meristematic cell using confocal images taken with a Leica Z8 microscopy. The end of the meristem zone was taken as the point where a meristematic cortical cell doubled in size than the previous one. Main root and total LR length were measured using the ImageJ program.

For hormonal treatments, 5 day old seedlings grown in MS1/2 were transferred to fresh medium containing mock, 10 nM of Indol-3-Acetic acid (IAA); 10 µM of Gibberellic acid 3 (GA3); 5 µM of Abscisic acid (ABA); 2.5 µM of cytokinin (CK, transZeatin); 2.5 µM of 1-Aminocyclopropane-1-carboxylic acid (ACC) or 5 µM of strigolactone (SL). All hormones were purchased from Duchefa, except cZ and tZ that were purchased from OlChemIm Ltd.

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Mitotic cells quantification

Mitotic cell number were quantified using the CYCB1::CYCB1-GUS marker line. These plants were grown in MS1/2 containing 625µM (+Pi) or 5 µM (5Pi) phosphate for 8 days. Afterwards, these seedlings were transferred to fresh plates containing the similar medium but supplemented with either the solvent (DMSO) or cZ or tZ (1 or 2.5 µM) during 24 hours. Afterwards, seedlings were stained for GUS activity and mitotic stained cells were quantified as described previously (Jurado *et al.*, 2008).

Hormone profiling

Several plant hormones including several CK trans- and cis-zeatin (tZ and cZ), dihydrozeatin (DHZ), trans- and cis-zeatin riboside (tZR and cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenosine (BAR), meta-topolin (mT), meta-topolin riboside (mTR), ortho-topolin (oT) and ortho-topolin riboside (oTR)] were quantified using mass spectrometry based methods (Methods S1). cZ and tZ were individually analyzed by their different retention times (Fig. S1b,c).

Free phosphate and anthocyanins quantification

Inorganic phosphate was quantified as described previously (Ames, 1966). The free phosphate was reference to milligram of fresh root or shoot weight. Due to the Pi level variability detected between different experiments, we also reference Pi level to the control as indicated. Arabidopsis plants were grown on MS1/2 medium for 12 days. Anthocyanin were extracted and quantified as described by (Swain & Hillis, 1959). The amounts of anthocyanin were calculated as absorbance at 530 per mg of tissue used. The results correspond to the average of five different biological replicates. Ionomic analyses were done as described previously by (Silva-Navas *et al.*, 2015).

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Gene Ontology and Statistical Analyses

Gene ontology (GO) was done using the AgriGO tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php). The analyses were done using the Fisher test with Yekutieli multitest adjustment and FDR under dependency and a significance of 0.05 with a minimum number of mapping entries of 5. The GO terms were later readjusted

using the REVIGO tool using medium similarity and p-values correction (Log10 p-val) and FDR < 0.05. The overall data were statically analyzed using Graphpad5 software. One-way ANOVA with Tukey's post hoc test were used for testing differences between multiple samples. Different letters were used to indicate means that differ significantly. Statistical calculations were performed using PRISM5 (GraphPad, San Diego, CA, USA). Comparisons between two groups were performed with student t-test, while multi group comparisons were performed using one-way analysis of variance (ANOVA), followed by Turkey's test. The p-values of < 0.05 were considered statistically significant.

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Results

Light limits root growth during phosphate starvation

As roots are underground organs, we decided to analyze Pi starvation responses in dark-grown roots (DGR) using the D-Root device (Silva-Navas *et al.*, 2015). In DGR, Pi deficiency slightly inhibited root growth compared with those grown in Pi-supplemented medium, but significantly less than Pi-starved light-grown roots (LGR) (Fig. 1a,b). Pi starvation reduced root meristem size in LGR by almost 50% (Fig. 1c, Fig. S2a), while this reduction was only of 12% or 8% in DGR seedlings grown without Pi or with low Pi (5 μM) respectively (Fig. 1c). Pi-starved LGR increased LR density, mainly due to the significant reduction in primary root length (Fig. 1d). In contrast DGR showed a slight, but significant, reduction in LR density only when Pi was completely removed, but not when media contained a low amount (5 μM) of Pi (Fig. 1d). Nevertheless, we found a higher total number of emerged eLR in DGR than in LGR seedlings (Fig. 1d). We also found that root

illumination accelerated root hair development in response to Pi starvation, which started to develop closer to root meristem (Fig. 1e; Fig. S2b) and reduced root hair length (Fig. 1e; Fig. S2c).

In response to Pi starvation plants increase the root/shoot weight ratio (Raghothama, 1999). Our analyses showed that LGR seedlings indeed increased in the root/shoot weight ratio by 2-fold (Fig. 1f), but interestingly Pi-starved DGR seedlings showed an increase of more than 3-fold (Fig. 1f), likely due to their bigger root system (Fig. 1a and 1b). Next, we quantified the free and total (free plus organic) phosphate level and found a significant reduction in Pi-starved plants. However, we did not find significant differences in total or free Pi levels between LGR or DGR seedlings (Fig. 1g; Fig. S3). Finally, we found that anthocyanin content, a physiological marker for Pi starvation, was significantly higher in LGR plants than in DGR under low Pi feeding (Fig. 1h).

It has been reported that high iron levels may contribute to root growth inhibition during Pi starvation (Ward *et al.*, 2008). Ionomic analyses showed that phosphate deficiency decreased iron levels in roots of both LGR and DGR seedlings, iron level was almost 3-fold higher in LGR than in DGR (Fig. S3). In addition, the levels of several other elements were also altered (Fig. S3), suggesting that Pi starvation unbalances plant nutrition.

Gene expression profile of dark grown roots during phosphate starvation

As the majority of the Pi starvation transcriptomic analyses have been done using illuminated roots, we decided to carry out a comparative transcriptomic analysis in response to Pi deficiency in DGR seedlings. We found that Pi starvation resulted in upregulation of

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2201 transcripts and downregulation of 1447 transcripts (+/- 2fold; FDR<0.01) (Table S1b). Among them, 36.6% of the up-regulated genes and 18% of the down-regulated contained at least one PSBS1 (phosphate starvation binding site 1) (Rubio *et al.*, 2001; Bustos *et al.*, 2010) motif in the promoter (1500 bp upstream from ATG) (Table S1c). Gene ontology analyses of up-regulated genes showed an enrichment of genes involved in responses to phosphate starvation, oxidative stress, lipid metabolism, protein phosphorylation, desiccation or salt stress among other (Table S1b). Among the metabolic changes, the most significant were the down-regulation of genes encoding enzymes involved in light reactions and the upregulation of genes encoding enzymes involved in cell wall remodeling, lipid metabolism and flavonol or sulfur-containing glucosinolates biosynthesis (Fig. S4, Table S1d). On the other hand, the down-regulated genes grouped into the functional categories of iron metabolism and chelating, immune response, salicylic and auxin response or metabolic processes among others (Table S1b, Fig. S4). To estimate the effect of root illumination in this molecular response, we compared our data with the one reported by Mora-Macias *et al* (Mora-Macias *et al.*, 2017), in which roots were grown in presence of light, finding that only 29% up-regulated and 22 % of the down-regulated transcripts were common between both the datasets (Fig. S5). Gene ontology analyses showed that the specific genes in our dataset were enriched in genes involved in root hair differentiation and development as well as in lipid and phosphorous metabolism or protein phosphorylation. In addition, genes involved in stress responses such as water deprivation, oxidative reactions, salt and osmotic stress were enriched (Fig. S5). Taken together, these data indicated that light influences the molecular and morphological response to Pi starvation in roots. Therefore, in this work, unless indicated, all the analyses have been done in DGR seedlings.

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Hormonal regulation of phosphate accumulation

As hormonal signaling modulates plant adaptation to different environments, we decided to analyze the effect of phytohormones on Pi accumulation in roots or shoots. Most of the hormones analyzed had no or a modest effect on free Pi level or Pi root/shoot allocation (Fig. 2a). However, CK treatment significantly increased free Pi root/shoot ratio, increasing the level in roots and, notably, decreasing Pi levels in shoots independently of the phosphate in the medium (Fig. 2a,b). Furthermore, this ratio was much higher in Pi-starved seedlings mainly due to a significant increase in Pi levels in the roots (Fig. 2a,b).

Phosphate starvation alters hormone levels

Hormone profiling of roots and shoots of DGR seedlings revealed that Pi-deficiency increased the level IAA, ABA and JA in shoots, while the level of SA was slightly increased in roots (Fig. S6a). However, the most striking change was in the level of different types of cytokinins. In Pi-starved plants, we found that in both roots and shoots the levels of tZ went down significantly whereas that of cZ significantly increased (Fig. 2c). Similar effect was also observed for tZR and cZR. However, this trend was not observed for free or riboside-conjugated isopentenyladenine (iP and iPR).

IPT3 and *IPT5,* which are involved in tZ biosynthesis, were down-regulated by Pi starvation (Fig. S6b). Conversely, the level of *IPT2* and *IPT9,* involved in cZ synthesis, did not change (Fig. S6b). We found that the expression of *LOG2* and *LOG5* (*LONELY GUY* Cytokinin-Activating 2 and 5), involved in downstream steps of zeatins biosynthesis (Kurakawa *et al.*,

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2007), were up-regulated by Pi starvation (Fig. S6b). These data suggest that Pi-starved plants increase cZ/tZ ratio by repressing tZ synthetic genes and by activating downstream steps of the biosynthesis of cZ. However, we cannot rule out a posttranslational regulation of enzymes involved in cZ biosynthesis.

We found that cZ levels were slightly, but significantly, lower than in *phr1* roots, while the level of tZ was increased by almost 2-fold in *phr1* Pi-starved seedlings (Fig. S7a). However, in shoots, Pi-starved *phr1* seedlings showed a significant reduction in the level of cZ and cZR compared to control shoots (Fig. S7b). Furthermore, iP levels did not change in *phr1,* but iPR levels significantly increased in *phr1* compared to control during the Pi starvation response. This data is suggestive that cZ and cZR accumulation is partially dependent on *PHR1* function.

We also found that control and *phr1* seedlings accumulated higher amount of Pi in roots than in shoots in response to both cZ or tZ (Fig. S8a). However, during Pi-starvation, Pi allocation was significantly higher in *phr1* roots than in control after treatment with both cZ or tZ (Fig. S8b), indicating that *PHR1*-zeatin signaling is needed to maintain a correct Pi homeostasis and allocation.

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Zeatins controls root growth and cell proliferation under low phosphate conditions

Due to the differential accumulation of cZ and tZ, we decided to evaluate the role of these isomers during the Pi starvation response. First, we found that tZ-treated plants showed more root growth inhibition than cZ, irrespective of the amount of Pi in the medium (Fig. 3a,b). It is known that CK treatment reduce the size of the root meristem (Dello Ioio *et al.*,

2007). We found that both cZ and tZ, which similarly induced the CK-response reporter TCSn-GFP (Liu & Muller, 2017) (Fig. S9a), reduced the meristem size, but tZ significantly more than cZ (Fig. 3c). This phenotype correlates with a higher reduction in cell division in tZ-treated seedlings, in both high and low Pi conditions (Fig. 3d; Fig. S9b). Furthermore, in a medium containing Pi, cell elongation in the root transition zone was reduced by tZ, but not by cZ (Fig. 3e, Fig. S9a). In Pi-starved plants, the cell length in the root transition zone was significantly reduced. Remarkable, cZ treatment, but not tZ, reverted this cell length reduction (Fig. 3e, Fig. S9a).

To analyze the effect of these zeatin isomers on root and LR development, SKP2B::GUS, a lateral root marker (Manzano *et al*., 2012) was used. As shown in Fig. 4, tZ, and cZ inhibited the root growth (both primary and lateral root) and the number of LRP and eLR. This inhibition was more pronounced for tZ than cZ, especially in Pi-starved roots (Fig. 4a; Fig. S9c). We also found that compared to primary root growth, LR growth was more sensitive to a low concentration of both cZ and tZ during Pi starvation (Fig. 4a). It is noticeable that, even though tZ was more effective in inhibiting LRP specification, both cZ and tZ have additive function in this process, preferentially during Pi starvation (Fig. 4b).

cZ and tZ have a differential effect on gene expression

We showed that Pi starvation changed the cZ/tZ balance. To further explore the role of both isomers at the molecular level, we compared cZ- and tZ-treated root transcriptomes. In high Pi conditions, we found that cZ induced 986 and repressed 780 genes while tZ induced 1442 and repressed 1471 genes. Among the induced ones, 569 genes were common between cZ

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and tZ, but there were more than 400 and 800 genes that were specifically induced by cZ or tZ respectively (Fig. 5a; Table S2). It is remarkable that out of the specifically up-regulated by cZ, 30.2 % were also up-regulated by Pi starvation while only 5% were down-regulated (Fig 5a). Conversely, out of the specifically up-regulated by tZ, only 6.7% were up-regulated by Pi starvation while a higher number (21.2%) were down-regulated. Interestingly, opposite trend was observed in the specifically down-regulated by cZ or tZ (Fig 5b). Taken together, these findings suggest that that tZ acts somewhat as antagonist of Pi starvation response while cZ seems to act as agonist of this response.

Among the transcripts specifically induced by cZ, we found genes involved in cell wall loosening and organization and multidimensional and unidimensional cell growth (Fig. 5c) that might be involved in root growth. Among the cZ repressed genes it should be mentioned the down-regulation of photosynthesis, or response to abscisic acid among other (Fig. S10a). On the other hand, tZ induced genes related to ion transport or iron binding (Fig. S10b). It is noticeable that, among the tZ-repressed transcripts, several genes involved in cell division and DNA replication (Fig. 5b; Table S2), that might explain the stronger effect on root growth inhibition executed by this isomer (Fig. 4a,b), were identified. In addition, we found a large number of genes that was up or down-regulated by both cZ and tZ, but to a different extent, indicating that cZ and tZ modulate common pathways with different intensity, but also specific genes.

Next, we analyzed the effect of cZ and tZ during the Pi starvation response (Table S2). We found that during the Pi starvation, cZ and tZ also affected the expression of a number of common genes but also some specific ones (Fig. 6). Among the specific genes, cZ up-regulated the expression of flavonol biosynthetic genes, cell wall, stress responses or auxin

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response genes (Fig. 6b). In this regard, we found that Pi starvation increased the levels of two flavonols, quercetin and kaempferol, which are involved in root growth control (Silva-Navas *et al.*, 2016). These flavonols accumulated in the differentiation zone of the root tip and in mature zones of the root, but they were excluded from young growing LR (Fig. S11). Genes specifically up-regulated by tZ during Pi starvation were mainly related to auxin transport and lipid metabolism or regulation of hormonal levels (Fig. 6c). Regarding the effect of cZ and tZ on the expression of genes down-regulated by Pi starvation, we found similar trend; genes specifically regulated by each isomers and also common ones. The cZ down-regulated were enriched in stress response and primary metabolism, while the tZ repressed one into glucosinolate biosynthesis, iron binding or oxide-reductase activities among others (Fig. 6e).

In addition to the specific gene regulation, we identified transcripts, either induced or repressed by Pi starvation, which expression level was differentially modulated (at least by ±20%) by cZ or tZ (Fig. S10c). The expression level of Pi starvation up-regulated genes was, in general, more reduced by tZ than by cZ, suggesting that tZ exert a stronger repression on PSR genes (Fig. S10c). In the case of Pi starvation down-regulated genes, we found tZ, in general, exacerbated the repression of these genes, while cZ effects was more moderate (Fig. S10c). Taken together, these data suggest that tZ reduces the transcriptional Pi-starvation response.

As zeatins seem to regulate Pi root/shoot allocation, leading to higher accumulation in roots than in shoot, we analyzed the root-to-shoot Pi translocator *PHO1*, involved in Pi loading into xylem in the root (Hamburger *et al.*, 2002). We found that zeatins inhibited PHO1::GUS reporter expression (Fig. S12a-b). Gene expression analysis showed that

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transcript level of *PHO1,* and to lesser extend *PHO1-like* (*PHO1:H3*), was also significantly reduced upon cZ or tZ treatment (Fig. S12b).

cZ controls root/shoot fresh weight and phosphate ratio under low phosphate conditions

Phosphate starvation increased fresh weight root/shoot ratio (Raghothama, 1999). We found that cZ treatment of DGR seedlings significantly increased the root/shoot ratio by 1.7 and 1.4-fold respectively in high Pi or low Pi medium (Fig. 7a,b). In contrast, this ratio was not increased by tZ treatment neither in high-Pi nor low-Pi medium (Fig. 7b), suggesting a specific role of cZ in this differential growth. Furthermore, treatment with both cZ or tZ increased Pi content in roots and decreased it in shoots, leading to a significant increase in the Pi root/shoot ratio (Fig. 7c-d), that is dependent on CK-signaling, as the double mutant for CK receptor *ahk3 ahk4* did not show change the Pi root/shoot ratio (Fig. 7c,d). We did not find differences in Pi allocation between cZ and tZ, but cZ showed always higher, although no significant, levels (Fig. 7c). Taken together, these data seem to indicate that zeatins function in the allocation of Pi in the plant.

***IPT2* and *IPT9* modulate phosphate starvation level and allocation**

The IPT2 and IPT9 enzymes are involved in cZ synthesis. The *ipt2 ipt9* double mutant, which shows a reduction in root growth and LR, has no detectable cZ-type cytokinins, except a residual cZOG (Kollmer *et al.*, 2014). We found that *ipt9* and *ipt2 ipt9* accumulated significantly more Pi in shoots than control seedlings grown in a Pi-containing media (Fig.

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S13a). When seedlings were grown in a low Pi, *ipt2* did not increase the Pi level in the root, although Pi root/shoot ratio did not change (Fig. 8a; Fig. S13b). However, it is noticeable that *ipt9* and *ipt2 ipt9,* which increased the Pi content in the shoot but not in the root, showed a reduced Pi root/shoot ration (Fig 8a; Fig. S13b). In addition, contrary to control and *ipt2*, which elongated root hairs in response to Pi starvation, the *ipt9* and *ip2 ipt9* mutants showed a shorter root hair length (Fig. 8b).

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cZ increases root hair size under low Pi

Phosphate deprivation increase the number and size of root hair to enlarge root system absorbing surface (Peret *et al.*, 2011). As Pi starvation increases the cZ/tZ ratio we analyzed the effect of both isomers in root hair elongation in control plants as well as in the double cytokinin receptor mutant *ahk3 ahk4* (Franco-Zorrilla *et al.*, 2002). In control seedlings, both cZ and tZ increased root hair elongation in the mature root (Fig. 8c,d) and close to the root tip (Fig. S11c). This elongation was significantly higher in cZ-treated seedlings grown with or without Pi (Fig. 8d). In Pi starved plants, cZ treatment, but not tZ, increased the root hair elongation in a *AHK3- AHK4*-dependent manner (Fig. 8c,d).

Discussion

In nature, soils are highly heterogeneous in mineral composition. Plant have evolved different root system architectural traits to search for rich nutrient areas and to cope with adverse environmental conditions (Giehl & von Wirén, 2014). Due to this necessity of

adaptation to different environments, roots show a high developmental plasticity, being able to arrest or to promote main root and LR growth throughout their life-span. The mechanisms underlying Pi starvation signaling and responses have been studied in different species, including the model plant Arabidopsis. However, the majority of these analyses were done growing the root system in presence of light. We previously showed that root illumination generates a stress (Silva-Navas et al., 2015; Silva-Navas et al., 2016; Manzano et al., 2017). In this work, we demonstrate that illumination is an additive stress to Pi starvation. Comparative analyses in dark-grown roots show that Pi starvation affects the expression of a number of genes involved in development or responses to stress, highlighting the link between root development and abiotic stress. In addition, we have also uncovered the important role for cZ in Pi starvation responses.

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Phosphate starvation response is influenced by light

Phosphate starvation triggers several adaptive responses to improve Pi mobilization, usage and uptake. In addition, plants use alternative metabolic pathways to limit the consumption of Pi in cells. It has been shown that Pi deficiency reduces root and shoot growth, increase in root/shoot growth ratio, LR density and root hairs (Lynch & Brown, 2001; Lopez-Bucio *et al.*, 2003; Gruber *et al.*, 2013). Pi deficiency also induces a determinate root growth arrest that leads to a progressive loss of dividing cells, exhausting the root meristematic activity (Sanchez-Calderon *et al.*, 2005). Roots are underground organs that normally do not perceive light. Here we show that root illumination has significant impact on the Pi starvation response at morphological, physiological and molecular levels. Our data clearly show that some of these responses are significantly influence by light. Recently it has been

shown that blue light is responsible for shortening roots under low Pi regime (Yeh *et al.*, 2017). Blue light stimulates flavonols production in roots, and this flavonols accumulation directly correlates with a reduction in cell division and premature cell differentiation in a cytokinin dependent manner (Silva-Navas *et al.*, 2016). Here we show that Pi starved roots accumulated higher flavonols in the root tip as well as in the mature areas of the roots but not in the LR formation areas, suggesting that flavonols might, at least in part, negatively modulate root growth during de adaptation to Pi starvation.

Phosphate starvation has a significant impact on global gene expression (Misson *et al.*, 2005; Bustos *et al.*, 2010; Woo *et al.*, 2012; Secco & Whelan, 2014; Mora-Macias *et al.*, 2017). These analyses, together with genetic studies (Rubio *et al.*, 2001; Catarecha *et al.*, 2007; Ticconi *et al.*, 2009; Nussaume *et al.*, 2011; Gamuyao *et al.*, 2012; Wang *et al.*, 2012; Ruan *et al.*, 2015), have shed light into the molecular mechanisms that regulate the Pi starvation response. It is known that environmental conditions might affect plant nutrition and growth. Particularly, salt and drought affect Pi starvation (Baek *et al.*, 2016). The identification of these cross-talks have allowed the finding of new transcription factors that seem to modulate the Pi starvation response in plants (Baek *et al.*, 2017). Interestingly, using the D-Root system that provides a more natural environment for investigating root traits, we are uncovering novel genes/pathways regulating Pi starvation response or that might be balance during the Pi starvation, such as water deprivation or salt stress. Therefore, a better understanding of these genes/pathways will be crucial for future crop improvement programs including developing strategies for improving plant growth under low Pi regime.

cZ as Pi starvation response hormone

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CK are an important class of phytohormones that control cell proliferation and differentiation and regulate several aspects of plant growth and development including delay of senescence, shoot/root growth balance, transduction of nutritional clues, etc. (Sakakibara, 2006). Different hormonal pathways have been implicated in modulating root system during Pi starvation responses, including auxin and cytokinin (Franco-Zorrilla *et al.*, 2002; Jiang *et al.*, 2007; Perez-Torres *et al.*, 2008; Rouached *et al.*, 2010). Pi-starved plants increase root hair density and length as well as LR density to enlarge the nutrient absorbing surface. However, although LR density is higher, the total number of LR and their lengths are significantly increased in DGR plants. Auxin and CK have opposite roles in LR formation promoting or repressing LR development respectively (Li *et al.*, 2006; Laplaze *et al.*, 2007; Du & Scheres, 2018). Local CK biosynthesis in LRP creates a signal that restricts the formation of new LRP in neighboring pericycle cells and seems to suppress the initiation of new LRs (Chang *et al.*, 2015). It is remarkable that in Pi-starved roots the level of two of these IPTs, *IPT3* and *IPT5*, decreases (Fig. S6b). This reduction might favor the formation of more LRs, a phenotype associated with Pi starvation. Furthermore, tissue specific expression experiments demonstrate that xylem-pole pericycle cells, but not LRP in early stages, are sensitive to CK (Laplaze *et al.*, 2007). Similar conclusion was reached by analyzing mutants and overexpression lines of CK biosynthetic genes (Kuroha *et al.*, 2009). Together, these data suggest that CK are important for both LR positioning and number. We show that tZ has more pronounced effect on reducing the LRP formation compare to cZ. This suggests that Pi-starved plants increase the cZ/tZ ratio for maintaining a balance between reducing CK signaling but maintaining root growth and LR development to increase root surface.

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CKs are perceived by a small family of three receptors AHK2, AHK3, and AHK4 (Lomin *et al.*, 2012). A triple *ahk2,ahk3,ahk4* mutants show small-dwarf phenotype with a severe root growth reduction and short root meristems (Nishimura *et al.*, 2004). Though these receptors can partially complement each other's function, based on their specific expression patterns, it seems that they are not completely redundant (Nishimura *et al.*, 2004). *AHK4*, which is more expressed in roots, is implicated in the Pi starvation response (Franco-Zorrilla *et al.*, 2002) and may likely to be part of a molecular mechanisms for adaptation to external nutritional clues. It is possible that the presence of AHK4 modulates the sensitivity of particular cells to CK. However, other possibility is that AHK4 has higher specificity for a particular type of CK. There are different zeatin isomers, cis- and trans-zeatin or cis- and trans-zeatin-riboside, which are synthesized through different pathways. The levels of tZ and cZ do not show any correlation with plant evolution (Gajdosova *et al.*, 2011), and is thought that cZ accumulation is related to environmental responses such as abiotic stresses, biotic interaction, or pathogen and herbivore attacks (Havlova *et al.*, 2008; Vyroubalova *et al.*, 2009; Jiang *et al.*, 2013; Schäfer *et al.*, 2015). In Arabidopsis, the majority of cZ is formed by isopentenylation of tRNA by *AtIPT2* and *AtIPT9* (Miyawaki *et al.*, 2006). Here we show that Pi starvation resulted in down regulation of two of tZ biosynthesis genes but no changes in the expression of cZ biosynthesis genes *IPT2* and *IPT9.* However, the increase in *LOG* genes, involved in later steps of zeatin synthesis, might be relevant to increase cZ levels. Interestingly, tRNA modifications are often correlated with specific stresses response (El Yacoubi *et al.*, 2012). During Pi starvation, transfer RNA (tRNA)-derived small RNAs are generated and might have important biological functions in the Pi deficiency response (Hsieh *et al.*, 2010; Megel *et al.*, 2015;

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Park & Kim, 2018). Thus, it is tempting to speculate that tRNA halves might be used for prenylation and cZ formation in response to Pi starvation.

cZ are widely present in plants but, due to its low affinity for CK receptors, their biological relevance has been questioned. In recent years it has been shown that cZ is able to bind to and activate the CHASE-domain containing histidine kinases (CHKs) and activate downstream CK response genes (Romanov *et al.*, 2006; Stolz *et al.*, 2011). In Arabidopsis, AHK2 and AHK3 receptors show higher affinity for cZ than AHK4, however in all cases cZ affinity was several fold lower than its trans-isomer (Romanov *et al.*, 2006; Stolz *et al.*, 2011). This work has clearly highlighted the role of cZ in Pi starvation response. We show that cZ regulates distinct set of genes, which are not regulated by tZ. Also cZ, but not tZ, differentially regulates root growth in response to Pi starvation. Therefore, increasing cZ/tZ ratio during Pi starvation may trigger a specific molecular response that is needed for a proper plant adaptation to low levels of phosphate. Thus, cZ plays an important role in Pi starvation response by regulating root hair elongation, allocating Pi preferentially in root and increasing root/shoot weight ratio for P foraging.

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CKs have been proposed to modify the sink/source nutrient mobilization and shoot/root ratio (Werner *et al.*, 2008). Under Pi starvation, CKs seems to play an important role in controlling the PSR systemically, reducing the expression level of PSR genes (Martin *et al.*, 2000; Franco-Zorrilla *et al.*, 2002; Wang *et al.*, 2006). CK treatment increases intracellular free Pi content, likely by mobilizing organic-P (Wang *et al.*, 2006). Based on these data and our physiological and transcriptomic results we believe that cZ plays an important function in the plant response to Pi starvation. The fact that cZ repression of PSR genes is lower than tZ will be important to balance Pi starvation response, reaching an

equilibrium between root/shoot growth-Pi absorption and PSR genes expression level and root growth under Pi deprivation. In line with this idea, both cZ and tZ reduce root meristem size but only cZ seems to increase cell elongation during Pi starvation, favoring root growth. Thus the cZ biological function is relevant for maintaining optimal root growth during Pi starvation to keep searching for new Pi sources with the aim of sustaining plant fitness. Concomitantly, cZ also effectively represses *PHO1* gene, blocking the translocation of Pi from root to shoot, favoring the Pi allocation in roots. Taken together, all these processes increase free Pi content in root cells to maintain a minimum growth under low Pi conditions.

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the GEO Data Bank GSE131096 (-Pi_vs+Pi comparison) and GSE131056 (5Pi_vs+Pi comparison combined with cZ or tZ treatments).

Authors´ contribution

JS-N, CC and JCP planned and designed the research. JS-N, AS, CC, AZ and S N-N performed experiments. JG-M, RB, RS and JCP analyzed data. JS-N, RS and JCP wrote and edited the manuscript.

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Fig. S1: Light intensity and quality used to grow Arabidopsis seedlings and cZ and tZ identification

Fig. S2: Light is an additional stress to phosphate starvation

Fig. S3 Root illumination alters ions accumulation in root and shoot.

Fig. S4 Phosphate deficiency affects Arabidopsis metabolism.

Fig. S5 Root illumination alters root gene expression during the Pi starvation response

Fig. S6 Phosphate starvation alters hormonal levels in root and shoot and the expression of cytokinin biosynthesis genes.

Fig. S7 The cZ/tZ balance is partially regulated by *PHR1*.

Fig. S8 cZ and tZ increase Pi accumulation in *phr1*.

Fig. S9 cZ and tZ affects differentially to cell division and cell elongation and lateral root primordia formation.

Fig. S10 cis-zeatin and trans-zeatin regulate common and specific genes.

Fig. S11 Phosphate starvation increases flavonols levels in roots.

Fig. S12 Cytokinins negatively regulates *PHO1*.

Fig. S13: *IPT2* and *IPT9* are important for Pi accumulation in roots during Pi starvation.

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Fig. Legends

Fig. 1: Light modifies phosphate starvation responses.

(a) Phenotype of 12 day old Arabidopsis seedling grown in MS1/2 with 625 µM of phosphate (+Pi), without phosphate (-Pi) or 5 µM phosphate (5Pi) with the root system in presence of light (LGR-light grown roots-white box) or in darkness (DGR-dark grown roots-black box). Scale bar: 0.5 cm. **(b)** Root length of 12 day old Arabidopsis grown as described in (a). n=20. **(c)** Root meristem length of 8 day old DGR Arabidopsis seedling gown in MS1/2 with different Pi concentrations. n=14. **(d)** Density of emerged lateral root of 12 day old LGR or DGR Arabidopsis seedlings grown in MS containing different concentration of Pi. n=20. Numbers inside the bars correspond to the mean value of eLR per plant ± SD. **(e)** Representative pictures root hairs in the root tip of 8 day old LGR (white box) or DGR (black box) seedlings grown in MS1/2 containing different concentrations of Pi. Scale bar: 500 µm. **(f)** Fresh weight root/shoot ratio of 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. **(g)** Free phosphate (Pi) content in roots (R) or shoot (S) of 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. **(h)** Anthocyanin content in 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. Values shown means and error bars

correspond to standard deviation. Significance was analyzed by ANOVA and Tukey HSD post-test. P < 0.01.

Fig. 2. Effect of hormones on plant phosphate distribution and hormone levels.

(a) Phosphate accumulation in roots and shoots in dark-grown Arabidopsis seedlings cultivated 5 days in MS1/2 with 625 µM of Pi (+Pi) or 5 µM of Pi (5Pi) and 5 extra days in a similar medium with or without the indicated hormones. **(b)** Pi Root/Shoot ratio calculated with data in (a). Values are represented as relative to mock. IAA: Indol-Acetic acid; GA3: Gibberellic acid 3; ABA: Abscisic acid; CK: citoquinina (tZeatin); ACC: 1-Aminocyclopropane-1-carboxylic acid; SL: strigolactone. Values shown means of 5 replicates ± SD. Significance was analyzed by ANOVA and Tukey HSD post-test. P < 0.05. Letters compared roots and numbers compared shoots. **(c)** Level of cis-Zeatin (cZ), trans-Zeatin (tZ), cis-Zeati-Riboside (cZR), trans-Zeatin-Riboside (tZR), isopentenyladenine (iP) and isopentenyladenine–riboside (iPR) was quantified in roots (R) or shoots (S) of 10 day old Arabidopsis seedlings grown in MS1/2 containing 625 µM (+Pi), 0 µM (-Pi) or 5 µM (5Pi) of phosphate. Values shown means of four replicates ± SD. Significance was analyzed by ANOVA and Tukey HSD post-test. P < 0.01.

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Fig. 3: cis-zeatin control root growth under low Pi.

(a) Representative pictures of Arabidopsis seedlings grown in MS1/2 with high phosphate (625 µM; +Pi) or low phosphate (5 µM; 5Pi) for 5 days and then treated for 5 extra days with 2.5 µM of cZ or tZ. Scale bar: 0.5 cm. **(b)** Root length of seedlings grown as described in (a). n=30. **(c)** Root meristem size of Arabidopsis seedlings grown in +Pi or 5Pi media for 7 days and then treated with 2.5 µM of cZ or tZ for 24 hours. n=12. **(d)** Number of GUS stained cells in the root meristem of CYCB1-GUS seedling grown in +Pi or 5Pi media for 7 days and then treated with or without cZ or tZ (1 or 2.5 µM) for 24 hours. n≥20. **(e)** Cell length of the first four cells after the last root meristematic cell of control seedlings grown as in (c). n=14. Significance was analyzed by ANOVA and Tukey HSD post-test. P < 0.01.

Fig. 4: Differential inhibitory effect of cZ and tZ on root growth.

(a) Main Root length or total lateral root length of DGR Arabidopsis seedlings grown in MS1/2 with 625 µM (+Pi) or 5 µM (5Pi) of phosphate. After 5 days, seedlings were transferred to similar medium containing DMSO (mock) or different concentrations of cis-zeatin (cZ), trans-zeatin (tZ) (in µM) and cultivated for another 5 days. Measures for the main root length were done from the root tip to the point of transference. **(b)** Number of lateral root primordia (LRP) or emerged lateral root (eLR) in seedlings grown as in (a). Values shown means ± SD. n≥20. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$.

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Fig. 5: cis-zeatin and trans-zeatin regulate common and specific genes. (a,b) Venny´s diagrams of up-regulated (a) or down-regulated (b) genes by cZ or tZ treatment. Percentage into boxes indicate the amount of cZ- or tZ-specific genes that are induced (green) or repressed (blue) by Pi starvation. **(c)** Gene ontology analysis of specific genes induced by cZ in a Pi containing medium (417 genes). **(d)** Gene ontology analysis of specific genes repressed by tZ in a Pi containing medium (880 genes).

Figure 6: Effect of cZ and tZ on the expression of phosphate starvation response genes.

(a,b) Venny´s diagrams showing Pi starvation up-regulated or down-regulated genes that are specifically controlled by cZ or tZ. **(b,e)** Bar charts showing the most significant gene ontology categories of the Pi starvation up-regulated genes that are specifically regulated by cZ (b) or tZ (c) or down-regulated genes specifically by cZ (d) or tZ (e).

Fig. 7: Zeatins control root/shoot ratio and Pi allocation.

(a) Root and shoot weight of Arabidopsis seedlings grown in MS1/2 with 625 µM (+Pi) or 5 µM (5Pi) of phosphate for 5 days and then treated with 1 µM of cZ or tZ for 5 extra days. n= 5 replicates/12 seedlings per replicate. **(b)** Weight Root/shoot ratio calculated with data from (a). **(c)** Pi content in roots or shoots of control or *ahk3 ahk4* double mutant seedlings grown as in (a). n= 4 replicates. Values are represented relative to mock in +Pi in control or double mutant. **(d)** Root/shoot Pi ratio calculated from data in (c). Significance was

analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. Letters compared roots and numbers compared shoots.

Fig. 8: IPT2 and IPT9 activity is important for Pi root/shoot allocation and root hair elongation.

(a) Root/shoot Phosphate content ratio in 10 day DGR control, *ipt2*, *ipt9* or *ipt2 ipt9* mutants grown in MS1/2 medium containing 626 µM of Pi (+Pi) or 5 µM of phosphate. The measures correspond to 4 replicates containing 14 seedlings each. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. **(b))** Representative pictures of root hairs close to the root tip of DGR, *ipt2*, *ipt9* or *ipt2 ipt9* seedlings grown in MS1/2 medium containing 626 µM of Pi (+Pi) or 5 µM of phosphate for 10 days. Scale bar: 500 µm. **(c)** Representative pictures of root hairs from the mature area of DGR roots of control and double mutant *ahk3 ahk4* grown in medium containing 625 µM (+Pi), 0µ (-Pi) or 5 µM (5Pi) of phosphate for 6 days and then transferred to a similar medium containing mock or 2.5 µM of cZ or tZ for 2 extra days. Pictures were taken in the zone of transference**.** Scale bar: 500 µm. **(d)** Root hair length of seedlings grown as in (c). $n = 10$ different roots; total number of root hairs ≥ 450. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$.

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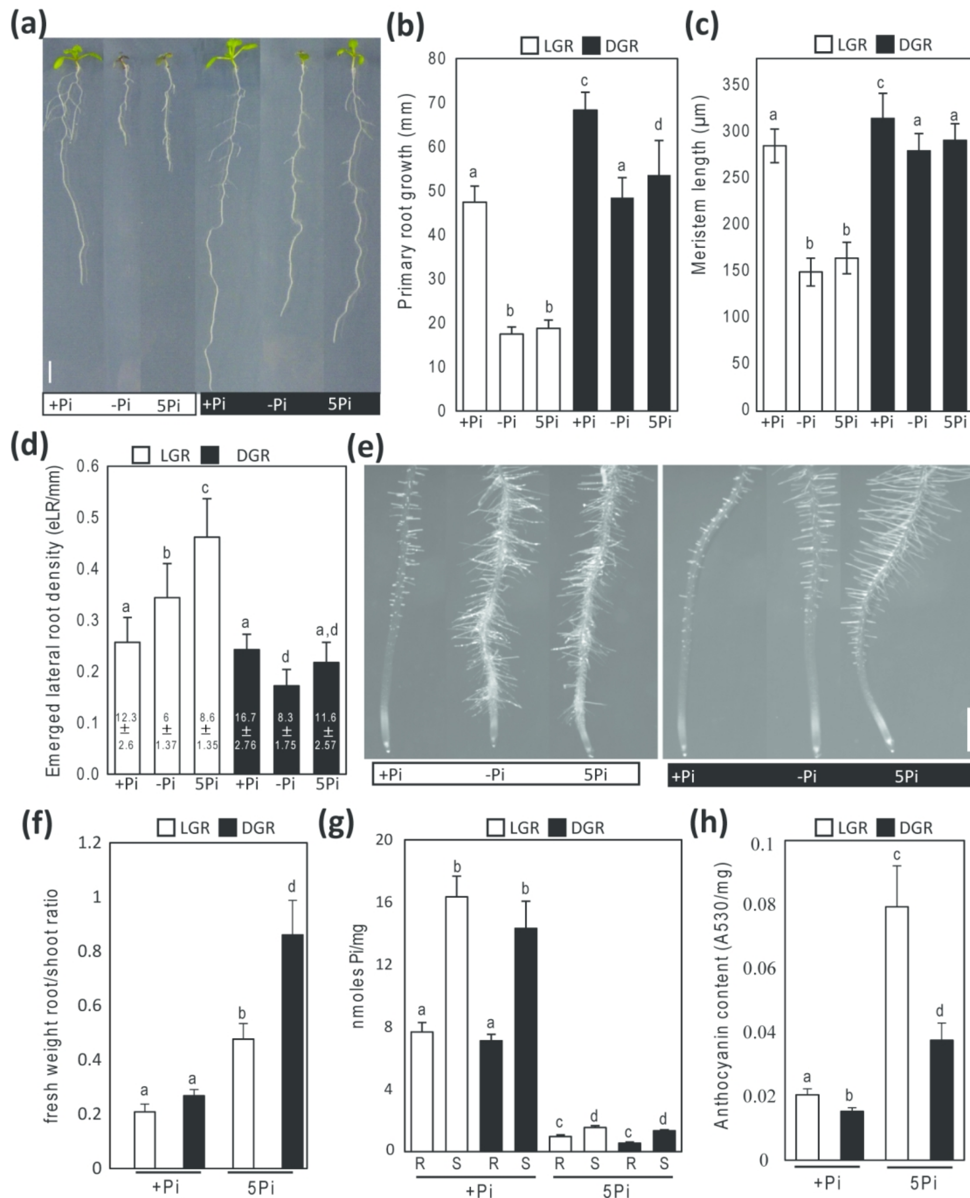

Fig. 1: Light modifies phosphate starvation responses.
(a) Phenotype of 12 day old Arabidopsis seedling grown in MS1/2 with 625 µM of phosphate (+Pi), without phosphate (-Pi) or 5 µM phosphate (5Pi) with the root system in presence of light (LGR-light grown roots-white box) or in darkness (DGR-dark grown roots-black box). Scale bar: 0.5 cm. (b) Root length of 12 day old Arabidopsis grown as described in (a). n=20. (c) Root meristem length of 8 day old DGR Arabidopsis seedling gown in MS1/2 with different Pi concentrations. n=14. (d) Density of emerged lateral root of 12 day old LGR or DGR Arabidopsis seedlings grown in MS containing different concentration of Pi. n=20. Numbers inside the bars correspond to the mean value of eLR per plant ± SD. (e) Representative pictures root hairs in the root tip of 8 day old LGR (white box) or DGR (black box) seedlings grown in MS1/2 containing different concentrations of Pi. Scale bar: 500 µm. (f) Fresh weight root/shoot ratio of 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. (g) Free phosphate (Pi) content in roots (R) or shoot (S) of 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. (h) Anthocyanin content in 10 day old LGR or DGR Arabidopsis seedlings grown in MS1/2 containing 626 µM of Pi (+Pi) or 5 µM of Pi. n=10. Values shown

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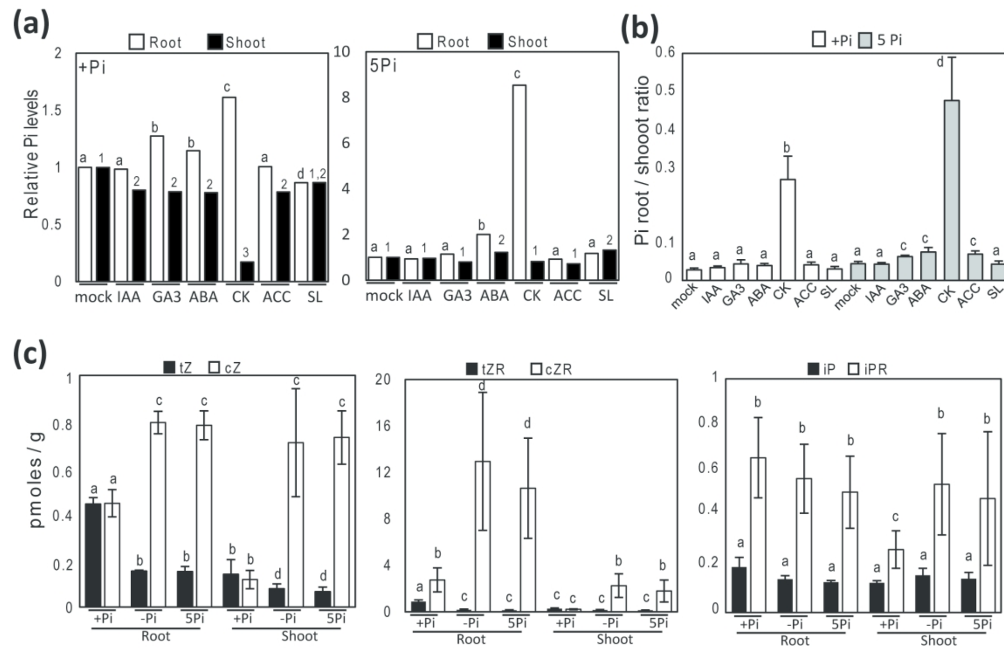

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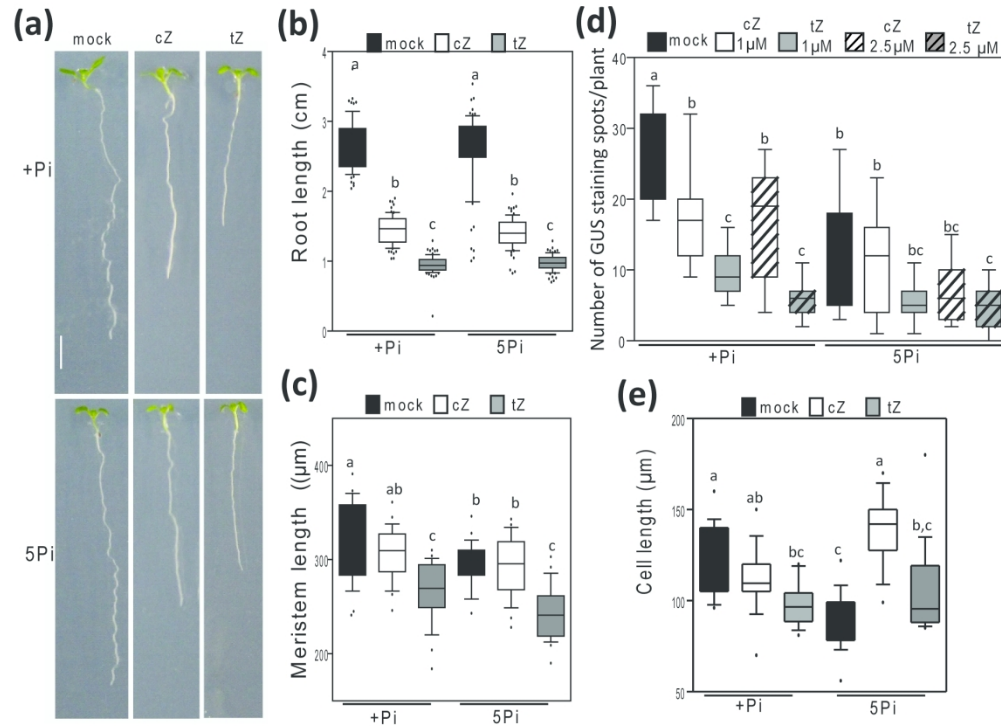
(a) Phosphate accumulation in roots and shoots in dark-grown Arabidopsis seedlings cultivated 5 days in MS1/2 with 625 µM of Pi (+Pi) or 5 µM of Pi (5Pi) and 5 extra days in a similar medium with or without the indicated hormones. (b) Pi Root/Shoot ratio calculated with data in (a). Values are represented as relative to mock. IAA: Indol-Acetic acid; GA3: Gibberellic acid 3; ABA: Abscisic acid; CK: citoquinina (tZeatin); ACC: 1-Aminocyclopropane-1-carboxylic acid; SL: strigolactone. Values shown means of 5 replicates ± SD. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. Letters compared roots and numbers compared shoots. (c) Level of cis-Zeatin (cZ), trans-Zeatin (tZ), cis-Zeati-Riboside (cZR), trans-Zeatin-Riboside (tZR), isopentenyladenine (iP) and isopentenyladenine–riboside (iPR) was quantified in roots (R) or shoots (S) of 10 day old Arabidopsis seedlings grown in MS1/2 containing 625 µM (+Pi), 0 µM (-Pi) or 5 µM (5Pi) of phosphate. Values shown means of four replicates ± SD. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.01$.



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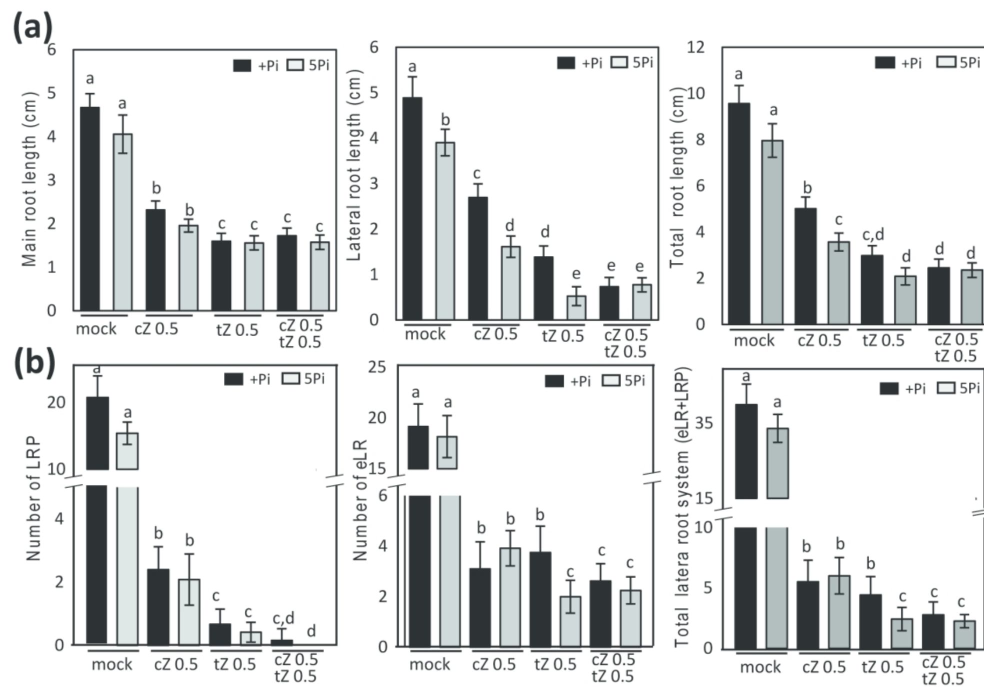

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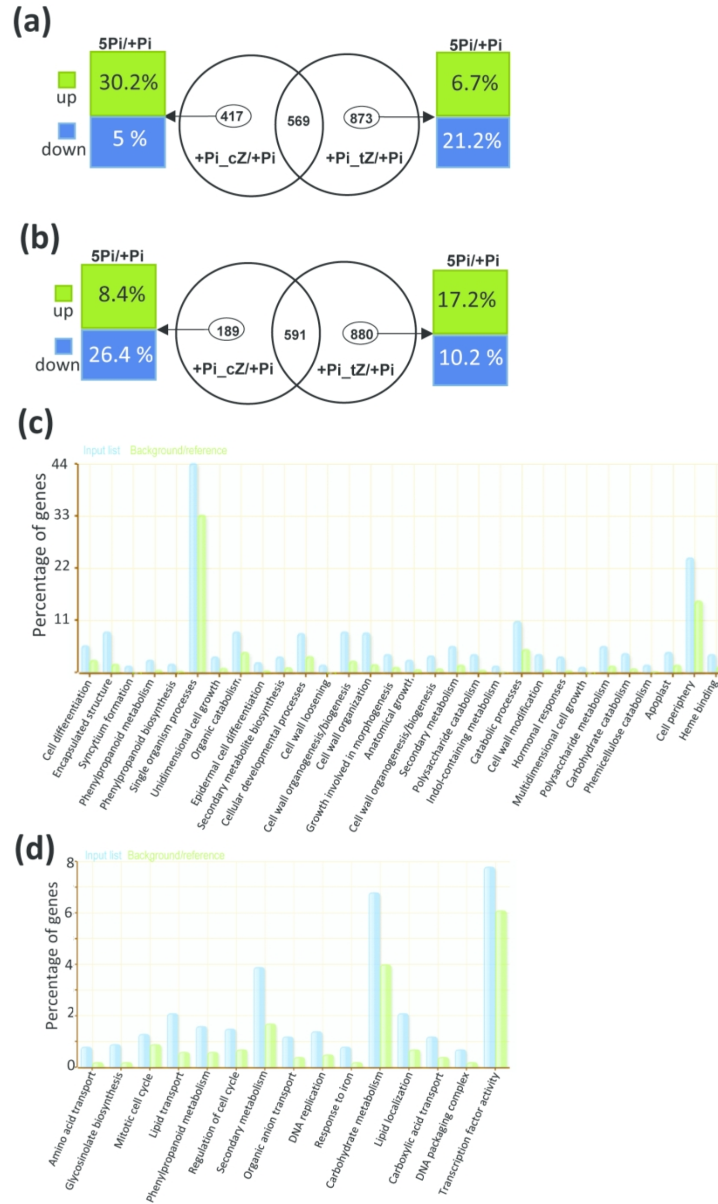

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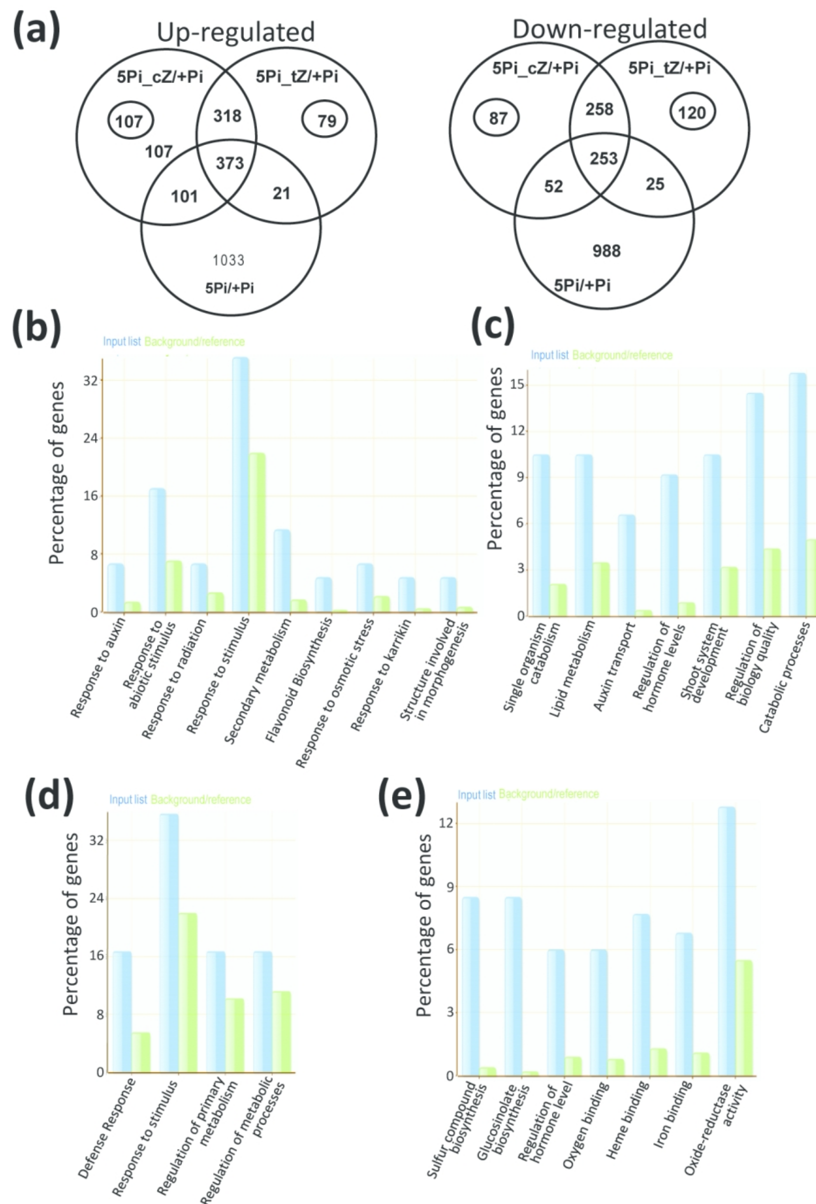

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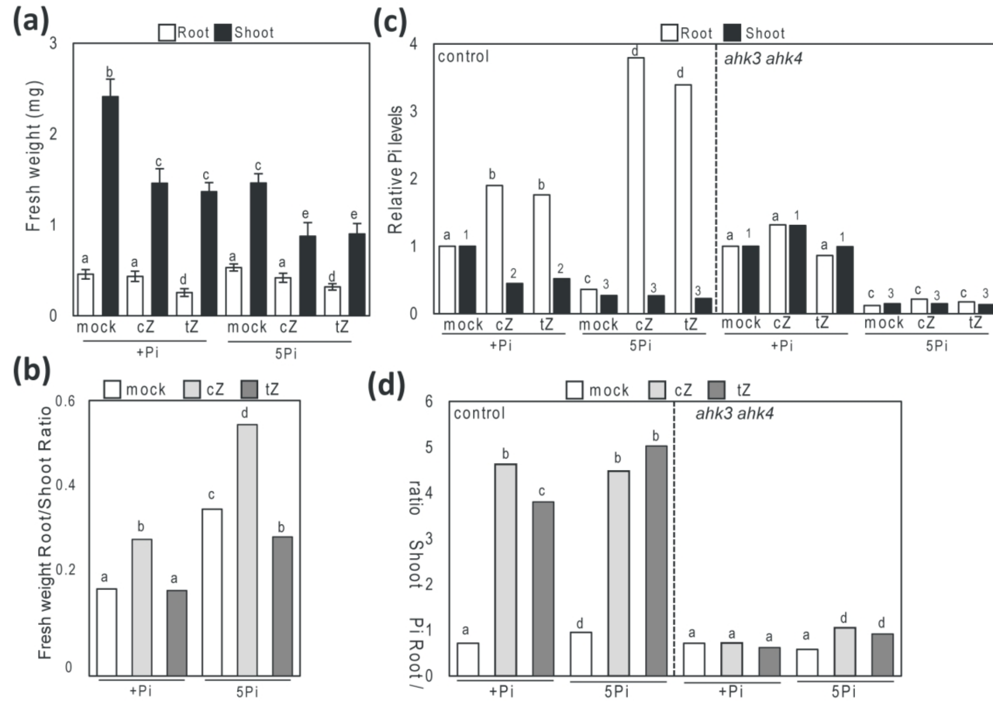

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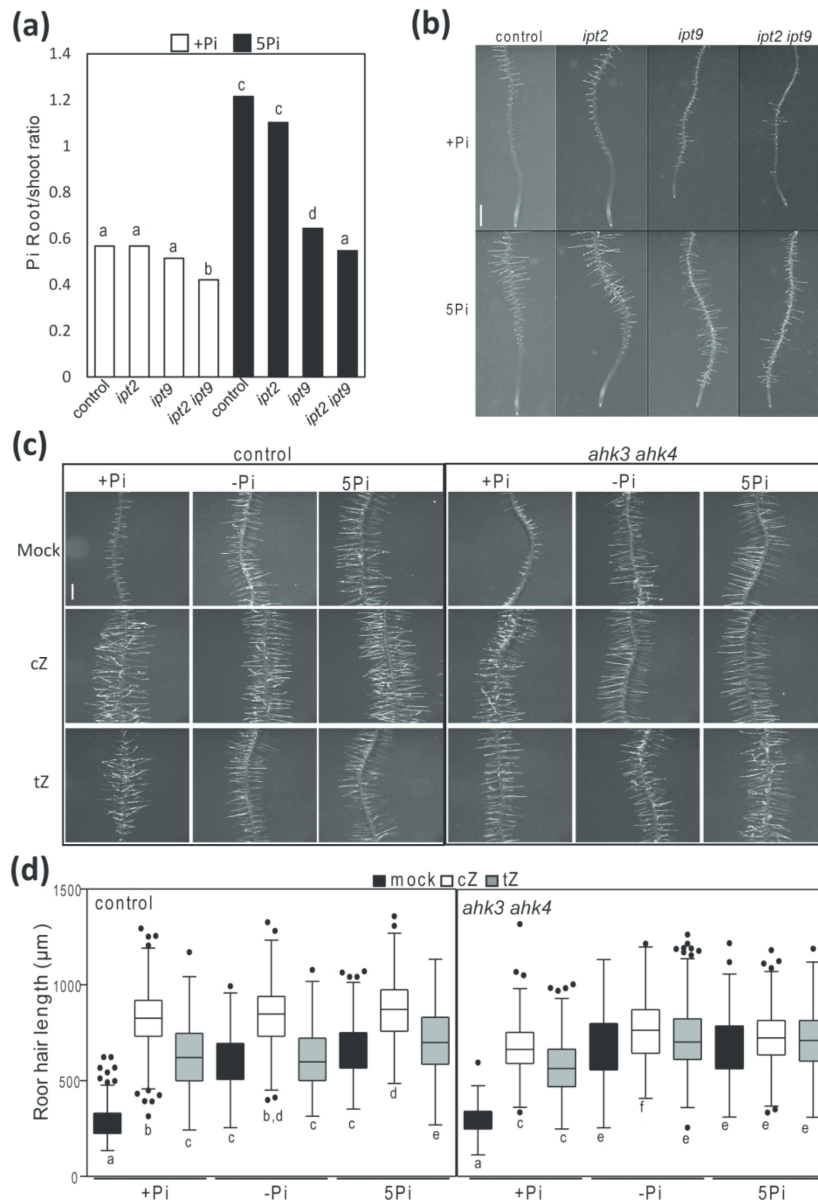

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