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ORIGINAL ARTICLE

Phosphoenolpyruvate carboxylase (PEPC) and PEPC-kinase (PEPC-k) isoenzymes in *Arabidopsis thaliana*: role in control and abiotic stress conditions

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Abstract

Main conclusion **Arabidopsis *ppc3* mutant has a growth-arrest phenotype and is affected in phosphate- and salt-stress responses, showing that this protein is crucial under control or stress conditions.**

Phosphoenolpyruvate carboxylase (PEPC) and its dedicated kinase (PEPC-k) are ubiquitous plant proteins implicated in many physiological processes. This work investigates specific roles for the three plant-type PEPC (PTPC) and the two PEPC-k isoenzymes in *Arabidopsis thaliana*. The lack of any of the PEPC isoenzymes reduced growth parameters under optimal growth conditions. PEPC activity was decreased in shoots and roots of *ppc2* and *ppc3* mutants, respectively. Phosphate starvation increased the expression of all *PTPC* and *PPCK* genes in shoots, but only *PPC3* and *PPCK2* in roots. The absence of any of these two proteins was not compensated by other isoforms in roots. The effect of salt stress on *PTPC* and *PPCK* expression was modest in shoots, but *PPC3* was markedly increased in roots. Interestingly, both stresses decreased root growth in each of the mutants except for *ppc3*. This mutant had a stressed phenotype in control conditions (reduced root growth and high level of stress molecular markers), but was unaffected in their response to high salinity. Salt stress increased PEPC activity, its phosphorylation state, and L-malate content in roots, all these responses were abolished in the *ppc3* mutant. Our results highlight the importance of the PPC3 isoenzyme for the normal development of plants and for root responses to stress.

Keywords Anaplerotic function · Phosphate starvation · Protein kinase · Salt stress

Abbreviations

PEPC	Phosphoenolpyruvate carboxylase
PEPC-k	Phosphoenolpyruvate carboxylase kinase
PTPC	Plant-type phosphoenolpyruvate carboxylase
BTPC	Bacterial-type phosphoenolpyruvate carboxylase
RSA	Root system architecture
MDA	Malondialdehyde

Introduction

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is a cytosolic enzyme catalyzing the irreversible β-carboxylation of PEP in the presence of HCO_3^- to yield oxaloacetate (OAA) and Pi, using Mg^{2+} as a cofactor. PEPC is present in all plants, green algae and cyanobacteria, most archaea and non-photosynthetic bacteria, but is absent in animals and fungi (O'Leary et al. 2011). PEPC is best known for its role in C_4 and CAM photosynthesis where it carries out the initial fixation of atmospheric CO_2. However, PEPC also plays a wide range of roles in non-photosynthetic and photosynthetic tissues of C_3 plants by anaplerotically replenishing C_4-dicarboxylic acids utilized for both energy and biosynthetic metabolism (Chollet et al. 1996; Izui et al. 2004). PEPC is an enzyme regulated at many levels. Most

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PEPC are regulated positively (glucose-6P) or negatively (L-malate, L-aspartate) by allosteric effectors, especially at suboptimal pH values that approximate the physiological pH of the cytosol (Chollet et al. 1996; Doubnerova and Ryslava 2011). Furthermore, plant PEPCs are regulated by reversible phosphorylation of a conserved Ser residue located at the N-terminal domain (Vidal and Chollet 1997). The phosphorylation of PEPC is catalyzed by PEPC-kinase (PEPC-k) and this reaction reduces the sensitivity to negative allosteric effectors and enhances its activation by glucose-6P (Nimmo et al. 1987; Takahashi-Terada et al. 2005). PEPC-k activity seems to be regulated only at the level of synthesis/degradation, in response to light in C_4 (Echevarría and Vidal 2003; Shenton et al. 2006; Monreal et al. 2010a) and C_3 (Gousset-Dupont et al. 2005) plants, and by a circadian mechanism in CAM plants (Taybi et al. 2000). Monoubiquitination (Uhrig et al. 2008; Shane et al. 2013; Ruiz-Ballesta et al. 2014) or interaction with anionic phospholipids (Monreal et al. 2010b) have recently been described as mechanisms introducing posttranslational modifications to PEPC. Interestingly, both modifications result in the inhibition of PEPC activity at least in vitro. PEPC and PEPC-kinase are encoded by small gene families. In *Arabidopsis thaliana* these families consist of 4 genes for PEPC (3 PTPCs (*PPC1-3*) and 1 BTPC (*PPC4*) that lacks the regulatory Ser for phosphorylation) and 2 genes for PEPC-k (*PPCK1-2*) (Fontaine et al. 2002; Sánchez and Cejudo 2003). The different PEPC and PEPC-k isoforms are expressed in a tissue-specific manner (Sánchez and Cejudo 2003; Shi et al. 2015). *PPC2* transcripts are found in all organs suggesting that it is a housekeeping gene. In contrast *PPC3* gene is expressed in roots, whereas *PPC1* and *PPC4* transcripts are found in roots and flowers (Sánchez et al. 2006). Photosynthetic and non-photosynthetic PEPCs and PEPC-ks have been implicated in many responses to different types of abiotic stresses (Amzallag et al. 1990; Echevarría et al. 2001; Sánchez et al. 2006; Chen et al. 2007; Monreal et al. 2007b; Gregory et al. 2009; Doubnerova and Ryslava 2011).

Phosphorous is an essential macronutrient required for numerous functions in plants but is often a limiting nutrient in soil (Vance et al. 2003; Niu et al. 2013). Plants absorb phosphate in an oxidized anionic form ($H_2PO_4^-$; Pi), but in soil Pi is frequently complexed with Al^{3+} or Ca^{2+} cations and thus exists as insoluble and unavailable for plant uptake (O'Leary et al. 2011). P deficiency is considered as one of the greatest limitations in agricultural production (Niu et al. 2013). To overcome low Pi availability, plants have evolved an array of responses. Among these responses, plants modify their root system architecture (RSA) to explore the upper parts of the soil ("topsoil foraging"), excrete organic acids (mainly malate and citrate, to acidify the rhizosphere and chelate metal ions) and phosphatases, express Pi transporters or establish symbiotic associations with fungi (arbuscular mycorrizhae or ectomycorrhizae) (Péret et al. 2011). Phosphate deprivation results in a significant up-regulation of PEPC activity and phosphorylation in *Arabidopsis thaliana* cell suspensions and seedlings. The isoenzymes implicated seem to be PPC1 and the PPCK1 and PPCK2 kinases (Chen et al. 2007; Gregory et al. 2009; O'Leary et al. 2011). A higher PEPC activity in phosphate-limiting conditions could lead to a higher accumulation and excretion of organic acids. Moreover, PEPC, together with NAD-MDH and NAD(P)-ME can provide an alternative glycolytic bypass for the ADP-limited cytosolic pyruvate kinase (PK) to facilitate continuous supply of pyruvate for the tricarboxylic acid cycle. Simultaneously, PEPC releases Pi when carboxylating PEP (Gregory et al. 2009; Doubnerova and Ryslava 2011).

High soil salinity is one of the major abiotic factors limiting crop yield. Salt stress limits plant growth by increasing the osmotic potential of the soil, thus decreasing water uptake by the roots (Julkowska and Testerink 2015). High salinity causes ionic stress and water shortage that negatively impact on cellular ion homeostasis, membrane permeability, enzyme activity, and subsequently, basic metabolic processes such as photosynthesis and respiration (Hasegawa et al. 2000; Zhu 2001). One of the most extended responses of plants to salt stress is the synthesis and accumulation of different metabolites acting as osmolytes. These compounds range from saccharides (sucrose, trehalose), sugar alcohols (mannitol, sorbitol), amino acids (proline) and amines (glycine betaine, polyamines) that appear to influence water balance (Seki et al. 2007). The role of these compatible osmolytes is to protect enzymes and cellular structures and to scavenge hydroxyl radicals (Liu and Zhu 1997). In *Arabidopsis thaliana* plants, salt stress up-regulates *PPC1*, *PPC3* and *PPC4* but not *PPC2* expression in roots (Sánchez et al. 2006). Some studies have analyzed the effect of salt stress on PEPC-k and its mechanisms of synthesis in C_4 and CAM plants (García-Mauriño et al. 2003; Taybi et al. 2004; Monreal et al. 2007a, b, 2013a, b), but little or no information regarding this protein kinase is available for C_3 plants.

The specific induction of PEPC and PEPC-k expression/activity by these two important and common stresses prompted us to investigate deeper the role of these proteins in more detail. To this end, we took the advantage of the availability of Arabidopsis plants (SALK T-DNA lines) specifically mutated in the *PPC* (*PPC1-3*) and *PPCK* (*PPCK1-2*) genes. In addition to stressed conditions, we examined the role of each of these proteins in optimal conditions during the entire life cycle of the plants. This work contributes important information about the role of specific PEPC and PEPC-k isoenzymes for plant growth under non-optimal growth conditions.

Material and methods

Plant material and growth conditions

All *Arabidopsis thaliana* plants used in this study were in the Columbia (Col-0) background. Seeds of the mutant SALK lines *ppc1-015* (SALK_N671015), *ppc1-378* (SALK_N666378), *ppc1-593* (SALK_ N675593), *ppc2* (SALK_N670126), *ppc3* (SALK_N656338), *ppck1* (SALK_N616510), *ppck2-774* (SALK_N673774) and *ppck2-866* (SALK_N677866) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK; http://arabidopsis.info). Mutant lines were analyzed and homozygosity confirmed by PCR (see Supplementary Table S1 for the list of primers).

Seeds were surface sterilized with ethanol 70 % (v/v) for 10 min followed by 50 % (v/v) HClO solution for 10 min. Finally, seeds were rinsed 8–10 times with sterile water and then stratified for 3 days at 4 °C in the dark in 0.1 % (w/v) agar solution to synchronize germination.

Seeds were sown on 1 % agar plates (12 × 12 cm) supplemented with 0.5× Murashige and Skoog medium (MS) (pH 5.7) and 1 % sucrose (Tables 1, 2; Figs. 3, 8). Seeds were germinated in L/D cycles (12 h/12 h, and 22 °C/18 °C, respectively) with white light at 150 µmol photons m^{-2} s^{-1}. Seven days after germination, 12–15 seedlings (approx. 2 cm primary root length) were transferred to fresh plates without sucrose and with the MS content modified as indicated in the figure legends (without phosphate or supplemented with 100 mM NaCl). After 14 days of treatment, roots were scanned (resolution 400 dpi) and plants harvested. Primary root length and the number and length of lateral roots were recorded and quantified using EZ-Rhizo software (Armengaud et al. 2009).

For hydroponic cultures, seeds were sown in Eppendorf tubes containing 0.5× MS media and 0.65 % (w/v) agar. The tubes were placed in Araponics® systems, where the roots were submerged in the indicated treatment solutions. Plants were grown in short day conditions (8 h light/16 h dark; 22 °C/18 °C, respectively). For phosphate starvation, 7-day-old plants were grown on 0.5× MS media without phosphate for 4 weeks before harvesting. For salt stress treatments, 21-day-old plants were grown on 0.5× MS media before the addition of 100 mM NaCl to the media, and harvested after two further weeks. Every sample typically composed 16–32 plants. Samples were harvested, quick-frozen in liquid nitrogen and stored at −80 °C until use.

Enzyme extraction and analysis

Protein extracts were obtained by grinding 0.2 g FW of leaf or root tissue in 1 ml of extraction buffer containing 0.1 M Tris–HCl pH 7.5, 20 % (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, a protease inhibitor cocktail (Sigma), 10 mM KF and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000*g* for 2 min and the supernatant was filtered through Sephadex G-25.

PEPC activity was measured spectrophotometrically at optimal pH 8.0 using the NAD-MDH-coupled assay at 2.5 mM PEP (Echevarría et al. 1994). A single enzyme unit (U) is defined as the amount of PEPC that catalyzes the carboxylation of 1 µmol of phosphoenolpyruvate per minute at pH 8 and 30 °C. The phosphorylation state of PEPC was determined by the malate test (malate inhibition at the suboptimal pH of 7.3) and expressed as the IC_{50}. A high IC_{50} is correlated to a high degree of PEPC phosphorylation (Echevarría et al. 1994).

Protein quantification

Protein concentrations were determined using the method of Bradford (Bradford 1976) with bovine serum albumin as the standard.

Table 1 Comparative physiological analysis of Col-0 and *ppc3* roots under NaCl stress

	L-Malate (mg g^{-1} FW)	Proline (µmol g^{-1} FW)	MDA (nmol g^{-1} FW)	Catalase activity (U mg^{-1} protein)
Control				
Col-0	0.45 ± 0.01	0.87 ± 0.03	9.39 ± 1.90	20.12 ± 1.98
ppc3	0.25 ± 0.01*	5.55 ± 0.02*	12.09 ± 0.34	32.97 ± 4.54
NaCl				
Col-0	0.91 ± 0.06[a]	15.37 ± 0.24[a]	32.58 ± 0.19[a]	15.93 ± 0.02
ppc3	0.23 ± 0.01*	5.40 ± 0.03*	9.58 ± 0.61*[a]	11.09 ± 1.55*[a]

Plants were grown hydroponically (MDA and catalase) or in plates (L-malate and proline) for 2 weeks with or without 100 mM NaCl. Data represent mean ± SE ($n = 3$). Every sample was typically composed of 25 plants

* $P < 0.05$ versus Col-0

[a] $P < 0.05$ versus Control (*t* test)

Table 2 Comparative morphological analysis of Col-0 and *ppc3* roots and shoots under NaCl stress

	Shoot FW (mg)	Shoot DW (mg)	Root FW (mg)	Root DW (mg)
Control				
Col-0	3.13 ± 0.09	0.20 ± 0.01	0.85 ± 0.16	0.06 ± 0.01
ppc3	2.53 ± 0.15*	0.21 ± 0.01	0.18 ± 0.01*	0.06 ± 0.01
NaCl				
Col-0	2.7 ± 0.1[a]	0.19 ± 0.01	0.21 ± 0.02[a]	0.05 ± 0.01
ppc3	2.59 ± 0.09	0.17 ± 0.01	0.18 ± 0.01	0.04 ± 0.01

Col-0 and *ppc3* plants were grown in plates for 2 weeks with or without 100 mM NaCl. Shoots and roots were collected for FW and DW determination. Data are mean ± SE of 80 plants

* $P < 0.05$ versus Col-0

[a] $P < 0.05$ versus Control (*t* test)

Electrophoresis and Western blotting

Protein extracts were denatured by heating in the presence of loading buffer (100 mM Tris–HCl pH 8, 25 % (v/v) glycerol, 1 % SDS, 10 % β-mercaptoethanol, 0.05 % (w/v) bromophenol blue). Denatured proteins were separated by SDS-PAGE in a Mini-Protean® III-2D Cell (Bio-Rad) and electroblotted onto a nitrocellulose membrane in a semidry transfer blot system (Bio-Rad Laboratories). Polyclonal antibodies against native C_4-photosynthetic PTPC from sorghum leaves (anti-C_4 PTPC) were prepared as described in Pacquit et al. (1995). After PTPC detection, membrane was stripped and incubated with sucrose synthase (susy) antibodies as a loading control. Susy antibodies were kindly provided by Prof WC Plaxton from the University of Queens (Fedosejevs et al. 2014). Bands were subsequently detected using affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories).

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of frozen, powdered leaves or roots using the RNeasy Plant Mini kit (Qiagen). Extracted nucleic acids were DNase treated to exclude genomic DNA. RNA concentrations and quality were determined using a Nanodrop 2000 UV–Vis Spectrophotometer (Thermo Scientific). Reverse transcription reactions were performed using 1 µg of purified total RNA, 1 µl ImProm-IITM Reverse Transcriptase (Promega) and a reaction buffer containing 0.5 mM dNTP, 6 mM $MgCl_2$, 20 U recombinant RNasin® ribonuclease inhibitor and 0.5 µg oligo(dt)15.

qPCR experiments

Quantitative PCRs (qPCRs) were performed in a final volume of 20 µl consisting of 1 µl of the cDNA, 15 µM of the specific primers (see Supplementary Table S1), and 10 µl of SensiFAST SYBR No-ROX kit (Bioline). PCR was conducted on the MiniOpticonTM Real-Time PCR Detection System (Biorad), and the threshold cycles (Ct) were determined using Bio-Rad CFX Manager software for all treatments. To normalize the obtained values, ubiquitin expression was used as internal control.

Proline determination

Proline was quantified by the acid-ninhydrin procedure of Bates et al. (1973). Root (0.5 g) samples were ground with 3 % sulphosalicylic acid (10 ml) and clarified by centrifugation. Supernatant (2 ml) was mixed with the same volume of acid-ninhydrin and acetic acid, the mixture was kept at 100 °C for 1 h, and the reaction was finished in an ice bath. The reaction mixture was extracted with toluene (4 ml), and absorbance was read at 517 nm using toluene as a blank. The proline concentration was determined from a standard curve (Arias-Baldrich et al. 2015).

Determination of malondialdehyde (MDA)

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). 0.2 g tissue was homogenized with 2 ml of 20 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 5 min. 2 ml of thiobarbituric acid solution (0.5 % (w/v) in 20 % TCA) was added to a 1 ml aliquot of the supernatant. The mixture was centrifuged at 12,000*g* for 5 min and heated at 95 °C for 30 min. The sample was quickly cooled on ice and centrifuged at 12,000*g* for 5 min. The absorbance of the supernatant was determined at 532 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968) was used to calculate the MDA concentration, which was expressed as nmol g^{-1} FW.

Catalase activity (CAT)

Catalase (CAT) activity was measured spectrophotometrically following the method of Beers and Sizer (1952). 0.2 g

of roots was homogenized with liquid nitrogen and resuspended in 2 ml extraction buffer (60 mM potassium phosphate buffer pH 7, 0.1 mM EDTA, 2 mM DTT). After centrifugation at 17,000*g* for 10 min at 4 °C, proteins in the supernatants were precipitated by the addition of $(NH_4)_2SO_4$ to 60 % saturation and centrifugation at 15,000*g* for 5 min. The proteins in the pellet were resuspended in 250 µl extraction buffer, and filtered through Sephadex G-25 equilibrated with 60 mM potassium phosphate buffer. The assay medium consisted of 60 mM potassium phosphate buffer pH 7, 20 mM H_2O_2. The decrease of the H_2O_2 absorbance was followed for 120 s at 240 nm at 25 °C using the extinction coefficient of 39.58 M^{-1} cm^{-1}. One U of CAT is defined as µmol of H_2O_2 consumed per min.

Malate quantification

Malate was determined in crude extracts obtained by grinding 0.2 g fresh weight of root tissue with 1 ml of 7 % (v/v) perchloric acid. The acid suspension was neutralized with 15 % (v/v) tetramethylammonium chloride–KOH (1 M/5 N), and the residue was removed by centrifugation at 15,000*g* for 2 min. L-Malate concentration was determined by measuring the increase in absorbance at 340 nm due to the enzymatic reduction of NAD^+ according to Lowry and Passoneau (1972). The reaction was carried out in 1 ml of a reaction mixture containing an aliquot of the supernatant, 40 mM 2-amino-2-methyl-1 propanol, pH 9.9, 4 mM NAD^+, 4 mM Glu, 3.5 U of NAD-MDH, and 0.9 U of Asp transaminase.

Statistical analysis

Statistical analysis was performed using SigmaStat (Systat Software Inc). Differences between groups were tested by ANOVA. Differences between pairs of groups were tested by *t* test. The means were considered to be significantly different at $P < 0.05$.

Results

Impact of the lack of specific PEPC isoenzymes

In *Arabidopsis thaliana*, phosphoenolpyruvate carboxylase (PEPC) is encoded by a small multigene family composed of four genes, the PTPC variants *PPC1-3*, and *PPC4*, a

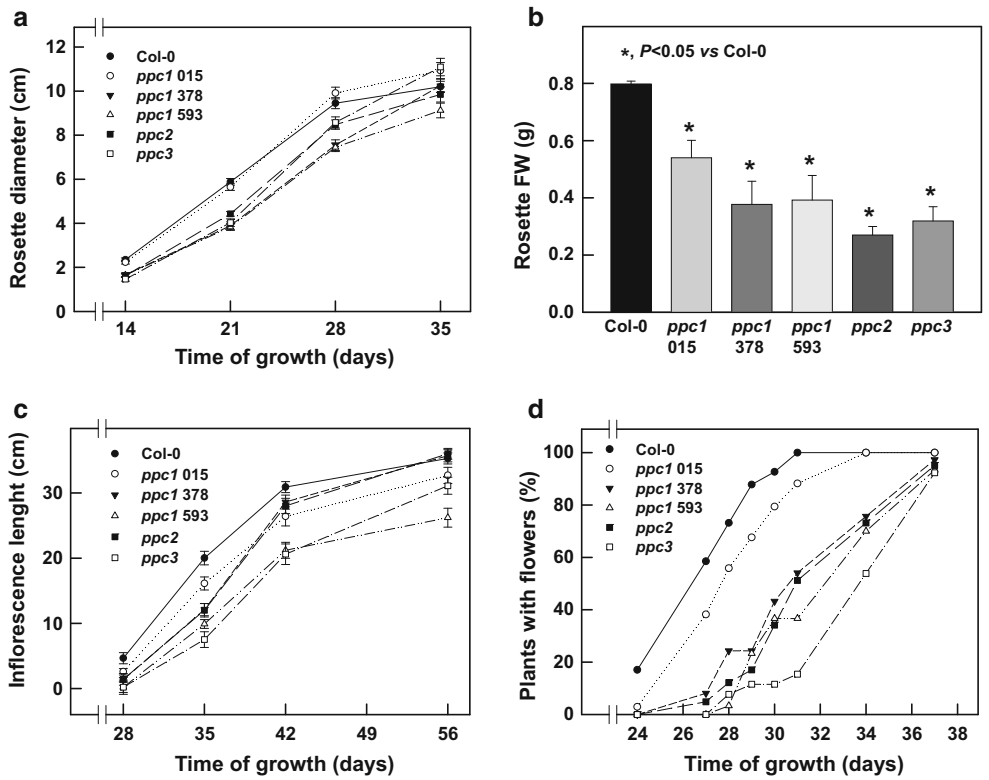

Fig. 1 Effect of *PTPC* gene mutation in *Arabidopsis thaliana* plants. After sterilization, seeds were sown directly in soil pots and plants were grown in an L/D white light cycle of 12 h/12 h. The *graphs* show the rosette diameter (**a**), the rosette fresh weight (**b**), the floral stem quantification (**c**) and the flowering time phenotype (**d**) of WT and SALK T-DNA plants. Values are expressed as the mean ± SE ($n = 40$). ANOVA showed significant differences among groups ($P < 0.001$)

BTPC lacking the canonical N-term domain that can be subjected to phosphorylation (Sánchez and Cejudo 2003). Some studies have examined the role of *PPC* genes in the behavior of *Arabidopsis* plants (Sánchez et al. 2006; Shi et al. 2015). However, none of them have examined the role of these genes during the whole life of the plant. Here, we examined the effect of these *PTPC* genes in the behavior of plants growing in optimal conditions during their entire life using different transgenic SALK lines. In most of the lines analyzed, the lack of PEPC isoenzymes delayed growth (Fig. 1a), decreased weight (Fig. 1b) and impacted on flowering by reducing the inflorescence length (Fig. 1c) and delayed flowering time (Fig. 1d).

The expression of *PPC* and *PPCK* genes was analyzed by qPCR. Transcripts of *PPC1*, *PPC3* and the two *PPCK* genes accumulated to higher levels in roots compared to shoots. *PPC2* was slightly but significantly more expressed in shoots than in roots, while *PPC4* was almost absent in both tissues in the optimal growth conditions employed (Fig. 2a). Next we evaluated the consequences of the lack of specific genes on PEPC activity in shoots and roots (Fig. 2b). In shoots, PEPC activity was markedly decreased only in *ppc2* plants. On the contrary, root PEPC activity was depressed only in *ppc3* plants (Fig. 2b). Western analysis showed that PEPC protein was markedly decreased in *ppc2* shoots (Fig. 2c). These results indicate that PPC2 is the prevalent PEPC protein in shoots and PPC3 in roots.

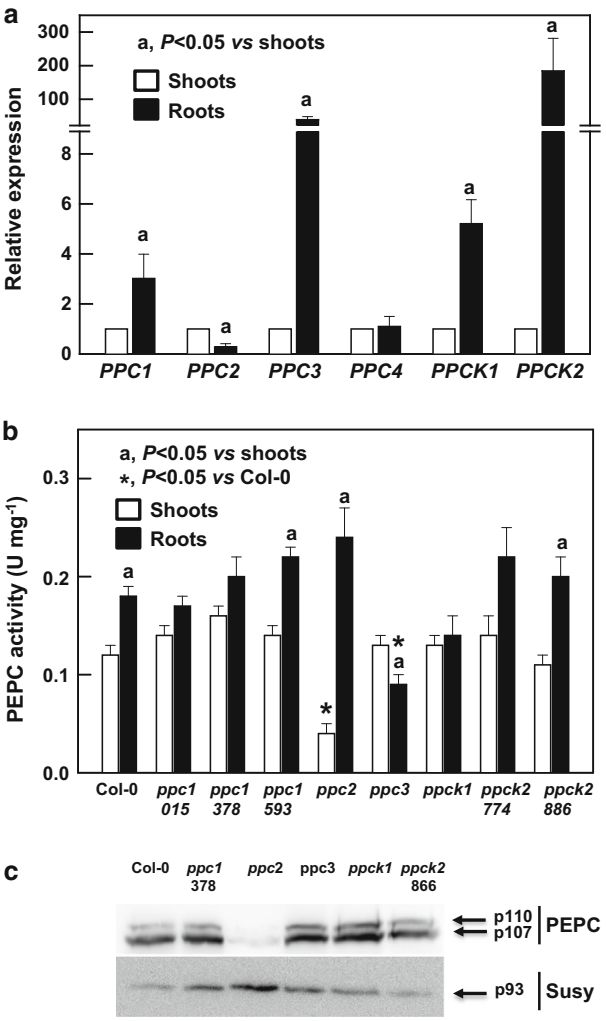

Fig. 2 Analysis of *PPC* and *PPCK* gene expression in Col-0 (**a**), and PEPC activity in shoots and roots (**b**). Data represent the mean ± SE ($n = 3$–6). Samples typically represent 40 pooled plants. *a* indicates a significant difference versus shoots ($P < 0.05$). *Indicates a significant difference versus Col-0 ($P < 0.05$). **c** PEPC (*above*) and sucrose synthase (susy) (*bottom*) proteins immunodetected in shoots of SALK T-DNA plants. ANOVA showed significant differences among groups ($P < 0.001$)

Phosphate starvation

Phosphate deficiency in the PEPC- and PEPC-k-defective plants (*ppc* and *ppck* mutants) decreased the length of the main root (Fig. 3a). In control conditions, *ppc3* had the shortest main root and the slowest growth rate. Consequently, phosphate deficiency in *ppc3* plants was negligible after 1 week, although deficiency was clearly observed after 2 weeks of treatment (Fig. 3b). The lack of phosphate supply modestly increased and markedly increased lateral root number (Fig. 3c) and lateral root density (Fig. 3d), respectively.

The up-regulation of specific PTPC and PPCK isoenzymes in response to phosphate starvation is well characterized for many plants (Fukayama et al. 2006; Chen et al. 2007, 2008a, b; Gregory et al. 2009). *P* deficiency increased the transcript level of all *PPC* and *PPCK* genes in shoots (Fig. 4a). Interestingly, the sequence GAATATTC, homologous to a *cis* element of phosphate starvation responsive genes (Rubio et al. 2001; Fukayama et al. 2006), was found in the 5′ region of *PPC1*, *PPC3*, *PPCK1* and *PPCK2* but not in *PPC2* or *PPC4* (Fig. S1). However, in roots, P deficiency markedly increased only *PPC3* and *PPCK2* transcript levels (Fig. 4b). In addition, these two genes had a much higher expression in roots than in shoots in P-sufficient plants.

Although PPC2 is the main PEPC isoenzyme detectable in shoots and *ppc2* has lower PEPC activity and protein level (Fig. 2b, c), P deficiency increased PEPC activity in *ppc2* at the same level as in Col-0 (Fig. 5a). In contrast, only a modest increase of PEPC activity was observed in roots of P-deficient *ppc3* plants (Fig. 5b).

In addition to increasing enzyme activity, P deficiency has also been shown to enhance PEPC's phosphorylation state, estimated in function of the sensibility of the enzyme to its allosteric inhibitor L-malate (IC_{50}) (Echevarría et al. 1990; Chen et al. 2007; Gregory et al. 2009). We found that

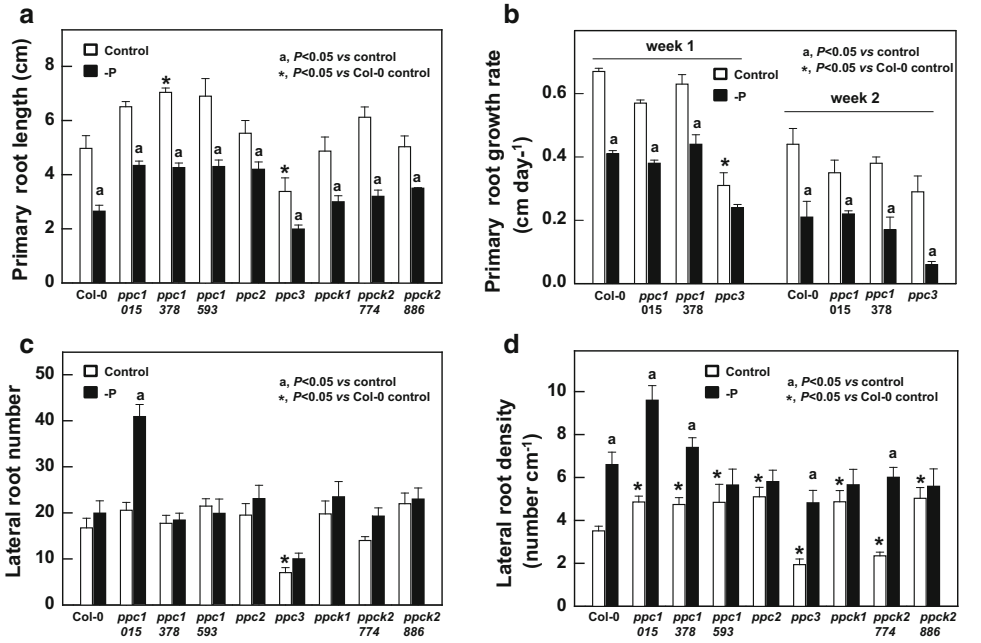

Fig. 3 Primary root length (**a**) and growth rate (**b**), and lateral root number (**c**) and density (**d**) were measured in *Arabidopsis* SALK lines growing in agar plates ("Materials and methods"). *Bars* indicate mean ± SE ($n = 3$). Samples typically represent 25 pooled plants. *a* indicates a significant difference versus control ($P < 0.05$). *Indicates a significant difference versus Col-0 in control conditions ($P < 0.05$). ANOVA showed significant differences among groups ($P < 0.001$)

P deficiency increased PEPC phosphorylation in shoots of Col-0, and this response was also observed in most of the *ppc* and *ppck* mutants (Fig. 6a). However, increased PEPC phosphorylation was not observed in the roots of the *ppc3* and *ppck2* mutants (Fig. 6b) where the IC_{50} index was not affected by the lack of phosphate.

Salt stress

Previous work by Sánchez et al. (2006) demonstrated that salt stress (170 mM NaCl) triggered the accumulation of *PPC1*, *PPC3* and *PPC4* transcripts but did not affect *PPC2* expression in roots. We found that after 4 weeks of treatment with 100 mM NaCl, salinity resulted in a modest increase of shoot *PPC* and *PPCK* expression levels (Fig. 7a). In roots, however, this salt treatment enhanced *PPC3* transcript levels markedly (Fig. 7b), thereby emphasizing the important role of PPC3 in responses to stress. *PPCK2* expression in roots was only slightly decreased by salt treatment.

As previously shown (Fig. 3), *ppc3* mutant plants have a small root system compared to Col-0 in optimal conditions (Fig. 8a, left). Salt stress decreased root length in Col-0 but had little effect on *ppc3* (Fig. 8a, right). Time-course analyses of root development, with and without NaCl, showed that Col-0 roots grew faster than *ppc3* roots (Fig. 8b), and the reduction caused by salinity was also manifested before. After 21 days of treatment, salt-treated Col-0 plants showed a marked reduction in root growth compared to control plants and this reduction in root growth was not observed in control and salt-treated *ppc3* plants. Salinity-treated Col-0 and *ppc3* plants showed reduced numbers of lateral roots, the latter having fewer lateral roots compared to Col-0 in control conditions (Fig. 8c). Similar to P deficiency, the phenotype of the roots of *ppc3* plants was more akin to Col-0 stressed plants than to non-stressed plants.

Previously we described a drastic increase in PEPC-kinase activity and PEPC phosphorylation under salinity in leaves of the C_4 plant *Sorghum bicolor* (Echevarría et al. 2001; García-Mauriño et al. 2003; Monreal et al. 2013a). However, for *Arabidopsis* plants, salinity increased PEPC activity (Fig. 9a) and phosphorylation (Fig. 9b) in roots, without any effect in shoots. Interestingly, this phenomenon was not found in *ppc3* plants. To further establish the role of PPC3 in *Arabidopsis* roots during salt stress, we measured L-malate levels (Table 1). The synthesis of L-malate was lower in *ppc3* plants compared to Col-0, suggesting that *ppc3* plants do not respond to salinity by increasing L-malate production. Stress markers such as proline, MDA or catalase activity were measured in Col-0 and *ppc3* roots under control and salt conditions (Table 1) and found higher in the *ppc3* line compared to Col-0 in optimal growth conditions, but unaffected by the stress in this mutant.

As already shown, *ppc3* plants are smaller than Col-0 growing in optimal conditions (Fig. 8a, left). However, although *ppc3* plants had lower shoot and root FW compared to Col-0, the shoot and root DWs were similar in the wild type and the mutant, indicating that *ppc3* plants are smaller than Col-0 due to lower water content (Table 2).

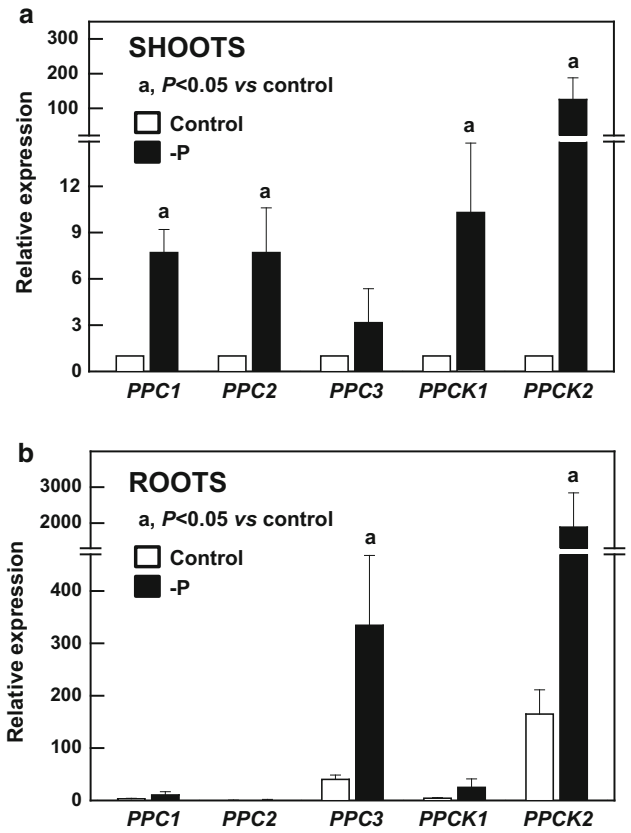

Fig. 4 Effect of phosphate starvation on *PPC* and *PPCK* genes in Col-0 shoots (**a**) and roots (**b**). Plants were grown hydroponically with or without phosphate in the media as indicated in "Materials and methods". The relative expression levels in shoots (**a**) and roots (**b**) were normalized for each gene to the shoot control condition. Data represent the mean ± SE ($n = 3$–6). Samples typically represent 30 pooled plants. *a* indicates a significant difference versus control ($P < 0.05$). ANOVA showed significant differences among groups ($P < 0.001$)

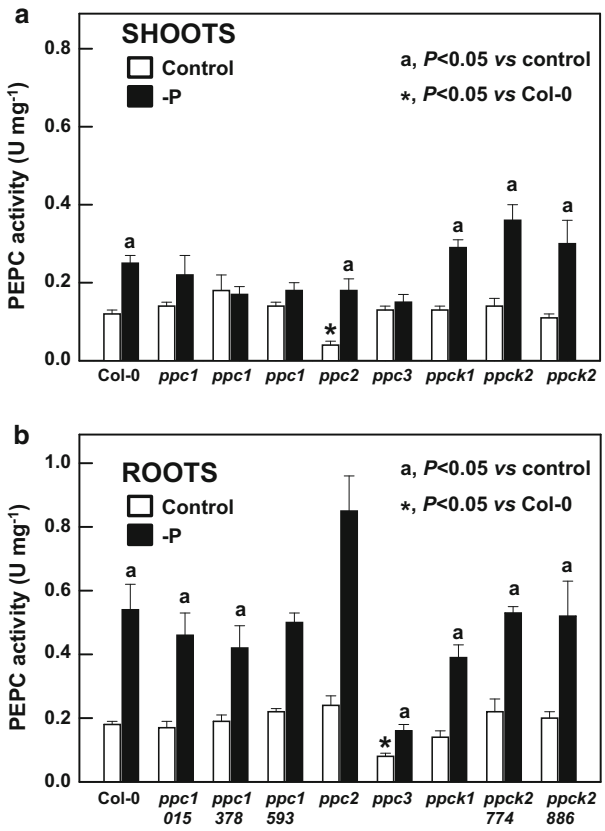

Fig. 5 PEPC activity in shoots (**a**) and roots (**b**). Col-0 and SALK lines were grown hydroponically as described in "Materials and methods". Data represent the mean ± SE ($n = 3$). Samples typically represent 30 pooled plants. *a* indicates a significant difference *versus* control ($P < 0.05$). *Indicates a significant difference versus Col-0 in control conditions ($P < 0.05$). ANOVA showed significant differences among groups ($P < 0.001$)

Discussion

Several groups have investigated PEPC and PEPC-k isoenzymes in *Arabidopsis thaliana* (Sánchez and Cejudo 2003; Sánchez et al. 2006; Chen et al. 2007, 2008a, b; Gregory et al. 2009; Shi et al. 2015). Most of the previous work has investigated *PPC* and/or *PPCK* gene expression in different plant organs, or in cell culture, and in different conditions, mainly salt and drought stress and P deficiency. Our approach was to consider flowering time, the duration of P deficiency and salt stress, and to employ SALK lines to evaluate the impact of the lack of specific PEPC and PEPC-k isoenzymes.

PTPC is composed of 3 isoenzymes in *Arabidopsis* plants referred to as PPC1-3 (Sánchez and Cejudo 2003). We show that the lack of any of these proteins has an impact on plant behavior and is required for optimum growth and development. However, all mutants were able to complete their life cycle and produce seeds when growing under optimal conditions. In roots, only *ppc3* mutant has a clear phenotype in optimal conditions, consisting of a root growth-arrest phenotype that features smaller primary roots and lower lateral root number and density. This clearly points to an important role for this protein in root development.

Transcripts of *PPC1*, *PPC3* and the two *PPCK* genes accumulated at higher level in roots than in shoots, with *PPC3* being the main PEPC isoform expressed in *Arabidopsis* roots. These results are in concordance with those reported by other authors (Fontaine et al. 2002; Gousset-Dupont et al. 2005; Sánchez et al. 2006; Gregory et al. 2009). The expression of *PPC3* and *PPCK2* genes was approximately 100 and 200 times higher, respectively, in roots than in shoots suggesting an important role for these isoenzymes in roots. Moreover, root PEPC activity was depressed only in *ppc3* plants, reinforcing the important role of this enzyme in *Arabidopsis* roots. In contrast, *PPC2*

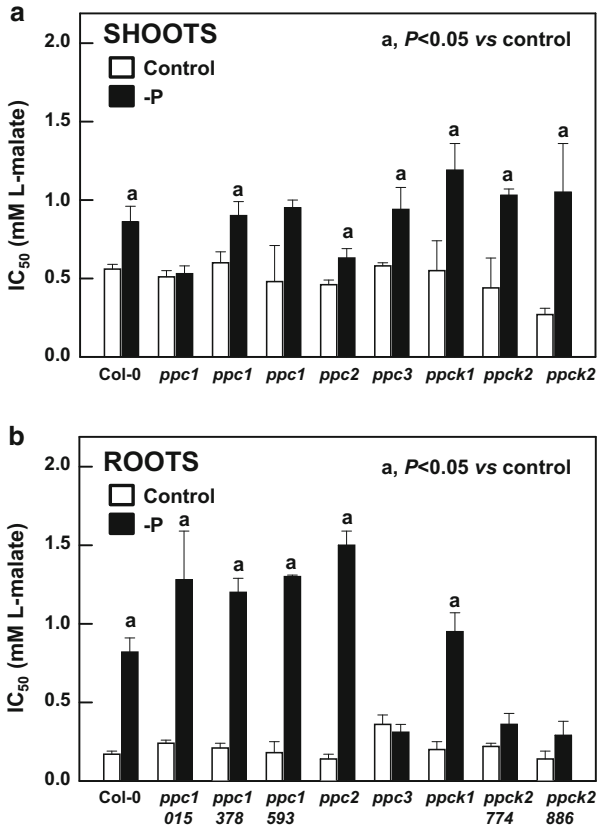

Fig. 6 Phosphorylation state of PEPC. Inhibition of the enzyme activity by L-malate (IC_{50}) was measured in shoots (**a**) and roots (**b**) of *Arabidopsis* plants growing hydroponically with (*white*) or without (*black*) phosphate in the media. Data represent the mean ± SE ($n = 3$). Samples typically represent 30 pooled plants. *a* indicates a significant difference versus control ($P < 0.05$). ANOVA showed significant differences among groups ($P < 0.001$)

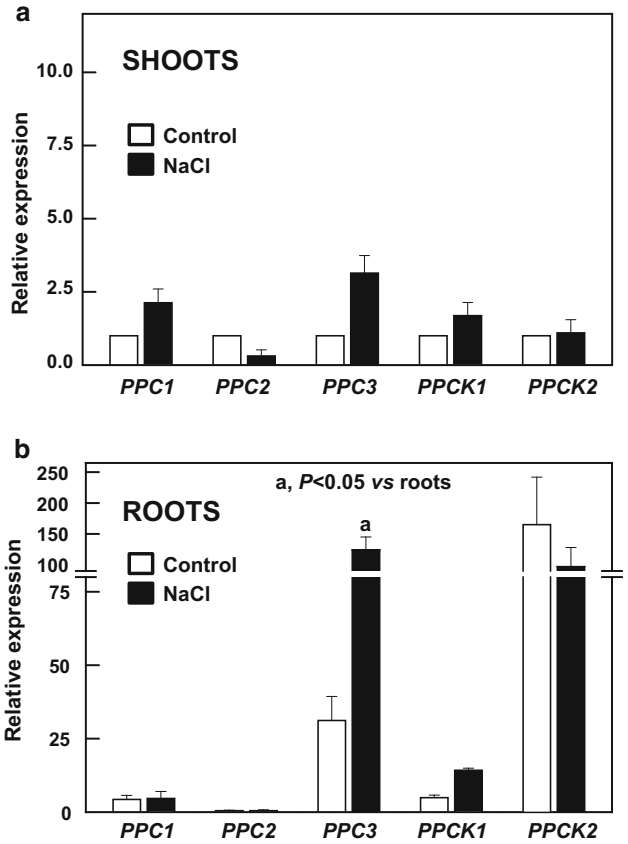

Fig. 7 Effect of salt stress on *PPC* and *PPCK* genes in Col-0 shoots (**a**) and roots (**b**). Plants were grown hydroponically under control or salt conditions as described in "Materials and methods". The relative expression levels in shoots (**a**) and roots (**b**) were normalized for each gene to the shoot control condition. Data are expressed as the mean ± SE ($n = 3$). Samples typically represent 25 pooled plants. *a* indicates a significant difference versus control ($P < 0.05$). ANOVA showed significant differences among groups (**a** $P < 0.01$; **b** $P < 0.001$)

is a ubiquitous isoform expressed across all tissues (Sánchez et al. 2006). In shoots in *ppc2* plants, PEPC activity was 30 % of wild-type levels, indicating that PPC2 isoenzyme is the major PEPC in *Arabidopsis* rosette. Indeed, Western blot analysis showed that PEPC protein was markedly decreased in *ppc2* shoots. Taken together, our data indicate that PPC2 is the most important PEPC isoform in shoots since (i) its absence is not substituted by other isoenzyme, (ii) it is the sole PEPC isoenzyme with higher levels of expression in shoots than in roots, and (iii) PEPC activity is severely decreased in *ppc2* shoots. *PPC4* was almost absent both in shoots and in roots, with ct values for qPCR much lower than the other genes analyzed, at least in optimal growth conditions (Sánchez et al. 2006).

PEPC and PEPC-k proteins have been implicated in many responses to both biotic and abiotic stress in C_4, CAM, and C_3 plants (Amzallag et al. 1990; Li and Chollet 1994; Popova et al.1995; González et al. 2003; Sánchez et al. 2006; Doubnerova and Ryslava 2011; O'Leary et al. 2011). A higher PEPC level (quantity, activity or both) is proposed to act in two main ways. First, a higher PEPC activity increases anaplerosis to support biosynthetic processes, or a bypass of PK to produce pyruvate; second, a high PEPC activity in roots leads to the synthesis of organic acids such as malate or citrate that are excreted to the rhizosphere to acidify the soil and chelate cations in nutrient deprivation and/or toxic metal stress (O'Leary et al. 2011). Furthermore, PEPC may also support the biosynthesis of biocompatible osmolytes such as proline upon drought or salt stress in many plant species (Chen et al. 2010; O'Leary et al. 2011). The functions of these enzymes are important not only for normal growth but also for responses to stress (Doubnerova and Ryslava 2011).

Phosphate deficiency is considered as one of the greatest limitations in agricultural production (Niu et al. 2013). It is a well-known factor that specific PTPC and PPCK

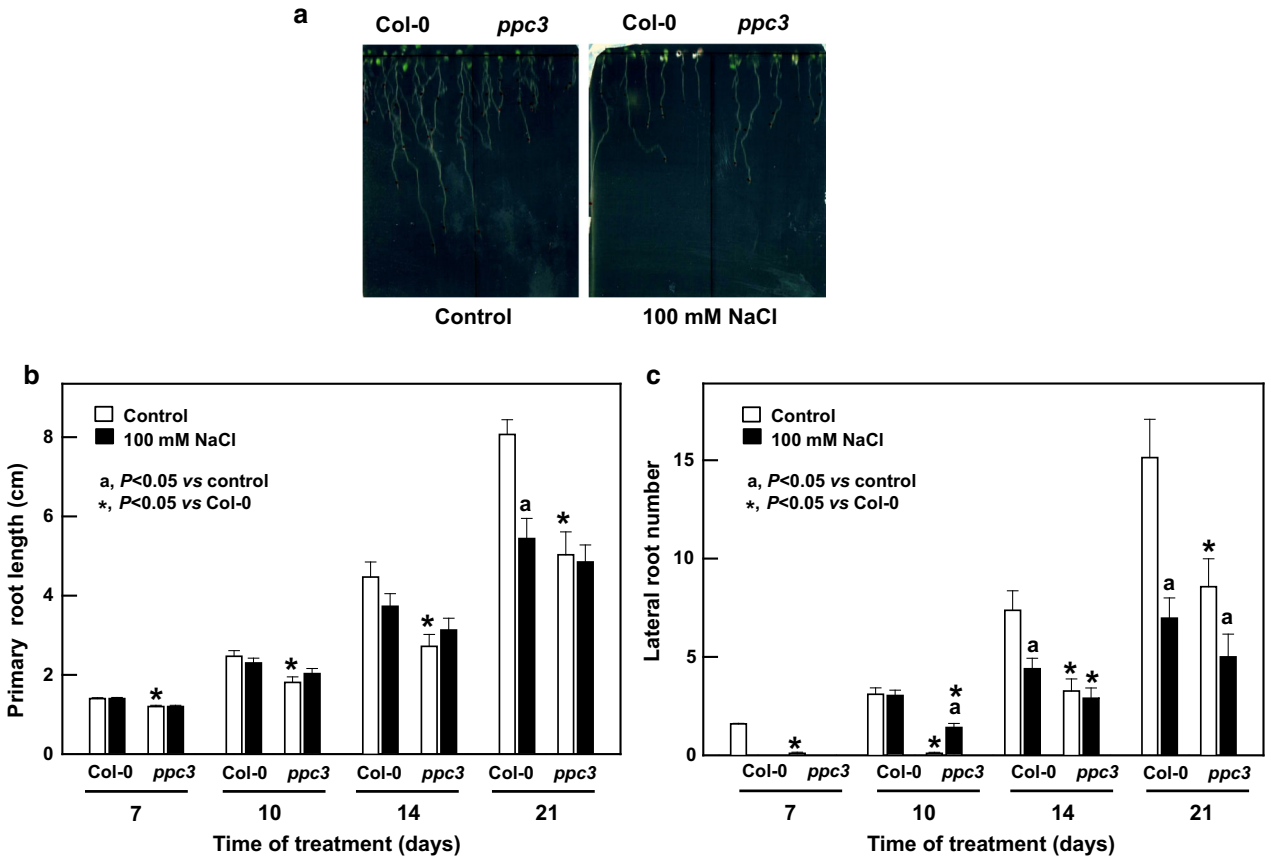

Fig. 8 PPC3 and salt stress. Aspect of *Arabidopsis* Col-0 and *ppc3* plants (**a**) growing in control (left) and salt stress (right) conditions. Primary root length (**b**) and lateral root number (**c**) of plants growing in control (*white*) or 100 mM NaCl (*black*) plates. Data represent the mean ± SE ($n = 3$). Samples typically represent 30 pooled plants. *Indicates a significant difference versus Col-0 ($P < 0.05$). *a* indicates a significant difference versus control ($P < 0.05$)

isoenzymes are upregulated in response to phosphate starvation in many plants (Fukayama et al. 2006; Chen et al. 2007, 2008a, b; Gregory et al. 2009). *Arabidopsis PPCK1* and *PPCK2* are among the most strongly induced genes in plants subjected to nutritional Pi deprivation (Morcuende et al. 2007; Muller et al. 2007). This model plant adapts its root system architecture (RSA) to phosphate deficiency through inhibition of primary root growth, increases in lateral root formation and growth and production of root hairs to promote topsoil foraging (Péret et al. 2011). We found that phosphate deficiency affected RSA in PEPC- and PEPC-k defective plants, including the *ppc3* mutant. This shows that a lack of PPC3 had a higher impact on standard root development than on morphological root responses to P deficiency.

Using heterotrophic *Arabidopsis* cells and seedlings, other groups have pointed to higher levels of expression of *PPC1* and two kinases, *PPCK1* and *PPCK2*, under phosphate starvation (Chen et al. 2007; Gregory et al. 2009). Our results extended these observations to show that P deficiency increased the transcript levels of all of the plant-type *PPC* and *PPCK* genes in shoots. However, the relevance of changes in *PPC3* expression is unclear due to the low expression levels of this gene in shoots. Moreover, P deficiency increases PEPC activity in shoots of *ppc1* and *ppc2* SALK lines, presumably by PPC1 compensating for the lack of PPC2 and vice versa. Therefore, PPC1 and PPC2 seem to have overlapping functions in shoots under P deficiency. Accordingly, the *ppc1/ppc2* double mutant has a clear growth-arrest phenotype and plants are unable to complete their life cycle (Shi et al. 2015). In contrast to shoots, the effect of P deficiency in roots was to increase the expression of the *PPC3* and *PPCK2* genes (*PPC* and *PPCK* genes are highly expressed in roots compared to shoots in optimal conditions). In this case, *PPC1* or *PPC2* cannot replace *PPC3* in roots, either in optimal, or under P deficiency conditions, further indicating the importance of this isoenzyme in roots. Our work highlights the relevance of *PPCK2* in the plants response to P deficiency. This isoenzyme has higher levels of expression in roots than in shoots, P deficiency increases the expression of *PPCK2* more so than *PPCK1*, and finally the absence of *PPCK2*

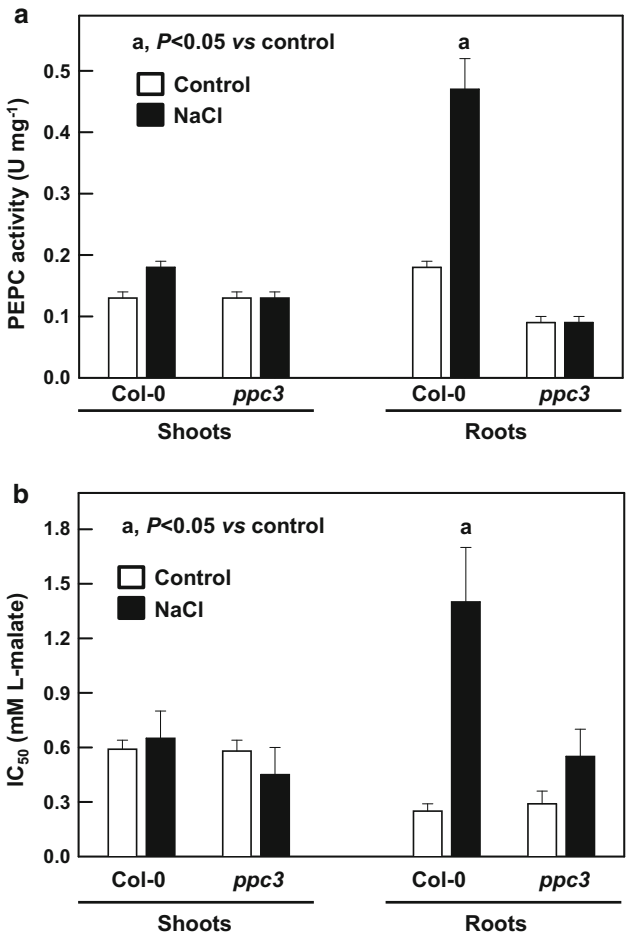

Fig. 9 PEPC activity (**a**) and IC_{50} (**b**) of Col-0 and *ppc3* plants growing hydroponically under optimal (*white bars*) or salt-stressed (*black bars*) conditions. Data represented are mean ± SE ($n = 3$). Samples typically represent 25 pooled plants. *a* indicates a significant difference versus control ($P<0.05$). ANOVA showed significant differences among groups (**a** $P < 0.01$; **b** $P < 0.001$)

cannot be replaced by *PPCK1* in P-deficient roots. Taken together, our data show that PPC3 and PPCK2 are the enzymes specifically upregulated by P deficiency in *Arabidopsis* roots and are responsible for PEPC's response to phosphate starvation.

Together with low-soluble phosphate in soils, high soil salinity is one of the major abiotic factors limiting crop yield. Salt stress has been reported to trigger the accumulation of *PPC1*, *PPC3* and *PPC4* but not *PPC2* transcripts in roots (Sánchez et al. 2006). Our results show that a long-term salinity treatment had little effect on *Arabidopsis thaliana* shoots in terms of *PPC* and *PPCK* gene expression, PEPC activity or phosphorylation state of the enzyme. However, salt stress has a marked effect in roots: higher *PPC3* expression, PEPC activity and IC_{50}—each of these effects was not found in the *ppc3* mutant line. All these results indicate once more the importance of the PPC3 protein in *Arabidopsis* roots under stress conditions. A hypothetical consequence of PEPC up-regulation in response to salt stress might be the synthesis of L-malate, since it can function as a vacuolar osmolyte (Doubnerova and Ryslava 2011), lowering the water potential of the cells, thereby promoting water uptake in the plant. L-Malate synthesis was lower in *ppc3* plants compared to Col-0, and consequently there was little L-malate induction in response to salinity treatment in *ppc3* plants compared to Col-0. Our results directly link *PPC* expression and PEPC activity and phosphorylation with L-malate production in roots in response to salinity.

Non-photosynthetic PEPC produces oxaloacetate that replenishes intermediates of the TCA and thus provides skeletons for biosynthesis of molecules and nitrogen assimilation (Doubnerova and Ryslava 2011). Many plant species accumulate high levels of proline in response to salinity stress. This compound is thought to protect plant tissues against osmotic stress since it is an osmolyte, a source of nitrogen compounds, a protectant for enzymes and cellular structures, and a scavenger for hydroxyl radicals (Liu and Zhu 1997). As expected, Col-0 plants responded with raised the levels of proline during salt stress treatment compared to control plants. However, proline levels in *ppc3* were similar both in optimal and in salt-treated plants. This might suggest a role for PPC3 in the synthesis of proline and thus in the response of the plant to salt stress. Transgenic *Arabidopsis* plants overexpressing C_4 PEPC from *Zea mays* were more tolerant to salt stress due to an increased ability to synthesize amino acids, including proline (Kandoi et al. 2016). On the other hand, we previously found that raised PEPC activity in Fe-deficient and/or salt-stressed barley leaves was not directly responsible for supplying C for proline synthesis (Arias-Baldrich et al. 2015). Whether this is also the case for PEPC activity in *Arabidopsis* roots is not currently clear. Interestingly, the levels of proline, together with other stress markers such as malondialdehyde (MDA) and catalase activity, were higher in *ppc3* plants compared to Col-0 in optimal growth conditions. Taken together, these results suggest that *ppc3* plants show the characteristics of stressed plants under non-stressful conditions. Although *ppc3* plants had lower shoot and root FW compared to Col-0, the shoot and root DWs were similar in the wild type and the mutant, indicating that the *ppc3* plants are smaller than Col-0 plants due to lower water content. Salinity reduces the water content of cells and tissues (Hu et al. 2005) and made that Col-0 plants reduced their FW but did not change the DW with respect to control plants.

In summary, our results show that *PTPC* is important, but not crucial, for optimal development of *Arabidopsis*. *PPC2* and *PPC3* are the main PEPC isoforms functional in *Arabidopsis* shoots and roots, respectively. Among all of

the PTPC and PPCK proteins, PPC3 and PPCK2 are the most important isoforms in roots, both for the control and phosphate conditions. In salt stress, only PPC3 seems to have an important role.

Author contribution statement ABF, SG-M and JAM conceived and designed research. ABF, NB, AS, AIN-I and CdlO conducted experiments. CE helped discussing and designing experiments. SG-M and JAM wrote the manuscript. All authors read and approved the manuscript.

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