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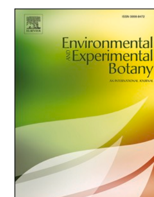

Responses to aluminum and cadmium of a RNAi sorghum line with decreased levels of phosphoenolpyruvate carboxylase 3 (PPC3)

Jesús Pérez-López, Jacinto Gandullo, Clara de la Osa, Ana B. Feria, Cristina Echevarría, José A. Monreal, Sofía García-Mauriño *

Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Avenida Reina Mercedes n° 6, 41012 Seville, Spain

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ABSTRACT

Phosphoenolpyruvate carboxylase (PEPC) is an enzyme family with central roles in carbon and nitrogen metabolisms. In order to obtain knowledge about the function of specific PEPC isoenzymes, we have characterized RNAi sorghum lines with decreased level of *PPC3* (*Ppc3* lines). PPC3 is the main root PEPC, and participates in responses to salinity and ammonium stress. This prompted us to study the responses of *Ppc3* lines to Al and Cd stress. Both Al and Cd treatments decreased germination rate, growth rate, and shoot and root biomass production. These detrimental effects were higher in *Ppc3* lines than in Wt. Root *PPC3* expression and PEPC activity was increased by Al and Cd in Wt, but not in *Ppc3* lines. The treatments also increased *PPC2* and *PPCK* gene expression, and PEPCk activity, but these changes did not substitute the lack of PPC3. The production and secretion of exudates by plant roots play an important role in plant tolerance to Al and Cd toxicity. Al (2 days of treatment) caused a 40-fold increase of the citrate content of root exudates, without differences between Wt and *Ppc3* lines. On the contrary, Cd (7 days of treatment) caused a 400-fold increase of citrate in Wt exudates, but only a 30-fold increase in *Ppc3* lines. These results indicate that although PPC3 is not necessary for early production of organic acids in root exudates, it is crucial for maintaining high level of synthesis and accumulation of citrate, and that PPC3 is the main PEPC isoenzyme responsible for this response to heavy metal stress.

1. Introduction

Sorghum (*Sorghum bicolor* L. Moench) is a C_4 cereal crop moderately resistant to drought and salinity. It is used as human food and for livestock feed, with the advantage that it is gluten-free. Worldwide, sorghum is the fifth most important cereal crop, providing food, feed, fiber, fuel, and chemical/biofuels feedstock across a range of environments and production systems (Kresovich et al., 2005). An increased demand for food, in parallel with decreased water resources for agriculture, is one of the most compelling challenges that society must face in the next future. In this framework, sorghum has great potential as smart food for the future. Using sorghum as experimental model, our research group has extensive experience in the study of the functions of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and the enzyme that phosphorylates it (PEPC-kinase, PEPCk), especially in stress responses (Echevarría et al., 2001; García-Mauriño et al., 2003; Monreal et al., 2013a, 2013b) and in seed development and germination (Ruiz-Ballesta et al., 2014, 2016).

PEPC is a key enzyme in the metabolism of carbon and nitrogen, with central roles in photosynthesis, respiration, amino acid synthesis, and development and germination of seeds. This enzyme catalyzes the addition of bicarbonate to PEP to form the four carbon compound oxaloacetate, which is reduced to malate by the enzyme malate dehydrogenase (MDH). PEPC is mostly acknowledged by its role in C_4 and CAM photosynthesis (Chollet et al., 1996; Nimmo, 2000), though it has also key functions in C_3 plants and C_3 tissues, such as seeds, fruits, roots, stomata, legume nodules, and others (O'Leary et al., 2011). *Sorghum bicolor* PEPC gene family (*PPC* genes) include five plant-type PEPC (*PTPC*) genes (*SbPPC1–5*: Sb10g021330, Sb02g021090, Sb04g00872, Sb07g014960 and Sb03g035090, respectively) and one bacterial-type PEPC (*BTPC*) gene (*SbPPC6*, Sb03g0084810) (Paterson et al., 2009). The photosynthetic C_4-type isoenzyme (PPC1) is expressed in shoots but

Abbreviations: PEPC, Phosphoenolpyruvate carboxylase; PEPCk, Phosphoenolpyruvate carboxylase kinase; *PPCn*, Gene encoding a specific PEPC isoenzyme; *PPCKn*, Gene encoding a specific PEPCk isoenzyme.

* Corresponding author.
E-mail address: sgarma@us.es (S. García-Mauriño).

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not in roots, and, exclusively, in leaf mesophyll cells. The non-photosynthetic PPC3 isoenzyme has a key role along seed development and germination (Ruiz-Ballesta et al., 2014, 2016), and in roots (Arias-Baldrich et al., 2017).

PEPC is subjected to different post-translational modifications (PTMs), such as phosphorylation, monoubiquitinatión, NO-related PTMs (S-nitrosylation, Tyr-nitration), and oxidative stress-associated PTMs (carbonylation) (Echevarría and Vidal, 2003; Ruiz-Ballesta et al., 2014, 2016; Arias-Baldrich et al., 2017; Baena et al., 2017). Protein phosphorylation is the most studied PTM of PEPC, which is phosphorylated at a conserved N-terminal serine residue by PEPC kinase (PEPCk). This PTM affects positively on the functional and regulatory properties of PEPC, decreasing its sensitivity to feedback inhibition by L-malate, and increasing its affinity for the allosteric activator glucose-6-phosphate and V_{max} (Echevarría and Vidal, 2003). *Sorghum bicolor* PEPCk gene family includes three genes (*SbPPCK1–3*; Sb04g036570, Sb04g026490 and Sb06g022690, respectively) (Wang et al., 2009). *SbPPCK1* is the photosynthetic isoform.

In order to obtain knowledge about the function of specific PEPC isoenzymes, we have characterized RNAi sorghum lines with decreased level of *PPC3* (*Ppc3* lines) (de la Osa et al., 2022). These lines showed decreased PEPC activity in roots and seeds, confirming the key role of PPC3 in these tissues. *PPC3* silencing had an important impact on plant growth: diminished biomass production in leaves and roots, delayed flowering, and reduced productivity in terms of number of seeds per plant. Silenced lines were more sensitive to salinity, with lower biomass and productivity, when were compared to Wt. *PPC3* silencing had a deep impact on the metabolome of the plant, even in control conditions, causing an imbalance in C and N metabolism with a drop in the level of organic acids and aminoacids in leaves, and the contrary in roots. In salinity, silencing of *PPC3* induced an even bigger imbalance of plant metabolism, more accused in roots than in leaves, indicating the key role of PEPC3 in plant responses to salt stress in this tissue (de la Osa et al., 2022). Salinity increased PEPC activity in roots, but not in *Ppc3* lines. This fact, together with the marked decrease of root PEPC activity in *Ppc3* lines, indicate that PPC3 is both the main root PEPC and that it participates in some root responses to stress. This prompted us to study the responses of *Ppc3* lines to other stresses that are sensed in roots, and which generate responses in this organ, such as Al and Cd stress.

Aluminium (Al) is the most abundant metal in the earth crust. After oxygen and silicon, Al is the most abundant chemical element, constituting about 8 % of the earth crust (Frieden, 1972). Fortunately, most Al in soils is found as aluminosilicate, which is usually non-toxic to living organisms (May and Nordstrom, 1991). However, under acidic conditions, toxic forms of Al (Al^{3+} cations) are mobilized into the soil solution and rapidly inhibit plant root growth, and result in poor crop growth and productivity (Kochian et al., 2004). Al toxicity is a major constraint on agricultural production in acidic soil throughout the world (Kochian, 1995). In addition, Al could enter the human body along with food consumption (Ghasemidehkordi et al., 2018), and accumulated in kidney, brain, and tissues, thus promoting many disorders or diseases, such as pulmonary alveolar proteinosis, toxic myocarditis, inflammatory bowel disease, Alzheimer's disease and others (Igbokwe et al., 2020).

Acid soils ($pH<5.5$) cover about 30 % of the world arable soils and up to 70 % of potentially arable land (von Uexküll and Mutert, 1995). Soil acidification is an increasing problem in the world because of acid rain, the removal of the natural plant coverage from large production areas, and the use of ammonium-based fertilizers (Pannatier et al., 2005). Inhibition of root elongation is one of the earliest and most distinct symptoms exhibited by plants exposed to micromolar concentrations of Al^{3+} in solution cultures (Horst et al., 2010; Sun et al., 2010; Kopittke et al., 2015). Consequently, nutrient and water uptake are decreased and a reduction in crop growth and productivity is observed.

To deal with Al stress, many plant species have evolved a series of strategies to adapt to the acidic soil environment. Al-induced organic acid anions efflux is the most important mechanism of Al resistance in a wide range of plant species, such as wheat, buckwheat, maize, soybean, common bean, and rye (Kochian et al., 2004). Secretion of organic acid anions avoid Al-toxicity due to the formation of nontoxic Al complexes. Malate and citrate are the most common Al-induced organic acid anions in plants (Ryan and Delhaize, 2010). Wheat and Arabidopsis roots release malate, while sorghum and maize roots mediate a citrate efflux in response to Al stress. In sorghum, Al resistance is associated to complexing and exclusion of Al in roots apices by Al-induced citrate secretion. There is a strong correlation between *SbMATE* (citrate efflux transporters) expression and Al resistance in sorghum (Kochian, 1995; Sivaguru et al., 2013; Carvalho et al., 2016). Secretion of malic and trans-aconitic acid by Al-exposed sorghum roots has also been reported (de de de Carvalho Goncalves et al., 2005). Production, accumulation, and exudation of organic acids were higher in the Al-tolerant cultivar compared with the Al-sensitive one. The role of PEPC contributing to organic acids synthesis is well documented. For example, a transgenic rice overexpressing C_4-PEPC showed increased tolerance towards Al toxicity (Begum et al., 2009). Similarly, enhanced expression of PEPC in response to Al was found in roots of tolerant soybean cultivars, as compared to sensitive cultures (Ermolayev et al., 2003).

The most common heavy metal pollutants available in soil for plants are Cd, Pb, Cr, Hg, As, Cu, Ni, and Zn (Adriano, 2001). Cadmium (Cd) is naturally present in soils, although the metal industry, landfills, mining, and manufacturing contribute to its accumulation in agricultural soil (Grant et al., 1998; Bigalke et al., 2017). Likewise, the utilization of synthetic phosphate fertilizers that contain Cd as an impurity is a common cause for the rise of Cd concentration in groundwater and soil (Kubier et al., 2019).

Cadmium negatively affects plant growth and development; it is an extremely noteworthy pollutant due to its high toxicity and large solubility (Hernandez et al., 1996). In addition, diet vegetables are the main entry pathway of Cd to human body. Cadmium is retained for many years in the human body, so consumption of foods high in Cd may induce chronic toxicity (FAO/WHO, 1995). Plant exposure to toxic levels of Cd suppresses germination and inhibits plant growth and production, interferes with seedling physiological processes, and reduces agricultural productivity (Guilherme et al., 2015; Raza et al., 2020). Cadmium decreases photosynthetic activity by inhibiting PSII (Mallick and Mohn, 2003) and Rubisco (Wahid et al., 2008), reducing stomatal opening (Perfus-Barbeoch et al., 2002), decreasing chlorophyll synthesis (Chaffei et al., 2004) and other effects.

Increased PEPC activity may alleviate Cd toxicity. Anaplerotic PEP carboxylation by PEPC not only results in malate and citrate accumulation, but also is involved in *de novo* production of amino acids for glutathione synthesis, the precursor for phytochelatin synthesis (Nocito et al., 2008). Cd stress enhanced the activity of PEPC in several plant species (wheat, bean, maize). In Arabidopsis, Cd increased PEPC activity and its phosphorylation state, and *PPC* and *PPCK* gene expression (Willick et al., 2019). In this study, the Arabidopsis *atppc3* mutant was the most sensitive to Cd, showing the relevance of this isoenzyme upregulation for acclimation to Cd stress. Although there is no phylogenetic relationship between *AtPPC3* and *SbPPC3*, this result shows that specific PEPC isoenzymes could be responsible for specific plant responses to stress.

This paper investigates the responses to Al and Cd in Wt and *Ppc3* RNAi lines, in order to i) identify specific roles for PPC3 in these responses; ii) gain knowledge about key points of PEPC-related responses that could help to improve plant tolerance to Al and/or Cd toxicity.

2. Materials and methods

2.1. Plant material and growth conditions

Sorghum (*Sorghum bicolor* L.) Wt and *Ppc3* plants used in this study correspond to the public genotype P898012. Seeds were surface sterilized with 50 % (v/v) bleach and 0.1 % Triton X-100 for 30 min and

rinsed 8–10 times with sterile water. Seeds were placed in moist sterile filter papers for 3 days in darkness at 25 °C. For hydroponic experiments, 3 seedlings were transferred to 1 L polyethylene pots filled with nitrate-type nutrient solution (Hewitt, 1966) and grown in 12 h light/dark cycles (25 °C, 60 % relative humidity, and 20 °C, 70 % relative humidity, for each photoperiod, respectively), 350 µmol m^{-2} s^{-1} PAR light intensity.

Vector construction, *Agrobacterium*-mediated transformation, selection of transformed *Ppc3* plants, and classification of transgenic T1 lines are described in de la Osa et al. (2022). T2 and T3 were used in this work. Plants were analysed in order to confirm the insertion of the T-DNA and the degree of silencing.

Plant material (roots and shoots) was collected after 3 weeks of growth. The first fully developed leaf and the full root biomass were regularly sampled. The increase of leaf or root length was calculated as the length at the end of the treatment minus the length at the beginning of it. Fresh and dry biomass of leaves and roots were measured at the end of treatments.

For aluminum treatment, plants were grown hydroponically in Hewitt medium for 11 days, then plants were transferred to medium containing 1 mM $CaCl_2$, 5 µM H_3BO_3 at pH 4.5, with or without 1 mM aluminum, for 48 h. The addition of Al to plant culture medium could cause phosphorous depletion (Wallihan, 1948). Meanwhile, changes in both PEPC and PEPCk activity have been reported under phosphate starvation (Chen et al., 2007; Gregory et al., 2009; O'Leary et al., 2011). In order to avoid indirect effects on PEPC activity and/or phosphorylation caused by phosphate starvation, the selection of Al concentration was in accord to the following conditions: i) The effect of Al should be shown in short-time treatments, which could be applied to a minimum growth solution without phosphate; ii) Al should decrease the rate of root elongation; iii) Al should increase root PEPC activity. In 48 h-treatments, 0.25–2 mM Al decreased root elongation (Fig. 1A). The same concentrations increased root PEPC activity (Fig. 1B), with a maximum about 1 mM. This was the concentration selected for next assays. In some experiments that required longer treatments, a lower concentration (0.1 mM) was used.

For cadmium treatment, plants were grown hydroponically in Hewitt medium for 14 days; then, plants were treated with or without 100 µM of cadmium acetate in Hewitt medium for 7 days. The selection of the concentration of Cd used in this work was according to specialized bibliography and taking into account the requirement for induced PEPC activity in roots. Cd increased PEPC activity (Fig. 2A) and the amount of immunoreactive protein (Fig. 2B) in a dose-dependent manner. The higher concentration (0.1 mM) has been used in most experiments in this work.

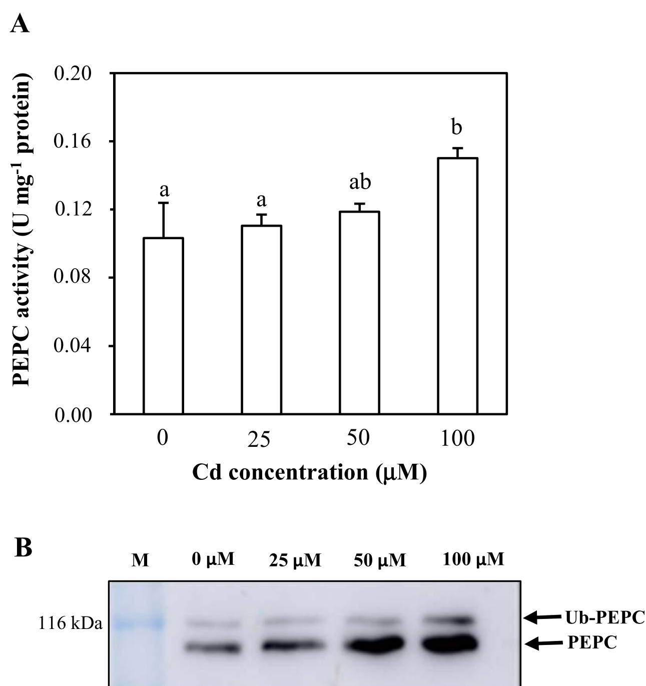

Fig. 2. Effect of increasing Cd concentrations on root PEPC activity. Sorghum plants (2 weeks) were supplied with 0, 25, 50, and 100 µM cadmium acetate for 1 week. (A) PEPC activity of root extracts. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, $P < 0.05$). (B) Immunoblots (50 µg protein of root extracts) revealed with anti-PEPC antibodies. M, molecular weight marker. Arrows show PEPC in gel.

2.2. Gas exchange measurements

Net photosynthetic rate (A) and stomatal conductance (gs) were determined in the youngest fully developed leaf at 350 µmol m^{-2} s^{-1} PAR, a CO_2 concentration of 400 ppm, and ambient relative humidity, using a portable photosynthesis analyser LI-6400XT (LI-COR).

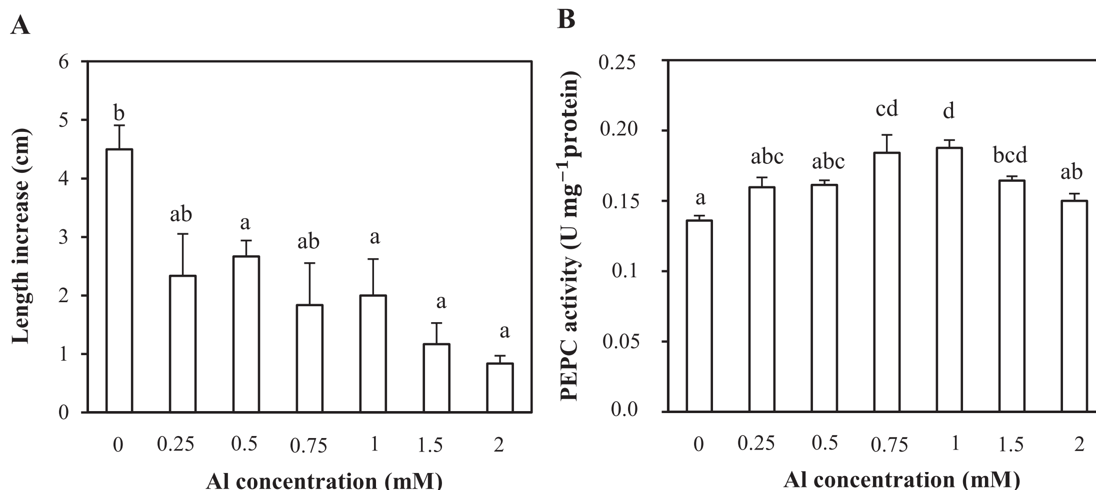

Fig. 1. Effect of Al on root growth and root PEPC activity. Wt sorghum plants (2 weeks) were supplied with increasing concentrations of $AlCl_3$ (0, 0.25, 0.5, 0.75, 1, 1.5 and 2 mM) for 48 h. (A) Total length increase of roots. (B) Specific PEPC activity of root extracts. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, $P < 0.05$).

2.3. Photosynthetic pigments

Chlorophylls and carotenoids were extracted with 80 % acetone from plant leaves. The absorbance (A) of the supernatants was measured spectrophotometrically at 470, 646.8 and 663.2 nm. The concentration of chlorophyll a (*Chl a*), chlorophyll b (*Chl b*), total chlorophyll (*Chl a+b*) and total carotenoids (*Car*) was calculated as described by Lichtenthaler (1987):

$$Chl\ a\ (\mu g\ ml^{-1}) = (12{\cdot}25 \times A_{663{\cdot}2}) - (2{\cdot}79 \times A_{646{\cdot}8})$$

$$Chl\ b\ (\mu g\ ml^{-1}) = (21{\cdot}50 \times A_{646{\cdot}8}) - (5{\cdot}10 \times A_{663{\cdot}2})$$

$$Chl\ a{+}b\ (\mu g\ ml^{-1}) = (7{\cdot}15 \times A_{663{\cdot}2}) + (18{\cdot}71 \times A_{646{\cdot}8})$$

$$Car\ (\mu g\ ml^{-1}) = [(1000 \times A_{470}) - (1{\cdot}82 \times Chl\ a) - (85{\cdot}02 \times Chl\ b)]/198$$

2.4. Protein extraction and enzymatic assays

Protein extracts were obtained by grinding 0.1 g or 0.4 g fresh weight from leaves or roots, respectively, in 1 ml of extraction buffer containing: 0.1 M Tris-HCl pH 7.5, 5 % (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu g\ ml^{-1}$ chymostatin, 10 μM leupeptin, and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 17,000xg for 5 min at 4 °C, and the supernatant was used for determination of enzymatic activities. Protein concentrations were determined using the method of Bradford (1976) with BSA as standard.

PEPC activity was measured spectrophotometrically at pH 8 and 2.5 mM PEP as described in 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, 30 °C.

PEPCk activity was measured by staining phosphorylated proteins with Pro-Q Diamond. The *in vitro* PEPCk activity of sorghum leaves or roots was measured in aliquots of protein extracts (50 μg) that were incubated in a reaction medium containing 100 mM Tris-HCl, pH 7.5, 20 % (v/v) glycerol, 5 mM $MgCl_2$, 0.25 mM P1P5-di(adenosine-5')-pentaphosphate (adenylate kinase inhibitor), 1 mM EGTA and 0.4 units of nonphosphorylated purified sorghum C_4 PEPC. The phosphorylation reaction was initiated by the addition of 1 mM ATP. The reaction was stopped by boiling the samples for 3 min at 90 °C in the presence of loading buffer. The denatured proteins were separated by SDS-PAGE. Fluorescent staining of SDS-PAGE gels was performed using Pro-Q Diamond phosphoprotein stain and SYPRO Ruby protein stain (Invitrogen-Molecular Probes, Carlsbad, CA, USA), following the manufacturer's instructions. After electrophoresis, the gels were stained with Pro-Q Diamond phosphoprotein stain and analyzed with a phosphorimager (Fuji FLA-5100; Fuji, Tokyo), and then stained with SYPRO Ruby and re-imaged. The positive control for the proQ diamond stain was phosphorylated ovalbumin, which is included in molecular weight marker (lane M), and is the single protein stained by proQ in this lane.

2.5. Electrophoresis and protein gel blot analysis

Protein samples were denatured by heating in the presence of loading buffer (100 mM Tris-HCl, pH 8, 25 % [v/v] glycerol, 1 % [w/v] SDS, 10 % [v/v] β-mercaptoethanol, and 0.05 % [w/v] bromophenol blue). Denatured proteins (10 μg for leaves extracts and 50 μg for roots extracts) were separated by SDS-PAGE in a Mini-Protean® Tetrasystem (Bio-Rad) and electroblotted onto a nitrocellulose membrane in a semidry transfer blot system (Trans-Blot® Turbo, Bio-Rad). Polyclonal antibody against native C_4-photosynthetic PEPC from sorghum leaves (anti-C_4 PTPC) were prepared as described in Pacquit et al. (1995). These antibodies recognize both photosynthetic and non-photosynthetic PEPCs (Ruiz-Ballesta et al., 2016). Immunolabeled proteins were detected by a chemiluminescence detection system (SuperSignal West Pico Rabbit IgGs; Thermo Scientific) according to the manufacturer's instructions in an Amersham Imager 600 (GE-Healthcare). The immunoreactive polypeptides were quantified *via* analysis of the scanned blots using Image StudioTM Lite software (LI-COR).

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of frozen, powdered leaves or roots using the Plant RNA Isolation Mini Kit (Agilent Technologies). Extracted nucleic acids were DNase treated to wipe out genomic DNA. RNA concentration was determined using Nanodrop 2000 (Thermo). Reverse-transcription reactions were performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche),and HISCRIPT II 1st Strand cDNA Synthesis Kit (Vazyme), following the manufacturer's instructions, with 1 μg of purified total RNA. cDNA synthesized was used for qPCR experiments.

2.7. Quantitative real time PCR (qPCR)

Quantitative PCR reactions (qPCR) were performed in a final volume of 20 μL consisting of 1 μL of the cDNA, 15 μM of the specific primers (Supplemental Table S1), and 10 μL of SensiFAST SYBR No-ROX kit (Bioline). PCR was conducted on the Light Cycler 480 II Real-Time PCR System (Roche), and the threshold cycles (Ct) were determined using the Light Cycler 480 software for all treatments. To normalize the obtained values, actin was used as internal control in each sample. Relative gene transcript abundance data were obtained through Livak method ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen, 2001), assigning the relative value 1 for transcript abundance in Wt leaves or roots in control conditions.

2.8. Quantification of malondialdehyde (MDA)

The level of lipid peroxidation was determined by measuring MDA concentration with the thiobarbituric (TBA) reactive substances method (Hodges et al., 1999). Fresh tissue samples (0.1 g) were ground in ethanol:water 80:20 (v/v) and the homogenates were centrifuged at 10, 000g for 5 min. Aliquots of 0.3 ml were added to 0.7 ml of TBA [5 % TBA (w/v) in 20 % TCA (w/v)] and to 0.7 ml of 20 % TCA. The mixture was heated at 100 °C for 30 min, and then cooled in an ice bath. After centrifugation at 10,000g for 10 min, the absorbance of the supernatant was measured at 532 nm. MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The value was corrected for non-specific absorption subtracting the absorbance obtained with TCA from that of TBA.

2.9. Quantification of proline

Proline was extracted by mixing 0.1 g FW aliquots with 2 ml of ethanol:water 80:20 (v/v). The resulting mixture was left overnight a 4 °C, and then centrifuged at 14000xg 5 min. Proline was quantified by the method of Carillo and Gibon (2011). Aliquots of 50 μL were added to 100 μL of reaction mix [ninhydrin 1 % (w/v) in acetic acid 60 % (v/v), ethanol 20 % (v/v)] by well, mixed and heated at 95∘C, 20 min. After cooling at room temperature, 100 μL were transferred to microplate wells and absorbance at 520 nm read with a microplate reader. Proline concentration was determined from a standard curve.

2.10. Iron, zinc, calcium, magnesium and cadmium measurements

For calcium, magnesium, iron, zinc and cadmium quantification, leaves and/or roots from 3 plants were dried in an oven at 60 °C for 48 h, and grinded with a mortar and a pestle. Subsequently, 0.25 g of each sample were mineralized in a carbolite furnace at 550 °C for 8 h, resuspended in 12 ml HCl 1 N, and heated in a heating plate at 90 °C for 15 min. Elements were quantified by Atomic Absorption using an iCE

3500 spectrometer (Thermo Scientific) at the Agronomic Research Services (CITIUS, University of Seville). The certified standard reference material "1573a tomato leaves" (NIST, National Institute of Standards and Technology, USA) was used to validate the method.

2.11. Quantification of organic acids in root exudates

To obtain the root exudates from aluminium treatments, plants were grown for 10 days in Hewitt medium prior to be transferred to tubes containing 35 ml of medium composed of 1 mM $CaCl_2$, 5 μM H_3BO_3, at pH 4.5, with or without 1 mM aluminium. Samples correspond to the exudates produced by roots during 48 h within this medium. To obtain the root exudates from cadmium treatments, plants were grown in Hewitt medium for 5 days, and then transferred to Hewitt medium with or without 100 μM of cadmium acetate for 5 days. Plants were then relocated to tubes containing 35 ml of medium composed of 1 mM $CaCl_2$, 5 μM H_3BO_3, at pH 4.5, with or without 100 μM of cadmium acetate. Samples correspond to the exudates produced by roots during 48 h within this medium. In both cases, each sample was composed of exudates from one plant growing in 35 ml of medium. Exudates were lyophilized prior to GC-MS analysis.

Organic acids from sorghum root exudates were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) at the Mass Spectrometry Services (CITIUS, University of Seville). Metabolites were extracted by adding 1 ml of methanol:water (70/30, v/v) to 30 mg lyophilized root exudate. Samples were centrifuged at 13,500xg for 10 min at 4 °C, and 800 μL of supernatant collected and stored at − 80 °C until use. Samples were derivatized by methoxilation with methoxilamine in pyridine 20 mg ml^{-1} (1 h, 40 °C) and then trimethylsilylated with MSTFA (1 h, 40 °C), according to the method described by Cerdán-Calero et al. (2012). The derivatives were further analyzed by GC-MS. Each sample was analyzed twice. GC-MS was performed with a triple quadrupole mass spectrometer coupled to a gas chromatograph instrument TSQ8000 (ThermoScientific) equipped with ZB-5 ms column (30 m x 0.25 mm). The temperature programmed for separating the MEOX-trimethylsilylated derivatives was isothermal at 70 °C for 5 min, followed by a 4 °C min-1 gradient up to 325 °C. The ionization potential was 70 eV.

Identification of metabolites was carried out by the use of standards, and analysed by the Thermo TraceFinder EPSLC software (https://www.thermofisher.com/es/es/home/industrial/mass-spectrometry/liq-uidchr omatography-mass-spectrometry-lc-ms/lc-ms-software/lc-ms-data-acquisitionsoftwar e/tracefinder-software.html). Quantification of metabolites was carried out using the Qual Browser of the Xcalibur data system (Thermo).

2.12. Statistical analysis

All data were analyzed by ANOVA. Then, differences between group means were compared by Duncan's or Games-Howell multiple range test. A P value of < 0.05 was considered to be statistically significant. All analyses were conducted using SPSS Statistics 21 (IBM, Armonk, NY, United States).

3. Results

3.1. Aluminium effects on sorghum

3.1.1. Effect of Al on the rate of germination

The effect of Al on the germination rate of seeds from Wt, *Ppc3–1* and *Ppc3–2* plants was evaluated by determining the average of the different stages of germination in a pool of seeds at a given time post-imbibition (0, 24, 48, 72, and 96 h), as described in Ruiz-Ballesta et al. (2016). Al caused a modest reduction of the germination rate (Supplementary Table S2). At 72 h, the effect of 1 mM Al was higher on *Ppc3–1* and *Ppc3–2* than on Wt (Fig. 3).

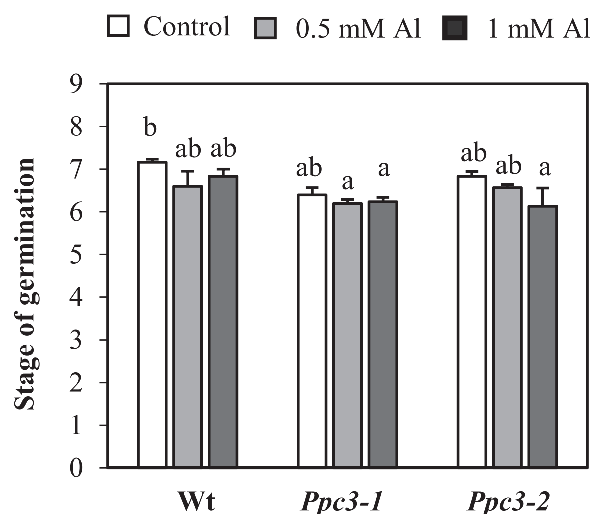

Fig. 3. Effect of Al on germination rate. Seeds (10 seeds/plate) were placed in Petri dishes, on moist sterile filter paper with or without 0.5 or 1 mM $AlCl_3$. Plates were kept in dark at 25 °C. Data show the stage of germination (mean ± SE, n = 3) after 72 h of imbibition. Different letters indicate statistically significant differences (Duncan, P < 0.05).

3.1.2. Effect of Al treatment on vegetative development

Inhibition of root growth is the primary symptom of Al stress in plants (Panda et al., 2009). Binding of Al to roots could be visualized by pyrocatechol violet staining (Watanabe et al., 1998). The higher staining corresponded to root apex and root cap (Supplementary Fig. S1). The effect of Al on early development of sorghum seedlings was investigated by adding 1 mM Al to germinating (2 days) seeds (Fig. 4). Al decreased the elongation rate of Wt seedlings (Fig. 4A). This rate was much lower in *Ppc3–1* plants, and only modestly further decreased by Al treatment. Nevertheless, *Ppc3–1* roots were 59 % shorter than Wt. Relative to their respective controls, Al reduced root length by 32 % in Wt and by 17 % in Ppc3–1 (Fig. 4B).

Next step was to subject two-week-old plants to Al treatment (Table 1). Al decreased biomass production (FW, fresh weight, and DW, dry weight) and length increase of leaves and shoots. In roots, no differences between lines was observed on FW basis. On the contrary, root DW was lower in transgenic plants, both with and without Al. The most remarkable effect of Al was on shoot and root elongation, which was decreased by 60 % (leaves) and 85 % (roots) in *Ppc3* lines, and by 40 % (leaves) and 56 % (roots) in Wt.

Al had little influence on net photosynthesis and stomatal conductance (Supplementary Table S3). This result indicates that Al decreased biomass production and rate of elongation due to its effects on root growth, and not on photosynthesis. Two stress markers, malondialdehyde (MDA) and proline, were measured in leaves (Supplementary Table S3). Basal MDA and proline levels were higher in *Ppc3–1* and *Ppc3–2* plants, accordingly to the "stressed phenotype" observed for these lines (de la Osa et al., 2022). Al treatment did not change MDA content, and increased proline exclusively in Wt. The amount of MDA and proline was lower in roots than in leaves, and no noteworthy changes were observed.

Several abiotic stresses induce anthocyanin synthesis due to its antioxidant activity (Naing and Kim, 2021). Anthocyanin content was increased by Al treatment in both Wt and *Ppc3* lines (Table 2). In leaves, anthocyanin increased by 20 % in Wt, 90 % in *Pc3–1*, and 28 % in *Ppc3–2*. In roots, anthocyanin increased by 70 % in Wt, and about 45 % in *Ppc3* lines.

3.1.3. Effect of Al treatment on PEPC and PEPCk activity and on PPC and PPCK gene expression

We have previously reported that PPC3 is the main isoenzyme

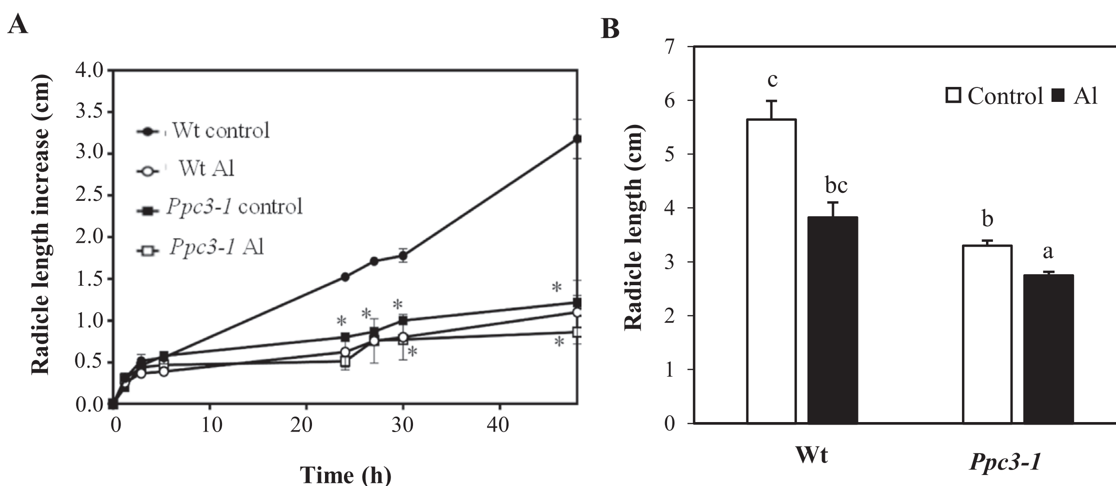

Fig. 4. Effect of Al on radicle growth rate and length. Sorghum seedlings were hydroponically grown with 1 mM $CaCl_2$ and 5 μM H_3BO_3, pH 4.5, with or without 1 mM $AlCl_3$, for 48 h. (A) Rate of root length increase. Data are means ± SE (n = 3). *, P < 0.05 *versus* control (*t test*). (B) Root length at the end of treatment. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, P < 0.05).

Table 1
Effect of Al on sorghum growth and biomass production. Plants (2 weeks) were grown with 1 mM $CaCl_2$ and 5 μM H_3BO_3, pH 4.5, with or without 1 mM $AlCl_3$, for 48 h. Then, leaf and root length increase, fresh weight (FW) and dry weight (DW) were measured. Data are means ± SE (n = 9, leaves; n = 3, roots).

		Leaves			Roots		
		Length increase (cm)	FW (g)	DW (g)	Length increase (cm)	FW (g)	DW (g)
Wt	Control	7.89 ± 0.78^{b}	1.28 ± 0.04^{b}	0.19 ± 0.007^{c}	4.5 ± 0.408^{c}	2.15 ± 0.094^{b}	0.17 ± 0.019^{c}
	$AlCl_3$	4.72 ± 0.69^{ab}	0.98 ± 0.043^{a}	0.15 ± 0.007^{ab}	2 ± 0.623^{ab}	1.02 ± 0.019^{a}	0.091 ± 0.015^{ab}
Ppc3–1	Control	6.72 ± 0.47^{b}	1.16 ± 0.06^{b}	0.179 ± 0.014^{bc}	2.67 ± 0.54^{bc}	1.78 ± 0.135^{b}	0.114 ± 0.003^{b}
	$AlCl_3$	3 ± 0.59^{a}	0.93 ± 0.029^{ab}	0.143 ± 0.005^{a}	0.33 ± 0.272^{a}	0.98 ± 0.036^{a}	0.074 ± 0.004^{a}
Ppc3–2	Control	5.78 ± 1.35^{b}	1.17 ± 0.107^{b}	0.177 ± 0.014^{bc}	3.33 ± 0.72^{bc}	1.97 ± 0.26^{b}	0.118 ± 0.014^{bc}
	$AlCl_3$	2 ± 0.36^{a}	0.98 ± 0.024^{ab}	0.144 ± 0.005^{a}	0.67 ± 0.27^{a}	0.99 ± 0.064^{a}	0.053 ± 0.015^{a}

[a,b,c]Different letters indicate statistically significant differences within each column (Games-Howell, P < 0.05)

Table 2
Effect of Al on anthocyanin content of leaves and roots. Plants (2 weeks) were grown with 1 mM $CaCl_2$ and 5 μM H_3BO_3, pH 4.5, with or without 1 mM $AlCl_3$, for 48 h. Then, anthocyanin content was measured in leaves and roots. Data are means ± SE (n = 3).

		Leaf anthocyanin (μg g^{-1} FW)	Root anthocyanin (μg g^{-1}FW)
Wt	Control	1.18 ± 0.03^{a}	1.77 ± 0.05^{a}
	$AlCl_3$	1.43 ± 0.04^{a}	3.03 ± 0.25^{b}
Ppc3–1	Control	1.24 ± 0.19^{a}	1.9 ± 0.05^{a}
	$AlCl_3$	2.34 ± 0.25^{b}	2.8 ± 0.16^{b}
Ppc3–2	Control	2.27 ± 0.28^{b}	1.92 ± 0.15^{a}
	$AlCl_3$	2.91 ± 0.22^{b}	2.79 ± 0.12^{b}

[a,b]Different letters indicate statistically significant differences within each column (Duncan, P < 0.05)

responsible for root PEPC activity (de la Osa et al., 2022). Accordingly, in *Ppc3–1* lines PEPC activity and amount of PEPC protein was about 70 % lower in roots of both *Ppc3* seedlings (Fig. 5A) and 2 week old plants (Fig. 5B). Al caused a 2-fold increase of PEPC activity and protein in Wt plants, but not in *Ppc3–1* and *Ppc3–2* lines. Two PEPC bands were detected in immunoblots (Fig. 5C and D). The upper band corresponds to monoubiquitinated PEPC. The effect of *PPC3* silencing, and the effect of Al, was similar on monoubiquitinated and no-ubiquitinated PEPC. On the contrary, *PPC3* silencing and Al treatment had no significant effect on PEPC in leaves, although PEPC activity showed a descending trend (Supplementary Fig. S2).

The expression of *PPC* genes was measured in roots (Fig. 5E) and leaves (Supplementary Fig. S3). *PPC1* corresponds to the photosynthetic C_4-type isoenzyme, and it is only expressed in leaves. *PPC5* (C_3-type) and *PPC6* (bacterial-type) had very low level of expression in roots and leaves. For this reason, the genes evaluated were *PPC2*, *PPC3* and *PPC4* in roots and leaves, and *PPC1* in leaves. *PPC3* expression in roots was lower in *Ppc3–1*, as expected, and its expression showed a 4-fold increase with Al only in Wt (Fig. 5E). In addition, Al treatment caused a 4-fold increase of *PPC2* expression in Wt and a 5-fold increase in *Ppc3–1*. The effect of Al on *PPC2* expression in *Ppc3–1* did not compensated for the lack of PPC3 at the level of root PEPC activity (Fig. 5A and B). In leaves, Al treatment decreased *PPC1* and increased *PPC2* expressions in Wt, with little effect on *Ppc3–1* (Supplementary Fig. S3). The effect on PPC1 explains the downward tendency of leaf PEPC activity in response to Al (Supplementary Fig. S2).

PEPCk activity was measured with the Pro-Q Diamond phosphorylation dye technology for the fluorescent detection of phosphoproteins directly in SDS-PAGE gels (Steinberg et al., 2003) (Fig. 6 and Supplementary Fig. S4). Fluorescence signal intensity correlates with the number of phosphorylated residues, and results are normalized to total protein with SYPRO Ruby gel stain. Root PEPCk activity was increased by Al treatment by 200 % in *Ppc3–1* and by 175 % in Wt (Fig. 6A). On the contrary, Al increased by 25 % leaf PEPCk activity only in Wt (Fig. 6B). With respect to *PPCK* gene expression, Al treatment increased *PPCK2* and *PPCK3* expressions in roots, both in Wt (3-fold) and *Ppc3–1* (5-fold) (Fig. 6C). In leaves, the main effect of Al was to cause a 5-fold increase of *PPCK1* transcript level in Wt (Fig. 6D). These results indicate that the isoenzymes responsible for Al-increased PEPCk activity were *PPCK2* and *PPCK3* in roots, and *PPCK1* in leaves.

3.1.4. Effect of Al treatment on root exudate composition

The effect of PPC3 silencing on the composition of root exudates was studied (Fig. 7). Al treatment increased 40-fold the citrate content in

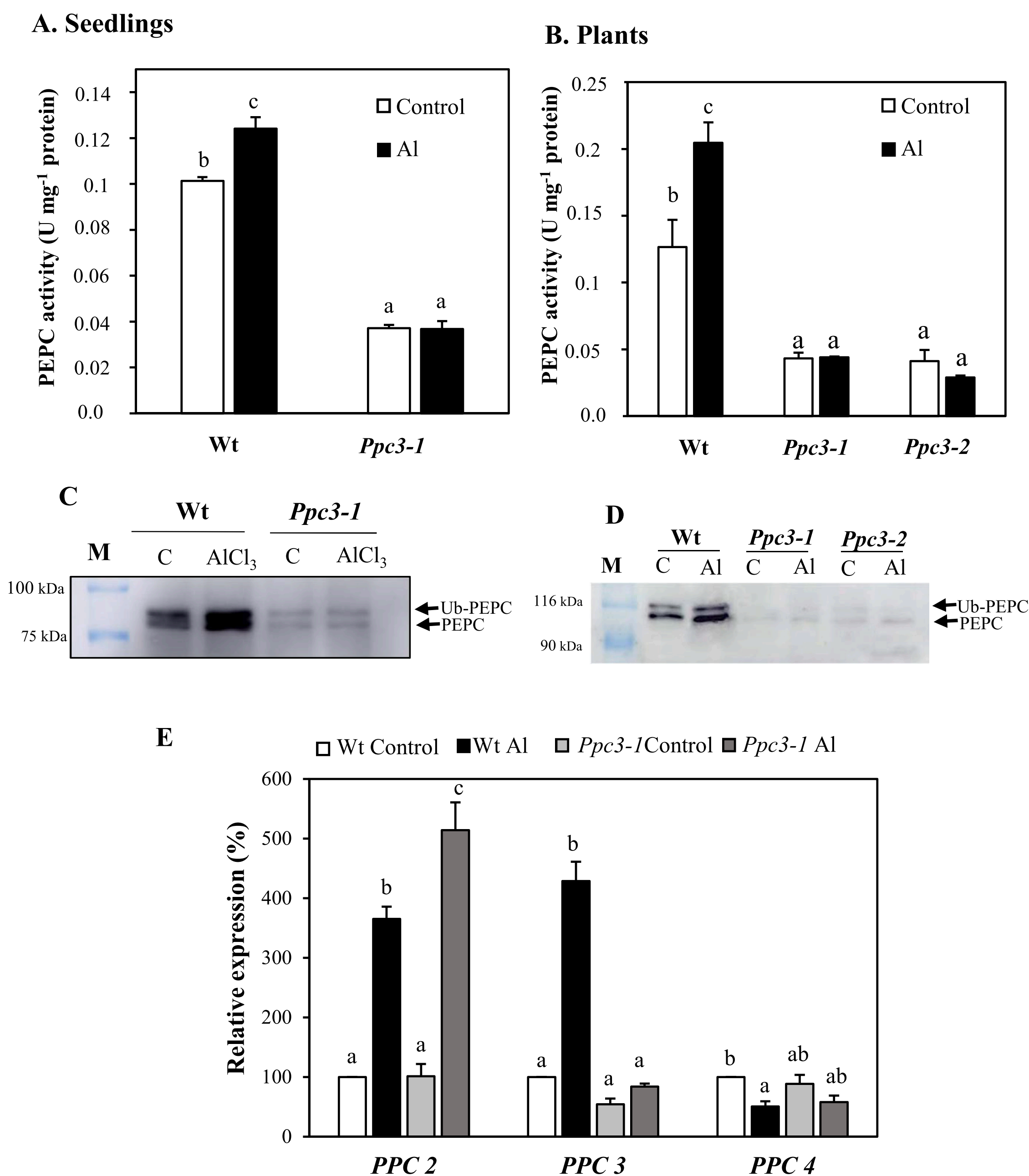

Fig. 5. Effect of Al on root PEPC activity and *PPC* gene expression. (A) Sorghum seedlings (3 days) were supplied with 1 mM $AlCl_3$ for 1 day. (B) Sorghum plants (2 weeks) were supplied with 1 mM $AlCl_3$ for 2 days. (C) and (D) Immunoblots (50 μg protein, extracts from radicles and roots, respectively) revealed with anti-PEPC antibodies. M, molecular weight marker. Arrows show PEPC in gel. (E) Sorghum plants (2 weeks) were supplied with 1 mM $AlCl_3$ for 2 days. The expression of *PPC2*, *PPC3* and *PPC4* was measured using *Sbactin* as internal control. Data are % with respect Wt without Al. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, $P < 0.05$).

root exudates, both in Wt and *Ppc3–1* and *Ppc3–2* lines. On the contrary, Al decreased malate (in Wt) and lactate (in *Ppc3–1* and *Ppc3–2*) in the exudates. These results show that, at least in short time treatments, the lack of PPC3 did not diminish the amount of citrate in root exudates.

3.2. Cadmium effects on sorghum

3.2.1. Effect of Cd on the rate of germination

The supply of Cd to germinating sorghum seeds reduced the germination rate in a dose-dependent mode (Fig. 8 and Supplementary Table S4). The decrease was more remarkable in *Ppc3–1* and *Ppc3–2* than in Wt.

3.2.2. Effect of Cd on vegetative development

Cd decreased the elongation of leaves and roots, both in Wt and *Ppc3–1* (Supplementary Fig. S5). As previously stated, the elongation rate of Ppc3–1 was lower than the Wt, and further lowered by Cd. The effect of Cd on length, FW and DW is shown in Table 3. Cd decreased all of them in leaves and roots. Cd decreased by 52 % and by 67 % leaf length in Wt and *Ppc3* lines, respectively.

Previous results had shown that *Ppc3* lines had decreased stomatal opening with a smaller effect on net photosynthesis (de la Osa et al., 2022). This effect could be seen in Fig. 9A (A, net photosynthetic activity) and Fig. 9B (gs, stomatal conductance). Both A and gs were decreased by Cd, but without differences between sorghum lines. Cd also decreased chlorophyll content (Figs. 9C, 9D and 9E), without effect on

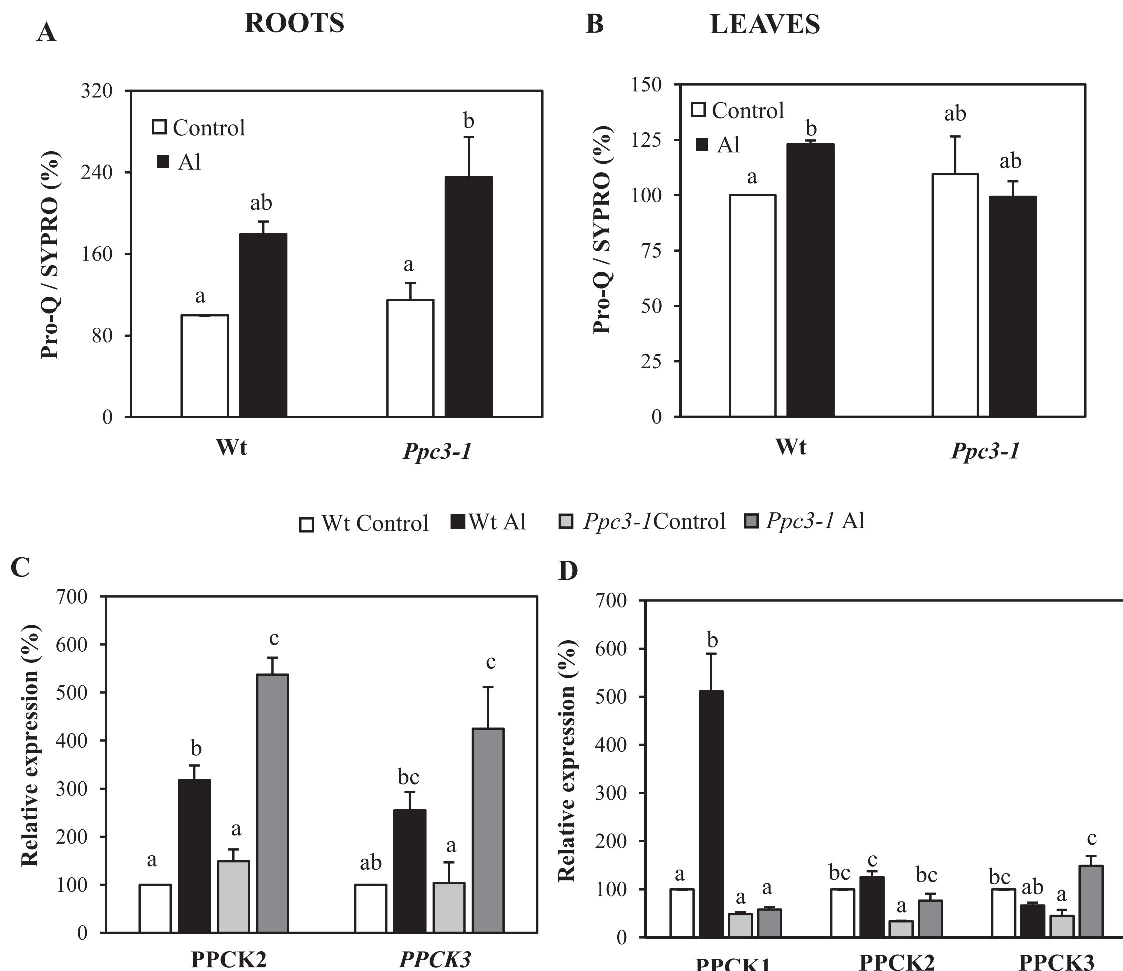

Fig. 6. Effect of Al on PEPCk activity and *PPCK* gene expression. Sorghum plants (2 weeks) were supplied with 1 mM $AlCl_3$ for 2 days. PEPCk activity of leaves and roots was measured in protein extracts (50 µg) with 0.4 U of purified C_4 PEPC. Pro-Q Diamond and SYPRO Ruby stained gels are shown in Fig. S4. (A) Ratio Pro-Q / SYPRO of root samples. (B) Ratio Pro-Q / SYPRO of leaf samples. The expression of *PPCK1, PPCK2*, and *PPCK3* was measured using *Sbactin* as internal control. (C) Roots. (D) Leaves. Data are % with respect Wt without Al. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, $P < 0.05$).

carotenoids (Fig. 9F). The effect of Cd on chlorophyll b was higher in *Ppc3–1* and *Ppc3–2* (Fig. 9D), indicating a higher sensibility to Cd in these two lines.

The stress markers MDA (Supplementary Fig. S6A) and proline (Fig. S6B) were increased by Cd in *Ppc3–1* and *Ppc3–2*. On the contrary, they were lower with than without Cd in Wt. This result suggests that, in response to Cd, Wt had initiated mechanisms that decreased stress level, and that *Ppc3–1* and *Ppc3–2* had not this capability. For example, Cd causes oxidative stress, and, in response to this metal, plants increase the activity of antioxidant enzymes (Zhang et al., 2005). Alternatively, the stress degree caused by Cd in *Ppc3–1* and *Ppc3–2* could be much higher than in Wt, and the anti-stress mechanisms, although activated, would be insufficient.

One of the causes for Cd toxicity in plants is that Cd interferes with the uptake, transport and utilization of several macro- and micronutrients (Li et al., 2012). The amount of Ca, Mg, Fe and Zn was measured in root and leaf extracts (Fig. 10). In roots, the supply of Cd increased by 85 % and 40 % the Fe content (Fig. 10A), and by 100 % and 35 % the Zn content (Fig. 10B), in Wt and in Ppc3 lines, respectively. On the contrary, Cd decreased root Ca (Fig. 10C) and Mg (Fig. 10D) but without differences between lines. In leaves, Cd decreased Fe (Fig. 10E), Zn (Fig. 10F) and Mg (Fig. 10H) content, and increased Ca (Fig. 10G). Differences between lines were smaller than in roots. With Cd, the level of leaf Fe in *Ppc3* lines was moderately higher than in Wt.

The expression of Strategy I (*SbIRT1*, Fe^{2+} transporter; *SbNRAMP1*, metal transporter) and Strategy II genes (*SbIRO2*, bHLH transcription factor; *SbYS1*, iron-phytosiderophore transporter) was measured in sorghum roots (Supplementary Table S5). The more noteworthy Cd effects were enhancing the expression of *SbNRAMP1* and *SbIRO2*. In this last case, the level of expression was much higher in Cd-treated *Ppc3* plants than Wt. These results show that Fe accumulation in roots could be due to indirect Cd effects on Fe homeostasis genes, and that Fe homeostasis is disturbed in *Ppc3* lines.

3.2.3. Effect of Cd on PEPC and PEPCk activity and on PPC and PPCK gene expression

Cd increased by 50 % root PEPC activity in Wt, but not in *Ppc3–1* and *Ppc3–2* (Fig. 11A and B), which had a lower PEPC activity than Wt, as expected. At *PPC* gene level, Cd caused a 2-fold increase of the expression of *PPC2* and 3-fold of *PPC3* in Wt, but only increased the expression of *PPC2* in *Ppc3–1* (Fig. 11C). This result shows that PPC3 is the main responsible for increased root PEPC activity with Cd, and that augmented *PPC2* expression could not replace the lack of PPC3. Due to the extremely low expression of *PPC4*, it was not included in Fig. 11C. In leaves, Cd increased modestly PEPC activity, without differences between lines (Supplementary Fig. S7A and S7B).

PEPCk activity was increased by 30 % by Cd in roots of *Ppc3–1* and not in Wt (Fig. 12A and Supplementary Fig. S8). In addition, Cd

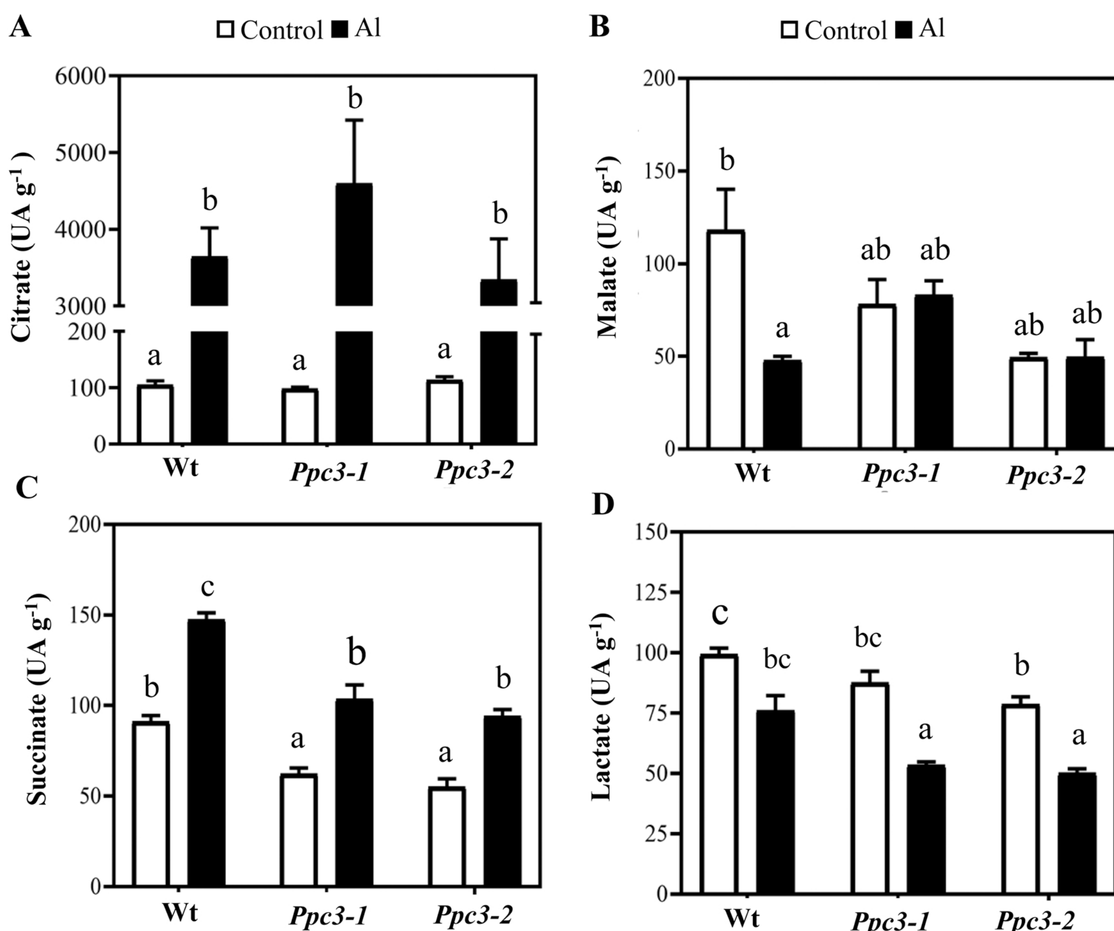

Fig. 7. Measurement of organic acids in root exudates. Sorghum plants (10 days) were transferred to 50 ml Falcon tubes containing 35 ml of 1 mM $CaCl_2$ and 5 μM H_3BO_3, pH 4.5, with or without 1 mM $AlCl_3$, for 48 h, and organic acids were quantified by mass spectometry. (A) Citrate. (B) Malate. (C) Succinate. (D) Lactate. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Games-Howell, P < 0.05).

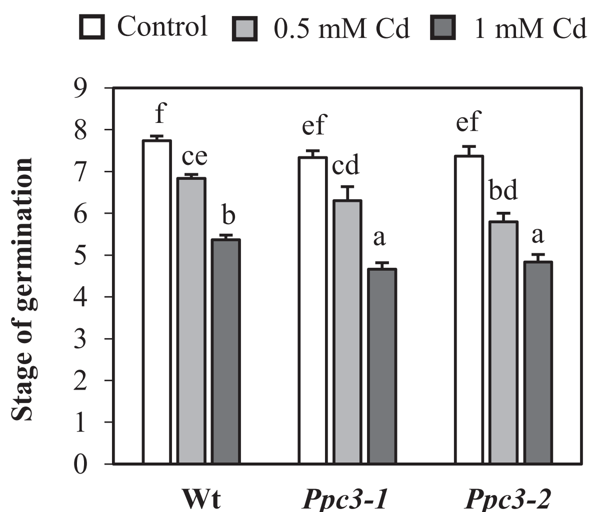

Fig. 8. Effect of Cd on germination rate. Seeds (10 seeds/plate) were placed in Petri dishes, on moist sterile filter paper with or without 0.5 or 1 mM cadmium acetate. Plates were kept in dark at 25 °C. Data show the stage of germination (mean ± SE, n = 3) at 96 h. Different letters indicate statistically significant differences (Duncan, P < 0.05).

increased the expression of *PPCK2* and decreased the expression of *PPCK3* (Fig. 12B). Consequently, the increased PEPCk activity in *Ppc3–1* roots was caused by enhanced *PPCK2* expression. In leaves, Cd increased *PPCK1* expression in *Ppc3* lines, and *PPCK2* expression in all lines (Supplementary Fig. S9).

3.2.4. Effect of Cd on root exudate composition

The effect of Cd on the composition of root exudates was determined similarly as Al effect, except that 5 days pretreatment, with or without 0.1 mM Cd, was applied before maintaining the roots submerged for 2 days in the medium, with or without Cd, used for collecting the exudates. In addition, PEPC activity was measured in roots extracts to confirm that Cd increased PEPC activity exclusively in Wt, and that the activity of Wt was higher than in *Ppc3–1* and *Ppc3–2* (not shown). Cd caused a 400-fold increase of citrate, in Wt exudates (Fig. 13A). In opposition, Al caused only a 30-fold increase of citrate in Ppc3 lines. This result unequivocally establishes a link between the citrate and malate content in root exudates and PPC3 activity. On the other hand, Cd decreased the amount of succinate (Fig. 13C) and lactate (Fig. 13D) in root exudates, without differences between sorghum lines.

Cd content in roots was measured to evaluate the impact of changes in organic acid content of root exudates (Table 4). After 1 week of treatment, Cd quantity was higher in Wt than in *Ppc3–1* and *Ppc3–2* lines. Afterwards, there was an increase of Cd absorption by *Ppc3–1* and *Ppc3–2*, and after 2 weeks, their content of Cd equaled Wt. To achieve this, Cd accumulation was 2-fold of that of Wt in silenced lines. This result is in accord with the relevance of PPC3 to maintain prolonged

Table 3
Effect of Cd on sorghum growth and biomass production. Plants (2 weeks) were supplied with 100 μM cadmium acetate for 1 week. Then, leaf and root length increase, fresh weight (FW) and dry weight (DW) were measured. Data are means ± SE (n = 9, leaves; n = 3, roots).

		Leaves			Roots		
		Length increase (cm)	FW (g)	DW (g)	Length increase (cm)	FW (g)	DW (g)
Wt	Control	21.4 ± 0.74[c]	2.7 ± 0.13[b]	0.35 ± 0.02[b]	9.1 ± 0.8[d]	5.2 ± 0.34[d]	0.39 ± 0.03[d]
	Cd	10.3 ± 2[b]	1.3 ± 0.1[a]	0.19 ± 0.01[a]	5.6 ± 0.48[a]	2.1 ± 0.2[b]	0.16 ± 0.02[ac]
Ppc3–1	Control	18.3 ± 0.78[c]	2.3 ± 0.17[b]	0.27 ± 0.03[b]	6.4 ± 0.38[bc]	4.5 ± 0.55[c]	0.2 ± 0.02[bc]
	Cd	6.2 ± 2.6[a]	1.02 ± 0.09[a]	0.15 ± 0.01[a]	4.4 ± 0.31[a]	1.34 ± 0.1[a]	0.1 ± 0.02[a]
Ppc3–2	Control	18.4 ± 1.86[c]	2.79 ± 0.33[b]	0.32 ± 0.03[b]	8.2 ± 0.5[bd]	5.54 ± 0.43[d]	0.28 ± 0.03[b]
	Cd	5.92 ± 1.8[a]	1.07 ± 0.04[a]	0.17 ± 0.01[a]	4.3 ± 0.7[a]	1.72 ± 0.13[b]	0.13 ± 0.01[ac]

[a,b,c]Different letters indicate statistically significant differences within each column (Duncan, P < 0.05)

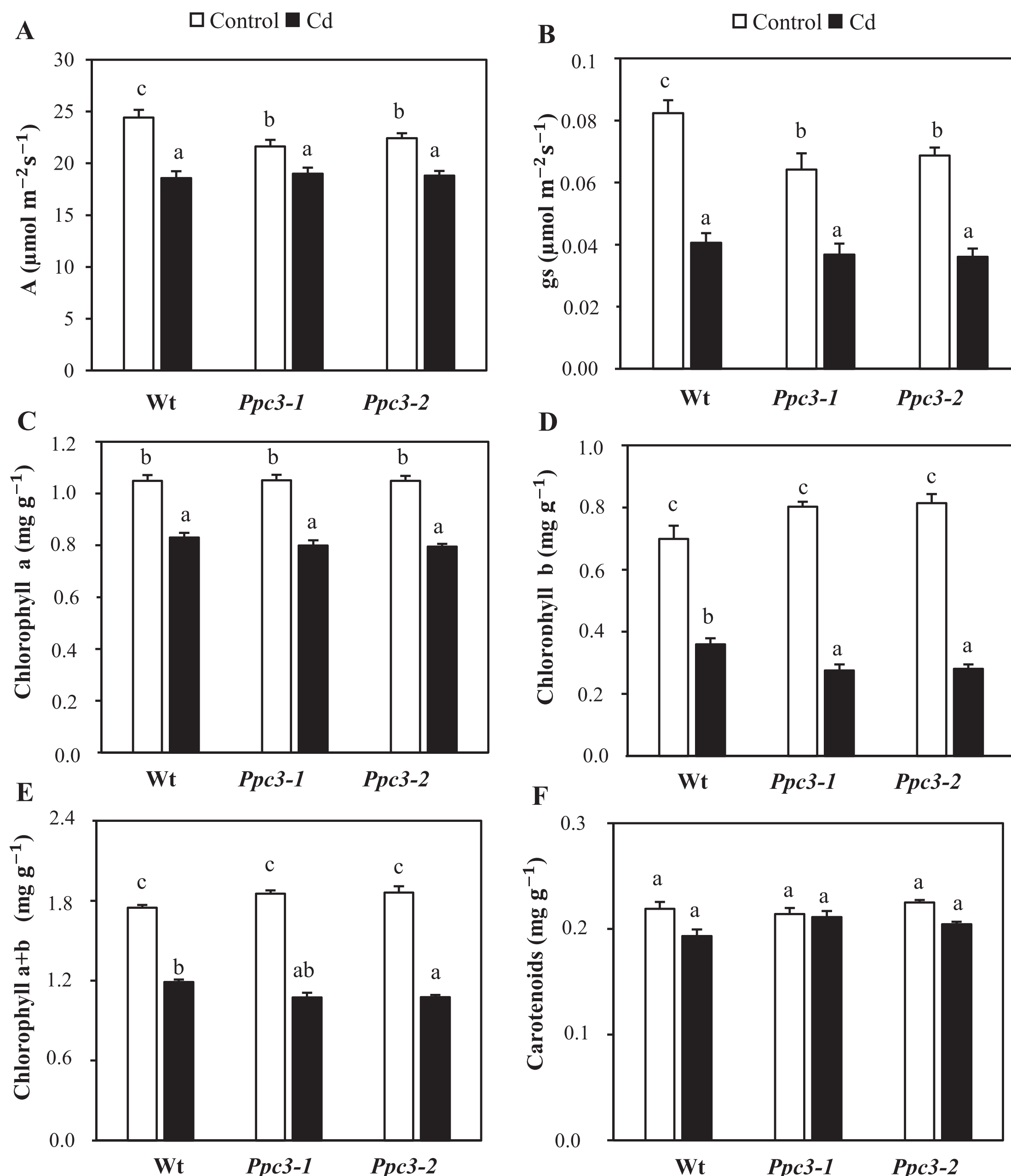

Fig. 9. Effect of Cd on photosynthetic activity, stomatal conductance and photosynthetic pigments. Sorghum plants (2 weeks) were supplied with 0.1 mM cadmium acetate for 1 week. (A) Net photosynthesis (A); (B) stomatal conductance (gs). Data are means ± SE (n = 3). (C) Chlorophyll a, (D) Chlorophyll b, (E) Chlorophyll a+b, and (F) carotenoids. Data are means ± SE (n = 6). Different letters indicate statistically significant differences (Duncan, P < 0.05).

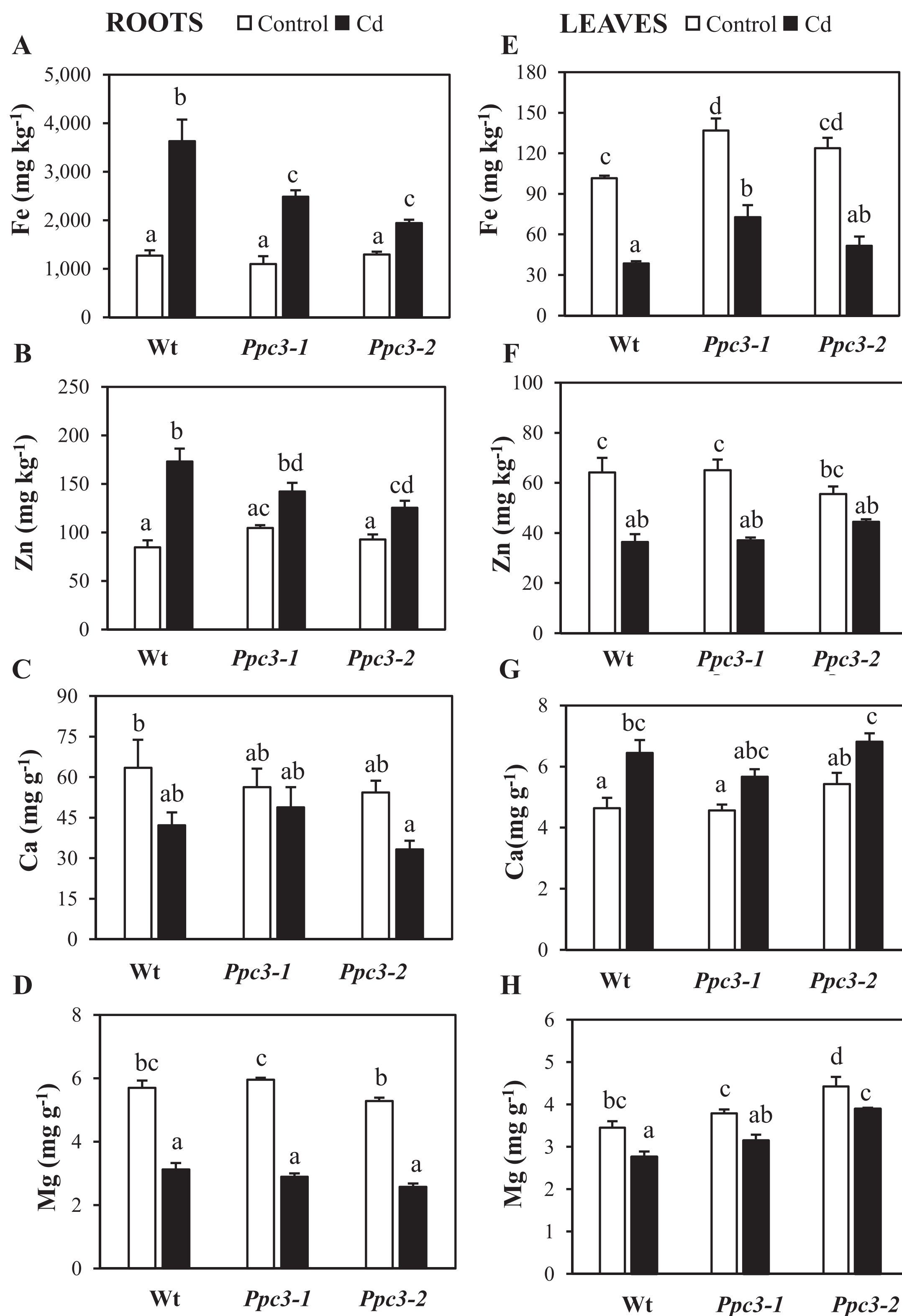

Fig. 10. Effect of Cd on Fe, Zn, Ca and Mg content of roots and leaves. Sorghum plants (2 weeks) were supplied with 0.1 mM cadmium acetate for 1 week and elements were quantified by Atomic Absorption Spectroscopy in samples of roots and leaves. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, P < 0.05).

citrate production, guaranteeing Cd exclusion at long term.

4. Discussion

This paper investigates sorghum responses to Al and Cd, both in Wt and RNAi *Ppc3* lines. In sorghum, PPC3 is the main isoenzyme contributing to root PEPC activity, which is greatly diminished in *Ppc3* lines (de la Osa et al., 2022). PPC3 has two main roles in sorghum roots. First, is important for the early development of the root and the lack of PPC3 negatively affects the rate of initial root growth in sorghum

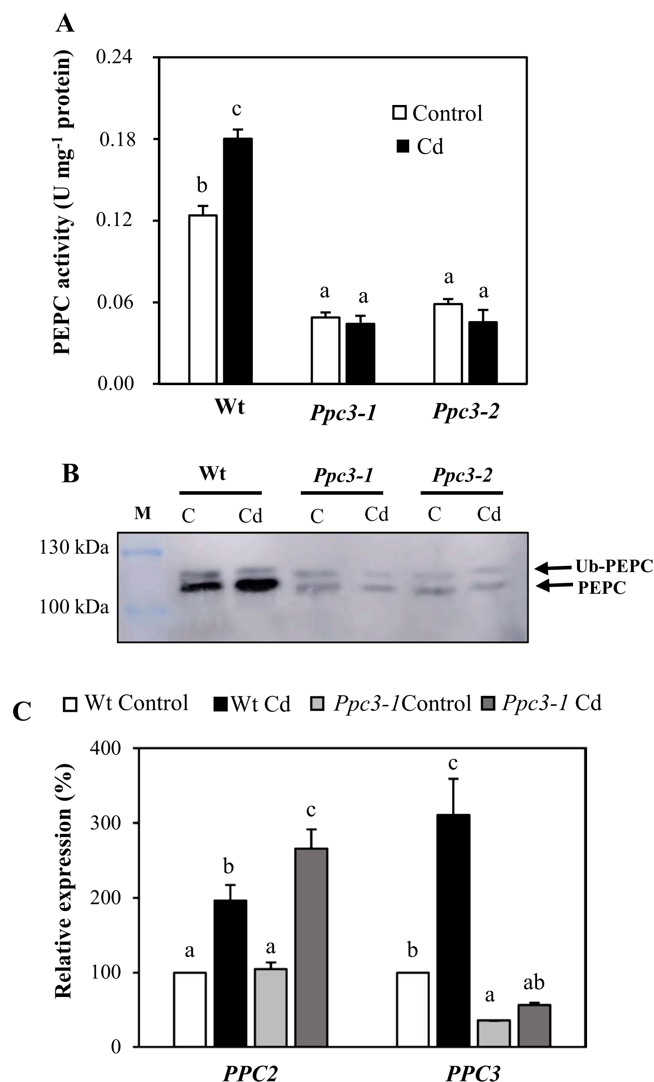

Fig. 11. Effect of Cd on root PEPC activity and PPC gene expression. Sorghum plants (2 weeks) were supplied with 0.1 mM cadmium acetate for 1 week. (A) PEPC activity in root extracts. (B) Immunoblots (50 μg protein) from plants grown with (Cd) or without (C, control) 0.1 mM cadmium acetate) revealed with anti-PEPC antibodies. M, molecular weight marker. Arrows show PEPC in gel. (C) The expression of *PPC2*, and *PPC3* was measured using *Sbactin* as internal control. Data are % with respect Wt without Cd. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, P < 0.05).

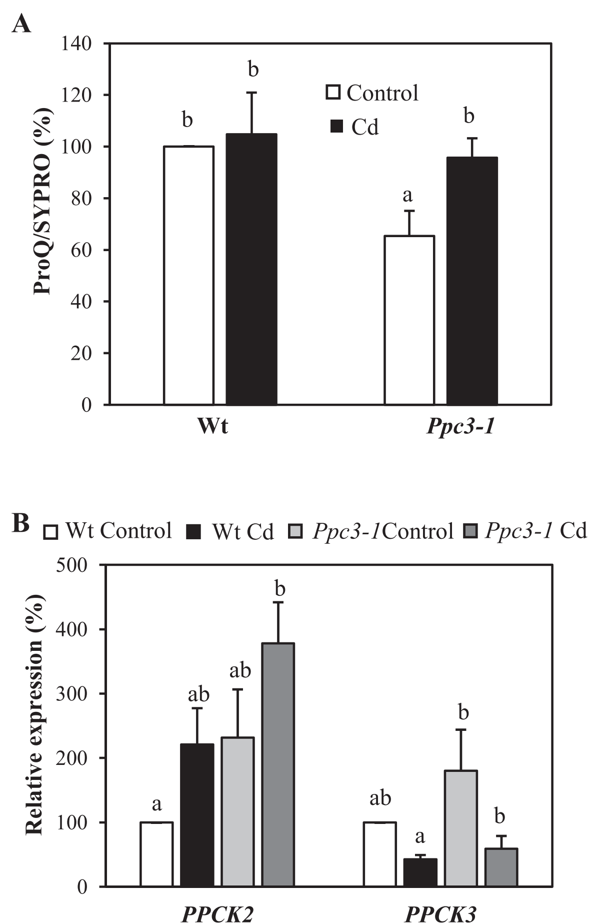

Fig. 12. Effect of Cd on root PEPCk activity and *PPCK* gene expression. Sorghum plants (2 weeks) were supplied with 0.1 mM cadmium acetate for 1 week. (A) Ratio Pro-Q / SYPRO of root samples. PEPCk activity was measured in protein extracts (50 μg) with 0.4 U of purified C_4 PEPC. Pro-Q Diamond and SYPRO Ruby stained gels are shown in Fig. S8. (B) The expression of *PPCK2*, and *PPCK3* was measured in roots using *Sbactin* as internal control. Data are % with respect Wt without Cd. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Duncan, P < 0.05).

seedlings (de la Osa et al., 2022). Second, it is the main responsible for the increase of root PEPC activity in responses to stresses such as salinity (de la Osa et al., 2022) and ammonium stress (Arias-Baldrich et al., 2017). In this work, we show that Al and Cd increased root PEPC activity and *PPC3* expression, and that the rise did not occur in *Ppc3* lines. In these lines, Al and Cd increased root *PPC2* expression, but it did not compensate the lack of PPC3 at the level of PEPC activity. These results confirm the relevance of PPC3 in root responses to stress. In the same line, we have found that these lines are defective in responses to P deficiency and in the capability to solubilize insoluble phosphorous. Although an increase of root PPC3 activity occurs in responses to several stresses in which PEPC participates, some specific aspects of the response could be associated to specific stresses. For example, ammonium stress increased the monoubiquitination of sorghum root PEPC (Arias-Baldrich et al., 2017), a posttranslational modification that has not been reported neither in salinity nor in responses to Al or Cd.

In addition, results in this work show that Fe homeostasis is altered in *Ppc3* lines, and it is probably due to malfunctioning of responses to Fe deficiency. Two accessions of *Noccaea caerulescens* that differed in foliar Cd accumulation, varied also in the way they manage Fe under Cd stress. (Halimaa et al., 2019). One of the accessions had higher levels of leaf Fe, which caused oxidative stress, and decreased the tolerance to Cd. Accordingly, *Ppc3–1* line had more Fe in leaves, and increased level of stress markers, MDA and proline. The iron content increased in the roots of the Cd hyperaccumulator *Noccaea caerulescens* that were exposed to 50 μM Cd (Halimaa et al., 2019). Transcriptional analysis revealed increased expression of genes related to Fe homeostasis (iron deficiency-regulated bHLH transcription factors, ferric reduction oxidase FRO2, iron transporters IRT1 and OPT3, and nicotianamine synthase NAS1). These changes were due to Fe shortage caused by Cd exposure, and to overcompensation of the Fe deficiency response. Although sorghum displays a Strategy II response, Fe deficiency has been reported to increase the expression of both Strategy I and Strategy II genes (Wairich et al., 2019). In this work, Cd increased the expression of *SbNRAMP1* (Strategy I gene) and *SbIRO2* (Strategy II) gene. The expression of *SbIRO2* was much higher in Cd-treated *Ppc3* plants than Wt, pointing towards differences in Fe homeostasis because of diminished PPC3 activity.

The *PPC* gene family has four members in *Arabidopsis thaliana*. *At*PPC2 transcripts are found in all organs, suggesting that it is a

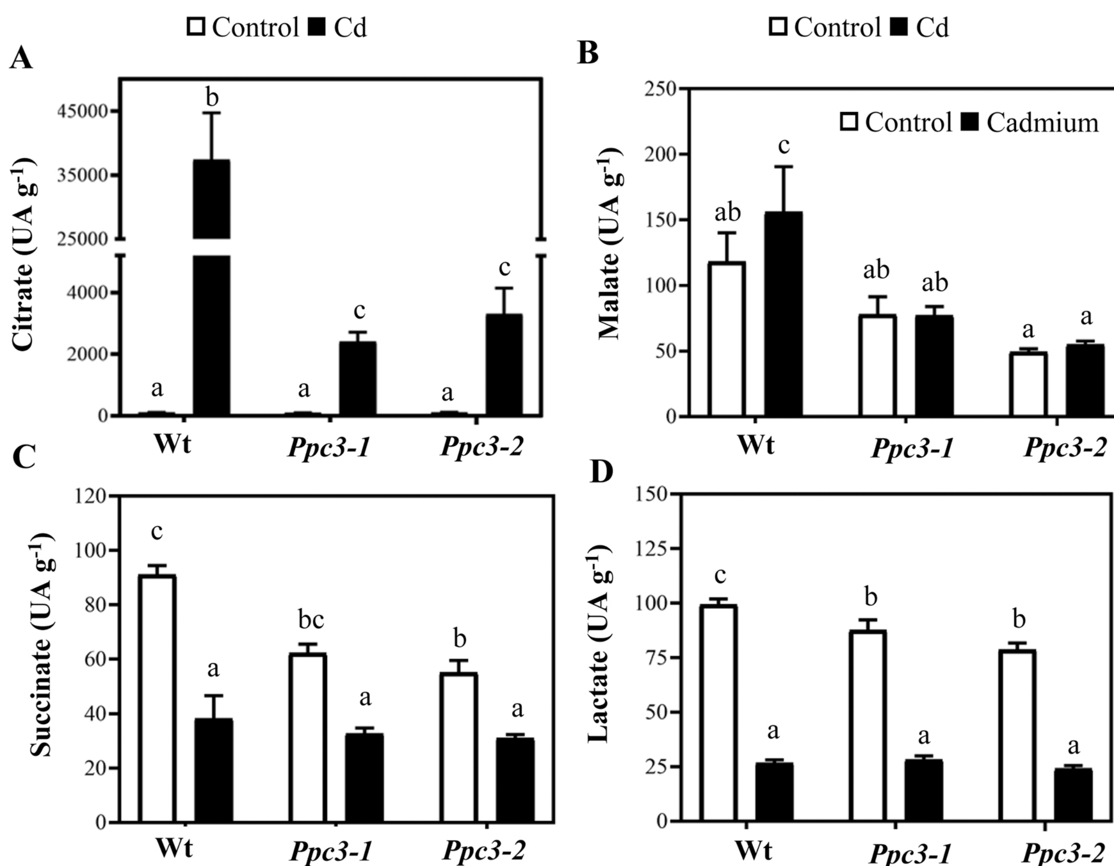

Fig. 13. Measurement of organic acids in root exudates. Sorghum plants (5 days) were kept with Hewitt medium, with or without 100 μM of cadmium acetate, for 5 days. Then, plants were placed in 50 ml Falcon tubes containing 35 ml of 1 mM $CaCl_2$ and 5 μM H_3BO_3, pH 4.5, with or without 0.1 mM cadmium acetate, and exudates were collected for 48 h. Organic acid were quantified by mass spectometry. (A) Citrate. (B) Malate. (C) Succinate. (D) Lactate. Data are means ± SE (n = 3). Different letters indicate statistically significant differences (Games-Howell, P < 0.05).

Table 4
Cadmium content in roots. Plants (2 weeks) were supplied with 100 μM cadmium acetate for 1 and 2 weeks. Cd was quantified by Atomic Absorption Spectroscopy at the end of each week. Data are means ± SE (n = 3).

	Cd in roots (mg kg^{-1})		
	Week 1	Week 2	Week 2-Week 1
WT	3429 ± 143^{b}	3974 ± 321^{b}	545
Ppc3–1	2479 ± 106^{a}	4040 ± 519^{b}	1280
Ppc3–2	2257 ± 49^{a}	3754 ± 249^{b}	1497

[a,b]Different letters indicate statistically significant differences within each column (Duncan, P < 0.05)

housekeeping gene. *AtPPC3* gene is expressed in roots, and *AtPPC1* and *AtPPC4* in roots and flowers (Sánchez et al., 2006). Analysis of single knock out lines for PEPC isoenzymes showed that root PEPC activity was depressed only in *atppc3* plants (Feria et al., 2016). Exposure to Cd of Arabidopsis plants increased *AtPPC* expression and PEPC activity in roots (Willick et al., 2019). Two T-DNA insertional mutant lines *atppc1* and *atppc3* showed enhanced sensitivity to Cd, and the level of transcripts of *AtPPC1* and *AtPPC3* increased in response to Cd in Wt. This shows the relevance of *AtPPC1* and *AtPPC3* upregulation for acclimation to Cd stress in Arabidopsis; meanwhile, in sorghum this response is associated mainly to *SbPPC3*.

Salinity and ammonium stresses increased sorghum root PEPC activity, and root and leaf PEPCk activity (Echevarría et al., 2001; Monreal et al., 2013a; Arias-Baldrich et al., 2017). In shoots, increased PEPCk activity was associated to PPCK1, the photosynthetic isoenzyme. In roots, increased PEPCk activity was dependent on PPCK2 isoenzyme. Similar effects were produced by Al and Cd on root PEPCk activity, which was increased by Al and Cd, and related to PPCK2 isoenzyme. It is noteworthy that the increase of *PPCK2* expression was higher in *Ppc3–1* than in Wt, but it could not compensate for the lack of PPC3.

Some of the detrimental effects of Al and Cd were more severe in Ppc3 lines than in Wt, indicating that the formers were more sensible to these metals. For example, decreased germination and elongation rate in Al treated plants, or decreased germination rate, decreased chlorophyll b content, and increased MDA and proline synthesis in Cd treated plants. This could be due to the global metabolic disturbance, both in roots and leaves, caused by the lack of PPC3 (de la Osa et al., 2022). These alterations affect the efficiency of stress responses, making *Ppc3* plants more sensible to stresses such as salinity, and Al or Cd toxicity. In addition, the absence of synthesis of citrate or malate linked to PPC3 activity will directly disturb mechanisms that increase plant resistance to Al and Cd toxicity, and that are related to root exudates.

Organic acid anions are important chelators of toxic metals, including aluminum and cadmium. In sorghum, citrate (Kochian, 1995; Sivaguru et al., 2013; Carvalho et al., 2016), and in minor degree malate (de de de Carvalho Goncalves et al., 2005), are the main organic acids related to metal tolerance. Sorghum uses the exclusion strategy, secreting citrate that complex extracellularly with Al or Cd, and thus decreasing their uptake (Ma, 2000). Citrate and malate are also important for detoxification of intracellular Cd, by forming Cd-citrate and Cd-malate complexes that are stored in vacuole (Sanità di Toppi and Gabbrielli, 1999; Zhu et al., 2011). In this work, we have shown that both Al and Cd increase the amount of citrate, and, at lesser extent, malate, in root exudates. With Al treatment (2 days), a 40-fold increase of citrate in root exudates was produced, although no differences

between Wt and *Ppc3* lines were detected. In control conditions, *Ppc3* lines had higher root citrate synthase activity and citrate content than Wt (de la Osa et al., 2022). This basal citrate could be sufficient to maintain the quantity of this organic acid in root exudates at the same level than Wt, despite of decreased synthesis as consequence of diminished PPC3 activity. With Cd treatment (7 days), the amount of citrate increased by 400-fold in Wt root exudates, but only by 30-fold in *Ppc3* lines. These results indicate that although PPC3 is not necessary for early production of organic acids in root exudates, it is crucial for maintaining high level of synthesis and accumulation of citrate, and that PPC3 is the main PEPC isoenzyme responsible for this response to heavy metal stress.

5. Conclusion

The results confirm the relevance of PPC3 in sorghum roots and their responses to stress. Specifically, in the synthesis of citrate, which is the main organic acid responsible for resistance to both Al and Cd in sorghum. Although PPC3 activity is not necessary for early citrate synthesis in response to these metals, it is indispensable for maintaining high amount of citrate in root exudates at longer term.

CRediT authorship contribution statement

Jesús Pérez-López performed most of experiments and formal analysis. **Jacinto Gandullo** contributed to some experiments, formal analysis, and critical revision of results. **Clara de la Osa** and **Ana Belen Feria** contributed to some experiments and critical revision of results; **Sofía García-Mauriño**, **José Antonio Monreal** and **Cristina Echevarría** designed the research and obtained funding. **Sofía García-Mauriño** wrote the original manuscript and revised it with the input of all the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.105139.

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