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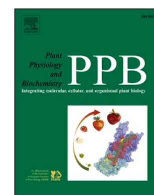

Phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxylase kinase isoenzymes play an important role in the filling and quality of *Arabidopsis thaliana* seed

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A B S T R A C T

Three plant-type phospho*enol*pyruvate carboxylase (*PPC1* to *PPC3*) and two phospho*enol*pyruvate carboxylase kinase (*PPCKs: PPCK1* and *2*) genes are present in the *Arabidopsis thaliana* genome. In seeds, all *PPC* genes were found to be expressed. Examination of individual *ppc* mutants showed little reduction of PEPC protein and global activity, with the notable exception of PPC2 which represent the most abundant PEPC in dry seeds. *Ppc* mutants exhibited moderately lower seed parameters (weight, area, yield, germination kinetics) than wild type. In contrast, *ppck1*-had much altered (decreased) yield. At the molecular level, *ppc3*-was found to be significantly deficient in global seed nitrogen (nitrate, amino-acids, and soluble protein pools). Also, N-deficiency was much more marked in *ppck1*-, which exhibited a tremendous loss of 95% and 90% in nitrate and proteins, respectively. The line *ppck2*-had accumulated amino-acids but lower levels of soluble proteins. Regarding carboxylic acid pools, Krebs cycle intermediates were found to be diminished in all mutants; this was accompanied by a consistent decrease in ATP. Lipids were stable in *ppc* mutants, however *ppck1*-seeds accumulated more lipids while *ppck2*-seeds showed high level of polyunsaturated fatty acid oleic and linolenic (omega 3). Altogether, the results indicate that the complete PEPC and PPCK family are needed for normal C/N metabolism ratio, growth, development, yield and quality of the seed.

1. Introduction

Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the quasi-irreversible biosynthesis of OAA from PEP in the cell cytosol (Chollet et al., 1996). This is a crucial step for primary metabolism in all plants as it is believed to sustain Krebs cycle activity (anaplerotic function) in mitochondria. In addition, in CAM and C_4 plants, PEPC is directly involved in the photosynthetic fixation of CO_2 (Echevarría et al., 1994; Chollet et al., 1996). The anaplerotic role implies the maintenance of energy and metabolite production for, notably, nitrogen assimilation in the GS/GOGAT cycle (Plaxton and Podestá, 2006; O'Leary et al., 2011). The PEPC protein is subject to in vivo reversible phosphorylation of a conserved, N-terminal Ser residue that modulates a sophisticated metabolite control of the enzyme (Echevarría et al., 1994; Echevarría and Vidal, 2003; O'Leary et al., 2011); Malate (inhibitor) and glucose-6-phosphate (activator) are such metabolite players (Echevarría et al., 1994; Chollet et al., 1996; Feria et al., 2008). PEPC phosphorylation is catalyzed by a dedicated PEPC-kinase (PPCK); this post-translational modification (PTM) activates the enzyme and contributes to a decrease in its sensitivity to feedback inhibition by L-malate (Chollet et al., 1996; Echevarría and Vidal, 2003). In seeds, this post-translational regulation of PPC has been described in wheat, barley, amaranthus, sorghum (Osuna et al., 1996; Feria et al., 2008; Álvarez et al., 2011; Ruiz-Ballesta et al., 2016, respectively) and castor oil seeds

Abbreviations: PEPC, Phosphoenolpyruvate carboxylase; PPC, Phosphoenolpyruvate carboxylase isoenzymes and genes; PPCK, Phospho*enol*pyruvate carboxylase kinase; WT, wild type; DPA, days post anthesis; Wbp1, Beta subunit of the oligosaccharyl transferase glycoprotein complex.

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(O'Leary et al., 2011). Additionally, the seed PEPC (both from C_3 and C_4 plants) has been demonstrated to be a target for monoubiquitination (Uhrig et al., 2008; Ruiz-Ballesta et al., 2014, 2016). The physiological role of this PTM is still unclear and this new and attractive domain of investigation needs more detailed studies, though an impact on the properties of PEPC was proposed (O'Leary et al., 2011; Ruiz-Ballesta et al., 2016). Also, very attractive, is the question of its possible regulatory interaction with the phosphorylation process (O'Leary et al., 2011; Ruiz-Ballesta et al., 2016).

Molecular biology and genetic-based studies have described a small family of PEPC and PPCK proteins (Lepiniec et al., 1993; Chollet et al., 1996; Hartwell et al., 1999; Sullivan et al., 2004; Shenton et al., 2006). In *Arabidopsis thaliana*, three genes *AtPPC1-3,* encode plant-type PEPCs and two genes: *AtPPCk1* and *AtPPCk2* encode the dedicated PPCKs (Fontaine et al., 2002; Meimoun et al., 2009). A bacterial-type PEPC exists in *Arabidopsis thaliana*. It has a major impact in plant stress physiology, and hence it will not be considered in this work (Sánchez et al., 2006; Feria et al., 2016).

PPC and *PPCK* transcripts are widely distributed through the entire plant, with unequal distribution in tissues and organs (Sanchez and Cejudo, 2006; Arias-Baldrich et al., 2017). In Arabidopsis: 1) *PPC1* and *PPC2* are the exclusive leaf mRNAs (Shi et al., 2015); 2) *PPC3* is more specifically expressed in roots (Feria et al., 2016), especially when plants are exposed to NaCl excess or Pi shortage stresses and 3) *PPCK1* and *PPCK2* transcripts are much more abundant in roots than in shoots (Feria et al., 2016). In Feria et al., 2016, we used a series of T-DNA tagged plants (SALK lines) to study the effects of the lack of *PPC* and *PPCK* genes on growth and development in Arabidopsis. We showed that any individual mutation impacted on plant growth, e.g., decrease in weight and inflorescence length, and delay in flowering (Feria et al., 2016), thereby indicating that normal levels of PEPC and its phosphorylation machinery are needed for optimal growth. However, despite a decrease in weight and inflorescence, all mutants produced flowers and viable seeds. Interestingly Shi et al. (2015) shows that a mutant combining the double loss of PPC1 and -2, via T-DNA insertions (SALK lines), was unable to complete its life cycle. In leaves, this genetic perturbation caused starch and sucrose accumulation, decreased nitrate levels and suppressed ammonium assimilation. The double mutant alterations were reversed in a complementation test, thereby establishing the cardinal role of the anaplerotic step catalyzed by PEPC, which cannot be compensated by another alternative metabolic pathway (Shi et al., 2015). Also, the suppression of the *PPCK* affected plant growth (Feria et al., 2016), which was in good agreement with previous data from Meimoun et al. (2009) where the growth rate of knockout plants was slowed down, but flowers and seeds were still produced.

In Feria et al. (2016), we focused interest on roots and shoots of Arabidopsis *ppc* and *ppck* mutants. In the present paper, we concentrate on PEPC and PPCK functions in seeds. The presence of PEPC has been described in barley (*Hordeum vulgare;* Feria et al., 2008), wheat (*Triticum aestivum;* Osuna et al., 1996), castor oil seeds (Uhrig et al., 2008) and sorghum seeds (Nhiri et al., 2000; Ruiz-Ballesta et al., 2016), where it has been established that PEPC phosphorylation occurred in situ during early germination. Seed formation needs a very active primary C/N metabolism and energy to build up structures, including embryo formation and endosperm cellularization, together with accumulation of storage reserves (lipids, proteins, starch …) as resources for the forthcoming germination. In the present work, mutant lines were grown to seeds, and phenotypic and molecular alterations that correlated with the absence of a given PEPC/PPCK in different plant locations where it is normally expressed were determined. The results show alterations in various parameters of the seeds produced by the *ppc* mutants, however an important alteration of the yield and the filling of the seeds was found in the *ppck* mutants, giving for the first time a special relevance to the phosphorylation of PEPC in the processes of yield and quality of the seeds.

2. Materials and methods

2.1. Plant material and growth conditions

All *Arabidopsis thaliana* plants used in this study were in the Columbia ecotype (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 analysed and homozygosity confirmed by PCR (Feria et al., 2016).

Seeds were germinated on soil (COMPO AGROSIL®: peat, perlite, CaO, pH 5/6.5, and nutrients) and were grown for two months in a long day regime of 16 h light/8 h darkness at a temperature of 22 °C/18 °C, respectively. Mature seeds were collected at the end of the desiccation period and stored at 4 °C. All seeds used were obtained from the same round of growth.

For germination studies (Fig. 6), seeds were surface sterilized (2% (v/v) bleach 20 min, 2 times sterile water, and 20 mM HCl and rinsed 8–10 times with sterile water) and stratified for 3 d at 4 °C in the dark. After stratification, seeds were sown on 1% agar plates with water, or supplemented with ½ strength Murashige and Skoog medium (MS) (pH 5.7), to test if application of exogenous nitrate can accelerate the germination of control seeds. Seeds were germinated in L/D cycles 12 h (22 °C, 60% relative humidity) in a white light regime of 150 μmol photons m^{-2} s^{-1} and 12 h of dark (18 °C, 70% relative humidity). The average germination stage was calculated at each post-imbibition time point (Ruiz-Ballesta et al., 2016). Developmental stages were classified as follows: testa rupture (stage I), radicle protrusion (stage II), primary root (0.3–0.5 mm in length, stage III), appearance of first root hairs before greening of cotyledons (stage IV).

2.2. Enzyme extraction and analysis

Protein extracts were obtained by grinding 50 mg of dry seeds in a mixer mill (MM-301 Retsch) with 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-Aldrich), 10 mM kF and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C.

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.

2.3. Electrophoresis and immuno-blotting

Protein extracts were denatured by heating (90 °C for 5 min) 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, 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 PEPC from sorghum leaves (PEPC-IgGs) were prepared as described in Pacquit et al. (1995). After PEPC detection, membranes were stripped and probed with Wbp1(Beta subunit of the oligosaccaryl transferase complex) antibodies as a loading control. Wbp1 antibodies (Wbp1-IgGs) were kindly provided by Prof Manuel Muñiz from Seville University, Sevilla, Spain. Anti-pSer13-IgG (anti-pSer13) was raised against a synthetic phosphopeptide corresponding to the N-terminal seryl phosphorylation site of a sorghum C3 PEPC sequence [phosphorylated on the regulatory serine: Cys-ERLS (**pS**)IDAQLR] (Lepiniec et al., 1993; Ruíz-Ballesta et al., 2014). Bands were subsequently detected using affinity-purified

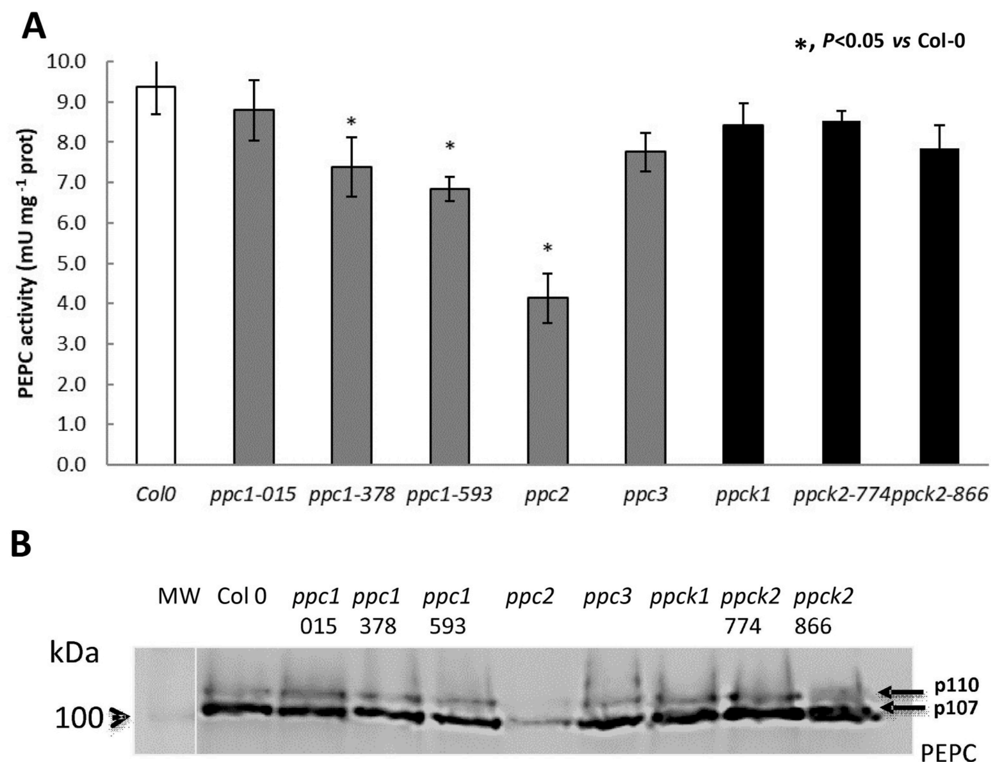

Fig. 1. Analysis of PEPC activity and polypeptides in extracts of dry seeds from Col-0 and *ppc/ppck* mutants (SALK lines). (**A**) PEPC specific activity (mU mg protein^{-1}). (**B**) Crude extracts were subjected to 8% SDS-PAGE (150 μg protein lane^{-1}) and immunoblotting using anti-PEPC.

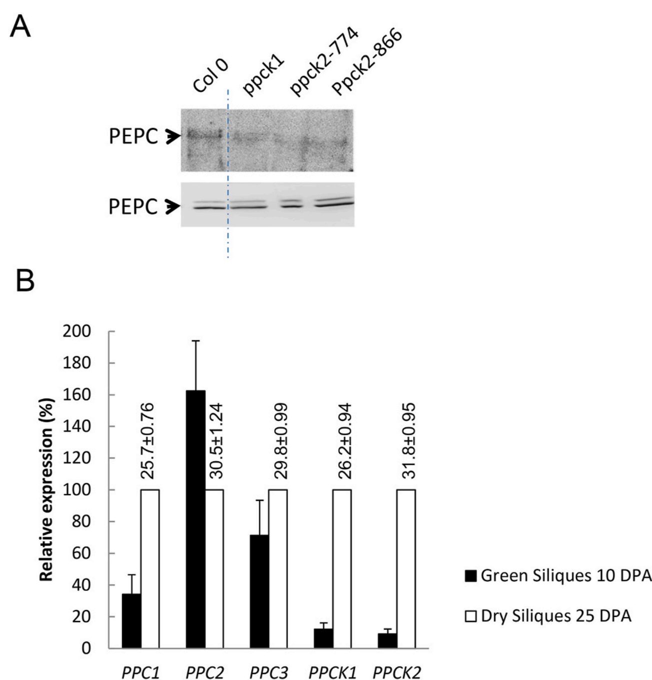

Fig. 2. Analysis of phosphorylated PEPC in *ppck* mutants by immunoblotting using specific anti phosphorylation site antibodies (anti-pSer13, see M and M). Crude extracts from dry seeds of Col-0 and *ppck* mutants were subjected to 8% SDS-PAGE (200 μg protein lane^{-1}) and transferred onto nitrocellulose membranes for gel blots and revealed with anti-pSer13 or anti-PEPC antibodies respectively (**A**). qRT-PCR analysis of *PPC1*, *PPC2*, *PPC3*, *PPCK1* and *PPCK2* gene expression in 10 DPA and 25 DPA siliques of Col-0 plants (**B**). *At*Actin RNA was used as internal control in each sample. Data were normalized to dry siliques. Cts (cycles) values obtained were indicated on the dry silique bars. Results are means ± SE of at least three biological replicates.

goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories).

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of frozen, powdered siliques 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.

2.5. qPCR experiments

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 (Feria et al., 2016) 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 actin expression was used as an internal control (*actin* rRNA: forward 5′-CCAGCTCATCTGTTGAGAAG-3′, reverse 5′-CCTGTGAACAATCGATGGAC-3′).

2.6. Nitrates, amino acids and proteins quantification

NO_3^- concentration was measured using the Miranda method using the vanadium chloride base (Miranda et al., 2001). Total amino acid content was measured using the ninhydrin method as described by Rosen et al. (1957). Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as the standard. Average N content was assumed to be 16% of protein weight (Kjeldahl,

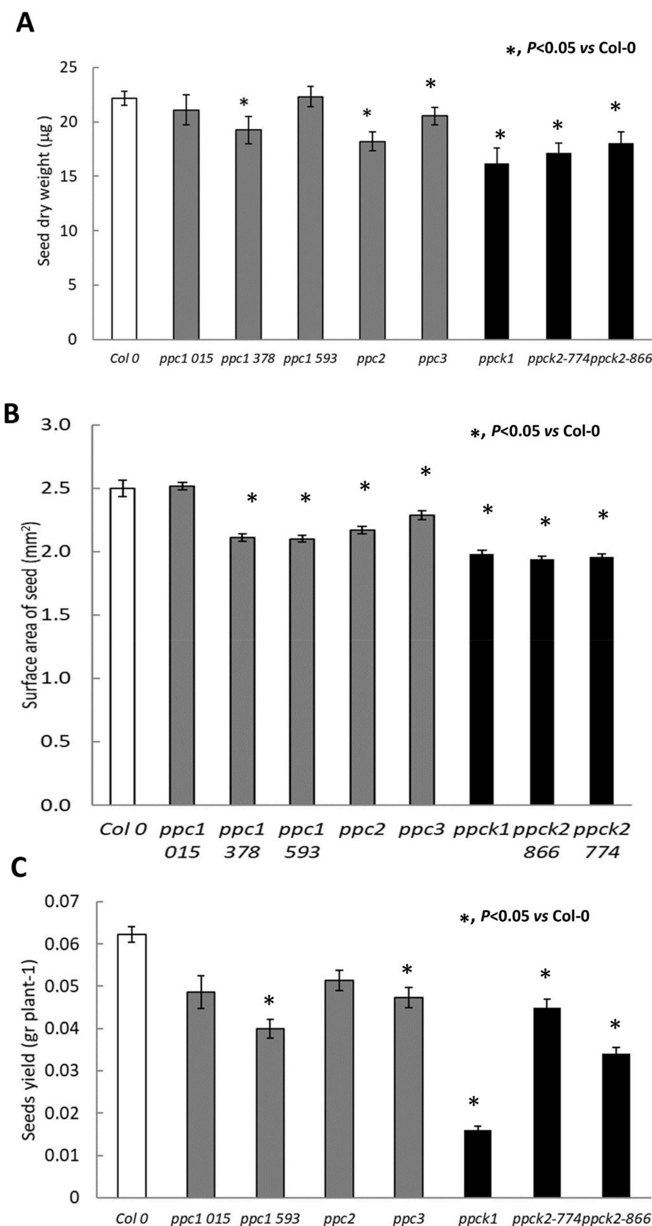

Fig. 3. Phenotypic analyses in dry seed *ppc* and *ppck* mutants of Arabidopsis. (**A**) Dry weight quantification. The weight of a dry seed was estimated from 1 mg of seeds pooled from 40 plants. Data represent the mean ± SE ($n = 10$). (**B**) Relative surface area was measured on seeds placed on agar plates (according to the area of the ellipse A = π*R*r). Bars indicate mean ± SE ($n = 80$). (**C**) Yield was quantified from the total weight of seeds per 30 plants. Values are expressed as the mean ± SE ($n = 40$). * Asterisks indicate a significant difference *versus* control ($P\langle 0.05$, Student's t-test).

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2.7. % C/N quantification

Analysis of the total C and N from three replicates of 150 mg of dried seed sprayed in liquid nitrogen were performed by a LECO Elemental CNS-Trumac Autoanalyzer at the Mass Spectrometry Services (CITIUS, University of Sevilla).

2.8. Organic acids separation and quantification

Metabolite analysis by GC-MS was carried out by a method modified from that described previously (Xu et al., 2006). Seeds (50 mg) were ground with a pre-chilled mixer mill (MM-301 Retsch) and homogenized with 1.5 ml 0.5 M HCl. The mixture was extracted for 20 min at 80 °C with shaking. After centrifugation at 2.500*g* for 20 min, the supernatant was collected, filtered (0.45 μmØ *Q-Max®Syringe Filter*) and stored at 4 °C prior to analysis. Quantitative analysis used liquid chromatography (HPLC) coupled with ion trap (GCMS-QP2010 Plus Shimadzu) and it was carried out by the IBVF chromatography service (CSIC, Sevilla).

2.9. Fatty acids separation and quantification

Fatty acid analyses were performed on pools of 2 mg of seeds as previously described (Garcés and Mancha, 1993). Fatty acid methyl esters (FAMEs) were obtained by heating the samples at 80 °C for 1 h in methanol: toluene: H_2SO_4 (88:10:2 by vol.) using 17:0 as internal standard to give reproducible information about the loss of analytes during extraction and isolation. Total seed fatty acid content and composition was determined by fused silica capillary (Supelco 2380) GC/MS (Agilent Technologies 6890 Series GC System) coupling analysis.

2.10. ATP quantification

Frozen samples (0.1 g of dry seeds) were ground and homogenized in a mixer mill (MM-301 Retsch) with 1 ml of 0.6 M $HClO_4$ and incubated for 30 min at room temperature. The acid suspension was adjusted to pH 7.6 with 2 M K_2CO_3, and the residue was removed by centrifugation. Supernatants were stored at −20 °C until use. The assay was performed with a luciferin-luciferase ATP bioluminescent assay kit (Sigma), using a Luminoskan TL (LabSystems Oy, Helsinki, Finland) luminometer (García-Mauriño et al., 2005).

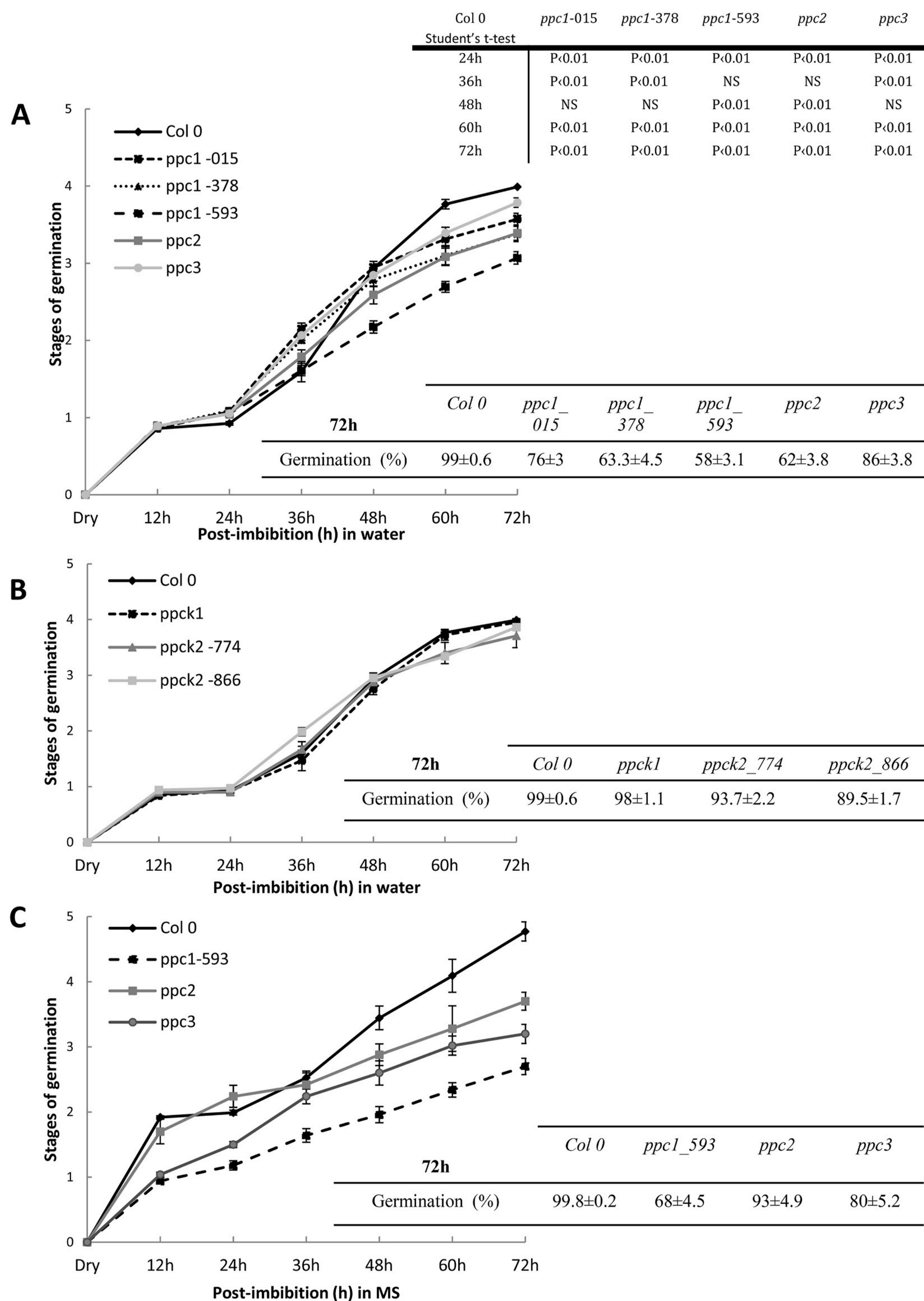
2.11. Statistical analysis

The statistical analysis of the results was performed using statistical software (SigmaStat, Systat Software Inc). Statistical differences between groups were tested by ANOVA. Statistical differences between selected pairs of groups were tested by Student's *t*-test. The means are considered to be significantly different at $P \leq 0.05$.

3. Results

3.1. Characterization of PEPC/PPCK in dry seed extracts

First, we characterized PEPC activity from Col-0 dry seeds. Crude extracts were obtained as described in the materials and methods section. Soluble extracts of proteins (mainly 12 S globulin_cruciferin and 2 S albumin_arabin, the two major storage proteins in *A. thaliana* seed, (Heath et al., 1986), contained 9–10 mU of PEPC activity per mg of protein (Fig. 1A, Col-0 line). Dry seed crude extracts from all SALK lines contained slightly lower amounts of PEPC activity except for the *ppc2*⁻ line, where it was markedly decreased (approximately a 55% loss) (Fig. 1A). This was confirmed by immunoblot analysis (Fig. 1B) where it is shown that PEPC amount in *ppc2*⁻ are highly decrease. This was not due to a decrease in the loaded protein since a statistic of four similar experiments also demonstrated the significant decrease of PEPC in *ppc2* mutant (Fig. S1A). In addition, the quantification of the signal of Wbp1, used as loading control, show a similar amount of protein in the different lanes (Fig. S1B). Therefore, PPC2 seems to be the more abundant PPC form in dry seed, although the remaining PEPC isoenzymes in *ppc2*⁻ still yielded about 40% of total wild type catalytic activity. In addition, immunoblot analysis revealed the presence of a doublet of PEPC subunits (approximately 107 and 110 kDa) in dry seeds (Fig. 1B) both, in wild type and mutant, which indicates that this PTM was not affected by the mutations. In previous works on castor oil seeds, sorghum and Arabidopsis it has been established that the upper band is a post-translational monoubiquitination of the lower (Ruiz- Uhrig et al.,



Col 0 Student's t-test	*ppc1*-015	*ppc1*-378	*ppc1*-593	*ppc2*	*ppc3*
24h	P‹0.01	P‹0.01	P‹0.01	P‹0.01	P‹0.01
36h	P‹0.01	P‹0.01	NS	NS	P‹0.01
48h	NS	NS	P‹0.01	P‹0.01	NS
60h	P‹0.01	P‹0.01	P‹0.01	P‹0.01	P‹0.01
72h	P‹0.01	P‹0.01	P‹0.01	P‹0.01	P‹0.01

72h	*Col 0*	*ppc1_ 015*	*ppc1_ 378*	*ppc1_ 593*	*ppc2*	*ppc3*
Germination (%)	99±0.6	76±3	63.3±4.5	58±3.1	62±3.8	86±3.8

72h	*Col 0*	*ppck1*	*ppck2_774*	*ppck2_866*
Germination (%)	99±0.6	98±1.1	93.7±2.2	89.5±1.7

72h	*Col 0*	*ppc1_593*	*ppc2*	*ppc3*
Germination (%)	99.8±0.2	68±4.5	93±4.9	80±5.2

Fig. 4. Kinetics of germination rate for seeds of wild-type, *ppc* (**A**) and *ppck* (**B**) mutant lines, in the absence, were imbibed in water, or presence (**C**) of medium containing nitrogen, for 72 h on agar plates. Stages of germination are described in the Materials and Methods. Data of germination efficiency was inserted as a table in each Fig. A, B and C, and represent the % germination at 72 h post-imbibition. Values are expressed as the average of eight replicates ± SE of 25 germinated seeds.

2008; Ballesta et al., 2014; and Baena et al., 2021 respectively).

To test the impact of *PPCK* genes silencing in the level of the in vivo phosphorylation of PPC a Western-blot was performed, where the crude extract from dry seed were analyses. Two independent membranes were probed with anti-PPC antibodies and with pSer13 antibodies respectively, being pSer13 specific antibodies against the phosphorylated form of the enzyme (see M. and M.). We found a reduced level of the in vivo phosphorylation of PEPC of about 60% in both *ppck* mutants in relation with the wild type (Fig. 2A, pSer13) showing the efficiency of the silencing of each PPCK in PEPC phosphorylation. However, about a 40% of phosphorylation can be detected in each *ppck* mutant due, probably, to the presence of the alternative non silenced *PPCK* (Fig. 2A, pSer13). These results also evidence that the two PPCKs are able to phosphorylate the enzyme during the development of the seed to produce a phosphorylated PEPC in dry seed. Finally, the result presents a PEPC already phosphorylate in dry seeds (Fig. 2A, Col 0). This is in contrast with cereal

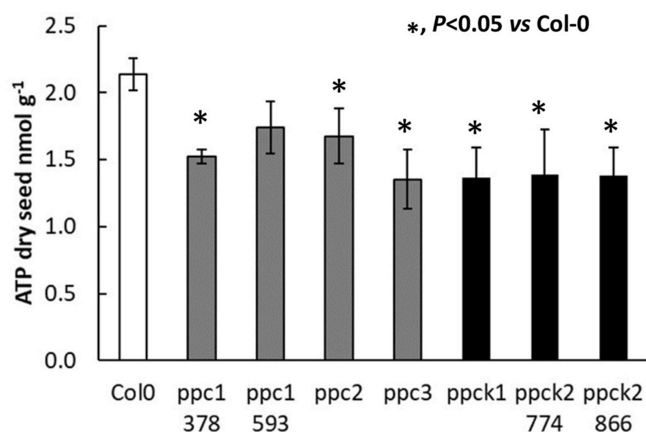


Fig. 5. Adenosine 5′-triphosphate (ATP) in dry seed from Arabidopsis wild-type and mutant knockout lines. Values are expressed as the mean ± SE (n = 5). * Asterisks indicate a significant difference *versus* control (P⟨0.05, Student's t-test).

seed (wheat, barley, and sorghum; Osuna et al., 1996; Feria et al., 2008 and Ruiz-Ballesta et al., 2016 respectively) were the PEPC is phosphorylated during imbibition. However, it agrees with *Amaranthus edulis* seeds where the PEPC is already phosphorylated in the dry seed (Álvarez et al., 2011).

Dry seed is the end of the developmental process of the seeds. Therefore, it was important to elucidate the mechanisms concerning the previous developmental phases, in terms of the content of *PPC* mRNAs. Here we used the siliques instead of seed because it is very difficult to separate the silique to the seed at 10 DPA to have enough amount to the mRNA assay. To this end, we performed qPCR experiments on both 25 DPA and 10 DPA silique extracts. This approach detected the presence of all *PPC* transcripts, with *PPC2* being the dominant species at 10 DPA (Fig. 2B). Regarding *PPCKs*, *PPCK1* and *PPCK2* was much more abundant in 25 than in 10 DPA silique suggesting an important role of these genes in the dry silique state (Fig. 2B). Finally, *PPCK1* transcripts seem to be more abundant than *PPCK2* in dry seeds with Ct values of 26 and 31 respectively.

3.2. Silencing of PPC or PPCK genes impact on seed phenotype (weight, surface and yield)

Previous work by Feria et al. (2016) reported that all *ppc* mutants showed reduced growth parameters (e.g., size and fresh weight of the rosette), thereby indicating that each individual PPC isozymes is needed for normal growth and development. However, single PPCs were not so indispensable that their loss caused a lethal phenotype, since, with

Fig. 6. Fatty acid content in wild-type and mutant dry seeds. (**A**) TAG content. (**B**)The ratio of polyunsaturated to other C18 fatty acid species (PUFA/NPUFA ratio) was calculated as follows: (C18:2, C18:3 in mol%)/(C18:0, C18:1 in mol %). (**C**) The ratio of very long chain to long chain fatty acid species (VLCFA/LCFA ratio) was calculated as follows: (C20:0, C20:1, C20:2, C22:0, C22:1 in mol%)/(C16:0, C18:0, C18:1, C18:2, C18:3 in mol %). (**D-F**) Dry seed fatty acid composition (FAMES) *18:1n9* (oleic acid or ω-9) (**d**), *18:2n6* (linoleic acid or ω-6) (**E**), *18:3n3* (linolenic acid or ω-3) (**F**). Values are the means ± SE of five replicates carried out with seeds from distinct individuals. * Asterisks indicate a significant difference *versus* control (P⟨0.05, Student's t-test).

delay, all produced flowers and seeds (Feria et al., 2016). Although, all analysed SALK lines showed delayed growth and development (Feria et al., 2016). In this work we analyze the weight, surface area and yield of seeds produced by the different mutants and found that seeds from the *ppc* mutants had significant differences (between 20 and 33%) regarding weight and surface, compared to the control (Fig. 3A and B). In the same way, seed yield was found to be highly reduced compared to the wild type, particularly in the case of the *ppck1*- line where the yield was reduced by almost a 63% (Fig. 3C). However, the weight was only modestly decreased (25%) (Fig. 3A). This observation indicates that the absence of PPCK1 was correlated with growth defaults that highly impact on seed yield. The results also show for the first time the important role of PPCK and PEPC phosphorylation in the yield of seeds in *Arabidopsis thaliana*.

3.3. Impact of the mutations on seed germination

With regard to the alterations detected in some characteristics (growth, development) of mutant seeds, it was expected to find an impact of the lack of PEPC or PPCK isoenzymes on the ability of seeds to germinate. Fig. 4 shows the % of germination seeds at 72 h post-imbibition. Significant differences were obserbed between *ppc* mutants and the wild type (Fig. 4A). However, these differences were lower in *ppck* mutants. In addition to the germination efficiency, the kinetic of germination (when a seed reaches a specific stage of germination I, II, III or IV; see M and M) was also recorded. In fact, although the germination process began equally in all *ppc* mutants (Figs. 4A, 12 h and 24 h), all of them subsequently showed a significant decrease in their germination stages compared to the wild type (Figs. 4A, 36 and 48, 60 and 72 h). At 72 h seeds from wild type have reached stage IV while *ppc* mutants are in lower stages of germination. Surprisingly, *ppck* mutant genotypes displayed little alteration of their germination rates. Eventually, all *ppck*- lines reached the IV germination stage at 72 h (Fig. 4B). Data of Fig. 4 was also represented as the % of germination at each stage of germination in supplemental Fig. S2.

Previous studies indicated that exogenously provided nitrate acts as a signal molecule favoring germination of Arabidopsis seeds (Alboresi et al., 2005). We tested the occurrence of such a positive effect of nitrate on the germination of our Arabidopsis seeds. We noted that exogenous nitrate application slightly accelerated the germination rate of control seeds, but at lower level in mutant seeds (Fig. 4C). We have calculated the mean rate of germination from the slope of each line, and we obtained a slope increase of 1.65% in the wild type while in mutants it decreased by up to 19% in the case of *ppc3*. The germination efficiency was also observed and we found a significant increase for *ppc2* mutant (from 62% to 93%; Fig. 4C), however *ppc1* and *ppc3* have low variation of its germination capacity (58%–63% for *ppc1* and 86%–80% for *ppc3*). These results point PPC1 and PPC3 as the enzyme implicate in the germination process. When seeds are deprived of one of these isoenzymes, exogenous nitrogen has a poor impact on the germination efficiency.

3.4. Impact of the mutations on the seed phenotype: molecular level

3.4.1. Nitrogen and the distribution of nitrogenous compounds in seeds

PEPC has an important role in the interaction of C/N metabolisms (O'Leary et al., 2011). We therefore studied the distribution of nitrogen-containing compounds and global N (nitrate, amino acids and soluble proteins) in seeds of the *ppc* and *ppck* SALK mutant lines (Table 1). Overall, *ppc*- lines showed a general increase in nitrate and amino-acid content, while protein content was found to be rather stable, except in *ppc3*- seeds with a 39% decreased in protein relative to wild type. To get a rough estimation of N-filling, we made the reasonable assumption that soluble proteins are a reliable gauge of the total reserve proteins accumulated in the seed. Relative contents of N were estimated as follows: nitrate and amino acids levels measured in nmol per seed stand for direct N concentration in these compounds. The N content in soluble proteins was assumed to be 16% (Kjeldahl, 1883), which is considered to be a good estimation for the comparison of this parameter between mutant seeds. This allowed us to establish that N content is considerably higher in soluble proteins than in amino acids and nitrate pools. For instance, for Col-0, the sum of the NO_3 (0.25 nmol) and amino acids (0.12 nmol) pool does not exceed 0.5 nmol (thus, 0.5 nmol of N), while protein amount is close to 2.2 μg, equivalent to 25 nmol N per seed (calculation: 2200 ng × 16% = 352 ng of N: 352/14 = 25 nmol N) (Table 1, Col-0). It can be concluded that amino acids and nitrate variations influence marginally in the global N content of the seed, which was mostly found to be driven by total protein content. Consequently, in terms of total N content, it was calculated that one seed contained approximately: wild type, 25.5 nmol N; *ppc1*, 22.3; *ppc2*, 25.7 and *ppc3*, 16.8 (Table 1). It is important to remark that the *ppc3*- mutant showed the most perturbed distribution. In this case, the high accumulation of nitrate + amino acids (0.97 nmol NO_3^- + 0.4 nmol amino acids = 1.37 nmol N) in one seed of this mutant did not explain the low level of protein and total N content, which was close to 15.4 and 16.8 nmol, respectively (compared to 25 and 25.5 for the wild type).

Despite the fact of the important alteration in N found in *ppc3*- mutant, much more surprising is to see the high alteration found in the N content of *ppck1*- and *ppck2*- lines (Table 1). First, *ppck1*- seeds showed an enormous deficit of soluble proteins, hence of N content. The amino acids pool of *ppck1*- was found to be slightly higher than the control, but it was in contrast to the tremendous global lack of N, around 23 nmol per seed less N content than wild type (90% decrease compared to wild type seeds; Table 1). Measuring the % N/C *ppck1*- had 21.74% less of this ratio than wild type ($0.069 \pm 0.4*10^{-3}$, Col0 & $0.054^{a} \pm 6.8*10^{-3}$, *ppck1*-; "a" indicate a significant difference *versus* control $P \leq 0.05$). Both, *ppck2*- lines were also considerably altered in the global N content. However, with an apparent contrasting behavior. In this case, a strong accumulation of amino acids attenuated the loss of N, although the seeds were still markedly N-deficient, with less than half of the wild type total N for *ppck2-774*.

3.4.2. Distribution of C compounds (Krebs cycle and fatty acids)

Pools of Krebs cycle intermediates: α-ketoglutarate, malate, succinate, and fumarate were found to be significantly decreased in *ppc* mutants (around 50%), showing that the lack of any PPC isoenzyme

Table 1
Nitrogen-containing compounds and total N (nmol) in one Arabidopsis dry seed. In the case of amino acids calculations are based on 1 amino acid = 1 N, which obviously minimizes N concentration. Yet allows comparisons to be made. In the case of soluble proteins, the N content is estimated to be 16%, as classically used. Seed content was quantified as described in materials and methods. Every sample was typically composed of 1 mg of seeds. Values are expressed as the mean ± SE (n = 10). * Asterisks indicate a significant difference *versus* control ($P \langle 0.05$, Student's t-test).

Seed content (per dry seed)	Col-0	*ppc1_378-*	*ppc1_593-*	*ppc2-*	*ppc3-*	*ppck1-*	*ppck2_774-*	*ppck2_866-*
NO3 (nmol)	0.248 ± 0.03	0.199 ± 0.07	0.555 ± 0.07*	0.186 ± 0.03	0.973 ± 0.2*	0.015 ± 0.003*	0.01 ± 0.01*	0.08 ± 0.006*
aa (nmol)	0.123 ± 0.01	0.717 ± 0.14*	0.963 ± 0.2*	0.873 ± 0.11*	0.408 ± 0.1*	0.289 ± 0.01*	4.11 ± 0.17*	4.63 ± 0.26*
Protein (mg)	2.2 ± 0.04	1.87 ± 0.13	2.12 ± 0.03	2.16 ± 0.06	1.35 ± 0.12*	0.19 ± 0.02*	0.57 ± 0.04*	0.78 ± 0.03*
N protein content (nmol)	25.1	21.4	24.2	24.7	15.4	2.2	6.5	8.9
Total N (nmol)	25.47	22.32	25.72	25.76	16.78	2.50	10.62	13.61

affected negatively on the anaplerotic metabolism of the seeds (Table 2). In addition, *ppck* mutants also showed perturbations in the content of these Krebs cycle intermediates (Table 2, *ppck1*- and *ppck2*-). Furthermore, ATP variations paralleled the decrease in Krebs cycle intermediates for all *ppc* and *ppck* mutants (Fig. 5), as expected from the slowdown of the cycle due to the decrease in the anaplerotic function. Again, the most affected mutants were *ppc3*- and *ppck*-.

Regarding lipids (Fig. 6A), there are two significant variations: *ppck1*- which showed a 30% increase, thus indicating that more carbon amount is invested in the lipids of this mutant and *ppc1*-378- seeds that showed a statistically significant decrease of about 10%. The rest of the mutants had no significant variations. The ratio of PUFA (polyunsaturated fatty acids): NPUFA (nonpolyunsaturated fatty acids) was found to be more variable (Fig. 6B). All *ppc* mutants and *ppck1*- showed a significant reduction of this ratio, while *ppck2* mutants exhibited a 25% increase. Interestingly, this mutant had comparable amounts of omega 6 (ω-6) (*18:2 n6*, linoleic acid) but less omega 9 (ω-9) (*18:1 n9*, oleic acid) and more omega 3 (ω-3) (*18:3 n3*, linolenic acid) compared to control values (Fig. 6D, E, F). Finally, the ratio VLCFA (very long chain fatty acids/LCFA (long chain fatty acids) was barely altered by any *PPC/PPCK* mutation (Fig. 6C).

4. Discussion

Feria et al., (2016) has shown that the lack of any of the three PEPC isoenzymes of *Arabidopsis thaliana* impacts negatively on its growth and development. Despite this negative impact, all mutants were still able to complete their life cycle and produce seeds when growing under standard conditions. This is in contrast to the double *ppc1/2* mutant plants of 10-days-old seedlings obtained by Shi et al. (2015), where the life cycle was severely altered, displaying a growth-arrested phenotype. This phenotype was partially reversed by metabolite complementation, following addition of malate or glutamate to the medium, or completely rescued following gene complementation (Shi et al., 2015). The latter results demonstrate that a double mutation (*ppc*1/2) is necessary to severely disturb the life cycle of the Arabidopsis plant and that PPC3 alone cannot supply the different functions of these isoenzymes. In summary, Shi et al., (2015) show that the complete set of PEPC isoenzymes and the corresponding global PEPC activity are needed for the normal functioning of cell metabolism or/and growth.

In the present work, we have analysed simple mutants of SALK lines affected only in one *PPC* or *PPCK* genes in order to allow the plant to complete his life cycle and to produce seeds. We focused on seeds to evaluate the impact of *PPC/PPCK* mutation on the phenotype at both macro and molecular levels, and on their ability to germinate. In fact, it should be kept in mind that seed filling and development depends partially on shoots and roots, and, since PEPC/PPCKs are present in all tissues of the plant, a mutation is likely to impact on the metabolism of other organs apart from the seed. In addition, in wheat seeds, the presence of PEPC has been identified in virtually all tissues: aleurone layer, scutellum and endosperm (González et al., 1998). Whether a tissue is specifically equipped with a given isozyme, or several or all isozymes are present in all tissues, is not known. In the first case, the loss of a PEPC may lead a tissue to be deprived of its activity, with adverse consequences in terms of its contribution to seed metabolism. In the second case, remaining isozymes can compensate for the lack of the missing PEPC form. It was expected that this observation may also apply in Arabidopsis seed.

Extracts from dry seeds of the *ppc2* mutant showed a strongly reduced PEPC activity and a considerable loss of PEPC protein in immunoblotting images. Thus, it is possible to conclude that this PPC2 isozyme is the most abundant PEPC form of the seed, although the remaining PEPC activity of the other PEPC isoforms (PPC1 and PPC3) is not negligible, accounting for around 40% of total activity. As expected, the lack of the other PEPCs did not lead to a comparable decrease in activity, indicating that these proteins have lower quantitative impact. In summary, it can be proposed that all *PPC* genes are likely active to varying extents in all organs of Arabidopsis plants. However, the isoenzyme content is modulated in specific locations and in relation with the environment. Sanchez et al. (2006) showed a preferential distribution of PPC isoenzymes in the different organs, and specifically *PPC2* transcripts were found in all organs studied (seeds were not include in that work) of Arabidopsis, suggesting that it was a housekeeping gene. In contrast, *PPC3* gene was expressed in roots and, finally, *PPC1* in roots and flowers. Shi et al. (2015) have firmly demonstrated that PPC1 and 2 were the largely (96%) dominant forms in leaf, with essential roles in primary metabolism and plant physiology. We had shown that PPC3 is the root form responding to salt stress episodes (Feria et al., 2016), and the present work establishes PPC2 as the major seed form present in dry seed. In this organ, a preferential sublocation is probable. However, the specific localization remains unknown, and the possibility that the level of any one of the isoforms is incremented in relation to specific environmental signals cannot be ruled out.

The effect of *PPCK* silencing on the level of PEPC phosphorylation was also determined and found that these mutants have reduced level of phosphorylated PEPC compared with the control. However, each mutant maintains about the 40% of phosphorylated PEPC due, probably, to the presence of at least one PPCK in each mutant (Fig. 2A).

At a molecular level, we described the N balance in nitrate, amino acids and soluble proteins and, also, calculated a rough estimate of global seed nitrogen. Interestingly, it was shown that the most important pool of N is due to reserve proteins, as expected for a seed, so that the variations for nitrate and amino acids make a marginal contribution to seed N content. It was determined that the *PPC1* or *PPC2* mutation had comparable N content to the wild type. A simple interpretation involves isozyme substitution to carry out the anaplerotic function, then N assimilation and amino acids feeding of the seed by leaves. This is in agreements to the *ppc1* and *ppc2* simple mutant described in Shi et al. (2015), where it is shown in the leaves similar or slightly increased amounts of nitrate and amino acids compared with the wild type. In marked contrast, the *PPC3* mutation led to a significant reduction in N content per seed (16.8 nmol compared to 25.5 nmol in a Col-0 seed),

Table 2
Variation in the organic acid concentrations in *Arabidopsis* dry seeds. Concentration of a-ketoglutaric, malic, succinic and fumaric acid in dry seed from Arabidopsis wild-type and mutant knockout lines. Data represent the mean ± SE (n = 2–5). *Asterisks indicate a significant difference *versus* control (*P*⟨0.05, Student's t-test).

Organic Acids (mg per seed)	Col-0	*ppc1_015-*	*ppc1_378-*	*ppc1_593-*	*ppc2-*	*ppc3-*	*ppck1-*	*ppck2_774-*	*ppck2_866-*
α-Ketoglutaric	0.020 ± 1.2E10-3	0.011 ± 0.6E10-3*	0.008 ± 0.8E10-3*	0.013 ± 0.5E10-4*	0.011 ± 0.8E10-3*	0.011 ± 0.2E10-3*	0.009 ± 0.9E10-3*	0.009 ± 0.4E10-4*	0.010 ± 0.8E10-3*
Malic	0.047 ± 2.9E10-3	0.025 ± 2.9E10-3*	0.025 ± 1.3E10-3*	0.032 ± 7.7E10-3	0.022 ± 1.5E10-3*	0.031 ± 4.9E10-3	0.022 ± 8.6E10-3	0.023 ± 2.9E10-3*	0.022 ± 6.7E10-3
Succinic	0.060 ± 6.8E10-3	0.034 ± 5.7E10-3*	0.036 ± 3.8E10-3*	0.048 ± 9.9E10-3	0.033 ± 4.0E10-3*	0.040 ± 10.1E10-3	0.026 ± 7.1E10-3*	0.034 ± 4.5E10-3*	0.030 ± 6.1E10-3*
Fumaric	0.004 ± 0.2E10-3	0.002 ± 0.3E10-3*	0.002 ± 3.5E10-5*	0.003 ± 0.8E10-3	0.001 ± 4.9E10-5*	0.003 ± 0.8E10-3	0.002 ± 0.8E10-3	0.002 ± 4.8E10-5*	0.002 ± 0.6E10-3

which was likely to be mostly due to a 10 nmol (40%) decrease in soluble proteins. We had previously shown that PPC3 was the main PEPC isoform in the Arabidopsis root, and that it was accumulated in response to some stresses (+NaCl, -Pi; Feria et al., 2016). The absence of this isozyme may reduce N entry/assimilation into the roots (via a decrease in Krebs cycle intermediates), and/or translocation to seeds. However, the accumulation of NO_3^- and amino-acids in *ppc3*- seeds supports the proposal that there was a reduction in the endogenous flux to the synthesis of reserve proteins. Our data are in good agreement with Yamamoto et al. (2014), who stated that protein content and PEPC activity of wheat seeds are correlated parameters.

In the case of the *ppck1* mutant, we observed a tremendous decrease in N content per seed with a highly depressed protein pool and global N content. In relative values, global N content was only 10% of wild type (2.5 nmol *vs* 25.5 nmol) and a 21.74% lower its N/C ratio. In a previous work, that used T-DNA knockout mutants, Meimoun et al. (2009) established that *PPCK1* was the only *PPCK* gene expressed in Arabidopsis leaves, and that the mutant lacking this enzyme exhibited a dephosphorylated PEPC and a clear delay in growth and development. A decrease in carbon content as sucrose and glucose and a loss of carbon via photorespiration was also shown (Meimoun et al., 2009). In the present work, we show that *ppck1* mutant has the most altered phenotype in term of N and protein content and yield. Therefore, it can be suggested that the phosphorylation of the PEPC in the leaves, in the *ppck1* mutant described by Meimoun et al. (2009), is at the base of the important alteration of the accumulated protein in the seed of our *ppck1* mutant. This point to the PEPC phosphorylation in leaves as a cardinal event in the filling, yield and quality of the Arabidopsis seeds. It is known that leaves contribute importantly to N-filling of seeds (Díaz et al., 2008), and it is highly important for this process the anaplerotic mechanism that provides C skeletons for transamination and ammonia assimilation in the GS/GOGAT pathway. In this way, it has been shown that a C_4 phosphoenolpyruvate carboxylase-deficient mutant of *Amaranthus edulis* has a reduced amount of proteins in seeds (Álvarez et al., 2011).

The *PPCK2* mutation was also interesting since, in contrast to the *PPCK1* mutation, it displayed an accumulation of amino acids, which can account for, at least partially, a strongly reduced number of soluble proteins. The same trend was observed in the two different mutant SALK lines. However, in a similar manner to the phenotype of *ppck1*- seeds, the global N content in *ppck2*- seeds was low, around 50% of wild type. Therefore, again in this case, the main alteration is expected to occur at an exogenous level of N (nitrate, amino acids) feeding to the seeds. Since this alteration was in relation to the lack of PEPC phosphorylation by PPCK2 containing organs, the results strongly suggest roots as being responsible for the alteration. Therefore, either non-phosphorylated PEPC (*ppck2* mutant) or absent PPC3 (*ppc3* mutant) in the root compartment would be linked to higher accumulation of NO_3^- (*ppc3* mutant) or amino acids (*ppck2* mutants) during seed formation. In addition, the fact that the amino acids flux towards protein synthesis appears to be reduced in *ppck2*- seeds is consistent with that observed for *ppc3*- mutant.

Regarding C-compounds, we have analysed seed distribution of some Krebs cycle intermediates and fatty acid pools. Regardless of which *PPC* or *PPCK* was mutated, fumarate, α-ketoglutarate, malate and succinate were found to be significantly decreased, consistent with a flux reduction in the anaplerotic pathway. This may occur not only in seeds, but also in any source compartment of the plant that sends metabolites to the developing seed. Because the effect was observed for both *ppc* and *ppck* mutants, it can be proposed that the phosphorylation process is absolutely needed for a normal functioning of the anaplerotic function. An approximately 50% reduction of fumarate and malic acid was also observed in the T-DNA insertion *ppck1* mutant described by Meimoun et al. (2009).

Fatty acid pools were analysed and found to be poorly affected by the mutations with the notable exception of *ppck1*-. It can be proposed that the large reduction in storage proteins (see Table 1) could correspond to available carbon amounts that were used to build up bigger lipid pools in this mutant. Amazingly, seeds from *ppc* and *ppck1* mutants showed a general decrease in PUFA/NPUFA ratios, while *ppck2* mutants displayed higher amounts of unsaturated fatty acids as compared with Col-0 seeds (Fig. 6 B). It was found that the major unsaturated forms were the omega 3 fatty acids (18:3n3), with corresponding reduction in oleic acid (18; 1n9), but not omega 6 (18:2n6) pools, which remained quite stable (Fig. 6 D, E, F and see supplemental Fig. S3). Unsaturated lipids are considered to be better nutriment for human and animal health (Beermann et al., 2003), therefore these seeds represent improved quality in relation to the wild type.

Collectively, the molecular level analysis presented in this work describes by the first time that alteration in both, PEPC or PPCK changes the composition in N and C of seeds, highlighting the most altered phenotype of *ppck1* mutant and pointing to PEPC phosphorylation as an important event in the yield and quality of Arabidopsis seeds.

It is important to point some relationship between the molecular data and seed parameters. The decrease of C-compounds (Table 2) may be responsible for a reduction on seed weight of about 20%–33% in *ppc* and *ppck* mutants. The yield was also affected with a general reduction of 10%–38%, with the notable exception of *ppck1* mutant with a decrease in yield of 63% compared to the wild type. This strong decrease in yield is the consequence of altered metabolism in the leaves where phosphorylation of PEPC by PPCK1 may results in a key event of the anaplerotic function. PPCK1 is the only PPCK in leaves (Meimoun et al., 2009) and was not compensated by the presence of PPCK2. Therefore, *ppck1* mutant has an important restriction in the phosphorylation of PEPC, anaplerotic function and consequently, in protein synthesis. The consequence of this restriction was a very low yield and very low amount of protein in dry seed. In addition, the C-compounds in this *ppck1* mutant seem to be mobilized for lipid synthesis (Fig. 6A). The low level of C-compound also affects the ATP content in dry seeds being *ppc3*, *ppck1* and *ppck2* the most affected mutants.

Although the PPCK phosphorylates PEPC, most of the seed parameters and molecular changes were different between the mutant of these two enzymes, which could indicate that PEPC and PPCK might play roles independently. However, the point is that a PEPC isoenzyme can be compensated by other isoenzymes in different organs, e.g., both, PPC1 and PPC2 are present in the leaves (Shi et al., 2015). PPC3 is more specifically expressed in roots, however, PPC2 and PPC3 transcripts are also present (Feria et al., 2016). This PEPC activity compensation has been also shown in Fig. 1 for dry seeds. Even in *ppc*2 mutant, there is a rest of 40% of PPC activity. This is not the case for *ppck1* mutant. The fact that PPCK1 be the only PPCK with a role in leaves (Meimon et al., 2009) makes *ppck1* mutant phenotype deeply affected by the absence of PPCK1, which was not replaced by PPCK2. On the other hand, *ppc3* mutant had a stressed phenotype in control conditions (reduced root growth and high level of stress molecular markers; Feria et al., 2016) in consequence of its phenotype may be related to the altered absorption, anaplerotic function and transport of nitrogen in this mutant affecting the level of protein in dry seeds. In relation to the kinases both PPCK1and PPCK2 are present in the root (Feria et al., 2016).

The possibility that PPCK has other targets could also be considered. It has been described that the enzyme phosphoenolpyruvate carboxykinase plays a role in the metabolism of imported nitrogenous assimilated during the development of the seed. This enzyme is regulated by reversible phosphorylation, and it has a RKRS phosphorylation motive which could be phosphorylated by the kinases of the calcium-dependent protein kinase family (CDPK; Bailey et al., 2007). PPCK is included in the CDPK family although it is a $Ca2^+$ independent kinase ((Nimmo et al., 2001). Potentially, PPCK could phosphorylated the phosphoenolpyruvate carboxykinase. However, PPCK is a dedicated enzyme to PEPC with no other target known to date.

At the germination phase, an interaction with the shoot is no longer possible. The seed is now autonomous and can use nothing but what has

been left inside. It was observed that all *ppc* mutant seeds germinated with a decreased kinetics of germination (Fig. 4 and see Suppl. Fig. S2). All *ppc* mutants reach stage 2–3, with delay in growth of roots and coleoptile, while the wild type has already reached stage IV, (Figs. 4, 60 and 76 h). The efficiency of germination at this stage was also reduced in the mutants (Fig. 4, % of germination). These altered kinetic and efficiency of germination could be related with a decrease amount of C-compound and ATP (energy) in the seeds (Table 2 and Fig. 5). However, *ppck1*⁻ or *ppck2*⁻ seeds, which were deficient in reserve proteins, displayed normal germination kinetics. Although, this could be explained by the fact that *Arabidopsis thaliana* store their energy reserves in form of lipids (To at al., 2002) and total lipids level in these mutants (Fig. 6; TGA) are similar or increase compared to wild type seeds. Therefore, the energy level for germination of mutant seed should be similar to that of the control seeds. Alternatively, PEPC phosphorylation could not be required at this very early stage of germination or the remaining PPCK in each mutant could phosphorylate the PEPC to levels sufficient for germination to occur (Fig. 2 A). PEPC phosphorylation has been demonstrated to occur during germination of wheat (Osuna et al., 1996), barley (Feria et al., 2008), sorghum (Ruiz-Ballesta et al., 2016) and ricin (O'Leary et al., 2011). Nevertheless, there is no clear evidence for this mechanism to be essential for germination. Our previous work in *Amaranthus edulis* has demonstrated that PEPC were already phosphorylated in dry seeds, and PEPC phosphorylation did not occur in vivo during seed imbibition (Álvarez et al., 2011).

We have determined whether the addition of nitrate to Arabidopsis seeds during imbibition could have an accelerating effect in the germination process, as described by Alboresi et al. (2005). The positive effect of nitrate was visible for Col-0 seeds. Except for the *ppc*3 mutant, nitrate has a positive effect at early states of imbibition (12, 24 and 36 h) but no influence was detected after 2 days of imbibition unlike wild type. This was likely due to a diminished capacity for the anaplerotic function, and subsequently for nitrogen assimilation.

Finally, these data bring some valuable new elements to the interesting debate about the role of PEPC phosphorylation in plant physiology. Meimoun et al. (2009) showed that an Arabidopsis *ppck1* mutant displayed a clear alteration in growth and development. In the same line, our last work on Arabidopsis *ppc/ppck* mutants (Feria et al., 2016) showed similar results. In conclusion, our present work report that the presence of PPCK is highly required for normal plant life cycle, being PPCK1 the most involved in seed yield, and its presence a mandatory event for the filling and quality of the Arabidopsis seeds.

From a biotechnological point of view, it could be interesting to analyze whether PEPC/PPCK activity levels (organs and tissues) are optimized in Arabidopsis, or an overexpression of *PPC/PPCK* can stimulate growth and quality of Arabidopsis via an augmented anaplerotic capacity. According to the results presented in this work, bioengineering of crop plants with PEPC/PPCK as targets could be beneficial to control the nutritional quality of their seeds.

5. Conclusion

Macro and molecular phenotypic analysis of *Arabidopsis thaliana* knockout mutants show that PEPC and PPCK isoenzymes play a main role in the optimal functioning of C/N-metabolism, yield, and quality of seeds.

Author contribution statement

AB-F and C-E conceived and designed research. AB-F, I-RB, G-B and N-RL conducted experiments. AB-F and J-V helped discussing and designing experiments. AB-F and J-V wrote the manuscript. All authors read and approved the manuscript.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.08.012.

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