

In vivo* regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved *Arabidopsis thaliana

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PEPC [PEP(phosphoenolpyruvate) carboxylase] is a tightly controlled cytosolic enzyme situated at a major branchpoint in plant metabolism. Accumulating evidence indicates important functions for PEPC and PPCK (PEPC kinase) in plant acclimation to nutritional P_i deprivation. However, little is known about the genetic origin or phosphorylation status of native PEPCs from $-P_i$ (P_i -deficient) plants. The transfer of *Arabidopsis* suspension cells or seedlings to $-P_i$ growth media resulted in: (i) the marked transcriptional upregulation of genes encoding the PEPC isoenzyme AtPPC1 (*Arabidopsis thaliana* PEPC1), and PPCK isoenzymes AtPPCK1 and AtPPCK2; (ii) >2-fold increases in PEPC specific activity and in the amount of an immunoreactive 107-kDa PEPC polypeptide (p107); and (iii) *in vivo* p107 phosphorylation as revealed by immunoblotting of clarified extracts with phosphosite-specific antibodies to Ser-11 (which could be reversed following P_i resupply). Approx. 1.3 mg of PEPC was purified 660-fold from $-P_i$ suspension cells to apparent homogeneity with a specific activity of 22.3 units \cdot mg⁻¹

of protein. Gel filtration, SDS/PAGE and immunoblotting demonstrated that purified PEPC exists as a 440-kDa homotetramer composed of identical p107 subunits. Sequencing of p107 tryptic and Asp-N peptides by tandem MS established that this PEPC is encoded by *AtPPC1*. P_i -affinity PAGE coupled with immunoblotting indicated stoichiometric phosphorylation of the p107 subunits of AtPPC1 at its conserved Ser-11 phosphorylation site. Phosphorylation activated AtPPC1 at pH 7.3 by lowering its K_m (PEP) and its sensitivity to inhibition by L-malate and L-aspartate, while enhancing activation by glucose 6-phosphate. Our results indicate that the simultaneous induction and *in vivo* phosphorylation activation of AtPPC1 contribute to the metabolic adaptations of $-P_i$ *Arabidopsis*.

Key words: *Arabidopsis*, gene expression, P_i starvation, mass spectrometry, phosphoenolpyruvate carboxylase kinase (PPCK), protein phosphorylation.

INTRODUCTION

PEPC [PEP (phosphoenolpyruvate) carboxylase] (EC 4.1.1.31) is a ubiquitous cytosolic enzyme in vascular plants that is also distributed widely in green algae and bacteria. It catalyses the irreversible β -carboxylation of PEP in the presence of HCO_3^- to yield oxaloacetate and P_i . PEPC plays a crucial role in C_4 and CAM (crassulacean acid metabolism) photosynthesis, where it catalyses the initial fixation of atmospheric CO_2 . PEPC also fulfils several important non-photosynthetic functions, in particular the anaplerotic replenishment of tricarboxylic-acid-cycle intermediates consumed during biosynthesis and N_2 assimilation. Owing to its location at a pivotal branchpoint in primary plant metabolism, PEPC is tightly controlled by a combination of fine metabolic controls, including allosteric effectors and reversible phosphorylation [1–3]. Allosteric inhibition by L-malate and activation by Glc-6-P (glucose 6-phosphate) are routinely observed, whereas phosphorylation at a conserved N-terminal serine residue of the 100–110-kDa PEPC subunit is catalysed by PPCK (PEPC kinase) [1–4]. Phosphorylation typically modulates PEPC sensitivity to

allosteric effectors by relieving its inhibition by L-malate while simultaneously enhancing activation by hexose-phosphates. To date, all plant PPCKs that have been studied are novel approx. 31-kDa protein kinases that are controlled mainly at the level of synthesis and degradation. PPCK synthesis is mediated by endogenous circadian rhythms in leaves that undergo CAM, by light-sensing mechanisms in C_4 leaves, or by the presence or absence of phloem-supplied sucrose in soya-bean root nodules or developing castor beans [3,5–7].

Phosphorus is an essential element for growth and metabolism because it plays a central role in nearly all metabolic processes. Plants preferentially absorb phosphorus from the soil in its fully oxidized anionic form, P_i ($H_2PO_4^-$; orthophosphate). Despite its importance, P_i is one of the least available nutrients in many terrestrial and aquatic environments [8]. In soil, P_i is frequently complexed with Al^{3+} , Ca^{2+} or Fe^{3+} cations and, therefore exists as insoluble mineral forms that render it unavailable for plant uptake. Agricultural P_i deficiency is alleviated by the massive application of P_i fertilizers, estimated to be approx. 40 million metric tons per year worldwide [8]. Therefore, studies of the remarkably adaptive mechanisms that contribute to the

Abbreviations used: Ab, antibody; anti-RcPEPC IgG, anti-(*Ricinus communis* PEPC) IgG; AtPPC1, *Arabidopsis thaliana* PEPC1; CAM, crassulacean acid metabolism; DTT, dithiothreitol; Glc-6-P, glucose 6-phosphate; MALDI, matrix-assisted laser-desorption ionization; MS medium, Murashige and Skoog medium; MS/MS, tandem MS; p107, 107-kDa PEPC polypeptide; oMALDI 2, orthogonal MALDI 2; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; $+P_i$, P_i -sufficient; $-P_i$, P_i -deficient; PP2A, protein phosphatase type-2A; PPCK, PEPC kinase; Q-TOF, quadrupole time-of-flight; QqTOF, quadrupole/quadrupole TOF; RT, reverse transcription.

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survival of $-P_i$ (P_i -deficient) plants could facilitate the development of rational strategies and molecular tools for engineering P_i -efficient transgenic crops. Although these adaptations are not identical in all plants, particular aspects are conserved in a wide variety of plants from very different environments [8–11]. For example, enhanced levels of PEPC mRNA, protein and/or enzyme activity during P_i deprivation have been reported for diverse species, including *Brassica nigra* (black mustard), *Brassica napus* (rapeseed), *Arabidopsis thaliana* (thale cress), *Cicer arietinum* (chickpea), *Triticum aestivum* (wheat), *Lupinus albus* (white lupin), *Nicotiana sylvestris* (tobacco) and *Lycopersicon esculentum* (tomato) [12–20]. It has been suggested that PEPC provides a metabolic bypass (together with malate dehydrogenase and NAD-malic enzyme) to the ADP-limited cytosolic pyruvate kinase to facilitate continued pyruvate supply to the tricarboxylic acid cycle, while concurrently recycling the PEPC byproduct P_i for its reassimilation into the metabolism of the $-P_i$ cells [4,9,12]. PEPC induction has also been correlated with the synthesis and consequent excretion of large amounts of malic acid and citric acid by roots during P_i stress [8,9,14–16]. This increases P_i availability to the roots by acidifying the rhizosphere to solubilize otherwise inaccessible sources of mineralized soil P_i [8].

Genome-sequence annotation demonstrated that *Arabidopsis* contains four genes that encode PEPCs: *AtPPC1*, *AtPPC2* and *AtPPC3*, which encode plant-type PEPCs, and *AtPPC4*, which encodes a distantly related bacterial-type PEPC [21]. The deduced *AtPPC1*–*AtPPC3* polypeptides have a predicted size of 107–110 kDa and share considerable (85–91%) sequence identity, as well as the conserved N-terminal serine phosphorylation site that is characteristic of plant-type PEPCs. Although poor correlations exist for the majority of P_i -starvation-inducible genes identified in independent transcriptomic studies of $-P_i$ *Arabidopsis*, several reports have consistently identified plant-type *AtPPC* genes as being P_i -starvation-inducible [14,22,23]. In addition, L-malate-inhibition studies of PEPC activity in desalted extracts from *Arabidopsis* suspension cells implied that PEPC becomes post-translationally activated by phosphorylation during P_i stress [23]. This was corroborated by the marked induction of PPCK polypeptides and/or transcripts by $-P_i$ *Arabidopsis* [14,22,23]. The overall goal of the present study was to describe the genetic origin, biochemical and structural properties, and *in vivo* phosphorylation status of PEPC in $-P_i$ *Arabidopsis*.

EXPERIMENTAL

Plant material

Heterotrophic *A. thaliana* (cv. Landsberg erecta) suspension cells were maintained in the dark at 25 °C in MS medium (Murashige and Skoog medium) (pH 5.7) containing 5 mM K_2HPO_4 (added from a sterile 1 M stock at the time of subculturing), as described previously [24,25]. Cells used in time-course studies were obtained by transferring 20 ml (0.04% packed-cell volume) of a 7-day-old suspension into 80 ml of fresh medium containing either 5 mM K_2HPO_4 [$+P_i$ (P_i sufficient)] or no K_2HPO_4 ($-P_i$). Cells used for PEPC purification were obtained by scaling up the culture volume: 100 ml of a 7-day-old $+P_i$ cell suspension was used to inoculate 400 ml of $-P_i$ media in 3.2-l Fernbach flasks. Cells were harvested by filtration through Whatman 541 filter paper on a Büchner funnel, frozen in liquid N_2 and stored at -80 °C. *Arabidopsis* (cv. Columbia) seedlings were cultivated axenically in 50 ml of half-strength MS liquid medium (pH 5.7) containing 1% (w/v) sucrose and 0.2 mM K_2HPO_4 , as described previously [25]. At 7 days, the medium was replaced with fresh medium containing 3 mM ($+P_i$) or 20 μ M ($-P_i$) K_2HPO_4 . At

14 days, the medium was replaced with fresh medium, and at 21 days, roots and shoots of the $+P_i$ and $-P_i$ seedlings were harvested, quick-frozen in liquid N_2 , and stored at -80 °C.

RNA isolation and semi-quantitative RT (reverse transcription)–PCR

Total RNA was extracted and purified from *Arabidopsis* suspension cells and seedlings, as described previously [25]. RNA samples were assessed for purity (via their A_{260}/A_{280} ratio) and integrity by resolving 1 μ g of total RNA on a 1.2% (w/v) denaturing agarose gel. Normalization of RNA for RT was performed for each sample by density measurement of actin 2 bands from the above gel (scanned using ImageJ software from the National Institutes for Health, U.S.A.). Gene-specific primers were used to amplify *Arabidopsis* PEPCs (*AtPPC1*, *AtPPC2* and *AtPPC3*) [21], PPCKs (*AtPPCK1* and *AtPPCK2*) [5] and actin 2 [25]. RNA (5 μ g) was reverse transcribed with Superscript III (Invitrogen) and non-competitive RT–PCR was performed as described in [26]. The amount of input cDNA necessary for non-saturating amplification for each primer pair was established by performing PCR using 0.17–50 ng of total RNA during first-strand cDNA synthesis.

Enzyme and protein assays and kinetic studies

PEPC activity was assayed at 25 °C by following NADH oxidation at 340 nm using a kinetics microplate reader (Molecular Devices) and the following optimized assay conditions: 50 mM Hepes/KOH (pH 8.0) containing 15% (v/v) glycerol, 2 mM PEP, 2 mM $KHCO_3$, 5 mM $MgCl_2$, 2 mM DTT (dithiothreitol), 0.15 mM NADH and 5 units·ml⁻¹ of desalted porcine muscle malate dehydrogenase. One unit of activity is defined as the amount of PEPC resulting in the production of 1 μ mol of oxaloacetate per min. All assays were linear with respect to time and concentration of enzyme assayed. Apparent V_{max} , K_m , and IC_{50} and K_a values (concentrations of inhibitors and activators producing 50% inhibition and activation, respectively) were calculated using the kinetics program as described by Brooks [27]. All kinetic parameters were the means of a minimum of three independent experiments and were reproducible within $\pm 10\%$ S.E.M. of the mean value. Stock solutions of PEP, amino acids and organic acids were made equimolar with $MgCl_2$ and adjusted to pH 7.5. Protein concentrations were determined using a Coomassie Blue G-250 colorimetric method, as described previously [13].

Preparation of clarified extracts used in time-course studies

Quick-frozen tissues were ground to a powder in liquid N_2 and homogenized [1:2 (w/v) for suspension cells and leaves; 1:3 (w/v) for roots] using a mortar and pestle and a small scoop of sand in ice-cold Buffer A, which contained 50 mM Hepes/KOH (pH 8.0), 5 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM $NaVO_3^-$, 1 mM $NaMoO_4$, 50 nM microcystin-LR, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mM DTT, 2 mM 2,2'-dipyridyl disulphide, 1 mM PMSF and 1% (w/v) poly(vinyl pyrrolidone). Homogenates were centrifuged at 4 °C and 15 000 g for 15 min, and the resulting clarified extracts were rapidly prepared for SDS/PAGE and immunoblotting, or assayed for total protein and PEPC activity.

Buffers used during PEPC purification

Buffers were degassed and adjusted to their respective pH values at 24 °C. All buffers contained protein-phosphatase inhibitors (5 mM EDTA, 5 mM NaPP_i, 25 mM NaF, 1 mM

NaMoO₄ and 1 mM Na₂VO₄), whereas 50 nM microcystin-LR was added to all pooled column fractions (to inhibit further any co-purifying protein phosphatase activity). Buffer B contained 50 mM imidazole/HCl (pH 7.1), 1 mM DTT and 25% (saturation) (NH₄)₂SO₄. Buffer C consisted of Buffer B lacking (NH₄)₂SO₄ but containing 10% (v/v) ethylene glycol. Buffer D contained 100 mM Tris/HCl (pH 8.0), 1 mM DTT and 20% (v/v) glycerol. Buffer E contained 50 mM imidazole/HCl (pH 7.5), 5 mM KCl, 5 mM L-malate, 1 mM DTT and 15% (v/v) glycerol.

Purification of PEPC from $-P_i$ *Arabidopsis* suspension cells

All chromatographic steps were carried out at room temperature (24 °C) using an ÄKTA FPLC system (GE Healthcare). Quick-frozen 7-day-old $-P_i$ *Arabidopsis* suspension cells (240 g) were ground under liquid N₂ using a mortar and pestle, homogenized in 300 ml of ice-cold Buffer A using Polytron, and centrifuged at 4 °C and 18 000 g for 20 min. The supernatant was brought to 30% (saturation) (NH₄)₂SO₄, stirred for 20 min at 4 °C and centrifuged as above. The supernatant was adjusted to 60% (saturation) (NH₄)₂SO₄, stirred and centrifuged as above. The 30–60% (saturation) (NH₄)₂SO₄ pellets were resuspended in 100 ml of Buffer B containing 2.5 $\mu\text{l} \cdot \text{ml}^{-1}$ ProteCEASE-100 (G-Biosciences). After 15 min of centrifugation at 17 500 g, the sample was loaded at 3 ml $\cdot \text{min}^{-1}$ on to a column (3.2 \times 11.5 cm) of butyl-Sepharose 4 Fast Flow (GE Healthcare) pre-equilibrated with Buffer B. The column was washed until the A₂₈₀ decreased to baseline, and PEPC was eluted with 500 ml of a linear gradient of decreasing concentrations of Buffer B (100–0%) and simultaneously increasing concentrations of Buffer C (0–100%).

Pooled peak PEPC-activity fractions were concentrated to 3 ml using an Amicon YM-30 ultrafilter unit (Millipore), and brought to 5 ml with Buffer D and 5 $\mu\text{l} \cdot \text{ml}^{-1}$ ProteCEASE-100. After centrifugation at 17 500 g for 15 min, the sample was brought to 20 ml with Buffer D and loaded at 2 ml $\cdot \text{min}^{-1}$ on to a column (1.6 cm \times 4.4 cm) of Fractogel EMD DEAE-650(S) (Merck) that had been pre-equilibrated with Buffer D. The column was washed with Buffer D until the A₂₈₀ decreased to baseline, and PEPC was eluted by applying a linear 0–250 mM KCl gradient (160 ml in Buffer D). Fractions with PEPC activity were pooled and concentrated to 0.5 ml with an Amicon Ultra-15 100-kDa cut-off centrifugal filter device. The sample was adjusted to 1.2 ml with Buffer E, centrifuged (14 000 g, 5 min) and applied at 0.3 ml $\cdot \text{min}^{-1}$ on to a Superdex-200 HR 16/60 gel-filtration column (GE Healthcare) that had been pre-equilibrated with Buffer E.

Peak activity fractions were immediately loaded at 0.75 ml $\cdot \text{min}^{-1}$ on to a Mono Q HR 5/5 column (GE Healthcare) pre-equilibrated with Buffer D. PEPC was eluted with a linear 0–300 mM KCl gradient (25 ml in Buffer D). Peak activity fractions were pooled, concentrated as described above to 0.7 ml, divided into 25- μl and 50- μl aliquots, frozen in liquid N₂, and stored at -80 °C. The purified PEPC was stable for at least 6 months when stored frozen.

Determination of native molecular mass via Superdex-200 gel filtration

Native molecular-mass estimation for PEPC was performed during FPLC on the Superdex-200 column, as described above. Native molecular mass was estimated from a plot of K_{av} (partition coefficient) against log of molecular mass for the following protein standards: thyroglobulin (669 kDa), apoferritin (443 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa) and ribonuclease (13.6 kDa).

In vitro dephosphorylation of PEPC

Clarified extracts were prepared in Buffer A lacking phosphatase inhibitors. Aliquots (39 μl) containing 200 μg of protein were incubated for 60 min at 30 °C with 8000 units $\cdot \text{ml}^{-1}$ of λ -phosphatase (New England BioLabs) with or without protein-phosphatase inhibitors (50 mM NaF, 10 mM NaVO₃⁻ and 50 mM EDTA) in a final volume of 50 μl according to the manufacturer's instructions. An aliquot of purified PEPC from the $-P_i$ cells was dephosphorylated in 50 μl using 10 m-units $\cdot \text{ml}^{-1}$ of the catalytic subunit of bovine heart PP2A (protein phosphatase type-2A) as described previously [28]. PP2A was purified from 2 kg of freshly harvested bovine heart tissue following the protocol described by Tran et al. [29].

Electrophoresis and immunoblotting

SDS/PAGE and subunit molecular mass determination was performed using Mini-PROTEAN 3 gel electrophoresis (Bio-Rad) at 200 V for 50 min as described previously [13]. P_i -affinity SDS/PAGE was conducted at 75 V for 5 h, with the modification that the resolving gel (10% acrylamide) contained 75 μM Phos-tagTM acrylamide (www.phos-tag.com) and 150 μM MnCl₂ [30]. Immunoblotting was performed by electroblotting proteins from gels on to PVDF membranes, followed by immunoreactive polypeptide visualization using an alkaline-phosphatase-conjugated secondary Ab (antibody) with chromogenic detection [13]. Mn²⁺ that was present following P_i -affinity PAGE was removed prior to electroblotting by incubating the gels for 10 min in transfer buffer containing 1 mM EDTA, and then for 10 min in transfer buffer lacking EDTA. Anti-RcPEPC [*Ricinus communis* (castor bean) PEPC] IgG was affinity-purified from corresponding rabbit immune serum raised against a homogeneous native Class 1 PEPC (RcPPC3) [26]. Anti-pSer-11 IgG immunoblots were probed with a polyclonal Ab raised against a synthetic phosphopeptide matching the conserved N-terminal Ser-11 phosphorylation domain of RcPPC3 (LEKLApSIDAQLR) [28]. The corresponding dephosphopeptide was used to block any non-specific antibodies raised against the non-phosphorylated sequence [28]. The relative amount of PEPC protein in clarified extracts from $+P_i$ compared with $-P_i$ cells and seedlings was estimated by quantification of the immunoreactive 107-kDa PEPC polypeptides (p107) on immunoblots by measuring A₆₃₃ using an LKB Ultrascan XL laser densitometer and Gel Scan software (version 2.1) (Pharmacia LKB Biotech). Derived A₆₃₃ values were linear with respect to the amount of the immunoblotted extract. All immunoblot results were replicated a minimum of three times, with representative results shown in the various Figures.

MS

Excised gel bands were destained, dried, reduced and alkylated as described in [31]. Subsequent digestion was performed at 37 °C overnight using 10 ng of sequencing-grade trypsin (Calbiochem) in 25 mM NH₄HCO₃ (pH 7.6) or 50 ng of Asp-N (Roche) in 10 mM Tris/HCl (pH 7.6). The resulting peptides were extracted and deposited on a MALDI (matrix-assisted laser-desorption ionization) target [31]. MALDI data were acquired using a QStar XL Q-TOF (quadrupole time-of-flight) mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an oMALDI 2 (orthogonal MALDI 2) source and a nitrogen laser operating at 337 nm. Peptide sequencing of selected ions was carried out by MALDI-Q-TOF/QqTOF (quadrupole/quadrupole TOF)-MS/MS (tandem MS) measurements using argon as the collision gas. All peptide-fingerprinting masses and MS/MS

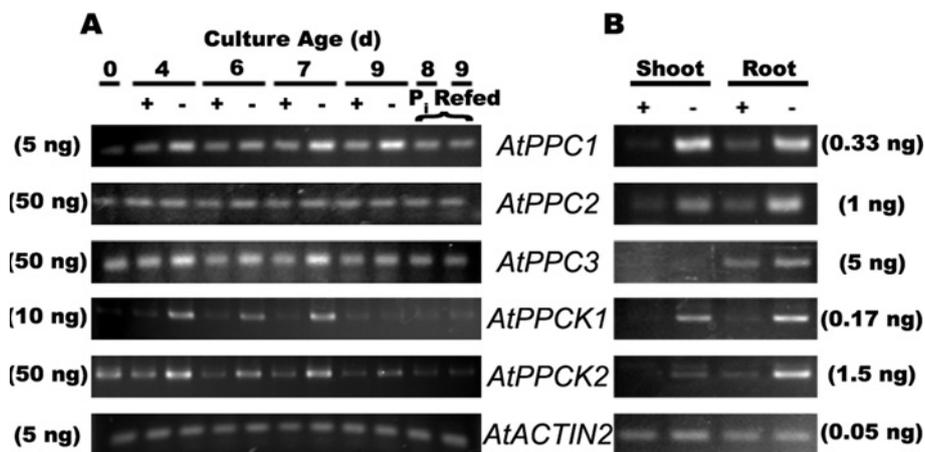


Figure 1 Semi-quantitative RT-PCR analysis of *AtPPC1–AtPPC3*, and *AtPPCK1* and *AtPPCK2* gene expression in + P_i and – P_i *Arabidopsis* suspension cells (A) and seedlings (B)

Levels of mRNA were measured using primers specific for *AtPPC1–AtPPC3*, *AtPPCK1* and *AtPPCK2*, and *AtACTIN2*. *AtACTIN2* was used as a reference to ensure equal template loading. All PCR products were measured at cycle numbers determined to be non-saturating. Template concentrations needed to achieve non-saturating conditions for primer pairs as tested for – P_i cells or roots of – P_i seedlings are indicated in parentheses. Control RT-PCR reactions lacking reverse transcriptase did not show any bands. 'P_i refed' (A) denotes 7-day-old – P_i suspension cells that were supplied with 2.5 mM P_i and cultured for an additional 24 and 48 h. d, day.

ion searches were performed with the Mascot search engine (MatrixScience, <http://www.matrixscience.com>) using the NCBI (National Center for Biotechnology Information) non-redundant database (NCBIInr, released 9 December 2008, which contains 7031513 protein sequences). These searches take into account one missed cleavage and the following modifications: carbamidomethylation; asparagine and glutamine deamidation to aspartic acid and glutamic acid; and N-terminal pyroglutamation and methionine oxidation. The mass tolerance between calculated and observed masses used for database searches was considered within the range of ± 50 p.p.m. for MS peaks and ± 0.1 Da for MS/MS fragment ions.

RESULTS

Semi-quantitative RT-PCR analysis of *AtPPC1–AtPPC3* and *AtPPCK1–AtPPCK2* transcripts in + P_i compared with – P_i *Arabidopsis* suspension cells and seedlings

AtPPC1, *AtPPC3*, *AtPPCK1* and *AtPPCK2* transcripts were elevated in the – P_i cells, and this was reversed within 1 day of P_i resupply (Figure 1A). Similarly, *AtPPC1*, *AtPPCK1* and *AtPPCK2* transcripts were induced in shoots and roots of the – P_i seedlings (Figure 1B). By contrast, *AtPPC2* transcripts were present at relatively low and constant levels in the + P_i and – P_i suspension cells, but appeared to be induced in the – P_i seedling tissues, whereas *AtPPC3* transcripts were detected in the roots but not in shoots. These results are in general agreement with a variety of transcriptomic studies of *AtPPC1–AtPPC3*, and *AtPPCK1* and *AtPPCK2* in *Arabidopsis* seedlings or cell cultures [14,21–23,32]. For example: (i) quantitative real-time PCR demonstrated that *AtPPC1* and *AtPPC2* transcripts were abundant in *Arabidopsis* leaves, whereas those encoding *AtPPC3* were negligible [32]; and (ii) *AtPPCK1* and *AtPPCK2* have been demonstrated to be amongst the most strongly induced genes in – P_i *Arabidopsis* [14,22,23].

Influence of P_i starvation on PEPC activity, amount and phosphorylation status in *Arabidopsis* cell cultures and seedlings

P_i deprivation resulted in a significant (>2-fold) up-regulation of PEPC specific activity in *Arabidopsis* suspension cells and

seedlings (which was reversed following P_i resupply) (Figures 2A and 2B). Laser-densitometric quantification of immunoblots revealed a good correlation between extractable PEPC activity and the relative amount of p107 (Figures 2A and 2B, insets). Analogous results have been described for the PEPC of – P_i white-lupin proteoid roots or *B. napus* suspension cells relative to corresponding + P_i controls [13,16]. Immunoblotting with anti-pSer-11 [28] indicated that P_i starvation resulted in enhanced p107 phosphorylation *in vivo*, and that this was also reversed following P_i resupply (Figure 2C). Immunodetection of p107 with anti-pSer-11 was eliminated when immunoblots were probed in the presence of anti-pSer-11 containing 10 $\mu\text{g} \cdot \text{ml}^{-1}$ of the corresponding blocking phosphopeptide and/or following pre-incubation of clarified extracts with λ -phosphatase for 60 min (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/420/bj4200057add.htm>).

PEPC purification from – P_i *Arabidopsis* cells and its identification as AtPPC1

To assess the influence of P_i deprivation on *Arabidopsis* PEPC more thoroughly, approx. 1.3 mg of PEPC was purified 660-fold from 240 g of 7-day-old – P_i suspension cells, with an overall recovery of 23% (Table 1). A single peak of PEPC activity was resolved during all FPLC steps. The final specific activity of approx. 22 units $\cdot \text{mg}^{-1}$ compares favourably with values reported for homogeneous PEPCs from a range of plant sources [1,13,26]. When the final PEPC preparation was denatured and subjected to SDS/PAGE, a single Coomassie-Blue-staining polypeptide was obtained that strongly cross-reacted with the anti-RcPEPC and that co-migrated with the p107 subunit of purified RcPEPC (Figure 3). A native molecular mass of 430 ± 15 kDa (mean \pm S.E.M., $n=3$) was estimated by gel-filtration FPLC on a calibrated Superdex-200 HR 16/60 column (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/420/bj4200057add.htm>). Thus, similar to most other plant PEPCs [1–3,13], the native enzyme from – P_i *Arabidopsis* suspension cells exists as a Class 1 PEPC homotetramer.

Discrimination between *Arabidopsis* AtPPC1–AtPPC3 via peptide mass fingerprinting is challenging, owing to the significant

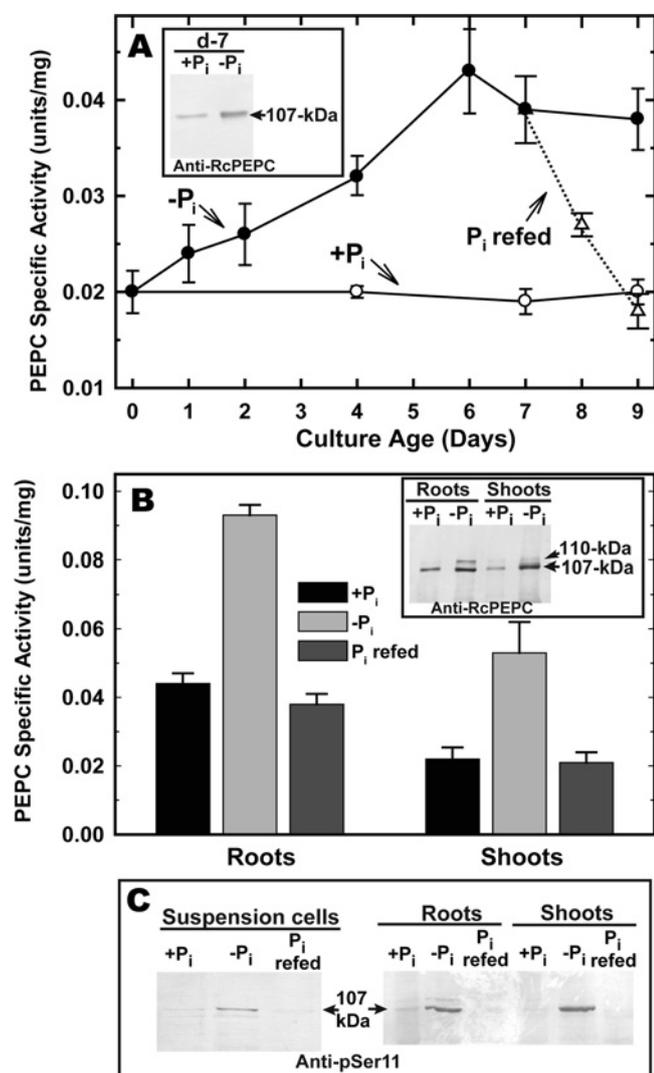


Figure 2 PEPC is reversibly up-regulated and phosphorylated by $-P_i$ *Arabidopsis*

All values represent the mean PEPC activities \pm S.E.M. of replicate determinations of clarified extracts from $n=4$ different cultures (A) or seedlings (B). At 0 days, 10-ml aliquots of suspension cells (A) cultured for 7 days in 5 mM P_i were subcultured into 40 ml of fresh MS medium containing 0 or 5 mM P_i ($-P_i$ and $+P_i$ respectively). Flasks of 7-day-old $-P_i$ cultures were resupplied with 2.5 mM P_i (P_i refed) and cultured for an additional 2 days as shown. (A, inset) Immunological detection of PEPC from the 7-day-old (d-7) $+P_i$ and $-P_i$ cells. Clarified extracts were subjected to SDS/PAGE (5 μ g of protein/lane) and blot-transferred on to a PVDF membrane. Blots were probed with affinity-purified anti-RcPEPC Ab [26] and immunoreactive polypeptides were detected using an alkaline-phosphatase-linked secondary Ab. Seedlings (B) were germinated and cultivated aseptically in liquid $0.5 \times$ MS medium containing 0.2 mM P_i for 7 days then transferred into fresh MS media containing 20 μ M P_i ($-P_i$) or 3 mM P_i ($+P_i$) and cultivated for an additional 14 days as described in the Experimental section. At 21 days, replicate $-P_i$ seedlings were resupplied with 3 mM P_i and cultivated for an additional 3 days (P_i refed). (B, inset) Immunological detection of PEPC from $+P_i$ compared with $-P_i$ roots and shoots (5 μ g of protein/lane) using anti-RcPEPC Ab as described above. (C) Clarified extracts from the $-P_i$, $+P_i$ and P_i -refed suspension cells or seedlings were subjected to SDS/PAGE and electroblotted on to a PVDF membrane. Immunoblots were probed with an anti-pSer-11 Ab raised against the conserved N-terminal Ser-11 phosphorylation domain of a plant-type RcPEPC isoenzyme, in the presence of 10 μ g \cdot ml $^{-1}$ of the corresponding dephosphopeptide [28]. Each lane of (C) was loaded according to the PEPC activity measured in the corresponding clarified extracts, as determined using the optimal assay conditions outlined in the Experimental section (0.2 m-units/lane for suspension cells; 0.7 m-units/lane for roots and shoots).

degree of sequence identity ($>85\%$) between these closely related isoenzymes (Figure 4). Nevertheless, the use of MALDI-TOF-MS for mass fingerprinting of tryptic peptides derived from a PEPC immunoprecipitate of *Arabidopsis* rosette leaves led to the identification of all three plant-type PEPC isoenzymes [32]. Determination of the AtPPC isoenzyme that the final PEPC preparation of the present study corresponded to was also attempted by peptide mass fingerprinting using MALDI-QqTOF-MS of both tryptic and Asp-N digests (combined 78% sequence coverage) (see Supplementary Tables S1 and S2 at <http://www.BiochemJ.org/bj/420/bj4200057add.htm>), but proved inconclusive. Thus we employed MALDI-QqTOF-MS/MS to sequence numerous peptides derived from the p107 obtained following SDS/PAGE of the final PEPC preparation. The 21 tryptic peptides that were sequenced all matched AtPPC1 [TAIR (The *Arabidopsis* Information Resource; www.arabidopsis.org) accession number At1g53310; 45% sequence coverage] (Supplementary Table S1), with nine peptides unique to AtPPC1 (Figure 4). Similarly, all 12 Asp-N peptides that were sequenced matched AtPPC1 (41% sequence coverage) (Supplementary Table S2), with six peptides unique to AtPPC1 (Figure 4). In contrast, no peptides that were exclusive to any other polypeptide (including AtPPC2 or AtPPC3) were detected in tryptic or Asp-N digests of p107. These results are consistent with those demonstrating that P_i deprivation of *Arabidopsis* suspension cells or seedlings resulted in the marked (i) transcriptional induction of *AtPPC1* (Figure 1) and (ii) accumulation of an anti-RcPEPC immunoreactive p107 (Figure 2). The collective results indicate that the simultaneous induction and *in vivo* phosphorylation of AtPPC1 form part of the metabolic adaptation of $-P_i$ *Arabidopsis*.

Purified AtPPC1 from $-P_i$ *Arabidopsis* cells is phosphorylated at Ser-11

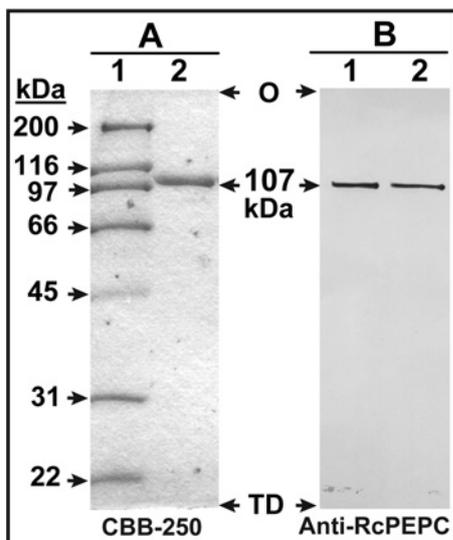
The use of anti-pSer-11 Ab for examining the phosphorylation status of p107 in purified AtPPC1 was complemented with: (i) an affinity-purified anti-RcPEPC Ab raised against a native PEPC (RcPPC3) from developing castor-oil seeds [28] that cross-reacts with phospho- and dephospho-forms of plant-type PEPCs; and (ii) P_i -affinity PAGE using Phos-TagTM acrylamide (Figures 5A and 5B). The Phos-TagTM ligand is a polyacrylamide-bound dinuclear Mn^{2+} complex that specifically captures phosphomonoester di-anions ($-OPO_3^{2-}$), thereby reducing phosphoprotein mobility during SDS/PAGE relative to the corresponding dephosphoprotein [30]. Pre-incubation of purified AtPPC1 with PP2A for 30 min resulted in the time-dependent disappearance of immunoreactive p107 when the immunoblots were probed with anti-pSer-11, but not with anti-RcPEPC (Figures 5A and 5B). P_i -affinity PAGE indicated that nearly 100% of the p107 subunits of purified AtPPC1 migrated as a putatively monophosphorylated species that was converted into the faster-migrating dephospho-p107 during its pre-incubation with PP2A (Figure 5B). Plant PPCKs have been suggested to phosphorylate specifically the serine residue in the conserved PEPC N-terminal phosphorylation motif: (E/D)-(K/R)-X-Z-SIDAQLR, where X denotes M/H/L and Z denotes A/Q/S/H [2]. As the Ala-Gln residues in this motif have been substituted with Val-His in AtPPC1 (Figure 4), it has been implied that AtPPC1 may be incapable of undergoing regulatory phosphorylation [33]. However, our results demonstrate that AtPPC1 is an effective *in vivo* substrate for the endogenous PPCK activity of $-P_i$ *Arabidopsis*.

It is notable that the aforementioned results were dependent upon the addition of a cocktail of protein-phosphatase inhibitors

Table 1 Purification of PEPC from 240 g of $-P_i$ *Arabidopsis* suspension cells

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units · mg ⁻¹)	Purification (fold)	Yield (%)
Clarified extract	375	120	6750	0.034	1.0	100
(NH ₄) ₂ SO ₄ fractionation	126	74.3	3260	0.023	0.7	62
butyl-Sepharose	5.0*	34.7	200	0.17	5.1	29
DEAE-Fractogel	1.2*	46.3	17.0	2.7	80	39
Superdex-200 FPLC	5.5	40.0	2.8	14.5	450	33
Mono Q FPLC	0.7*	28.0	1.3	22.3	660	23

*Concentrated pooled fractions.

**Figure 3** SDS/PAGE and immunoblot analysis of purified PEPC from $-P_i$ *Arabidopsis* suspension cells

(A) SDS/PAGE: lane 1 contains various molecular mass standards (3 µg) whereas lane 2 contains 2 µg of the pooled peak fractions from the final purification step (Mono Q FPLC). Protein staining was performed using Coomassie Brilliant Blue R-250 (CBB-250). (B) Immunoblotting was performed using an anti-RcPEPC Ab [26]; lanes 1 and 2 contain 25 ng of the final preparation of $-P_i$ *Arabidopsis* PEPC and homogeneous RcPEPC [26] respectively. O, origin; TD, tracking-dye front.

to all purification buffers and pooled column fractions generated during AtPPC1 isolation from the $-P_i$ cells (see the Experimental section). Our initial purification trials employed a protein-phosphatase inhibitor cocktail in the extraction buffer and 20 mM NaF in column running buffers, but resulted in final AtPPC1 preparations displaying sub-stoichiometric p107 phosphorylation (as assessed by immunoblotting with anti-pSer-11, P_i -affinity PAGE, or kinetic studies with or without PP2A treatment) (results not shown). The combined results indicate that an endogenous protein-phosphatase activity capable of catalysing the *in vitro* dephosphorylation of AtPPC1 co-purified with AtPPC1, and that this activity was not fully inhibited by 20 mM NaF. This also provides a possible rationale for the absence of detectable phosphorylation in homogeneous PEPC preparations isolated from heterotrophic $+P_i$ or $-P_i$ *B. napus* suspension cells using a protocol that employed only 20 mM NaF in column running buffers to control unwanted phosphatase activity [13]. Our results support recommendations that effective *in vitro* suppression of endogenous protein-phosphatase activity necessitates the inclusion of a wide assortment of protein-phosphatase inhibitors (e.g. NaF, MoO₄⁻, VO₃⁻, PP_i and microcystin-LR) in extraction and purification buffers [34].

Table 2 Kinetic properties of purified phosphorylated AtPPC1 compared with dephosphorylated AtPPC1

Dephospho-AtPPC1 was obtained following *in vitro* dephosphorylation of purified AtPPC1 with bovine PP2A as described in the Experimental section and the legend to Figure 5. K_a and IC₅₀ values were determined using subsaturating (0.25 mM) PEP. All values were determined at pH 7.3 and represent means ± S.E.M. of $n = 4$ separate determinations. All values are given in mM.

Parameter	Phospho-AtPPC1	Dephospho-AtPPC1
K_m (PEP)	0.18 ± 0.02	0.34 ± 0.01
K_a (Glc-6-P)	0.028* ± 0.001	ND*†
IC ₅₀ (L-Asp)	1.14 ± 0.01	0.52 ± 0.04
IC ₅₀ (L-malate) + 0 mM Glc-6-P	0.68 ± 0.04	0.30 ± 0.03
IC ₅₀ (L-malate) + 0.2 mM Glc-6-P	1.5 ± 0.04	0.61 ± 0.01
IC ₅₀ (L-malate) + 2 mM Glc-6-P	9.1 ± 0.3	5.5 ± 0.2

*Saturating Glc-6-P elicited 1.8- and 1.2-fold increases in the activity of phospho- and dephospho-AtPPC1 respectively.

†ND, not determined. Weak Glc-6-P activation prevented accurate determination of the K_a (Glc-6-P) value of dephospho-AtPPC1.

Kinetic properties

Similar to other plant PEPCs [1,13,28,35], the purified AtPPC1 exhibited: (i) a broad pH/activity profile with a maximum between pH 8.0 and 9.0; PEPC activity at pH 7.0 was approx. 50% of that occurring at pH 8.5; (ii) an absolute dependence on a bivalent metal cation, with Mg²⁺ or Mn²⁺ satisfying this requirement; and (iii) hyperbolic PEP-saturation kinetics (results not shown). The AtPPC1 displayed a K_m (PEP) value of 0.054 ± 0.005 mM (mean ± S.E.M., $n = 4$) at pH 8.5. It has been amply demonstrated that phosphorylation of plant PEPCs reduces the sensitivity of the enzymes to L-malate when assayed at subsaturating PEP and suboptimal, but physiological pH values ranging from approx. pH 7.0 to 7.4 [1–3,28,35]. Phosphorylation can also relieve L-Asp inhibition of plant PEPCs, while simultaneously promoting binding of its substrate PEP and activator Glc-6-P at physiological pH (e.g. pH 7.3) [28,35]. Results presented in Table 2 demonstrate that *in vivo* phosphorylation of AtPPC1 during P_i stress also activates this enzyme at pH 7.3 by significantly lowering its K_m (PEP) value and sensitivity to inhibition by L-malate and L-Asp, while increasing its activation by Glc-6-P.

DISCUSSION

It has been suggested that PEPC plays three important roles in plant acclimation to P_i deficiency: (i) anaplerotic production of large quantities of organic acids for excretion via the roots into the rhizosphere to facilitate soil P_i solubilization; (ii) part of a glycolytic bypass to the ADP-limited cytosolic pyruvate kinase; and (iii) metabolic P_i recycling from PEP [4,8–20,23].

many protein kinases that are allosterically controlled by second messengers such Ca²⁺ ions, control of plant PPCK activity, and hence the phosphorylation status of target PEPCs, appears to arise largely from PPCK synthesis/degradation [2,3,5]. Chen et al. [23] identified a transcription factor, BHLH32, that functions as a negative regulator of several P_i-starvation-inducible genes in *Arabidopsis*, including *AtPPCK1* and *AtPPCK2*. Characterization of additional components of the signal-transduction pathways that link nutritional P_i status with the control of *AtPPCK1*, and *AtPPCK1* and *AtPPCK2* transcription and translation, as well as *AtPPCK1*, and *AtPPCK1* and *AtPPCK2* turnover, will be a fruitful area for future research.

Important insights into the P_i-starvation response of *Arabidopsis* and other vascular plants have been provided by the classification of genes displaying altered transcription during P_i deprivation [11,14,19,36–41]. However, the integration of transcriptomics with metabolomics, proteomics and native-enzyme biochemistry will be needed to achieve a thorough understanding of the intricate mechanisms by which plant metabolism acclimates to nutritional P_i deficiency. Future investigations of these pathways should provide further links between the molecular and biochemical control of plant metabolism. This knowledge is relevant to the applied efforts of agricultural biotechnologists to engineer transgenic crops exhibiting an improved resistance to abiotic stress, including nutritional P_i deprivation.

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REFERENCES

- Chollet, R., Vidal, J. and O'Leary, M. H. (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 273–298
- Izui, K., Matsumura, H., Furumoto, T. and Kai, Y. (2004) Phosphoenolpyruvate carboxylase: a new era of structural biology. *Annu. Rev. Plant Biol.* **55**, 69–84
- Nimmo, H. G. (2003) Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. *Arch. Biochem. Biophys.* **414**, 189–196
- Plaxton, W. C. and Podestá, F. E. (2006) The functional organization and control of plant respiration. *Crit. Rev. Plant Sci.* **25**, 159–198
- Zhang, X. Q. and Chollet, R. (1997) Phosphoenolpyruvate carboxylase protein kinase from soybean root nodules: partial purification, characterization, and up/down-regulation by photosynthate supply from the shoots. *Arch. Biochem. Biophys.* **343**, 260–268
- Murmu, J. and Plaxton, W. C. (2007) Phosphoenolpyruvate carboxylase protein kinase from developing castor oil seeds: partial purification, characterization, and reversible control by photosynthate supply. *Planta* **226**, 1299–1310
- Fontaine, V., Hartwell, J., Jenkins, G. I. and Nimmo, H. G. (2002) *Arabidopsis thaliana* contains two phosphoenolpyruvate carboxylase kinase genes with different expression patterns. *Plant Cell Environ.* **25**, 115–122
- Vance, C. P., Uhde-Stone, C. and Allan, D. L. (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol.* **157**, 427–447
- Plaxton, W. C. (2004) Biochemical adaptations of phosphate starved plants. In *Encyclopedia of Plant and Crop Sciences* (Goodman, R., ed.), pp. 976–980, Marcel Dekker, New York
- Ticconi, C. A. and Abel, S. (2004) Short on phosphate: plant surveillance and countermeasures. *Trends Plant Sci.* **9**, 548–555
- Raghothama, K. G. and Karthikeyan, A. S. (2005) Phosphate acquisition. *Plant Soil* **274**, 37–49
- Duff, S. M. G., Moorhead, G. B., Lefebvre, D. D. and Plaxton, W. C. (1989) Phosphate starvation inducible 'bypasses' of adenylate and phosphate dependent glycolytic enzymes in *Brassica nigra* suspension cells. *Plant Physiol.* **90**, 1275–1278
- Moraes, T. F. and Plaxton, W. C. (2000) Purification and characterization of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures: implications for phosphoenolpyruvate carboxylase regulation during phosphate starvation, and the integration of glycolysis with nitrogen assimilation. *Eur. J. Biochem.* **267**, 4465–4476
- Morcuende, R., Bari, R., Gibon, Y., Zheng, W., Pant, B. D., Blasing, O., Usadel, B., Czechowski, T., Udvardi, M. K., Stitt, M. and Scheible, W. R. (2007) Genome-wide reprogramming of metabolism and regulatory networks of *Arabidopsis* in response to phosphorus. *Plant Cell Environ.* **30**, 85–112
- Neuman, G. and Romheld, V. (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant Soil* **211**, 121–130
- Johnson, J. F., Vance, C. P. and Allan, D. L. (1996) Phosphorus deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiol.* **112**, 31–41
- Uhde-Stone, C., Gilbert, G., Johnson, J. M., Lijtsen, R., Zinn, K. E., Temple, S. J., Vance, C. P. and Allan, D. L. (2003) Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. *Plant Soil* **248**, 99–116
- Penalzo, E., Munoz, G., Salvo-Garrido, H., Silva, H. and Corcuera, L. J. (2005) Phosphate deficiency regulates phosphoenolpyruvate carboxylase expression in proteoid root clusters of white lupin. *J. Exp. Bot.* **56**, 145–153
- Toyota, K., Koizumi, N. and Sato, F. (2003) Transcriptional activation of phosphoenolpyruvate carboxylase by phosphorus deficiency in tobacco. *J. Exp. Bot.* **54**, 961–969
- Pilbeam, D. J., Cakmak, I., Marschner, H. and Kirkby, E. A. (1993) Effect of withdrawal of phosphorus on nitrate assimilation and PEP carboxylase activity in tomato. *Plant Soil* **154**, 111–117
- Sanchez, R. and Cejudo, F. J. (2003) Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiol.* **132**, 949–957
- Müller, R., Morant, M., Jarmer, H., Nilsson, L. and Nielsen, T. H. (2007) Genome-wide analysis of the *Arabidopsis* leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiol.* **143**, 156–171
- Chen, Z. H., Nimmo, G. A., Jenkins, G. I. and Nimmo, H. G. (2007) BHLH32 modulates several biochemical and morphological processes that respond to P_i starvation in *Arabidopsis*. *Biochem. J.* **405**, 191–198
- Tran, H. T. and Plaxton, W. C. (2008) Proteomic analysis of alterations in the secretome of *Arabidopsis thaliana* suspension cells subjected to nutritional phosphate deficiency. *Proteomics* **8**, 4317–4326
- Veljanovski, V., Vanderbeld, B., Knowles, V. L., Snedden, W. A. and Plaxton, W. C. (2006) Biochemical and molecular characterization of ATPAP26, a vacuolar purple acid phosphatase up-regulated in phosphate-deprived *Arabidopsis* suspension cells and seedlings. *Plant Physiol.* **142**, 1282–1293
- Gennidakis, S., Rao, S., Greenham, K., Uhrig, R. G., O'Leary, B., Snedden, W. A., Lu, C. and Plaxton, W. C. (2007) Bacterial- and plant-type phosphoenolpyruvate carboxylase polypeptides interact in the hetero-oligomeric Class-2 PEPC complex of developing castor oil seeds. *Plant J.* **52**, 839–849
- Brooks, S. (1992) A simple computer program for the analysis of enzyme kinetics. *Biotechniques* **13**, 906–911
- Tripodi, K. E., Turner, W. L., Gennidakis, S. and Plaxton, W. C. (2005) *In vivo* regulatory phosphorylation of novel phosphoenolpyruvate carboxylase isoforms in endosperm of developing castor oil seeds. *Plant Physiol.* **139**, 969–978
- Tran, H. T., Ferrar, T. S., Ulke-Lemee, A. and Moorhead, G. B. (2007) Purification of PP2Ac from bovine heart. *Methods Mol. Biol.* **365**, 127–132
- Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. and Koike, T. (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell. Proteomics* **5**, 749–757
- Uhrig, R. G., She, Y. M., Leach, C. A. and Plaxton, W. C. (2008) Regulatory monoubiquitination of phosphoenolpyruvate carboxylase in germinating castor oil seeds. *J. Biol. Chem.* **283**, 29650–29657
- Gousset-Dupont, A., Lebouteiller, B., Monreal, J., Echevarria, C., Pierre, J. N., Hodges, M. and Vidal, J. (2005) Metabolite and post-translational control of phosphoenolpyruvate carboxylase from leaves and mesophyll cell protoplasts of *Arabidopsis thaliana*. *Plant Sci.* **169**, 1096–1101

- 33 Sánchez, R., Flores, A. and Cejudo, F. J. (2006) *Arabidopsis* phosphoenolpyruvate carboxylase genes encode immunologically unrelated polypeptides and are differentially expressed in response to drought and salt stress. *Planta* **223**, 901–909
- 34 Haystead, T. A. G. and Garrison, J. C. (2000) Study of protein phosphorylation in intact cells. In *Protein Phosphorylation: A Practical Approach* (2nd edn), (Hardie and D.G., ed., eds.), pp. 1–30, Oxford University Press, Oxford
- 35 Law, R. D. and Plaxton, W. C. (1997) Regulatory phosphorylation of banana fruit phosphoenolpyruvate carboxylase by a copurifying phosphoenolpyruvate carboxylase-kinase. *Eur. J. Biochem.* **247**, 642–651
- 36 Hammond, J. P., Broadley, M. R. and White, P. J. (2004) Genetic responses to phosphorus deficiency. *Ann. Bot.* **94**, 323–332
- 37 Hernandez, G., Ramirez, M., Valdes-Lopez, O., Tesfaye, M., Graham, M. A., Czechowski, T., Schlereth, A., Wandrey, M., Erban, A., Cheung, F. et al. (2007) Phosphorus stress in common bean: root transcript and metabolic responses. *Plant Physiol.* **144**, 752–767
- 38 Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N. et al. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc. Nat. Acad. Sci. U. S. A.* **102**, 11934–11939
- 39 Penalzoza, E., Gutierrez, A., Martinez, J., Munoz, G., Bravo, L. A. and Corcuera, L. J. (2002) Differential gene expression in proteoid root clusters of white lupin (*Lupinus albus*). *Physiol. Plant.* **116**, 28–36
- 40 Wasaki, J., Shinano, T., Onishi, K., Yonetani, R., Yazaki, J., Fujii, F., Shimbo, K., Ishikawa, M., Shimatani, Z. and Nagata, Y. et al. (2006) Transcriptomic analysis indicates putative metabolic changes caused by manipulation of phosphorus availability in rice leaves. *J. Exp. Bot.* **57**, 2049–2059
- 41 Wu, P., Ma, L., Hou, X., Wang, M., Wu, Y., Liu, F. and Deng, X. W. (2003) Phosphate starvation triggers distinct alterations of genome expression in *Arabidopsis* roots and leaves. *Plant Physiol.* **132**, 1260–1271

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SUPPLEMENTARY ONLINE DATA

In vivo regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved *Arabidopsis thaliana*

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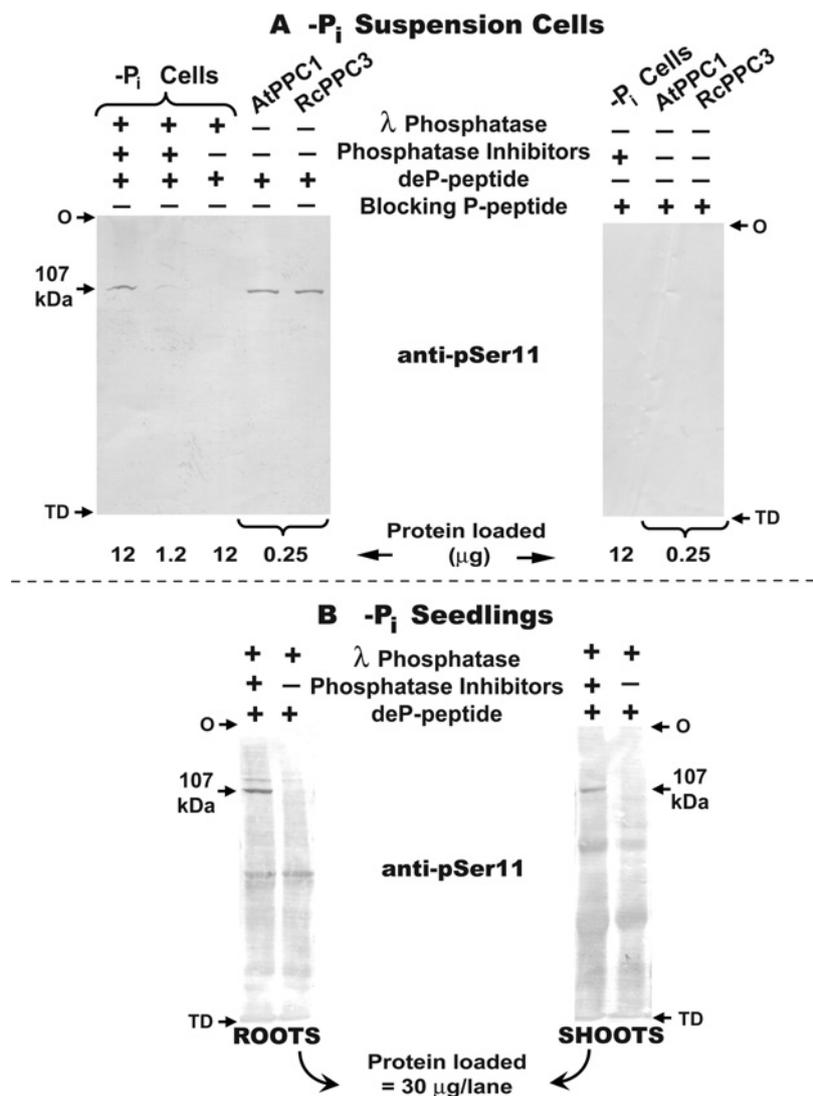


Figure S1 Anti-pSer-11 antibody specificity

Clarified extracts from 7-day-old -P_i suspension cells (A) or roots and shoots of 21-day-old -P_i seedlings (B) were incubated for 1 h with λ-phosphatase with or without protein phosphatase inhibitors as described in the Experimental section in the main article, subjected to SDS/PAGE and electroblotted on to a PVDF membrane. Immunoblots were probed with anti-pSer-11 IgG in the presence of 10 μg · ml⁻¹ of the corresponding dephosphopeptide or blocking phosphopeptide (P-peptide) [28] as indicated. AtPPC1 and RcPPC3 denote homogeneous native Class 1 PEPC preparations isolated from the -P_i *Arabidopsis* suspension cells (see Table 1 and Figure 2 in the main article) or the endosperm of developing castor-oil seeds respectively [26]. deP-peptide, dephosphopeptide; O, origin; P-peptide, phosphopeptide; TD, tracking-dye front.

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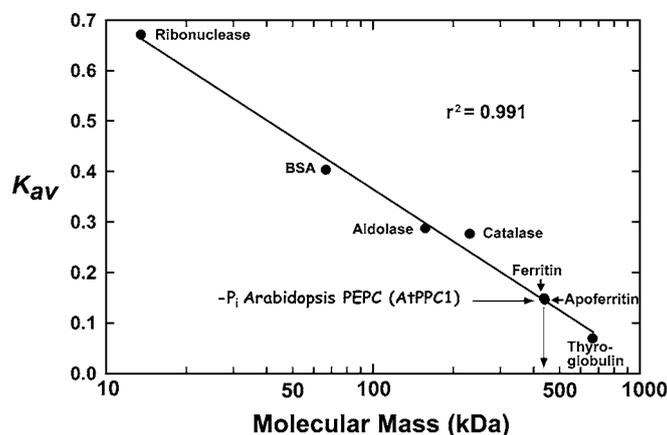


Figure S2 Native molecular-mass estimation for PEPC from $-P_i$ *Arabidopsis* suspension cells

Native molecular-mass estimation was performed during FPLC on a calibrated Superdex-200 HR 16/60 gel-filtration column as described in the Experimental section in the main article. r^2 , correlation coefficient.

Table S1 MALDI-QqTOF-MS identified tryptic peptides (derived from purified native PEPC from $-P_i$ *Arabidopsis* suspension cells) matching the deduced amino-acid sequence of AtPPC1

MS/MS-sequenced peptides are in bold font, with those unique to AtPPC1 italicized. Calc., calculated; meas., measured.

Position	<i>m/z</i> (Meas.)	[MH ⁺] (Calc.)	Delta	Peptide sequence
734-739	860.431	860.430	+0.001	FVEYFR
726-733	961.506	961.510	-0.004	SVVFQEPR
494-503	1129.628	1129.636	-0.008	RPLFGSDLPK
153-162	1148.587	1148.583	+0.004	LATPELEYGR
564-576	1267.686	1267.700	-0.014	LADLEAAPAAVAR
553-563	1325.785	1325.794	-0.009	QLLRVVPLFEK
189-199	1350.698	1350.719	-0.021	IRDCLAQLYAK
482-493	1445.761	1445.774	-0.013	RQEWLLSELGK
603-615	1478.766	1478.775	-0.009	DAGRLSAAWQLYK
770-781	1489.783	1489.795	-0.012	AIPWIFAWTQTR
782-795	1599.872	1599.879	-0.007	FHLPVWLGFGSAIR
711-725	1709.835	1709.841	-0.006	ALLDEMAVVATEEYR
726-739	1802.916	1802.922	-0.006	SVVFQEPRFVEYFR
360-375	1815.970	1815.996	-0.026	SIPTTEPYRVILGDVR
690-706	1852.956	1852.960	-0.004	FTAATLEHGMRPPISPCK
163-179	1878.976	1878.976	0.000	NQTVDLVLTAAHTQSVR
908-923	1919.028	1919.061	-0.033	IRDPSYHVTLRPHISK
734-749	1989.989	1990.006	-0.017	FVEYFRLATPELEYGR
163-180	2035.091	2035.104	-0.013	NQTVDLVLTAAHTQSVRR
360-377	2059.102	2059.118	-0.016	SIPTTEPYRVILGDVRDK
889-906	2159.085	2159.116	-0.031	LRDSYITLLNVCQAYTLK
644-665	2201.132	2201.167	-0.035	GGGPTHLLAILSQPPDTINGSLR
476-493	2262.100	2262.114	-0.014	EWSEERRQEWLLSELGK
97-116	2290.134	2290.164	-0.030	AFSHMLNLANLAEEVQIAYR
97-116	2306.159	2306.159	0.000	AFSHMLNLANLAEEVQIAYR
666-685	2364.100	2364.128	-0.028	VTVQGEVIEQSFGEELCFR
690-710	2421.215	2421.249	-0.034	FTAATLEHGMRPPISPCKPEWR
97-117	2446.239	2446.266	-0.027	AFSHMLNLANLAEEVQIAYRR
97-117	2462.225	2462.260	-0.035	AFSHMLNLANLAEEVQIAYRR
207-227	2503.256	2503.278	-0.022	QELDEALQREIQAAFRTDEIK
272-294	2577.202	2577.230	-0.028	VPYNAPLIQFSSWMGGDRDGNPR
229-250	2622.243	2622.211	+0.032	TPPTPQDEMRRAGMSYFHETIWK
711-733	2652.307	2652.333	-0.026	ALLDEMAVVATEEYRSVVFQEPR
934-957	2690.323	2690.384	-0.061	ELIELNPTSEYAPGLEDTLILTMK
228-250	2778.299	2778.312	-0.013	RTPTTPQDEMRRAGMSYFHETIWK
30-54	2984.489	2984.572	-0.083	LVEYDALLLDRFLDILQDLHGEDLR
302-330	3626.669	3626.562	+0.107	DVCLLARMMAATMYFNQIEDLMFEMSMWR

Table S2 MALDI QqTOF MS identified Asp-N peptides (derived from purified native PEPC from – P_i *Arabidopsis* suspension cells) matching AtPPC1

MS/MS-sequenced peptides are in bold font, with those unique to AtPPC1 italicized. Calc., calculated; meas., measured.

Position	<i>m/z</i> (Meas.)	[MH ⁺] (Calc.)	Delta	Peptide sequence
333–339	830.466	830.447	–0.019	DELARA
500–508	1015.546	1015.530	+0.016	DLPKTEEIA
340–348	1055.581	1055.522	+0.059	DEVHANSRK
470–478	<i>1112.521</i>	<i>1112.500</i>	+0.021	<i>DIGSYREWS</i>
801–809	1125.601	1125.583	+0.018	DVRNLHMLQ
149–158	1191.621	1191.589	+0.032	DLNKSPEEIF
512–522	1212.642	1212.625	+0.017	DTFHVIAELPA
52–61	1265.660	1265.637	+0.023	DIRETVQELY
138–148	1278.706	1278.694	+0.012	DLEETFKKLVG
224–234	<i>1281.690</i>	<i>1281.679</i>	+0.011	<i>DEIKRTPPTPQ</i>
2–12	1302.738	1302.719	+0.019	ANRLEKMASI
290–301	1340.709	1340.691	+0.018	DGNPRVTPVTR
880–890	1457.884	1457.869	+0.015	DPYLRQLRLR
566–580	<i>1529.851</i>	<i>1529.832</i>	+0.019	<i>DLEAAPAAVARLFSV</i>
13–26	<i>1576.893</i>	<i>1576.880</i>	+0.013	<i>DVHLRQLVPGKVSE</i>
210–223	1647.857	1647.844	+0.013	DEALKREIQAAFRT
13–27	1691.888	1691.907	–0.019	DVHLRQLVPGKVSSED
810–822	1739.861	1739.836	+0.025	DMYQHWPFRRVI
434–449	1851.064	1851.048	+0.016	DFLRQVSTFGLSLVRL
696–713	<i>2114.132</i>	<i>2114.132</i>	0.000	<i>EHGMRPPISPKEWRAIL</i>
581–598	2201.089	2201.080	+0.009	DWYKNRINGKQEVMIQYS
376–394	2262.162	2262.148	+0.014	DKLYHTRERAHQLLSNGHS
891–909	2315.232	2315.217	+0.015	DSYITTLNVCOAYTLKRIR
138–158	2451.258	2451.265	–0.007	DLEETFKKLVGDLNKSPEEIF
479–499	<i>2517.331</i>	<i>2517.320</i>	+0.011	<i>EERRQEWLLSELGKRPLFGS</i>
167–190	2781.612	2781.607	+0.005	DLVLAHTPTQSVRRSLLQKHGRIR
349–372	2836.469	2836.283	+0.186	DAAKHYIEFWKSIPITTEPYRVILG
235–259	3054.611	3054.581	+0.030	DEMAGMSYFHETIWKGVPKFLRRV
260–287	3106.553	3106.566	–0.013	DTALKNIGIEERVYPYNAPLIQFSSWMGG
470–499	3610.807	3610.803	+0.004	DIGSYREWSEERRQEWLLSELGKRPLFGS

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