RESEARCH PAPER

\textit{In vivo} monoubiquitination of anaplerotic phosphoenolpyruvate carboxylase occurs at Lys624 in germinating sorghum seeds

Isabel Ruiz-Ballesta\textsuperscript{1}, Ana-Belén Feria\textsuperscript{1}, Hong Ni\textsuperscript{2}, Yi-Min She\textsuperscript{2}, William Charles Plaxton\textsuperscript{3} and Cristina Echevarría\textsuperscript{1,}\textsuperscript{*}

\textsuperscript{1} Departamento de Biología Vegetal, Facultad de Biología, Universidad de Sevilla, Avda Reina Mercedes no. 6, 41012 Sevilla, Spain
\textsuperscript{2} Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, 3888 Chenhua Road, Shanghai 201602, China
\textsuperscript{3} Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada

\* To whom correspondence should be addressed. E-mail: echeva@us.es

Received 3 June 2013; Revised 13 October 2013; Accepted 21 October 2013

Abstract

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is an important cytosolic regulatory enzyme that plays a pivotal role in numerous physiological processes in plants, including seed development and germination. Previous studies demonstrated the occurrence of immunoreactive PEPC polypeptides of ~110 kDa and 107 kDa (p110 and p107, respectively) on immunoblots of clarified extracts of germinating sorghum (\textit{Sorghum bicolor}) seeds. In order to establish the biochemical basis for this observation, a 460 kDa PEPC heterotetramer composed of an equivalent ratio of p110 and p107 subunits was purified to near homogeneity from the germinated seeds. Mass spectrometry established that p110 and p107 are both encoded by the same plant-type PEPC gene (\textit{CP21}), but that p107 was \textit{in vivo} monoubiquitinated at Lys624 to form p110. This residue is absolutely conserved in vascular plant PEPCs and is proximal to a PEP-binding/catalytic domain. Anti-ubiquitin IgG immunodetected p110 but not p107, whereas incubation with a deubiquitinating enzyme (USP-2 core) efficiently converted p110 into p107, while relieving the enzyme's feedback inhibition by L-malate. Partial PEPC monoubiquitination was also detected during sorghum seed development. It is apparent that monoubiquitination at Lys624 is opposed to phosphorylation at Ser7 in terms of regulating the catalytic activity of sorghum seed PEPC. PEPC monoubiquitination is hypothesized to fine-tune anaplerotic carbon flux according to the cell's immediate physiological requirements for tricarboxylic acid cycle intermediates needed in support of biosynthesis and carbon–nitrogen interactions.

Key words: Development, germination, monoubiquitination, phosphoenolpyruvate carboxylase, post-translational modification, seeds, \textit{Sorghum bicolor}.

Introduction

Phosphoenolpyruvate (PEP) carboxylase (PEPC; EC 4.1.1.31) catalyses the irreversible β-carboxylation of PEP to yield oxaloacetate and P\textsubscript{i}. The enzyme plays a central role in the initial fixation of atmospheric CO\textsubscript{2} during C\textsubscript{4} and Crassulacean acid metabolism photosynthesis (Echevarría and Vidal, 2003). PEPC also fulfils a broad array of crucial
non-photosynthetic functions, particularly the anaplerotic replenishment of tricarboxylic acid (TCA) cycle intermediates consumed during biosynthesis and nitrogen assimilation (Echevarria and Vidal, 2003; O’Leary et al., 2011a). To achieve their diverse functions and complex regulation, vascular plant PEPCs belong to a small multigene family encoding several closely related PTPCs (plant-type PEPCs), as well as a single, distantly related BTPC (bacterial-type PEPC) (O’Leary et al., 2011a). PTPC genes encode 100–110 kDa polypeptides that typically assemble as Class-1 PEPC homotetramers.

A remarkable array of strategies has evolved to control plant PEPC activity post-translationally, including allosteric effectors (Echevarria et al., 1994), covalent modification via phosphorylation or monoubiquitination, and protein–protein interactions (Uhrig et al., 2008; O’Leary et al., 2011a, b). PTPC phosphorylation at its N-terminal seryl residue has been widely studied, and is catalysed by a dedicated calcium-independent PTPC protein kinase (PPCK). This enhances allosteric activation of Class-1 PEPCs by hexose-phosphates while reducing inhibition by l-malate and l-aspartate (O’Leary et al., 2011a). Class-1 PEPCs were recently discovered to be subject to reciprocal control by inhibitory monoubiquitination in germinating castor oil (Ricinus communis) seeds (COS) (Uhrig et al., 2008), as well as in immature protoxylem roots of harsh hakea (Hakea prostrata) (Shane et al., 2013). Extracts of germinating COS or immature protoxylem roots of harsh hakea contain an equivalent ratio of immunoreactive PTPC polypeptides having molecular masses of ~110kDa and 107kDa (p110 and p107, respectively) (Uhrig et al., 2008; Shane et al., 2013). PTPC purification and mass spectrometry (MS) revealed that: (i) both subunits arise from the same pTPC gene and interact to form a p110:p107 heterotetrameric Class-1 PEPC; and (ii) p110 is the monoubiquitinated form of p107 (Uhrig et al., 2008; Shane et al., 2013).

Monoubiquitination of COS or harsh hakea Class-1 PEPC is inhibitory as it increased their K_P (PEP) values while enhancing sensitivity to allosteric inhibitors. These studies have provided a new paradigm for the post-translational control of non-photosynthetic Class-1 PEPCs, as well as the first examples of regulatory monoubiquitination of a metabolic enzyme in nature. Similarly, a deubiquitinating enzyme, ataxin 3, is activated by monoubiquitination (Todi et al., 2009).

An immunoreactive PTPC ‘doublet’ highly reminiscent of partially monoubiquitinated COS or harsh hakea PEPC has frequently been observed on immunoblots of clarified extracts from diverse plant tissues including leaves, guard cells, roots, and fruits (Denecke et al., 1993; Law and Plaxton, 1995; De Nisi and Zocchi, 2000; Rao et al., 2006), and during the development or germination of cereal seeds such as barley, wheat, and sorghum (Osuna et al., 1996, 1999; Gonzalez et al., 1998; Nhiri et al., 2000; Feria et al., 2008). The overall aim of the present study was to test the hypothesis that Class-1 PEPC of germinating sorghum seeds is subject to in vivo monoubiquitination. The main PEPC isozone of germinating sorghum was identified as the product of a PTPC gene (CP21) and it was established that it exists as a heterotetramer comprised of an equivalent ratio of monoubiquitinated p110 and non-ubiquitinated p107 PTPC subunits. Detailed MS analyses demonstrated that monoubiquitination of the p110 subunit occurs at a conserved Lys624 residue that aligns with the Lys628 monoubiquitination site of Class-1 PEPC from germinated COS (Uhrig et al., 2008). Partial PEPC monoubiquitination was also shown to occur during sorghum seed development.

Materials and methods

Plant material

Sorghum [Sorghum bicolor (L.) Moench, var. PR87G57, Pioneer Hi-Bred Spain] seeds were sterilized and germinated as previously reported (Nhiri et al., 2000). Harvested seeds were frozen in liquid N2 and stored at –80 ºC until used. Sorghum plants were cultivated in a greenhouse under a 14 h day (25 ºC)/10 h night (18 ºC) cycle. Seeds were harvested at various stages of development, ground to a powder under liquid N2, lyophilized, and kept at 4 ºC for later analyses.

Antibodies

Polyclonal antibodies against: (i) native C4-photosynthetic PTPC from sorghum leaves (anti-C4 PTPC) were prepared as described in Pacquit et al. (1995); and (ii) synthetic peptides corresponding to the C-terminal [(Y) EDTLILTMKGIAAGMQNTG] and dephosphorylated N-terminal [ERHHSSIDAQLRLAPGKVSEER(Y)G] ends of C4-photosynthetic PTPC (anti-C19 and anti-N24, respectively), and the SIDAQLR phosphorylation motif (anti-SIDAQLR) common to all known PTPC sequences were from NEOSYSTEM S.A. (Strasbourg, France). Anti-pSer13-IgG (anti-pSer13) was raised against a synthetic phosphopeptide corresponding to the N-terminal seryl phosphorylation site of a sorghum C1 PTPC sequence [phosphorylated on the regulatory serine: Cys-ERLS(pSer13)DAQLR] (Lepinec et al., 1993). Anti-COS PTPC-IgG (anti-COS PTPC) was raised against native Class-1 PEPC of developing COS as described in Tripodi et al. (2005). Anti-ubiquitin IgG (anti-UB) was purchased from Millipore, Canada (catalogue no. 05-944).

Protein extraction

Frozen powder (0.4 g) from germinated sorghum seeds was ground in a chilled mortar with sand in an ice-cold buffer which contained 1 mM TRIS-HCl (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 20% (v/v) glycerol, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 10 μg ml–1 chymostatin, 10 μg ml–1 leupeptin, 50 mM KF, 1 mM Na2MoO4, 1 mM Na3VO4, and 50 mM microcystin-LR or okadaic acid. Homogenates were centrifuged at 17 000 g for 7 min. Clarified extract proteins were precipitated by the addition of 60% (saturation) (NH4)2SO4, pelleted by centrifugation at 10 500 g, resuspended in 200 μl of extraction buffer, and desalted by chromatography on a Sephadex G-25 column (1 × 5 cm) that had been equilibrated in extraction buffer lacking β-mercaptoethanol.

Electrophoresis and immunoblotting

Samples were subjected to SDS–PAGE [7.5% (w/v) acrylamide] and immunoblotting as previously described (Tripodi et al., 2005). Antigenic polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody with chromogenic detection (Tripodi et al., 2005). Alternatively, immunolabelled proteins were detected by a chemiluminescence detection system (SuperSignal West Pico Rabbit IgGs; Thermo Scientific) according to the manufacturer’s instructions in a Fujifilm LAS3000 mini-system.

Determination of PEPC activity and kinetic studies

PEPC activity was assayed at 340 nm using a Molecular Devices Spectramax Kinetics Microplate reader. Optimized assay conditions

444 | Ruiz-Ballesta et al.
were: 50 mM HEPES-KOH, pH 8.0, 2.5 mM PEP, 5 mM NaHCO$_3$, 5 mM MgCl$_2$, 0.15 mM NADH, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), and 5 U ml$^{-1}$ porcine heart malate dehydrogenase (0.2 ml final volume). One unit of activity is defined as the amount of PEPC catalysing the carboxylation of 1 μmol of PEP min$^{-1}$ at 25 °C. Protein concentrations were determined by the Coomassie Blue G-250 dye binding method using bovine γ-globulin as the standard (Tripodi et al., 2005).

Apparent $K_m$ and $IC_{50}$ (concentration of inhibitor producing 50% inhibition) values were calculated using a computer kinetics program as previously described (Tripodi et al., 2005). All kinetic parameters are the means of three independent experiments and are reproducible to within ±15% (SE) of the mean value. Stock solutions of metabolites were made equimolar with MgCl$_2$ and adjusted to pH 7.5.

**Buffers used during PEPC purification**

All buffers contained 50 mM HEPES-KOH (pH 8.0), 5 mM MgCl$_2$, 1 mM DTT, and a phosphatase inhibitor cocktail consisting of 5 mM EDTA, 1 mM EGTA, 25 mM NaF, 1 mM Na$_2$MoO$_4$, 1 mM Na$_3$VO$_4$, and 5 mM Na$_2$P$_2$O$_7$. Buffer A contained 20% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1% (w/v) insoluble poly(vinylpolypyrrolidone), 1 mM phenylmethylsulphonyl fluoride, and 2 mM 2,2'-dipyridil disulphide. Buffer B contained 25% (saturation) (NH$_4$)$_2$SO$_4$. Buffer C consisted of buffer B lacking (NH$_4$)$_2$SO$_4$ but containing 10% (v/v) ethylene glycol. Buffer D contained 20% (v/v) glycerol and 50 mM KCl. Buffer E contained 20% (v/v) glycerol and 100 mM KCl.

**PEPC purification**

All procedures were performed at room temperature unless otherwise noted. Frozen whole 48 h germinated sorghum seeds (300 g) were homogenized (1:2.5; w/v) in ice-cold buffer A using a Polytron wise noted. Frozen whole 48 h germinated sorghum seeds (6 g) were respectively incubated at 37 °C for up to 60 min with 5 μM and 20 μM ubiquitin-specific protease-2 core (USP-2) (Abcam, cat. # ab125735) in a final volume of 50 μl. The incubation buffer was 25 mM TRIS-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, 20% (v/v) glycerol, 1 mM DTT, 0.5 mM Na$_2$MoO$_4$, and 0.5 mM Na$_3$VO$_4$.

**Deubiquitination**

Clariified seed extracts (50 μg) or the purified PEPC from germinated seeds (6 μg) were respectively incubated at 37 °C for up to 60 min with 5 μM and 20 μM ubiquitin-specific protease-2 core (USP-2) (Abcam, cat. # ab125735) in a final volume of 50 μl. The incubation buffer was 25 mM TRIS-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, 20% (v/v) glycerol, 1 mM DTT, 0.5 mM Na$_2$MoO$_4$, and 0.5 mM Na$_3$VO$_4$.

**Mass spectrometry**

Coomassie Blue-stained polypeptides were excised from SDS gels, in-gel reduced, alkylated, and digested with trypsin using standard protocols (Uhrig et al., 2008). Tryptic peptides were extracted using acetonitrile/0.1% trifluoroacetic acid (TFA) (v/v, 60:40), and dried by a CentriVap refrigerated centrifugal concentrator (Labconco Corp.). Peptides were reconstituted in 4 μl of 0.1% formic acid (FA) and identified using two instrumentation methods: an Eksigent nanoLC 400 system coupled with a TripleTOF 5600+ quadrupole time-of-flight (TOF) mass spectrometer (AB Sciex, Concord, ON, Canada) at low-energy collision-induced dissociation (CID); and an EASY-nLC Orbitrap Fusion Tribid LC-MS system (Thermo Fisher, San Jose, CA, USA) at higher energy collision-induced dissociation (HCD) mode. The sample was trapped by a reverse-phase (RP) C$_{18}$ column (Chrom XP, 350 μm id×0.5 mm length, 3 μm, 120A) at 2 μl min$^{-1}$ of solvent A (0.1% FA) for 15 min, and then separated through a C$_{18}$ analytical column (75 μm id×150 mm, 3 μm, 120A) at 300 nI min$^{-1}$ for 60 min. The mobile phase was set up to a linear gradient from 5% to 50% solvent B (0.1% FA in acetonitrile), followed by 85% solvent B over 10 min for peptide elution. TripleTOF MS and MS/MS scans were acquired with a high resolution of 30,000, and the m/z regions of the MS survey scan and MS/MS were selected from m/z 350–1600 and m/z 100–1250, respectively, and by data-dependent scanning of the top 20 ions at multiply charged states of 2+, 3+, and 4+. Dynamic exclusion was set to a period of time at 30 s. For the purpose of comparison, similar analyses were performed on the gel-separated proteins using the Orbitrap Fusion Tribid LC-MS system under high-accuracy MS survey scan at a resolution of 120 000 followed by sequential HCD scans of the top 10 ions. MS/MS data from the two instruments were searched against the viridiplantae (green plants) protein sequences in the NCBI database using Mascot Server (version 2.4.0, Matrix Science, London, UK).

**Statistical analysis**

This was performed using statistical software (SigmaStat, Systat Software Inc., San José, CA, USA). Data were analysed using the Student’s t-test. Means were considered to be significantly different at $P < 0.05$.

**Results and Discussion**

**Influence of sorghum seed germination on PEPC subunit structure and post-translational modifications**

Preliminary immunoblot experiments corroborated an earlier report (Nhiri et al., 2000) that sorghum seed germination is accompanied by: (i) accumulation of comparable
amounts of pre-existing approximate 107kDa and inducible 110kDa immunoreactive PTPC polypeptides (p107 and p110, respectively) (Fig. 1B); and (ii) in vivo phosphorylation of p107 at its conserved N-terminal seryl residue (Fig. 1C; anti-pSer13). Both subunits are intact since the anti-C19 and anti-N24 (antibodies raised against synthetic peptides corresponding to the C-terminal [Y]942-EDTLILTMKGIAGMQNTG-960] and dephosphorylated N-terminal [4-ERHHSIDQLRAGKVSEE-24(YG)] ends, respectively, of C₄-photosynthetic PTPC) both cross-reacted with p110 and p107 (Fig. 1C). Incubation with USP-2, a recombinant human deubiquitinating enzyme, resulted in p110’s apparent deubiquitination (Fig. 1D). Subsequent experiments confirmed that p110 is a monoubiquitinated form of p107 (see below). As the USP-2 core lacks an N-terminal regulatory domain, it can deubiquitinate a large number of substrates in vitro, including monoubiquitinated Class-1 PEPCs from germinating COS and developing protod roots of harsh hakea (Uhrig et al., 2008; Shane et al., 2013).

**PEPC purification**

PEPC from the 48h germinated seeds was purified 391-fold to near homogeneity and an overall yield of 29% (Fig. 2A; Supplementary Table S1 available at JXB online). The final specific activity of 7.1 U mg⁻¹ compares favourably with the value of 9.6 U mg⁻¹ obtained with purified monoubiquitinated PEPC from germinating COS (Uhrig et al., 2008). The single peak of PEPC activity resolved during Superdex-200 gel filtration FPLC co-eluted with an equivalent ratio of immunoreactive p110 and p107 (Fig. 2). Similarly, SDS-PAGE and immunoblotting indicated that the final preparation consisted of equivalent amounts of protein-staining or anti-COS-PTPC-immunoreactive p110 and p107 that respectively co-migrated with the subunits of purified Class-1 PEPC from germinated COS (Fig. 3A, B). The enzyme’s native molecular mass was estimated by analytical gel filtration to be ~460kDa (Supplementary Fig. S1). In agreement with earlier results obtained with clarified sorghum seed extracts (Fig. 1D), p110, but not p107, of the purified PEPC was immunodecorated by anti-UB (Fig. 4A); this cross-reaction was eliminated together with the protein-staining p110 band upon USP-2 treatment of the final preparation (Fig. 4B). No higher molecular mass immunoreactive polypeptides indicative of polyubiquitination were observed when the final preparation was probed with anti-UB (Fig. 4C).

The p110 and p107 subunits of Class-1 PEPC from germinated COS are both truncated by 19 amino acids such that their N-terminal phosphorylation domain is absent; this process appears to occur in vivo and could therefore have physiological significance (Uhrig et al., 2008). In contrast, the p110 and p107 subunits of the purified sorghum seed PEPC appear to be intact since they both cross-reacted with the anti-N24, anti-SIDAO, and anti-C19 (Fig. 3C). Despite evidence for p107 phosphorylation in clarified extracts of 48h germinated sorghum seedlings (Fig. 1C; anti-pSer13) (Nhiri et al., 2000), the N-terminal seryl phosphorylation site of the p110 or p107 subunits of the final preparation did not appear to be phosphorylated, as indicated by immunoblotting with anti-pSer13 (Fig. 3C) (confirmed below via MS). However, both subunits readily incorporated ³²P when the final preparation was incubated with [γ-³²P]ATP and recombinant sorghum PPCK2 or PPCK3 (the physiological PPCKs that catalyse in vivo phosphorylation of sorghum PTPC) (Supplementary Fig. S2 at JXB online) (Echevarria and Vidal, 2003). In addition, both subunits of the in vitro phosphorylated enzyme efficiently cross-reacted with anti-pSer13 (Supplementary Fig. S2). Thus, phosphorylation and monoubiquitination of the same PTPC polypeptide are not mutually exclusive, at least in vitro.

![Fig. 1. Immunocharacterization of p110 and p107 PEPC subunits during sorghum seed germination. (A) Stages of seed germination; DS, dry seed. (B) Seed extracts were subjected to SDS–PAGE (8% gel, 40 μg of protein), electroblotted onto a PVDF membrane, and probed with anti-C₂ PEPC. (C) Immunoblots of 48h post-imbibition extracts were probed with anti-N24, anti-pSer13, and anti-C-19. (D) Monoubiquitination of p110. Clarified extracts from seeds imbibed in water for 48h were incubated in the presence or absence of 1 μM USP-2 core (Abcam) for 1h at 30 °C, and subjected to immunoblot analysis using anti-C₂ PEPC; 50 μg protein lane⁻¹.](image-url)
The $^{32}$P autoradiography also revealed PPCK2 and PPCK3 autophosphorylation (Supplementary Fig. S2), as previously described (Monreal et al., 2013). The overall results suggest that two different Class-1 PEPC populations might co-exist in germinating sorghum seeds (e.g. a monoubiquitinated dephosphorylated heterotetramer versus a phosphorylated homotetramer), or that a co-purifying phosphatase mediated in vitro p107 dephosphorylation during PEPC purification (despite the inclusion of a phosphatase inhibitor cocktail in all purification buffers). Further studies are needed to address the interplay between in vivo PTPC phosphorylation versus monoubiquitination in germinating sorghum seeds.

**Mass spectrometry**

Coomassie Blue R-250-stained p110 and p107 were excised from SDS gels of the final preparation and subjected individually to in-gel tryptic digestion and detailed MS analyses. Mascot analysis of p110 and p107 data sets derived from nanospray HPLC TripleTOF MS of their tryptic peptides (sequence coverage=81%) revealed that both subunits originated from the same sorghum CP21 PTPC gene (gi|242061132 or Sb8720). CP21 transcripts were also induced during sorghum seed germination (Supplementary Fig. S3 at JXB online). CP21 expression in both etiolated and green sorghum leaves, and also in roots depending on nitrogen supply, was previously reported (Lepiniec et al., 1993). The present results establish CP21 (predicted molecular mass 109.4 kDa) as the predominant PEPC isozyme expressed in germinating sorghum seeds. Consistent with earlier results: (i) p110 and p107 N-terminal tryptic peptides (residues 5–13) containing non-phosphorylated Ser7 were identified by Orbitrap LC MS/MS; and (ii) p110, but not p107, was ubiquitinated. Trypsin digestion of ubiquitin-conjugated proteins produces a signature peptide at their ubiquitination site containing a diglycine remnant derived from ubiquitin’s C-terminus and that remains attached to the target lysine residue via an isopeptide bond (Uhrig et al., 2008). MS/MS analysis of the quadruply charged p110 peptide ion of m/z 469.2522 corresponding to residues 617–631 showed that the masses of the C-terminal fragments up to y7 have a mass change of 16 Da corresponding to the oxidation of Met627, and that the ubiquitination site was localized at Lys624 based on the mass increase of 114 Da (characteristic mass increment indicating glycine–glycine attachment) on the larger fragments of y11 and y14 (Fig. 5A). LC MS/MS using a high resolution Orbitrap Fusion Tribid instrument identified a smaller ubiquitinated peptide fragment at residues 620–631 (Fig. 5B). Under high-resolution HCD fragmentation, the mass shift of 114 Da on both the N-terminal b5 ion and the C-terminal y8 ion unambiguously confirmed Lys624 as p110’s monoubiquitination site. This residue precisely aligns with the Lys628 monoubiquitination site previously determined for the p110 subunit of Class-1 PEPC from germinated COS (Uhrig et al., 2008). As this lysine residue appears to be absolutely conserved in all plant PTPCs, monoubiquitination at this site may be a universal PTM (post-translational modification) that occurs with the PTPC subunits of Class-1 PEPCs throughout the plant kingdom (Supplementary Fig. S4). It is also intriguing that this site is only several amino acid residues on the N-terminal side of a crucial and absolutely conserved PEP-binding/catalytic domain of all PEPCs (Supplementary Fig. S4).

![Fig. 2. Co-elution of PEPC activity with 110 kDa and 107 kDa PEPC polypeptides (p110 and p107, respectively) during Superdex-200 HR 16/60 gel-filtration FPLC of PEPC from germinating sorghum seeds. Aliquots (10 μl each) from various fractions were subjected to SDS–PAGE and immunoblot analysis using anti-COS PTPC. $V_0$ denotes the column’s void volume.](https://example.com/pepc_fig2.png)
Kinetic studies

The impact of various metabolite effectors (2 mM each) on the activity of the purified, monoubiquitinated heterotetrameric sorghum seed PEPC (UB-PEPC) versus the in vitro deubiquitinated homotetrameric enzyme (deUB-PEPC) was assessed using suboptimal assay conditions (0.1 mM PEP, pH 7.3 and 8.0). With the exception of l-malate, monoubiquitination of germinating sorghum seed PEPC (CP21) exerted little to no influence in the response to the metabolites tested (Supplementary Table S2 at JXB online). UB-PEPC displayed a significantly lower IC$_{50}$ for l-malate relative to deUB-PEPC (1.8 mM and 3.6 mM, respectively) (Table 1). In addition, the K$_m$(PEP) value of UB-PEPC appeared to be slightly higher than that of deUB-PEPC, although the differences were not statistically significant (Table 1). Similarly, monoubiquitination of COS and harsh hakea Class-1 PEPCs is inhibitory, as it enhances sensitivity to allosteric inhibitors such as l-malate and l-aspartate, while interfering with the enzyme’s ability to bind PEP (Uhrig et al., 2008; Shane et al., 2013).

Class-1 PEPC monoubiquitination also occurs in developing sorghum seeds

The Class-1 PEPC of developing COS endosperm exists as a 410 kDa homotetramer composed of identical, in vivo phosphorylated p107 subunits, and also as a novel 910 kDa Class-2 PEPC hetero-octameric complex in which the same Class-1 PEPC isozyme (RcPPC3) is tightly associated with four BTPC (RcPPC4) subunits (Tripodi et al., 2005; Gennidakis et al., 2007; O’Leary et al., 2011a). However, neither Class-1 nor Class-2 PEPC of intact, developing COS is monoubiquitinated (O’Leary et al., 2011a, b). In the cereal seeds wheat, barley, and sorghum, Class-1 PEPC has been defined as a phosphorylated enzyme in the middle of the developmental stages, dephosphorylated in dry seed, and then re-phosphorylated during germination (Osuna et al., 1996, 1999; Gonzalez et al., 1998; Nhiri et al., 2000; Feria et al., 2008). The presence of a Class-2 PEPC complex containing tightly...
PEP carboxylase monoubiquitination in sorghum seeds

associated PTPC and BTPC subunits has not yet been established in barley or sorghum seeds (Feria et al., 2008, and unpublished data, respectively), indicating clear differences in the PEPC biochemistry of developing castor (oil-storing) versus cereal (starch-storing) seeds. It was also of interest to explore whether PEPC monoubiquitination occurs during sorghum seed development. Immunoblot analysis of PEPC in clarified extracts prepared from whole seeds harvested at different stages post-anthesis revealed the presence of anti-COS PTPC-immunoreactive p110 and p107 subunits similar to those occurring in the germinating seeds. Incubation with USP-2 led to the disappearance of immunoreactive p110 at each stage of development (Fig. 6B, +USP-2). This indicates that p110 is also a monoubiquitinated version of p107 in developing sorghum seeds. This is the first report of PEPC monoubiquitination during seed development. However, elimination of photosynthate supply to developing COS by excision of intact fruit clusters caused the Class-1 PEPC’s subunits to be rapidly dephosphorylated, and then subsequently monoubiquitinated in vivo (O’Leary et al., 2011b).

**Concluding remarks**

It is notable that a recent survey of photosynthetic and non-photosynthetic tissues of the castor plant unexpectedly revealed that monoubiquitination, rather than phosphorylation, was the most prevalent PTM of Class-1 PEPC’s PTPC subunits that occurs in unstressed plants (O’Leary et al., 2011b). C₄ and Crassulacean acid metabolism photosynthetic PTPCs (including that of sorghum) also contain a lysine residue corresponding to Lys624 and Lys628 of the p110 subunit of germinating sorghum and COS PEPCs, respectively (Supplementary Fig. S4 at JXB online). However, it has not yet been established whether the PTPC subunits of C₄ or Crassulacean acid metabolism photosynthetic Class-1 PEPCs are also subject to in vivo monoubiquitination, in addition to their diurnal control by reversible phosphorylation.

The results of the current and previous studies extend the occurrence of in vivo PTPC activation and inhibition by phosphorylation and monoubiquitination, respectively, to starch-storing cereal seed Class-1 PEPCs during seed development.

**Table 1. Influence of USP-2-mediated deubiquitination on allosteric effector sensitivity of purified PEPC**

DeUB-PEPC was prepared by incubating UB-PEPC for 1 h with 20 μM USP-2 as described in the Materials and Methods. IC₅₀ values were determined using subsaturating 0.1 mM PEP at pH 8.0 and/or pH 7.3.

| Kinetic parameter | pH 7.3 | pH 8.0 |
|-------------------|--------|--------|
|                   | UB-PEPC | DeUB-PEPC | UB-PEPC | DeUB-PEPC |
| K₅₀(PEP)          | 0.039 ± 0.002 | 0.033 ± 0.003 | 0.044 ± 0.004 | 0.036 ± 0.005 |
| IC₅₀(malate)      | 1.81 ± 0.13* | 3.62 ± 0.24* | ND | ND |
| IC₅₀(isocitrate)  | 1.47 ± 0.17 | 1.37 ± 0.12 | ND | ND |

All values are given in mM and represent the means ±SE of n=3 independent experiments. The asterisk indicates statistically significant differences between DeUB-PEPC and UB-PEPC (P < 0.05). ND, not determined.
germination and development. Further experiments are necessary to establish how monoubiquitination and phosphorylation combine to fine-tune anaplerotic carbon flux according to cellular demands for C₄-C₆ carboxylic acids in developing versus germinating seeds. However, the inhibitory influence of monoubiquitination on the kinetic properties of sorghum (Table 1), COS, or harsh hakea Class-1 PEPCs is not dramatic (Uhrig et al., 2008; Shane et al., 2013), suggesting that this PTM may have an additional role, in vivo. Many studies of protein monoubiquitination in yeast and animal cells have revealed that this PTM typically functions to recruit client proteins containing a ubiquitin-binding domain. This mediates protein–protein interactions and protein localization to control diverse processes including endocytosis, protein kinase phosphorylation ‘cascades’, gene expression, and DNA repair and replication (Sadowski and Suryadhatu, 2012). Thus, future studies need to assess potential ubiquitin-binding domain proteins that might interact with monoubiquitinated Class-I PEPCs of vascular plants, as well as the possible influence of this PTM on the enzyme’s subcellular localization. Equally important will be to identify the specific E3 ligase isozyme and upstream signalling pathways that lead to widespread monoubiquitination of non-photosynthetic PTPCs in planta, as well as how this PTM is coordinated with PPCK-mediated phosphorylation–activation of PTPCs at their conserved N-terminal seryl residue.

**Supplementary data**

Supplementary data are available at *JXB* online.

Figure S1. Native molecular mass estimation for PEPC from 2-day germinated sorghum seeds.

Figure S2. The p110 and p107 subunits of purified Class-I PEPC from germinated sorghum seeds can be phosphorylated *in vitro* by recombinant PPCK2 and PPCK3.

Figure S3. Quantitative PCR (qPCR) analysis of *CP21* transcript levels in germinating sorghum seeds.

Figure S4. Select amino acid sequence alignment of PEPC from different sources to show the phosphorylation and monoubiquitination sites.

Table S1. Purification of PEPC from 300 g of 2-day-old germinated sorghum seeds.

Table S2. Influence of various metabolites on the activity of monoubiquitinated and *in vitro* deubiquitinated PEPC purified from germinated sorghum seeds.

**Acknowledgements**

We thank Dr Jean Vidal for assisting with the redaction of the manuscript and providing important scientific comments and discussion. We gratefully acknowledge Felipe Clemente and Dolores Gutierrez from Unidad de Proteómica, Centro de Genómica y Proteómica, Universidad Complutense de Madrid for helpful comments on MS analyses. This research was supported by the ‘Ministerio de Economía y Competitividad’ grant no. AGL2012-35708, by ‘La Junta de Andalucía’ grant no. P06-CVI-02186 and BIO298, and the ‘Plan Propio de la Universidad de Sevilla’. This work was also supported by grants from the Natural Science and Engineering Research Council of Canada and Queens’s Research Chairs program to WCP.

**References**

Denecke M, Schulz M, Fischer C, Schnabl H. 1993. Partial purification and characterization of stomatal phosphoenolpyruvate carboxylase from *Vicia faba*. *Physiologia Plantarum* 87, 1996–2012.

De Nisi P, Zocchi G. 2000. Phosphoenolpyruvate carboxylase in cucumber *Cucumis sativus* L. roots under iron deficiency, activity and kinetic characterization. *Journal of Experimental Botany* 51, 1903–1909.

Echevarría C, Pacquit V, Bakrim N, Osuna L, Delgado B, Arrio-Dupont M, Vidal J. 1994. The effect of pH on the covalent and metabolic control of C₄ phosphoenolpyruvate carboxylase from Sorghum leaf. *Archives of Biochemistry and Biophysics* 315, 425–430.

Echevarría C, Vidal J. 2003. The unique phosphoenolpyruvate carboxylase kinase. *Plant Physiology and Biochemistry* 41, 541–547.

Feria AB, Álvarez R, Cochereau L, Vidal J, García-Mauriño S, Echevarría C. 2008. Regulation of phosphoenolpyruvate carboxylase phosphorylation by metabolites and ABA during the development and germination of barley seeds. *Plant Physiology* 148, 761–774.

Genndidakis S, Rao S, Greenham K, Glen Uhrig R, O’Leary B, Snedden WA, Lu C, Plaxton WC. 2007. Bacterial and plant type phosphoenolpyruvate carboxylase polypeptides interact in the heterooligomeric Class-2 PEPC complex of developing castor oil seeds. *The Plant Journal* 52, 839–849.
Expression and localization of phosphoenolpyruvate carboxylase in developing and germinating wheat grains. *Plant Physiology* **116**, 1249–1258.

Law RD, Plaxton WC. 1995. Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit. *Biochemical Journal* **307**, 807–816.

Law RD, Plaxton WC. 1997. Regulatory phosphorylation of banana fruit phosphoenolpyruvate carboxylase by a copurifying phosphoenolpyruvate carboxylase-kinase. *European Journal of Biochemistry* **247**, 642–651.

Lepiniec L, Keryer E, Philippe H, Gadal P, Cretin C. 1993. Sorghum phosphoenolpyruvate carboxylase gene family, structure, function and molecular evolution. *Plant Molecular Biology* **21**, 487–502.

Monreal, JA, Arias-Baldrich C, Pérez-Montaño F, Gandullo J, Echevarría C, García-Mauriño S. 2013. Factors involved in the rise of phosphoenolpyruvate carboxylase-kinase activity caused by salinity in sorghum leaves. *Planta* **237**, 1401–1413.

Nhiri M, Bakrim N, Bakrim N, El Hachimi-Messouak Z, Echevarría C, Vidal J. 2000. Posttranslational regulation of phosphoenolpyruvate carboxylase during germination of Sorghum seeds, influence of NaCl and l-malate. *Plant Science* **151**, 29–37.

O’Leary B, Fedosejevs ET, Hill AT, Bettridge J, Park J, Rao SK, Leach CA, Plaxton WC. 2011b. Tissue-specific expression and post-translational modifications of plant- and bacterial-type phosphoenolpyruvate carboxylase isozymes of the castor oil plant, *Ricinus communis* L. *Journal of Experimental Botany* **62**, 5485–549.

O’Leary B, Park J, Plaxton WC. 2011a. The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological function and post-translational controls of non-photosynthetic PEPCs. *Biochemical Journal* **436**, 15–34.

Osuna L, González MC, Cejudo FJ, Vidal J, Echevarría C. 1996. In vivo and in vitro phosphorylation of the phosphoenolpyruvate carboxylase from wheat seed during germination. *Plant Physiology* **111**, 551–558.

González MC, Osuna L, Echevarría C, Vidal J, Cejudo FJ. 1998. Expression and localization of phosphoenolpyruvate carboxylase in developing and germinating wheat grains. *Plant Physiology* **116**, 1249–1258.

Law RD, Plaxton WC. 1995. Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit. *Biochemical Journal* **307**, 807–816.

Law RD, Plaxton WC. 1997. Regulatory phosphorylation of banana fruit phosphoenolpyruvate carboxylase by a copurifying phosphoenolpyruvate carboxylase-kinase. *European Journal of Biochemistry* **247**, 642–651.

Lepiniec L, Keryer E, Philippe H, Gadal P, Cretin C. 1993. Sorghum phosphoenolpyruvate carboxylase gene family, structure, function and molecular evolution. *Plant Molecular Biology* **21**, 487–502.

Monreal, JA, Arias-Baldrich C, Pérez-Montaño F, Gandullo J, Echevarría C, García-Mauriño S. 2013. Factors involved in the rise of phosphoenolpyruvate carboxylase-kinase activity caused by salinity in sorghum leaves. *Planta* **237**, 1401–1413.

Nhiri M, Bakrim N, Bakrim N, El Hachimi-Messouak Z, Echevarría C, Vidal J. 2000. Posttranslational regulation of phosphoenolpyruvate carboxylase during germination of Sorghum seeds, influence of NaCl and l-malate. *Plant Science* **151**, 29–37.

O’Leary B, Fedosejevs ET, Hill AT, Bettridge J, Park J, Rao SK, Leach CA, Plaxton WC. 2011b. Tissue-specific expression and post-translational modifications of plant- and bacterial-type phosphoenolpyruvate carboxylase isozymes of the castor oil plant, *Ricinus communis* L. *Journal of Experimental Botany* **62**, 5485–549.

O’Leary B, Park J, Plaxton WC. 2011a. The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological function and post-translational controls of non-photosynthetic PEPCs. *Biochemical Journal* **436**, 15–34.

Osuna L, González MC, Cejudo FJ, Vidal J, Echevarría C. 1996. In vivo and in vitro phosphorylation of the phosphoenolpyruvate carboxylase from wheat seed during germination. *Plant Physiology* **111**, 551–558.

Osuna L, Pierre JN, González MC, Alvarez R, Cejudo FJ, Echevarría C, Vidal J. 1999. Evidence for a slow-turnover form of the Ca<sup>2+</sup>-independent phosphoenolpyruvate carboxylase kinase in the aleurone endosperm tissue of germinating barley seeds. *Plant Physiology* **119**, 511–520.

Pacquit V, Giglioli N, Cretin C, Pierre JN, Vidal J, Echevarría C. 1995. Regulatory phosphorylation of Ca<sup>2+</sup> phosphoenolpyruvate carboxylase from sorghum. An immunological study using specific anti-phosphorylation site-antibodies. *Photosynthesis Research* **43**, 283–288.

Rao S, Reiskind J, Bowes G. 2006. Light regulation of the photosynthetic phosphoenolpyruvate carboxylase PEPC in *Hydrilla verticillata*. *Plant and Cell Physiology* **9**, 1206–1216.

Sadowiski M, Suryadinata R, Tan AR, Roesley SN, Sarcevic B. 2012. Protein monoubiquitination and polyubiquitination generate structural diversity to control distinct biological processes. *IUBMB Life* **64**, 136–142.

Shane MW, Fedosejevs ET, Plaxton WC. 2013. Reciprocal control of anaplerotic phosphoenolpyruvate carboxylase by in vivo monoubiquitination and phosphorylation in developing proteoid roots of phosphate-deficient Harsh Hakea. *Plant Physiology* **161**, 1634–1644.

Todi SV, Winborn BJ, Scaglione KM, Blount JR, Travis SM, Paulson HL. 2009. Ubiquitination directly enhances activity of the deubiquitinating enzyme ataxin-3. *EMBO Journal* **28**, 372–382.

Tripodi KE, Turner WL, Gennidakis S, Plaxton WC. 2005. In vivo regulatory phosphorylation of novel phosphoenolpyruvate carboxylase isoforms in endosperm of developing castor oil seeds. *Plant Physiology* **139**, 969–978.

Uhrig RG, She YM, Leach CA, Plaxton WC. 2008. Regulatory monoubiquitination of phosphoenolpyruvate carboxylase in germinating castor oil seeds. *Journal of Biological Chemistry* **283**, 29650–29657.