New insights into the post-translational modification of multiple phosphoenolpyruvate carboxylase isoenzymes by phosphorylation and monoubiquitination during sorghum seed development and germination

Isabel Ruiz-Ballesta1, Guillermo Baena1, Jacinto Gandullo1, Liqun Wang2,3, Yi-Min She2, William Charles Plaxton4 and Cristina Echevarría1,*

1 Departamento de Biología Vegetal, Facultad de Biología, Universidad de Sevilla, Avda Reina Mercedes nº 6, 41012 Sevilla, Spain
2 Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, 3888 Chenhua Road, Shanghai 201602, China
3 State Key Laboratory of Crop Genetics and Germplasm Enhancement, Soybean Research Institute, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China
4 Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada

* Correspondence: echeva@us.es

Received 23 December 2015; Accepted 11 April 2016

Editor: Gerhard Leubner, Royal Holloway, University of London

Abstract

Phosphoenolpyruvate carboxylase (PEPC; E.C. 4.1.1.31) was characterized in developing and germinating sorghum seeds, focusing on the transcript and polypeptide abundance of multiple plant-type phosphoenolpyruvate carboxylase (PTPC) genes, and the post-translational modification of each isoenzyme by phosphorylation versus monoubiquitination during germination. We observed high levels of SbPPC4 (Sb07g014960) transcripts during early development (stage I), and extensive transcript abundance of SbPPC2 (Sb02g021090) and SbPPC3 (Sb04g008720) throughout the entire life cycle of the seed. Although tandem mass spectrometry (MS) analysis of immunopurified PTPC indicated that four different PTPC isoenzymes were expressed in the developing and germinating seeds, SbPPC3 was the most abundant isozyme of the developing seed, and of the embryo and the aleurone layer of germinating seeds. In vivo phosphorylation of the different PTPC isoenzymes at their conserved N-terminal seryl phosphorylation site during germination was also established by MS/MS analysis. Furthermore, three of the four isoenzymes were partially monoubiquitinated, with MS/MS pinpointing SbPPC2 and SbPPC3 monoubiquitination at the conserved Lys-630 and Lys-624 residues, respectively. Our results demonstrate that monoubiquitination and phosphorylation simultaneously occur in vivo with different PTPC isozymes during seed germination. In addition, we show that PTPC monoubiquitination in germinating sorghum seeds always increases at stage II (emergence of the radicle), is maintained during the aerobic period of rapid cell division and reserve mobilization, and remains relatively constant until stage IV–V when coleoptiles initiate the formation of the photosynthetic tissues.

Key words: Germination, monoubiquitination, phosphorylation, phosphoenolpyruvate carboxylase, post-translational modifications, seed development, seeds, Sorghum bicolor, tissue-specific gene expression.
Introduction

Phosphoenolpyruvate (PEP) carboxylase (PEPC; EC 4.1.1.31) is an important, tightly regulated, cytosolic metabolic enzyme that plays a pivotal photosynthetic role in primary CO₂ fixation by C₃ and Crassulacean acid metabolism plants, but also has a variety of additional functions, including roles in seed development and germination (O’Leary et al., 2011a). It catalyses the irreversible β-carboxylation of PEP to form oxaloacetate (OAA) and orthophosphate (Pₗ) (Chollet et al., 2006). The plant-type PEPC (PTPC) genes encode closely related 100–110kDa polypeptides that typically exist as Class-1 PEPC tetramers that are post-translationally regulated by a combination of several allosteric effectors, including Glc-6-P (activation) and L-malate (inhibition), reversible phosphorylation (Nimmo, 2003; O’Leary et al., 2011a), and monoubiquitination (Uhrig et al., 2008, Shane et al., 2013; Ruiz-Ballesta et al., 2014). The metabolite control is integrated with the enzyme’s reversible phosphorylation at a highly conserved serine residue located near the N-terminus of its PTPC subunits (Chollet et al., 1996; Echevarria and Vidal, 2003; O’Leary et al., 2011a). Phosphorylation activates the enzyme by making it less sensitive to feedback inhibition by malate. Phosphorylation is catalysed by a PTPC-kinase (more commonly referred to as PEPC-kinase; PPCK), whereas dephosphorylation is catalysed by a protein phosphatase type-2A (Nimmo, 2000; Izui et al., 2004; O’Leary et al., 2011a). Both controls are interactive and thus allow a fine modulation of the enzyme’s kinetic properties as required by the physiological context (Echevarria and Vidal, 2003). Regulatory PTPC monoubiquitination has been demonstrated in the endosperm of germinating castor oil seeds (COS) (Uhrig et al., 2008), in developing proteoid roots of P-deficient harsh hakea (Shane et al., 2013), and in lily pollen (Igawa et al., 2010). A recent study by our group has shown monoubiquitination of SbPPC3 in sorghum, a cereal with starch-storing seeds (Ruiz-Ballesta et al., 2014). Monoubiquitination is inhibitory as it results in increased $K_m$ (PEP) values and enhanced sensitivity to allosteric inhibitors (Uhrig et al., 2008; Shane et al., 2013; Ruiz-Ballesta et al., 2014). In COS, PTPC monoubiquitination and phosphorylation appear to be mutually exclusive, while PTPC polypeptides are alternatively phosphorylated or monoubiquitinated in developing proteoid roots of harsh hakea (Shane et al., 2013). In addition, the elimination of photosynthate supply caused by depodding in COS triggers in vivo dephosphorylation and subsequent partial monoubiquitination of PTPC polypeptides (Uhrig et al., 2008; O’Leary et al., 2011b). Previous studies have indicated that PTPC polypeptides become in vivo phosphorylated in de-embryonated sorghum seed during imbibition (i.e. on the basis of a significant increase in the enzyme’s $IC_{50}$ for L-malate and in vivo $^{32}$P incorporation) (Nhiri et al., 2000). In other starch-storing cereal seeds such as barley and wheat, the phosphorylation of both PTPC polypeptides during germination has also been confirmed by immunoprecipitation, followed by SDS-PAGE and autoradiography of the in vivo $^{32}$P-labeled PEPC (Osuna et al., 1999; Feria et al., 2008). As far as we know, no previous study has focused on how these post-translational modifications (PTMs) are combined in the different subunits and isoenzymes of a PEPC’s protein family. Deciphering how monoubiquitination and phosphorylation influence each PTPC isoenzyme during the life cycle of sorghum seeds was a major objective of the current study.

Bacterial-type PEPC (BTPC) genes encode larger 116–118kDa polypeptides exhibiting low (<40%) sequence identity with PTPCs. BTPC lacks the conserved N-terminal seryl phosphorylation motif characteristic of PTPCs, and in vascular plants it appears to only exist as catalytic regulatory subunits of novel Class-2 PEPC heteromeric complexes composed of tightly associated PTPC and BTPC subunits (Igawa et al., 2010; O’Leary et al., 2011a; Park et al., 2012). Using western blot analysis and specific anti-BTPC-IgGs in crude extract of barley seeds, Class-2 PEPC complexes have not been identified (Feria et al., 2008). This is an interesting finding as the BTPC and Class-2 PEPC appear to play an important role during COS development (Uhrig et al., 2008). Assessing, by MS/MS and qRT-PCR, the possible occurrence of BTPC (SbPPC6) and/or Class-2 PEPC during the course of the life cycle of sorghum seeds was also an important objective of this work.

Sorghum (Sorghum bicolor L. Moench) is the world’s fifth most important cereal crop (www.fao.org) and provides food, feed, fiber, fuel, and chemical/biofuel feedstocks across a range of environments and production systems (Kresovich et al., 2005). Sorghum encodes five PTPC genes (Paterson et al., 2009): Sb02g021090 and Sb04g008720 encode housekeeping and inducible SbPPC2 and SbPPC3, respectively (Lepiniec et al., 1993), whilst Sb07g014960 and Sb03g035090 encode PTPC isoenzymes SbPPC4 and SbPPC5 but these have unknown functions. All of these are C₃-type PTPCs. Sb10g021330 encodes C₄ photosynthetic SbPPC1 subunits that are highly expressed in sorghum leaves. In addition, Sb03g008410 (SbPPC6) encodes a single BTPC (Table 1).

In this study, the biochemical and immunological properties of PTPCs during development and germination of starch-storing sorghum seeds were further characterized. We also elucidated the transcript abundance of PTPC genes, and determined subunit composition, relative abundance, and PTMs of each PTPC isoenzyme during germination. As far as we know, this represents the most detailed study to date that examines both phosphorylation and monoubiquitination of a PTPC protein family in any plant species. Furthermore, we show that enhanced PTPC monoubiquitination always accompanies radical emergence during seed germination, and that both processes are accelerated in parallel under aerobic conditions or elevated temperature.

Materials and methods

Plant material

Mature sorghum (Sorghum bicolor (L.) Moench, var. PR87G57 and var. PR88Y20, Pioneer Hi-Bred, Spain) seeds were sterilized and germinated for up to 96 h at 25 or 35 °C as previously reported (Nhiri
Table 1. The sorghum PEPC gene family. Names used in this work for the different PEPC genes, the equivalence with other names, and assigned functions described by Lepiniec et al. (1993, 1994), and orthologs established by Paterson et al. (2009). Sb, Sorghum bicolor; Sv, Sorghum vulgare; Zm, Zea mays; Os Oryza sativa.

| Name of gene | Accession number | Name by Lepiniec et al. (1993) and Ruiz-Ballesta et al. (2014) | Known function | Orthologs (Paterson et al., 2009) |
|--------------|-----------------|---------------------------------------------------------------|----------------|---------------------------------|
| SbPPC1       | Sb10g021330     | CP46                                                          | C₄ PTPC, photosynthetic C₄ gene | X63756 (SvPEPC1); NM001111948 (ZmPEPC1) |
| SbPPC2       | Sb02g021090     | CP28                                                          | C₄ PTPC, housekeeping gene | X59925 (SvPEPC4); NM001111968 (ZmPEPC2) |
| SbPPC3       | Sb04g008720     | CP21                                                          | C₄ PTPC, root-inducible gene | X65137 (SvPEPC3); NM001112033 (ZmPEPC3) |
| SbPPC4       | Sb07g014960     | -                                                             | C₄ PTPC, unknown so far | Os08g0366000 |
| SbPPC5       | Sb03g035090     | -                                                             | C₄ PTPC, unknown so far | Os01g0758300 |
| SbPPC6       | Sb03g038410     | -                                                             | BTPC, as Class-2 PEPC | Os01g0110700 |

*NCBI database

et al., 2000). For immunological studies or to analyze PEPC activity, the aleurone layers, starchy endosperm (aleurone-endosperm), and embryos were carefully dissected at the indicated times post-imbibition. Sterilized sorghum seeds were also submerged in distilled water (hypoxic condition) or placed on moistened filter paper (normoxic condition) at 25 °C for 48 and 72 h. Harvested tissues were frozen in liquid nitrogen and stored at −80 °C until used. Developing seeds were obtained from sorghum plants cultivated in a greenhouse under a 14 h day (25 °C)/10-h night (18 °C) cycle. Seeds were harvested at various stages of development, ground to a powder under liquid nitrogen, lyophilized, and stored at 4 °C for later analyses.

**Antibodies**

Polyclonal antibodies against native C₄-photosynthetic SbPPC1 from sorghum leaves (anti-C₄ PTPC) were prepared as described in Pacquít et al. (1995).

Anti-C19 and anti-N24 were raised, respectively, against synthetic peptides corresponding to the C-terminal ([Y] ETLILITMK GIAAGMNTG) and the dephosphorylated N-terminal [ERHHSIDALQR ALAPGKYSEE24(YGG)] ends of SbPPC1 (i.e., sorghum C₄-photosynthetic PTPC) as previously described (Ruiz-Ballesta et al., 2014). Anti-COS PTPC was raised against native Class-I PEPC purified from the endosperm of developing castor oil seeds as described in Tripodi et al. (2005).

**Immunohistochemistry**

Seeds germinated at 24 h post-imbibition were fixed in 4% (w/v) paraformaldehyde and in 0.25% (v/v) glutaraldehyde dehydrated with concentrations of ethanol, sections were blocked for 30 min post-imbibition and embedded in Paraplast Plus (Parrafin) as described by González et al. (1998). Sections (15 μm thick) were prepared using a microtome (model RM2165, Leica) and placed on poly-L-Lys-coated microscope slides. After deparaffinizing in xylol and rehydrating in decreasing concentrations of ethanol, sections were blocked for 30 min in TBS buffer containing 3% (w/v) BSA. Then, 500 μl of the same solution containing affinity-purified anti-C₄ PTPC (3 μg of protein) (Pacquít et al., 1995) or pre-immune serum was placed on the samples and incubated overnight at 4 °C. Unbound antibodies were removed by three 10-min washes in TBS. Tissue sections were then incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG)-IgG (Bio-Rad) for 2 h at 37 °C. The reaction of alkaline phosphatase was developed colorimetrically with a BCIP/NBT Liquid Substrate System (Sigma).

**Protein extraction**

Frozen powder (0.4 g) from whole developing or germinated sorghum seeds was ground in a chilled mortar with sand in 1 ml of an ice-cold buffer that contained 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EDTA, 20% (v/v) glycerol, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml⁻¹ chymostatin, 10 μM leupeptin, 50 mM KF, 1 mM Na₃MoO₄, 1 mM Na₃VO₄, and 50 mM microcin-LR. Homogenates were centrifuged at 17000 g for 7 min and the supernatants (0.5 ml) desalted through 3-ml Sephadex G-25 spin columns equilibrated in extraction buffer (lacking PVPP and FVPP) prior to enzymatic analysis.

**Electrophoresis and immunoblotting**

SDS-PAGE, non-denaturing PAGE and in-gel PEPC activity staining, immunoblotting, and visualization of antigenic polypeptides using an alkaline-phosphatase-conjugated secondary antibody with chromogenic detection were performed as previously described (Rivoal et al., 2002; Tripodi et al., 2005). Alternatively, immunoreactive polypeptides were quantified via analysis of the scanned blots using Multi-Gauge V3.0 software (Fujifilm).

**Determination of PEPC activity and protein concentration**

PEPC activity was assayed at 340 nm using a Molecular Devices Spectramax Kinetics Microplate reader. Optimized assay conditions were: 50 mM HEPES-KOH (pH 8.0), 2.5 mM PEP, 5 mM NaH₂CO₃, 5 mM MgCl₂, 0.15 mM NADH, 10% (v/v) glycerol, 1 mM DTT, and 5 units ml⁻¹ porcine heart malate dehydrogenase (0.2 ml final volume). One unit of activity is defined as the amount of PEPC catalysing the production of 1 μmol of oxaloacetate 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).

**Immunoprecipitation and immunoaffinity chromatography**

Immunoprecipitation of PEPC polypeptides from clarified seed extracts was performed using anti-C₄ PTPC as previously described (Osuna et al., 1999).

For immunoaffinity chromatography, frozen whole 48-h germinated sorghum seeds (5 g) were homogenized (1:2.5; w/v) using a Polytron PT-3100 homogenizer in ice-cold buffer A that contained 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 15% (v/v) glycerol, 5 mM MgCl₂, 100 mM KCl, 10 mM NaCl, 25 mM NaF, 1 mM NaVO₄, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-100, 2 mM 2,2'-dipyridyl disulfide, and 10 μl ml⁻¹ ProteCEASE-100 (G-BioSciences). Homogenates were centrifuged at 4 °C for 10 min at 48 500 g, and the supernatant fluid re-centrifuged for 5 min and filtered through a layer of Miracloth (Calbiochem). Clarified extracts were pre-cleared at 25 °C by eluting at 0.5 ml min⁻¹ through an AminoLink column (1 × 4 cm) that had been pre-saturated with 1 M Tris-HCl (pH 7.4). Unbound proteins were immediately absorbed at 1 ml min⁻¹ onto an anti-COS PTPC.
immunofinity column (1 × 2 cm; prepared as described by Uhrig et al., 2008) that had been pre-equilibrated with PBS. Flow-through fractions were collected by eluting the column at 1 mL min⁻¹ with PBS until A₂₈₀ decreased to baseline, and bound proteins were eluted at 0.5 mL min⁻¹ with 100 mM Gly-HCl (pH 2.8), and immediately neutralized (1-mL fractions collected into 0.1 mL of unbuffered 1 M Tris). A₂₈₀ absorbance fractions were concentrated to ~1 mg mL⁻¹ and analyzed for their polypeptide composition by SDS-PAGE and immunoblotting.

Mass spectrometry

In the initial MALDI-TOF MS analysis, PTPCs at the different stages of seed development and germination were extracted and purified by immunoprecipitation from the clarified extracts, followed by SDS-PAGE of the solubilized immunoprecipitates (Osuma et al., 1999). Protein-staining bands of interest were manually excised from micro-preparative gels using biopsy punches, then reduced, alkylated, and digested with trypsin according to Sechi and Chait (1998). Briefly, gel bands were washed twice with water, shrunk for 15 min with 100% acetonitrile, and dried in a Savant SpeedVac for 30 min. Then the samples were reduced with 10 mM dithioerythritol in 25 mM ammonium bicarbonate (NH₄HCO₃) for 30 min at 56 ºC and subsequently alkylated with 55 mM iodoacetamide in 25 mM NH₄HCO₃ for 15 min in the dark. Finally, samples were digested with 12.5 ng µL⁻¹ sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM NH₄HCO₃ (pH 7.6) overnight at 37 ºC. After digestion, 1 µL of each sample was spotted onto a MALDI plate and allowed to air-dry at room temperature. Then, 0.4 µL of 3 mg mL⁻¹ α-cyano-4-hydroxy-cinnamic acid as the matrix (Sigma) in 50% acetonitrile was added onto the spots and they were again allowed to air-dry at room temperature.

Sample analyses were performed on a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (AB Sciex, Toronto, Canada). The instrument was operated in the positive reflector mode with an accelerating voltage of 20 000 V. Mass spectra were calibrated internally using the peptides of trypsin autolysis. Mass spectrometric measurements yielded a list of monoisotopic masses of the observed peptides at a signal-to-noise ratio greater than 10, which were used for peptide mapping.

Peptides of interest were further analyzed by MS/MS in order to assess their sequences. Suitable precursor ions were selected from the MS spectra and broken down using collision induced dissociation (CID) with atmospheric air as the collision gas, at a collision energy of 1 kV and in the positive ion reflector mode. The precursor ions were selected within the mass windows of ±2 Da. The non-redundant NCBI database (downloaded on 24/2/2011; 13 135 398 sequences) and in-house databases with the sorghum entries from NCBIInr (71 862 sequences) and the sorghum PEPC sequence only were used for protein identification, and the mass spectrometric data were searched using the MASCOT 2.3 server through the Global Protein Server v3.6 (AB Sciex). The search parameters were set to no limitation in taxonomy, trypsin as the cleavage enzyme, carboxyamidomethylation on cysteine as the fixed modification, and the oxidation of methionine and ubiquitination (Gly-Gly adducts) of lysine, phosphorylation of serine/threonine as variable modifications. Mass tolerances were set up to 30 ppm and 0.3 Da for Orbitrap MS and MS/MS fragment ions, 20 ppm for Mass TOF and MS/MS scans were acquired with a high resolution of 35 000, and the mz regions of the MS survey scan and MS/MS were selected from mz 330–1600 and mz 100–1250, respectively, and by data-dependent scanning of the top twenty ions at multiply charged states of 2+, 3+ and 4+. Dynamic exclusion was set to a period of time of 30 s. For comparison purposes, similar analyses were performed on the gel-separated proteins using the Orbitrap Fusion Trubrid LC-MS system under high-accuracy MS survey scan at a resolution of 60 000 followed by sequential HCD scans at the twenty top ions. MS/MS data from the two instruments were searched against the viridiplantae (green plants) protein sequences in the NCBI database using the Mascot Server (version 2.4.0, Matrix Science, London, UK). The search parameters were restricted to tryptic peptides at a maximum of two missed cleavages. Cysteine carbamidomethylation was designated as a fixed modification, and deamidation of asparagine and glutamine, oxidation of methionine, phosphorylation of serine/threonine/tyrosine, ubiquitination of lysine were considered as variable modifications. Mass tolerances were set up to 30 ppm for TripleTOF 5600 MS ions and 0.05 Da for MS/MS fragment ions, 20 ppm for Orbitrap MS and 0.8 Da for the MS/MS fragments. Peptide assignments were filtered by an ion score cut off at 15, and the identified MS/MS spectra were also verified manually.

RNA Extraction and cDNA synthesis

Total RNA was isolated from 100 mg of frozen, powdered half-embryonated germinating seeds or whole developing seeds using the Qiagen Plant RNA Extraction (Intron Biotechnology). Extracted nucleic acids were DNase treated to eliminate genomic DNA. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse-transcription reactions were performed using 1 µg of purified total RNA, 1 µl ImProm-IITM Reverse Transcriptase (Promega) and a reaction buffer containing 0.5 mM dNTP, 6 mM MgCl₂, 20 units recombinant RNasin® ribonuclease inhibitor, and 0.5 µg oligo(dt)₁₅.

qRT-PCR

Quantitative polymerase chain reaction (qRT-PCR) was performed in a final volume of 20 µl consisting of 1 µl of the cDNA, 10 µl of SensiFAST SYBR No-ROX kit (Roche) and 15 µl of the gene-specific primers pairs as follows: ShPPC3 (forward 5’-TTGGGAAACGTTTTCTGAACCTTCTT-3’, reverse 5’-GGTTCGA AAGGGCCAAGCCAAAG-3’), ShPPC2 (forward 5’-CGCCCT CGCAACACTGGAACA-3’, reverse 5’-ACCCGGGAGTTGGA ACGTGT-3’), ShPPC4 (forward 5’-TGACGTCCTGGCAGCAC AGATG-3’, reverse 5’-GGTCCAAAGGCTTCAAGAACTGT C-3’), and 18 S rRNA (forward 5’-GGGGGAAACTTACCA CGCACTT-3’, reverse 5’-GGATGCGTCCGCGTACAGCTA-3’). qRT-PCR was conducted on the MiniOpticon™ Real-Time PCR Detection System (Bioread), and the threshold cycles (Ct) were determined using Opticon Monitor™ analysis software for all treatments. To analyze their sequences, suitable precursor ions were selected from the MS spectra and broken down using collision induced dissociation (CID) with atmospheric air as the collision gas, at a collision energy of 1 kV and in the positive ion reflector mode. The precursor ions were selected within the mass windows of ±2 Da. The non-redundant NCBI database (downloaded on 24/2/2011; 13 135 398 sequences) and in-house databases with the sorghum entries from NCBIInr (71 862 sequences) and the sorghum PEPC sequence only were used for protein identification, and the mass spectrometric data were searched using the MASCOT 2.3 server through the Global Protein Server v3.6 (AB Sciex). The search parameters were set to no limitation in taxonomy, trypsin as the cleavage enzyme, carboxyamidomethylation on cysteine as the fixed modification, and the oxidation of methionine and ubiquitination (Gly-Gly adducts) of lysine, phosphorylation of serine/threonine as variable modifications. Mass tolerances were set up to 30 ppm and 0.3 Da for Orbitrap MS and MS/MS fragment ions, 20 ppm for Mass TOF and MS/MS scans were acquired with a high resolution of 35 000, and the mz regions of the MS survey scan and MS/MS were selected from mz 330–1600 and mz 100–1250, respectively, and by data-dependent scanning of the top twenty ions at multiply charged states of 2+, 3+ and 4+. Dynamic exclusion was set to a period of time of 30 s. For comparison purposes, similar analyses were performed on the gel-separated proteins using the Orbitrap Fusion Trubrid LC-MS system under high-accuracy MS survey scan at a resolution of 60 000 followed by sequential HCD scans at the twenty top ions. MS/MS data from the two instruments were searched against the viridiplantae (green plants) protein sequences in the NCBI database using the Mascot Server (version 2.4.0, Matrix Science, London, UK). The search parameters were restricted to tryptic peptides at a maximum of two missed cleavages. Cysteine carbamidomethylation was designated as a fixed modification, and deamidation of asparagine and glutamine, oxidation of methionine, phosphorylation of serine/threonine/tyrosine, ubiquitination of lysine were considered as variable modifications. Mass tolerances were set up to 30 ppm for TripleTOF 5600 MS ions and 0.05 Da for MS/MS fragment ions, 20 ppm for Orbitrap MS and 0.8 Da for the MS/MS fragments. Peptide assignments were filtered by an ion score cut off at 15, and the identified MS/MS spectra were also verified manually.

RNA Extraction and cDNA synthesis

Total RNA was isolated from 100 mg of frozen, powdered half-embryonated germinating seeds or whole developing seeds using the Qiagen Plant RNA Extraction (Intron Biotechnology). Extracted nucleic acids were DNase treated to eliminate genomic DNA. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse-transcription reactions were performed using 1 µg of purified total RNA, 1 µl ImProm-IITM Reverse Transcriptase (Promega) and a reaction buffer containing 0.5 mM dNTP, 6 mM MgCl₂, 20 units recombinant RNasin® ribonuclease inhibitor, and 0.5 µg oligo(dt)₁₅.

qRT-PCR

Quantitative polymerase chain reaction (qRT-PCR) was performed in a final volume of 20 µl consisting of 1 µl of the cDNA, 10 µl of SensiFAST SYBR No-ROX kit (Roche) and 15 µl of the gene-specific primers pairs as follows: ShPPC3 (forward 5’-TTGGGAAACGTTTTCTGAACCTTCTT-3’, reverse 5’-GGTTCGA AAGGGCCAAGCCAAAG-3’), ShPPC2 (forward 5’-CGCCCT CGCAACACTGGAACA-3’, reverse 5’-ACCCGGGAGTTGGA ACGTGT-3’), ShPPC4 (forward 5’-TGACGTCCTGGCAGCAC AGATG-3’, reverse 5’-GGTCCAAAGGCTTCAAGAACTGT C-3’), and 18 S rRNA (forward 5’-GGGGGAAACTTACCA CGCACTT-3’, reverse 5’-GGATGCGTCCGCGTACAGCTA-3’). qRT-PCR was conducted on the MiniOpticon™ Real-Time PCR Detection System (Bioread), and the threshold cycles (Ct) were determined using Opticon Monitor™ analysis software for all treatments. To
normalize the obtained values, 18S ribosomal RNA was used as internal control in each sample. This gene displays a steady RNA level across the experimental conditions.

Statistical analysis

Statistical analysis was performed using SigmaStat (Systat Software Inc., San José, CA, USA). Data were analyzed using the Student’s t-test or with the Mann-Whitney U test. Means were considered to be significantly different at $P<0.05$.

Results and discussion

PEPC activity, and PTPC integrity and cellular localization

PEPC was characterized throughout the development, maturation, and germination of sorghum seeds, focusing on the enzyme’s activity, integrity, subunit composition, and immunolocalization. Stages of sorghum seed development and germination are described in Supplementary Fig. S1 (at JXB online); among these, stage IV of development marks the beginning of seed desiccation, whilst stage II of germination marks the protrusion of the radicle and the completion of germination (Bewley et al., 2013). During seed development, PEPC activity of desalted extracts increased by about 3-fold to reach a maximum level at stage II–III and then decreased (Fig. 1). During germination, PEPC activity in the embryo increased to a maximum at 72 to 96 h post-imbibition, while in the aleurone/endosperm it did not change significantly and was 10–15-fold lower than that of embryos (Fig. 1).

In germinating COS, the p110 and p107 PTPC polypeptides appear to be in vivo truncated by 19 amino acids such that their N-terminal phosphorylation domain is absent (Uhlig et al., 2008). In contrast, non-monoubiquitinated p107 and monoubiquitinated p110 PTPCs from germinating sorghum seeds at 48 h post-imbibition were shown to be intact and to therefore contain the phosphorylation motif (Ruiz-Ballesta et al., 2014). In this study, we demonstrate the integrity of PTPC polypeptides throughout the life cycle of sorghum seeds since the anti-C19 and anti-N24 both cross-reacted with the p110 and p107 (see Supplementary Fig. S2). PTPC was also immunolocalized in germinating sorghum seed tissues characterized by high metabolic activities (epicotyl, radicle, aleurone layer, scutellum, scutellum’s epithelium) (Fig. 2). Immunohistochemistry studies have localized PTPC in the protein bodies of wheat grains, where it was suggested to contribute to amino acid and protein biosynthesis during grain development (Araus et al., 1993). PTPC has also been localized in tissues of developing and germinating wheat grains characterized by high metabolic activity (González et al., 1998). It is noteworthy that PEPC was detected as an abundant protein in the aleurone layer using immunolocalization (Fig. 2E). Consistent with this result, the role of PEPC in malate production by the barley aleurone layer has been described by Macnicol and Raymond, (1998).

Collectively, these results indicated the stability and consistent functional relevance of PTPC throughout the life cycle of sorghum seeds.

Expression of PEPC genes in developing and germinating seeds

Sorghum PEPC is encoded by a small multigene family consisting of six genes: ShPPC2, ShPPC3, ShPPC4, ShPPC5 (all C3 PTPCs), and ShPPC1 (C4 photosynthetic PTPC) encode closely related PTPCs, whilst ShPPC6 encodes the distantly related BTPC (Table 1) (Paterson et al., 2009). In this present study, we used qRT-PCR to analyze the pattern of their transcript abundance. ShPPC2, ShPPC3, and ShPPC4 transcripts were present in developing and germinating sorghum seeds (Fig. 3). Transcripts of ShPPC2, the housekeeping PTPC (Lepiniec et al., 1994), were detected during seed development and its abundance increased slightly during the maturation phase (Fig. 3A). In the germinating seeds, ShPPC2 showed maximal transcript abundance at 24–48 h post-imbibition and then decreased (Fig. 3B). By contrast, ShPPC3 transcript levels were maximum at the beginning of seed development, during the period of cellularization (Bewley et al., 2013); this was followed by a decrease of ShPPC3 transcripts until stage III and then an increase during the desiccation period with a peak at stage V (Fig. 3C). ShPPC3 transcripts were also detected during germination with two important peaks at 24 and 72 h post-imbibition (Fig. 3D). Finally, ShPPC4 transcripts were quite abundant at the beginning of development, suggesting a possible specific role for ShPPC4 during the phase of cellularization (Fig. 3E). Cellularization is the massive production of new cells. Malate is a ubiquitous molecule that provides carbon skeletons and reducing power and is needed for the synthesis of storage end-products by developing seeds. ShPPC4 transcripts were also very abundant at 24–72 h post-imbibition in germinating seeds (Fig. 3F). By contrast, transcripts of ShPPC5 and ShPPC6 (BTPC) were very low during sorghum seed development and germination and, as expected, no ShPPC1 transcripts were detected (results not shown). These results show a novel pattern of the steady-state of transcript abundance of PTPC isozymes during the life cycle of sorghum seeds, with an important presence of ShPPC3 and also an important contribution of the ShPPC4 isogene to the cellularization stage of development (stage I) and during germination.

Different PTPC isoenzymes co-exist in developing and germinating seeds

Peptide mass fingerprinting by MALDI-TOF MS of the p110 and p107 polypeptides immunoprecipitated using anti-C3 PTPC revealed that different PTPC isoenzymes co-exist in developing and germinating sorghum seeds (Table 2; asterisks denote confirmation of the identification via LC MS/MS sequencing of tryptic peptides). ShPPC3 was the most abundant during the course of the life cycle of the seeds in both p107 and p110. ShPPC3 was highly abundant in the embryo (Table 2) and less abundant in the aleurone/endosperm of the germinating seed; however, ShPPC3 was the only isozyme detected in this tissue (Table 2). The housekeeping ShPPC2 (Lepiniec et al., 1993, 1994) was present in early development only as a p107 (i.e. probably non-monoubiquitinated) subunit
Ruiz-Ballesta et al. (Table 2, stages I and III) and was identified as a p110 and p107 subunit in stage VI (Table 2). SbPPC2 was also present as a monoubiquitinated p110 subunit in the embryo of dry and 48-h post-imbibition seeds, but was not identified in the aleurone (Table 2). Finally, the SbPPC4 isogene was shown to be translated into the corresponding p107 PTPC subunits in early development (Table 2, stages I and III), in agreement with the transcript profiling results of Fig. 3E. In addition, SbPPC4 was also detected in p110, indicating the probable monoubiquitination of this PTPC isoenzyme. Although the precise physiological function of SbPPC4 remains unclear, our results support a role for this isoenzyme during the early stages of seed development. During the phase of cellularization SbPPC4 is hypothesized to generate the C-skeletons needed as precursors for synthesis of new components such as amino acids/proteins, and membrane or storage lipids. Finally, SbPPC5 was detected only as p110 polypeptides in stage I (Table 2). SbPPC6 polypeptides were never detected by MS/MS analysis, in agreement with the absence of SbPPC6 expression during sorghum seed development and germination. Nor was a high molecular mass Class-2 PEPC hetero-octamer evident when clarified extracts from different stages of development and germination were subjected to non-denaturing PAGE followed by in-gel PEPC activity staining (see Supplementary Fig. S3). However, tetrameric and dimeric Class-1 PEPC oligomers were observed.

Fig. 1. Time course of PEPC activity in developing and germinating sorghum seeds. PEPC activity was assayed at optimal pH (8.0), 2.5 mM PEP, and 30 °C in clarified extracts from: (A) whole seeds during development; and (B) embryos or (C) aleurone-endosperm of germinating seeds. (A) PEPC activity is expressed on a per seed basis (mU seeds⁻¹) or on a protein basis (U mg⁻¹ protein). Results are means ±SE of three independent experiments. Mean values that are not significantly different (P<0.05, Student’s t-test) are indicated with the same letters. Data for aleurone-endosperm are not significantly different.

| Stages of Development | Whole Seeds | Embryo | Aleurone-Endosperm |
|------------------------|-------------|--------|-------------------|
| I                      |             | a, b   | b                 |
| II                     |             | a, b, c| c                 |
| III                    |             | a, b, c| b                 |
| IV                     |             | a, b, c| b                 |
| V                      |             | a, b, c| a                 |
| VI                     |             | a, b, c| b, c              |

Time course of PEPC activity in developing and germinating sorghum seeds. PEPC activity was assayed at optimal pH (8.0), 2.5 mM PEP, and 30 °C in clarified extracts from: (A) whole seeds during development; and (B) embryos or (C) aleurone-endosperm of germinating seeds. (A) PEPC activity is expressed on a per seed basis (mU seeds⁻¹) or on a protein basis (U mg⁻¹ protein). Results are means ±SE of three independent experiments. Mean values that are not significantly different (P<0.05, Student’s t-test) are indicated with the same letters. Data for aleurone-endosperm are not significantly different.
with the dimeric form being the most abundant in vitro (Supplementary Fig. S3A). This dimeric form was not altered by de-ubiquitination of the enzyme after incubation with 2 µM of the catalytic subunit of ubiquitin specific protease-2 (USP-2c) (Supplementary Fig. S3B).

A number of important points emerge from these results, as follows. (i) Multiple C₃ PTPC isoenzymes are present during the development and the germination of sorghum seeds. SbPPC3 appears to have an essential role as it is the isoenzyme most consistently present during the course of the life cycle of the seeds, and was the only one detected in the aleurone/endosperm. (ii) The SbPPC4 isozyme appears to play a major role during the very early stage of seed development, known as the phase of cellularization (Bewley et al., 2013). (iii) BTPC is not significantly expressed throughout the life cycle of the seeds, which is an important difference relative to developing COS, a non-green oil seed, in which p118 BTPC polypeptides, and thus Class-2 PEPC complexes are highly expressed (O’Leary et al., 2011b).

**Fig. 2.** Immunological localization of PTPC in germinating sorghum seeds. Grains were imbibed for 24 h. Sections (15 µm) were probed with: (A, B), preimmune serum, or (C–F) polyclonal anti-C₃ PTPC (SbPPC1). Abbreviations: al, aleurone layer; ep, epicotyl; ept, epithelium; sc, scutellum; se, starchy endosperm; r, radicle; p, pericarp.

Post-translational modifications of the different Class-1 PEPC isoenzymes during germination

In the endosperm of germinating COS, p110 PTPC subunits are monoubiquitinated but not phosphorylated (Uhrig et al., 2008; O’Leary et al., 2011b). On the other hand, simultaneous p110 monoubiquitination and p107 phosphorylation have been described as an alternative PTM pattern for Class-1 PEPC of immature proteoid (cluster) roots of harsh hakea (Shane et al., 2013). However, the coincident occurrence
of both PTMs in sorghum seed p110 and p107 PTPC (i.e. SbPPC3) subunits has only been shown in vitro (Ruiz-Ballesta et al., 2014). In this study, we show both PTMs also occur in vivo with three of the four PTPC isoenzymes present in germinating sorghum seeds. To this end, PTPC polypeptides of clarified extracts from 48-h post-imbibition germinating seeds were isolated by immunoaffinity chromatography with anti-COS PEPC (see Supplementary Fig. S4A, B). Coomassie blue R-250-stained p110 and p107 were excised from SDS gels of the immunopurified PTPC and individually subjected to in-gel tryptic digestion and detailed analysis by LC MS/MS. A Mascot database search of p110 and p107 datasets derived from nano HPLC TripleTOF MS of their tryptic peptides (Table 3) revealed the presence of SbPPC2, SbPPC3, and SbPPC4 PTPCs in p110 and p107, as well as SbPPC5 in p107. Consistent with our earlier results (Ruiz-Ballesta et al., 2014), p107 N-terminal tryptic peptides of SbPPC3 containing non-phosphorylated Ser7 were identified by Orbitrap LC MS/MS. However, the monoubiquitinated SbPPC3 p110 subunits were also determined to be phosphorylated at Ser7 (Table 3). Monoubiquitinated SbPPC2 and SbPPC4 (i.e. p110 subunits), and deubiquitinated SbPPC5 (i.e. p107 subunits) were also shown to be phosphorylated at their conserved N-terminal seryl phosphorylation sites; i.e. Ser13, Ser10, and Ser11 for SbPPC2, SbPPC4, and SbPPC5, respectively (Table 3). To the best of our knowledge, this is the first evidence that in vivo phosphorylation of different PTPC isoenzymes can simultaneously occur in any plant tissue. MS/MS also confirmed the monoubiquitination of SbPPC3 p110 subunits at Lys624 (Table 3), as previously reported (Ruiz-Ballesta et al., 2014). In addition, TripleTOF MS/MS analysis of the quadruply charged SbPPC2 p110 peptide ion of m/z 455.7462 (LC retention time 16.61 min) and the triply charged ion of m/z 507.9268 (LC retention time 18.01 min), corresponding to residues 623–637 (VAKDFGVKLTMFHGR) and 626–637 (DFGVKLTMFHGR), showed that the masses of the C-terminal fragments up to y7 matched the predicted values, but the mass shift of the fragment y8 by 114 Da indicated a ubiquitination site localized at Lys-630, where a characteristic mass increment of 114 Da indicates a Gly-Gly motif attachment of ubiquitin at the side chain (Supplementary Fig. S5A, B). LC MS/MS measurements of the doubly and
is the case with PTPCs from developing proteoid roots of harsh hakea (Shane et al., 2013), as well as various green and non-green tissues of the castor oil plant (Uhrig et al., 2008; O’Leary et al., 2011b).

Is PTPC monoubiquitination linked to specific stages and/or environmental conditions during sorghum seed germination?

Because of its recent discovery (Uhrig et al., 2008), there are few studies about the influence of environmental factors or biotic/abiotic stresses on PTPC monoubiquitination during seed development and germination. Elimination of photosynthesize translocation (a type of abiotic stress) from the shoot to the seed by depodding (fruit excision) of developing COS triggered rapid in vivo p107 dephosphorylation, followed by monoubiquitination of 50% of the p107 subunits to form p110 characteristic of germinating COS (O’Leary et al., 2011b).

Sorghum is a cereal cultivated in warm environments and seeds germinate well within a wide range of temperature from 25–35 °C, with an optimum of over 30 °C (Kanemasu et al., 1975). This allowed us to correlate the effect of temperature with germination and the pattern of PTPC monoubiquitination (i.e. appearance of p110 polypeptides on PTPC immunoblots). To this end, seeds from two sorghum varieties PR88Y20 and PR90Y35 were germinated in a temperature gradient from 20 to 35 °C. To minimize the influence of inherent (intrinsic) variation in the seed lots, seeds from each variety were divided into two pools and germinated in separate experiments.

Triply charged ions of m/z 769.3827 and m/z 513.2607 (LC retention time 23.22 min) using a high-resolution Orbitrap Fusion Tribrid instrument identified the ubiquitination of the oxidized peptide at residues 626–637 (Supplementary Fig. SSC, D). 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 Lys-630 as p110’s monoubiquitination site in SbPPC2. This residue precisely aligns with the Lys-624 and Lys-628 (a highly conserved PTPC residue that is immediately proximal to catalytic residues involved in PEP binding) monoubiquitination sites, respectively determined for p110 subunits of sorghum seed SbPPC3 PTPC (Table 3 and Ruiz-Ballesta et al., 2014) and PTPC (RePPC3) from germinated COS (Uhrig et al., 2008).

Collectively, these results establish for the first time several important points. (i) Different PTPC isoenzymes are co-expressed during sorghum seed germination and all of them except the p107 subunits of SbPPC3 are phosphorylated during germination. (ii) Monoubiquitination of the corresponding conserved Lys was confirmed by MS/MS analysis for SbPPC3, and determined in this study for SbPPC2, demonstrating that this PTM occurs with multiple PTPC isoenzymes at a conserved lysine residue within a single plant species. (iii) Monoubiquitination and phosphorylation can simultaneously occur on the same PTPC polypeptide and are therefore not mutually exclusive PTMs, as appears to be the case with PTPCs from developing proteoid roots of harsh hakea (Shane et al., 2013), as well as various green and non-green tissues of the castor oil plant (Uhrig et al., 2008; O’Leary et al., 2011b).

Table 2. Identification of p107 and p110 PTPC isofoms from developing and germinating sorghum seeds. Proteins were identified via MALDI-TOF/TOF MS peptide mass fingerprinting of tryptic peptides derived from immunoprecipitated samples of representative stages. Asterisks indicate identification via MS/MS sequencing of tryptic peptides and BLAST analysis. DPA, days post-anthesis.

| STAGE | Subunit | Protein name | Accession number a | Score | Sequence coverage (%) | N°. of Matching Peptides |
|-------|---------|--------------|--------------------|-------|----------------------|--------------------------|
| Dry seed | Aleurone/endosperm | p110 | PEPC SbPPC3* | gi|241931686 | 248 | 35 | 31 |
| Dry seed | Embryo | p110 | PEPC SbPPC3* | gi|241931686 | 310 | 45 | 30 |
| 48h post-imbibition | Aleurone/endosperm | p110 | PEPC SbPPC3 | gi|241931686 | 95 | 24 | 16 |
| 48h post-imbibition | Embryo | p110 | PEPC SbPPC3 | gi|241931686 | 305 | 44 | 38 |
| 48h post-imbibition | p107 | PEPC SbPPC2 | gi|241925556 | 54 | 15 | 14 |
| 48h post-imbibition | p107 | PEPC SbPPC2 | gi|241925556 | 55 | 15 | 13 |

aNCBI database
Table 3. Identification of PTMs of immunopurified PTPC polypeptides from 48 h germinated sorghum seeds using TripleTOF 5600 and Orbitrap Fusion mass spectrometry. pSer; phosphorylated-Ser; Ub, monoubiquitinated.

| Peptide | m/z(obs.) | Mr(exp.) | Mr(calc.) | ppm | Score | Peptide sequence | Modification sites |
|---------|-----------|----------|-----------|-----|-------|-----------------|---------------------|
| **p107**: PEPC SbPPC2, 107 kDa, NCBI access # gi|242048870, sequence coverage 90%. | 8–19 | 749.8647 | 1497.7148 | 1497.7010 | 9.2 | 18 | K.MERLS$\text{S}_{2}$DAQLR.M | pSer13 |
| 11–19 | 541.7654 | 1081.5169 | 1081.5169 | -0.5 | 49 | R.LSS$\text{S}_{2}$DAQLR.M | pSer13 |
| **p107**: PEPC SbPPC3, 107 kDa, NCBI access # gi|242061132, sequence coverage 87%. | 8–16 | 542.7460 | 1083.4764 | 1083.4784 | 1.8 | 34 | K.MAS$\text{S}_{2}$DAQLR.M | pSer10 |
| 8–16 | 550.7449 | 1099.4733 | 1099.4753 | 1.1 | 58 | R.NAVDKATS$\text{S}_{2}$DAQLR.L | pSer11 |
| **p107**: PEPC SbPPC4, 107 kDa, NCBI access # gi|242078871, sequence coverage 89%. | 14–18 | 527.9304 | 1580.7559 | 1580.7569 | 8.5 | 16 | K.MERLS$\text{S}_{2}$DAQLR.M | pSer13 |
| 14–18 | 791.3842 | 1580.7559 | 1580.7569 | 2.0 | 58 | R.NAVDKATS$\text{S}_{2}$DAQLR.L | pSer11 |
| **p107**: PEPC SbPPC5, 107 kDa, NCBI access # gi|242058871, sequence coverage 64%. | 4–17 | 527.9304 | 1580.7559 | 1580.7569 | 8.5 | 16 | K.MAS$\text{S}_{2}$DAQLR.M | pSer10 ; Met-Ox |
| 4–17 | 791.3842 | 1580.7559 | 1580.7569 | 2.0 | 58 | R.NAVDKATS$\text{S}_{2}$DAQLR.L | pSer11 |

and PR87G57, respectively characterized by rapid and slow germination velocities, were imbibed in water for up to 96 h at 25 and 35 °C. As defined by Bewley et al. (2013), germination begins with water uptake by the seed (imbibition, Phase I) and ends with the emergence of the radicle, which marks the end of Phase II and the beginning of Phase III (Bewley et al., 2013). Phase II is also associated with a slow rate of water uptake, where the little net movement of water into the seed is due mainly to a lowering of the solute potential (ψs), which drives an increase in volume that results in cracking of the testa. Germination does not include seedling growth. Accordingly, eight stages of germination were established in this study (see Supplementary Fig. S1). Among them stage II represents the emergence of the root, and the beginning of the post-germination Phase III (Bewley et al., 2013). Germination rates were calculated by determining the average of the different stages of germination in a pooled of seeds at a given time post-imbibition (0, 24, 48, 72, and 96 h). Figure 4 shows germination rate for the two sorghum varieties at 25 and 35 °C. Both varieties had a higher germination velocity at 35 than 25 °C, with the seeds germinating at 35 °C having an average lead of two stages over those incubated at 25 °C (Fig. 4). Moreover, PR88Y20 germinated more rapidly than PR87G57 at both 25 and 35 °C (Fig. 4). In parallel, clarified extracts from the same pool of seeds (the 15–20 seeds in the most advanced stages of germination at each time of imbibition) were analyzed by SDS-PAGE and immunoblotting with anti-CaPepC (SbPPC1) (Fig. 5). PR88Y20 at 25 °C attained maximal PTPC monoubiquitination (i.e. formation of p110) between 24 and 48 h post-imbibition (Fig. 5A, arrow), with a p110:p107 ratio of approximately 2. By contrast, maximal PTPC monoubiquitination in PR87G57 appeared to be delayed by up to 48–72 h, in accordance with its slower germination rate (Fig. 5A, dashed arrow), with a p110:p107 ratio of 3 at 48 h. PTPC monoubiquitination in seeds germinated
To further analyze this phenomenon, seeds were imbibed with water for periods up to 24 h and classified as germinated seeds (stage II) or non-germinated seeds (stage I) depending on the emergence of or not of the radicle. PTPC monoubiquitination was far more prevalent in germinating seeds where the emergence of the radicle occurred (Fig. 6A, Germ.). These results confirmed that PTPC monoubiquitination in germinating sorghum seeds coincides with metabolic events associated with the emergence of the radicle (stage II) and early seedling growth.

As the relative rates of glycolysis and gluconeogenesis and adenylate energy charge are influenced by oxygen availability (Bewley et al., 2013), it was also of interest to study the impact of hypoxia stress on PTPC monoubiquitination during seed germination. To this end, seeds were imbibed on moist filter paper (control, normoxic) or submerged in water (hypoxic).

At 48 h post-imbibition most of the normoxic seeds reached stage II, a smaller number remained at stage I, whereas others had attained stage III–IV (Fig. 6, control). However, the submerged seeds remained at stage I, at 72 h post-imbibition (Fig. 6, Hypoxia). The hypoxia-stressed seeds were not dead since they germinated well after they were transferred to normoxic conditions (data not shown). As expected, PTPC monoubiquitination of normoxic seeds markedly increased at stages II to III, but remained low in hypoxia-stressed seeds, even up to 72 h post-imbibition (Fig. 6B). These results establish that enhanced PTPC monoubiquitination is intrinsically linked to the germination of the seed and corresponding enhanced aerobic respiration, cracking of the testa, and the protrusion of the radicle. This makes sense since the ubiquitination machinery is ATP dependent, and abundant ATP probably requires abundant aerobic respiration (Callis, 2014).

In addition, even if germination did not occur (i.e. the radicle did not emerge; stage I), PTPC monoubiquitination was enhanced under normoxic relative to hypoxic conditions (Fig. 6B).

These are the first results showing that PTPC monoubiquitination in germinating sorghum seeds: (i) is very low during Phase I; (ii) always increases at stage II (emergence of the radicle); (iii) is maintained during the aerobic period of high activity of cell division and reserve mobilization; and (iv) remains until stage IV–V when coleoptiles are about 1 cm long and the formation of the photosynthetic tissues begins.

**Concluding remarks**

Deciphering the occurrence, roles, and PTMs of different PTPC isoenzymes during seed development and germination has been poorly studied. Monoubiquitination was recently discovered to be a novel but relatively common PTM of PTPC subunits of Class-1 PEPCs from diverse tissues and plant species (Uhrig et al., 2008; Igawa et al., 2010; O’Leary et al., 2011b; Ruiz-Ballesta et al., 2014; Shane et al., 2013). However, the occurrence of PTPC monoubiquitination and phosphorylation has been specifically described only in the limited examples of developing and germinating COS (Tripodi et al., 2006; Uhrig et al., 2008; O’Leary et al., 2011b), and during proteoid root formation in harsh
This present study describes the patterns of transcript levels and relative abundance of different PTPC isozymes during sorghum seed development and germination, as well as their PTM by monoubiquitination and phosphorylation during germination. The results reveal new and important points; for example, the high level of *SbPPC4* transcripts during early development, at stage I (Fig. 3). This is the first report in which a possible role of this PTPC isozyme during cellularization of the developing seed (Bewley et al., 2013) is suggested. The absence of *BTPC* transcripts and protein represent an important difference with developing COS, a non-green oil seed in which BTPC is highly expressed and appears to play an important role as a catalytic and regulatory subunit of the unusual

![PTPC monoubiquitination](image-url)

Fig. 6. PTPC monoubiquitination occurs in viable seeds that have increased aerobic respiration and reserve mobilization associated with radicle emergence. (A) Seeds (var. PR88Y20) at 24h post-imbibition were separated into two pools: germinated seeds (where the testa was cracked with protrusion of the radicle; Germ) and non-germinated seeds (where the testa was not disrupted, No Germ.). Clarified extracts were subjected to 8% SDS-PAGE and analyzed by immunoblotting with anti-C4 PTPC. (B) Seeds were imbibed submerged in water (Hypoxia) or on moistened filter paper (Control, normoxic). At 48h post-imbibition control seeds mostly ranged from stage II to IV of germination, although some remained at stage I (Control, stages I, II, III and IV), while hypoxic seeds remained at stage I even at 72h post-imbibition. Results in the plot represent the ratio of p110:p107 in control and hypoxic seeds at stage I, 48h post-imbibition, and represent means ±SE of at least three independent experiments. Mean values that are significantly different (*P*<0.01, Student’s *t*-test) are indicated with different letters.
Class-2 PEPC hetero-octameric complex (Uhrig et al., 2008; O’Leary et al., 2011a, b). ShPPC2 and ShPPC3 transcripts and ShPPC2 and ShPPC3 isoenzymes were extensively detected throughout the life cycle of sorghum seeds (Fig. 3 and Table 2). As ShPPC3 expression in roots depends on nitrogen supply (Lepiniec et al., 1993), we propose an important role of this enzyme in providing the precursors needed for amino acid synthesis and transamination reactions in the germinating seed. In addition, ShPPC3 was the only isoenzyme found in the aleurone/endosperm, where it is proposed to play an important role in regulating the production of malic acid, which is either excreted for the acidification of the starchy endosperm (Macnicol and Raymond, 1998), or transported to the growing seedling for the anaplerotic function (Osuna et al., 1996, 1999). Among the immunopurified PTPCs revealed by MS/MS analysis ShPPC2, ShPPC3, and SbPPC4 are monoubiquitinated in vivo. These results provide the first evidence that monoubiquitination is not restricted to a single PTPC isoenzyme within a specific plant species or physiological context, but can occur with multiple PTPC isozymes in vivo. ShPPC2, ShPPC3, and ShPPC4 were simultaneously monoubiquitinated and phosphorylated (Table 3), showing that both PTMs can occur in vivo in the same PTPC polypeptide (Table 3); i.e., they are not mutually exclusive PTMs, as appears to be the case in castor oil plants (Uhrig et al., 2008; O’Leary et al., 2011b). Class-1 PEPC phosphorylation at the single highly conserved serine residue close to the N-terminus of the protein uniformly results in the enzyme activation at physiological pH by decreasing and increasing its sensitivity to allosteric inhibitors and activators, respectively (Echevarria et al., 1994). By contrast, PTPC monoubiquitination has an opposite kinetic effect (Uhrig et al., 2008; Shane et al., 2013). In germinating COS and developing proteoid roots of P-deficient hakea, both PTPC PTMs appear to be exclusive, leading to a clear picture about the impact of each PTM on the activity of the enzyme. However, we show in this study that monoubiquitination and phosphorylation can simultaneously occur with three of the four PTPC isoenzymes expressed in sorghum seeds. This suggests that a combination of both PTMs may serve to fine tune the precise kinetic and regulatory properties for each PTPC isoenzyme at each specific stage of development and germination. However, it is at present difficult to establish the precise impact of phosphorylation versus monoubiquitination in each PTPC isoenzyme at the specific stage of the life cycle of sorghum seed. The use of phosphorylation-site-specific PTPC antibodies (i.e. anti-pSer13 and anti-pSer7 for ShPPC2 and ShPPC3, respectively), which we are presently characterizing, will help us to evaluate the specific pattern of in vivo phosphorylation for the two predominant PTPC isoenzymes in sorghum seeds. In this study we have documented the remarkable versatility and complexity of in vivo PTMs of different PTPC isoenzymes during the life cycle of sorghum seeds. This is also the first study where the extent of PEPC monoubiquitination was observed to increase at stage II of germination. This stage corresponds to radicle protrusion and the beginning of the post-germination phase (Phases II and III, respectively, as described by Bewley et al., 2013), and also to initiation of the second respiratory burst due to the cracking of the testa (Bewley et al., 2013).

Future studies are needed in order to clarify the extent of PEPC monoubiquitination in other seeds, its relation with the efficiency of germination, and its possible regulation by metabolites and other elements of the signal transduction pathway that lead to this PTM. It will also be important to assess whether this PTM influences PEPC’s stability, protein–protein interactions, and/or subcellular location. It will be equally important to identify how this PTM is coordinated with PPCK-mediated phosphorylation-activation of PTPCs at their conserved N-terminal seryl residue. We are currently attempting to disrupt PPCK and ShPPC3 expression in transgenic sorghum in order to study the impact of the absence of PTPC phosphorylation (PPCK silenced), or the ShPPC3 PTPC isoenzyme on seed development and germination.

### Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Stages of development and germination in sorghum seeds.

**Figure S2.** Pattern of PTPC subunit structure and integrity during sorghum seed development and germination.

**Figure S3.** Non-denaturing PAGE followed by in-gel PEPC activity staining of sorghum seed extracts.

**Figure S4.** Immunopurification of PTPC (p110 and p107) from germinating sorghum seeds.

**Figure S5.** The p110 subunit of the ShPPC2 isoenzyme immunopurified from germinating sorghum seeds (48 h post-imbibition) is monoubiquitinated at Lys-630.

### Acknowledgements

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 the MS analyses shown in Table 1. We thank Dr Jean Vidal for providing important scientific comments and discussion, Dr Rosario Álvarez and Dr José Antonio Monreal for collaborating in the immunohistochemistry detection and oligonucleotide design, respectively. 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 Queen’s Research Chairs program to W.C.P., and the 100 Technical Talents Program of the Chinese Academy of Sciences to Y. M. She.

### References

Araus JL, Bort J, Brown RH, Bassett CL, Cortadellas N. 1993. Immunocytochemical localization of phosphoenolpyruvate carboxylase and photosynthesis gas-exchange characteristics in ears of *Triticum aestivum* Desf. *Planta* 191, 507–514.

Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H. 2013. *Seeds: physiology of development, germination and dormancy*, 3rd edn. New York, NY: Springer.
Callis A. 2014. The ubiquitination machinery of the ubiquitin system. The Arabidopsis Book, 06, e0174. doi: 10.1199/tab.0174.

Chollet R, Vidal J, O’Leary M. 1996. Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annual Review of Plant Physiology and Plant Molecular Biology 47, 273–298.

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

Echevarría C, Pacquit V, Bakrim N, Osuna L, Delgado B, Arriocarca. 2001. Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. Archives of Biochemistry and Biophysics 313, 425–430.

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.

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

Igawa T, Fujiwara M, Tanaka I, Fukao Y, Yanagawa Y. 2010. Characterization of bacterial-type phosphoenolpyruvate carboxylase expressed in male gametophyte of higher plants. BMC Plant Biology 10, 200.

Izu K, Matsumura H, Furumoto T, Kiy S. 2004. Phosphoenolpyruvate carboxylase: a new era of structural biology. Annual Review of Plant Biology 55, 69–84.

Kanemasu ET, Bark DL, Chin Choy E. 1975. Effect of soil temperature on sorghum emergence. Plant Soil 43, 411–417.

Kresovich S, Barbazuk B, Bedell JA, et al. 2005. Toward sequencing the sorghum genome. A U.S. National Science Foundation--Sponsored Workshop Report. Plant Physiology 138, 1898–1902.

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

Lepiniec L, Vidal J, Chollet R, Gadad P, Crétin C. 1994. Phosphoenolpyruvate carboxylase: structure, regulation and evolution. Plant Science 99, 111–124.

Macnicol PK, Raymond P. 1998. Role of phosphoenolpyruvate carboxylase in male production by the developing barley aleurone layer. Physiologia Plantarum 103, 132–138.

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.

Nimmo HG. 2000. The regulation of phosphoenolpyruvate carboxylase in CAM plants. Trends in Plant Science 5, 75–80.

Nimmo HG. 2003. Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. Archives of Biochemistry and Biophysics 414, 189–196.

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, 5485–5495.

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 phosphoenolpyruvate carboxylase from wheat seeds during germination. Plant Physiology 111, 551–558.

Osuna L, Pierre JN, González MC, Álvarez R, Cejudo FJ, Echevarría C, Vidal J. 1999. Evidence for a slow-turnover form of the Ca²⁺-independent phosphoenolpyruvate carboxylase kinase in the aleurone endosperm tissue of germinating barley seeds. Plant Physiology 119, 511–520.

Pacquit V, Giglioli N, Crétin C, Pierre JN, Vidal J, Echevarría C. 1995. Regulatory phosphorylation of C₄ phosphoenolpyruvate carboxylase from sorghum. An immunological study using specific anti-phosphorylation site-antibodies. Photosynthesis Research 43, 283–288.

Park J, Khuu N, Howard ASM, Mullen RT, Plaxton WC. 2012. Bacterial- and plant-type phosphoenolpyruvate carboxylase isozymes from developing castor oil seeds interact in vivo and associate with the surface of mitochondria. The Plant Journal 71, 251–262.

Paterson AH, Bowers JE, Bruggmann R, et al. 2009. The Sorghum bicolor genome and the diversification of grasses. Nature 457, 551–556.

Rivoal J, Turpin DH, Plaxton WC. 2002. In vitro phosphorylation of phosphoenolpyruvate carboxylase from the green alga Selenastrum minutum. Plant Cell Physiology 43, 785–792.

Ruiz-Ballesta I, Feria AB, Ni H, She YM, Plaxton WC, Echevarría C. 2014. In vivo monoubiquitination of anaplerotic phosphoenolpyruvate carboxylase occurs at Lys624 in germinating sorghum seeds. Journal of Experimental Botany 65, 443–451.

Sechi S, Chait BT. 1998. Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. Analytical Chemistry 70, 5150–5158.

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.

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.