RESEARCH PAPER

Proteolytic cleavage of Arabidopsis thaliana phosphoenolpyruvate carboxykinase-1 modifies its allosteric regulation

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Abstract

Phosphoenolpyruvate carboxykinase (PEPCK) plays a crucial role in gluconeogenesis. In this work, we analyze the proteolysis of Arabidopsis thaliana PEPCK1 (AthPEPCK1) in germinating seedlings. We found that the amount of AthPEPCK1 protein peaks at 24–48 h post-imbibition. Concomitantly, we observed shorter versions of AthPEPCK1, putatively generated by metacaspase-9 (AthMC9). To study the impact of AthMC9 cleavage on the kinetic and regulatory properties of AthPEPCK1, we produced truncated mutants based on the reported AthMC9 cleavage sites. The Δ19 and Δ101 truncated mutants of AthPEPCK1 showed similar kinetic parameters and the same quaternary structure as the wild type. However, activation by malate and inhibition by glucose 6-phosphate were abolished in the Δ101 mutant. We propose that proteolysis of AthPEPCK1 in germinating seedlings operates as a mechanism to adapt the sensitivity to allosteric regulation during the sink-to-source transition.

Keywords: Arabidopsis thaliana, gluconeogenesis, metacaspase, phosphoenolpyruvate carboxykinase, proteolysis, seedlings.

Introduction

Phosphoenolpyruvate (PEP) is a metabolic hub that connects various pathways, including glycolysis, gluconeogenesis, and metabolism of organic and amino acids (Chiba et al., 2015). Among the enzymes that metabolize PEP, phosphoenolpyruvate carboxykinase (PEPCK) is particularly relevant, due to its numerous physiological functions (Leegood and Walker, 2003; Latorre-Muro et al., 2018; Wang and Dong, 2019). Based on the phosphate donor, PEPCKs are classified as ATP (EC 4.1.1.49), GTP (EC 4.1.1.32), or PPI dependent (EC 4.1.1.38), all with different evolutionary origin (Matte et al., 1997; Fukuda et al., 2004; Aich and Delbaere, 2007; Chiba et al., 2015).

ATP-dependent PEPCK is found in bacteria, yeasts, and plants. This enzyme catalyzes the reversible decarboxylation of oxaloacetate (OAA) to form PEP, according to the reaction: OAA + ATP ↔ PEP + ADP + CO2. Although this reaction is fully reversible in vitro, it is generally accepted that it proceeds...
towards OAA decarboxylation in vivo (Johnson et al., 2016). PEPCK requires two divalent cations to catalyze the reaction: one Mn²⁺ ion which acts as an essential activating cofactor that promotes OAA decarboxylation and stabilizes the enolate ion during catalysis and one Mg²⁺ cation which forms the metal nucleotide complex that constitutes the active form of the substrate (Goldie and Sanwal, 1980; Burnell, 1986; Matte et al., 1997; Johnsen et al., 2016).

ATP-dependent PEPCK is a cytosolic enzyme (Ito et al., 2011; Tsiatsiani et al., 2013) with different physiological roles in plants: (i) it is part of the CO₂-concentrating mechanisms operating in C₄ and Crassulacean acid metabolism (CAM) photosynthesis (Edwards et al., 1971; Reiskind and Bowes, 1991; Martin et al., 2011); (ii) it participates in biotic and abiotic stress responses (Sáez-Vásquez et al., 1995; Chen et al., 2000, 2002; Saito et al., 2008; Penfield et al., 2012; Choi et al., 2015); (iii) it is involved in nitrogen and amino acid metabolism, especially during fruit development (Walker et al., 1999; Lea et al., 2001); and (iv) it is involved in gluconeogenesis during seed germination, channeling carbon released from fatty acid reserves to form sugars, until the photosynthetic apparatus is fully developed (Rylott et al., 2003; Penfield et al., 2004; Malone et al., 2007; Graham, 2008; Eastmond et al., 2015).

The occurrence and regulatory effects of proteolysis on PEPCK have remained obscure. Walker et al. (1995) found that a discrete proteolytic cleavage at the N-terminus of PEPCK occurred in crude extracts from cucumber cotyledons and leaves of C₄ and CAM species; however, proteolysis of the cucumber PEPCK did not significantly alter its activity (Walker and Leegood, 1995). Two PEPCK isoforms of different molecular mass (74 kDa and 65 kDa) were found in Ananas comosus (pineapple) leaves. The shorter version was purified to homogeneity and biochemically characterized, but study of the large isoform was not possible because it was recalcitrant to purification (Martin et al., 2011).

A large-scale study conducted by Tsiatsiani et al. (2013) identified AthPEPCK1 as a substrate of Arabidopsis thaliana (Arabidopsis) metacaspase-9 (AthMC9). This cysteine protease cleaved AthPEPCK1 at the N-terminus, which seemed to boost PEPCK activity. In crude extracts, PEPCK activity was reduced in the mc9 mutant and increased in the MC9-overexpressing lines (Tsiatsiani et al., 2013). AthMC9 is localized in the nucleus, cytosol, and apoplast (Vercammen et al., 2006; Kwon and Hwang, 2013; Tsiatsiani et al., 2013), and is implicated in cell death regulation in many physiological situations, for example during plant immune response (Kim et al., 2013; Shen et al., 2019) and plant vascular development (Escamez et al., 2016, 2019).

Arabidopsis has two ATP-PEPCK genes, pck1 (AT4G37870) and pck2 (AT5G65690), encoding AthPEPCK1 and AthPEPCK2, respectively. We have recently reported the biochemical properties of these proteins, which are finely regulated by numerous metabolites. Mainly, they are inhibited by glucose 6-phosphate (Glc6P), shikimate, and inorganic pyrophosphate (PPi), and activated by malate (Rojas et al., 2019). In this work, we focused on the combined effects of allosteric regulation and proteolysis on the activity of AthPEPCK1, which plays a critical role during seed germination (Rylott et al., 2003; Penfield et al., 2004). We used recombinant proteins to study in detail the biochemical effects of the AthMC9-mediated cleavage of AthPEPCK1. Our results show that proteolysis of the N-terminus modifies the allosteric regulation of AthPEPCK1, which could have important metabolic implications during the sink-to-source transition associated with seedling development.

Materials and methods

Reagents

ATP, ADP, PEP, OAA, NADH, G6P; L-malic acid, pyruvate kinase, and L-malic dehydrogenase were from Sigma Aldrich. L-Lactate dehydrogenase was from Roche. All other reagents were of the highest available quality.

Plant material and growth conditions

All experiments were performed with Arabidopsis Col-0. The pck1 mutant corresponds to the SALK_072899C T-DNA insertion. Seeds were disinfected with 70% (v/v) ethanol for 5 min, then treated with 10% (v/v) bleach for 10 min and washed three times with sterile, distilled water. Seeds were soaked in sterile 0.1% (w/v) agar and stratified at 4 °C in the dark for 2 d. In germinating assays, seeds were sown on a mesh soaked with 0.5× Murashige and Skoog medium in 16 cm diameter Petri dishes and transferred to growth chambers (Raineri et al., 2016). In mature leaf assays, plants were grown on soil in 8 cm diameter ×7 cm height pots, one plant per pot. In all cases, plants were grown at 23 °C and 120 µmol m⁻² s⁻¹, with a long-day photoperiod (16 h light and 8 h dark). The seeds employed were harvested, dried in darkness at room temperature, and stored at 4 °C. In all experiments, samples were taken, immediately frozen with liquid nitrogen, and stored at −80 °C until use.

Protein extraction

Plant material was homogenized in a pre-cooled mortar with liquid nitrogen. For denaturing protein extraction, 20 mg of FW tissue was extracted with 200 µl of denaturing sample buffer, consisting of 2% (w/v) SDS, 20% (w/v) glycerol, 1.4 M 2-mercaptoethanol, 125 mM Tris–HCl pH 6.8, and 0.05% (w/v) bromophenol blue. After adding the buffer, samples were vortexed and heated for 5 min at 95 °C with agitation. Samples were cooled to room temperature and centrifuged at 21 000 × g for 10 min to separate the protein extract from tissue debris. For ultra-denaturing protein extraction, 20 mg of FW tissue were extracted with 500 µl of native buffer, consisting of 100 mM Bicine-KOH pH 9.0, 10% (w/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1X Set III protease cocktail (Merck, 539134), 1 mM NaF, 1 mM Na₂Mo₄, and 1 mM Na₃VO₄. After adding the extraction buffer, samples were vortexed and incubated on ice for 10 min and then centrifuged at 21 000 × g for 10 min. Then, the protein extract was separated from tissue debris and transferred to a new tube.
Production and purification of recombinant proteins

The production of recombinant AthPEPCK1 was performed as previously described (Rojas et al., 2019). The sequence coding for AthMC9 (At5g4200) was cloned using cDNA from Arabidopsis seedlings with the primers AthMC9-f0 (GGAGCCGGATCCACAAAGGATGG TCAAG, the BamHI site is underlined) and AthMC9-r (GAATTCTC AAGGTTGAGAAAGGAAGCTCG, the EcoRI site is underlined). The amplified sequence was inserted in-frame with an N-terminal His6-tag between the BamHI and EcoRI sites of the petDuet-1 vector (Novagen). Protein expression was performed in Escherichia coli BL21 (DE3) (Invitrogen) grown in LB medium supplemented with 100 µg ml–1 ampicillin and induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside for 16 h at 18 °C with agitation. Cells were harvested by centrifugation and resuspended in lysis buffer [25 mM Tris–HCl pH 8.0, 300 mM NaCl, 5% (v/v) glycerol, and 10 mM imidazole]. Cells were disrupted by sonication and centrifuged at 12,000 g for 15 min at 4 °C. The crude extract was loaded on an IDA-Ni2+ column, previously equilibrated with lysis buffer. The recombinant protein was eluted with lysis buffer supplemented with 300 mM imidazole and 10% (v/v) glycerol.

Protein methods

Total proteins were quantified with the Bradford assay (Bradford, 1976), using a standard curve constructed with BSA.

Protein electrophoresis was performed under denaturing conditions (SDS–PAGE), according to the method described by Laemmli (1970). For immunodetection, proteins were transferred to 0.45 µm nitrocellulose membranes (Amersham) at 180 mA for 60 min. Membranes were incubated overnight at 4 °C with purified anti-AthPEPCK1 antibodies diluted 1:1000 and then incubated for 1 h at room temperature with goat-anti-rabbit IgG H&L conjugated to horseradish peroxidase (Abcam, ab6721) diluted 1:10 000. Protein bands were revealed with SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fischer Scientific), ab6721) diluted 1:10 000 and then incubated for 1 h at room temperature with goat-anti-rabbit IgG H&L conjugated to horseradish peroxidase (Abcam, ab6721) diluted 1:10 000. Protein bands were revealed with SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fischer Scientific), following the manufacturer's instructions. Radiographic films (AgFA) were exposed for between 2.5 min and 5 min for the detection of AthPEPCK1. Antibodies were stripped with 100 mM glycine pH 2.5 to analyze protein loading. Membranes were thoroughly washed and then incubated overnight with anti-TaqGAPDH antibodies diluted 1:5000. All subsequent steps were performed as previously described. In this case, exposure times were between 30 s and 1 min.

Enzyme activity assays

Recombinant AthPEPCK1 and the Δ19 and Δ101 truncated mutants were assayed spectrophotometrically as previously described (Rojas et al., 2019), with the concentrations of substrates indicated below.

Carboxylase activity of PEPCK in crude extracts was measured using 100 mM HEPES-NaOH pH 7.0, 4 mM 2-mercaptoethanol, 0.2 mM NADH, 4 mM MgCl2, 1 mM MnCl2, 100 mM KHCO3, 0.2 mM ADP, 10 mM PEP and 1 U of malate dehydrogenase. Assays were performed in 250 µl at 30 °C and were corrected for PEP carboxylase activity by omitting ADP from the reaction mixture, as previously done by Martin et al. (2007). Activity was calculated by measuring the change in absorbance at 340 nm due to NADH consumption.

Kinetic parameters were calculated using the software GraphPad Prism (Version 5). Activity data were plotted against the concentration of the variable substrate or effector and fitted to a modified Hill equation:

\[ v = \frac{v_\text{max}}{K_v + (1-F)\cdot C^{n_H}/(k^{n_H}+C^{n_H})} \]

where \( v \) is the initial velocity; \( v_\text{max} \) is the velocity in the absence of the substrate or effector being analyzed; \( V \) is the maximal velocity (\( V_\text{max} \)); activation or inhibition; \( C \) is the concentration of substrate or effector under study; \( k \) is the concentration of substrate or effector producing half of the maximal velocity (\( K_v \)), activation (\( A_v \)) or inhibition (\( I_v \)); and \( n_H \) is the Hill coefficient.

One unit is defined as the amount of enzyme that catalyzes the formation of 1 µmol of product (PEP or OA) per minute under the specified assay conditions (of decarboxylation or carboxylation, respectively). All assays were performed using standard conditions (see above). Before performing these experiments, effectors were tested on coupled enzymes to avoid unwanted effects.

Recombinant AthMC9 protein was assayed as described previously by Verschuren et al. (2004), with minor modifications. Reactions were done with 50 mM MES-KOH pH 5.5, 150 mM NaCl, 300 mM sucrose, 10 mM DTT, 0.3 µg µl–1 AthMC9, and 0.15 µg µl–1 AthPEPCK1. Aliquots were taken at different time intervals and analyzed by SDS–PAGE and PEPC kinase activity.

Native molecular mass determination

Protein molecular mass was determined by gel filtration chromatography using a Superdex 200 10/300 column (GE Healthcare) equilibrated with 50 mM HEPES pH 8.0 and 100 mM NaCl. A calibration curve was constructed with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and RNase (13.7 kDa). Molecular mass values were calculated as (Ve−V0)/(Vt−V0), where Ve is the elution volume of the protein, V0 is the elution volume of dextran blue (Promega), and Vt is the total volume of the column.

Protein thermal shift assays

Protein thermal shift assays were performed as previously described (Rojas et al., 2019). Assays were performed in a final volume of 20 µl with 0.15 mg ml–1 protein, 4X Sypro Orange (Sigma), and 25 mM HEPES-NaOH pH 7.0 in Microamp fast 96-well PCR plates (Applied Biosystems). All the reactions were performed with their corresponding controls (without protein or effector). Plates were sealed with Microseal adhesive film (Bio-Rad) and heated in a StepOne Real-Time PCR System (Applied Biosystems) from 25 °C to 99 °C, with increments of 0.4 °C. Changes in fluorescence were monitored simultaneously. The wavelengths for excitation and emission were 490 nm and 575 nm, respectively. The melting temperature (\( T_m \)) of each sample was calculated by plotting the first derivative of the fluorescence emission (–dF/dT) as a function of temperature (\( T \)) and identifying the minimum of the curve. The shift in the melting temperature (\( \Delta T_m \)) was calculated subtracting \( T_m \) from the control without effector.

Statistical analysis

To compare means, a t-test for two independent samples or one-way ANOVA was performed using Minitab 17 statistical software. When comparing kinetic parameters of substrate and effectors curves, the adjusted models were compared with an extra-sum-of-squares F-test (Motulsky and Christopoulos, 2003; Hall and Langmead, 2010) using GraphPad Prism (Version 5).
Results

Proteolysis of \textit{AthPEPCK1} in germinating seedlings

Transcriptomic data retrieved from the eFP browser (Winter \textit{et al.}, 2007) showed that the expression of \textit{AthPEPCK1} and \textit{AthPEPCK2} in germinating seedlings and mature leaves was considerably different (Supplementary Fig. S1). \textit{AthPEPCK1} transcripts were 620-fold higher than \textit{AthPEPCK2} transcripts in germinating seedlings at 48 hours after imbibition (HAI; Supplementary Fig. S1). \textit{AthPEPCK1} transcripts were 10-fold higher in germinating seedlings than in mature leaves, while \textit{AthPEPCK2} transcripts were relatively low in germinating seedlings and below the detection limit in mature leaves (Supplementary Fig. S1). Based on this information, our experiments were focused on \textit{AthPEPCK1}. To analyze the integrity of this protein at different developmental stages, we extracted proteins under denaturing conditions from germinating seedlings harvested at 48 HAI and mature leaves from 32-day-old rosettes (Fig. 1A). \textit{AthPEPCK1} codes for a protein of 73.5 kDa, which further arranges as a hexamer of $\sim$440 kDa (Rojas \textit{et al.}, 2019). We found that \textit{AthPEPCK1} was partially proteolyzed in germinating seedlings but not in mature leaves, although the amount of \textit{AthPEPCK1} was lower in the latter (Fig. 1A).

Some proteases can be active during protein extraction, even with sample buffer containing SDS (Plaxton, 2019). To discard the possibility that \textit{AthPEPCK1} from germinating seedlings was degraded during the extraction, we compared four extraction methods, namely sample buffer, sample buffer supplemented with 2 M urea, 10\% (w/v) TCA, and 10\% (w/v) TCA in acetone. The protocols based on TCA are considered as ultra-denaturing extraction methods. Supplementary Fig. S2A shows that limited proteolysis of \textit{AthPEPCK1} occurred \textit{in vivo} and was not an artifact of the extraction procedure. In all our experiments, we found that the majority of \textit{AthPEPCK1} was present \textit{in vivo} as a non-proteolyzed form, thus we had to adjust exposure times to visualize the proteolyzed forms (Supplementary Fig. S2B).

Polyclonal antiserum raised against recombinant \textit{AthPEPCK1} cross-reacts with \textit{AthPEPCK2} (78.4\% sequence identity, data not shown). Considering the differences in the relative abundances of \textit{AthPEPCK1} and \textit{AthPEPCK2} transcripts in germinating seedlings (Supplementary Fig. S1), we assumed that the main isoform detected in Fig. 1A was \textit{AthPEPCK1}. To test our hypothesis, we analyzed protein extracts obtained under denaturing conditions from germinating seedlings of wild-type (WT) plants and the \textit{pck1} knockout mutant. Seedlings were grown under long-day conditions and samples were harvested at time intervals from 4 to 72 HAI. Figure 1B shows that \textit{AthPEPCK1} proteolysis peaked at 24–48 HAI in WT plants, whereas no protein bands were detected in the \textit{pck1} mutant at any time point. The proteolysis of \textit{AthPEPCK1} was also observed in WT seedlings grown in total darkness (skotomorphogenesis), even though the amount of \textit{AthPEPCK1} was significantly lower than in seedlings grown under long-day conditions (Fig. 1B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Proteolysis of \textit{AthPEPCK1} in germinating seedlings. (A) Analysis of \textit{AthPEPCK1} in germinating seedlings and mature rosettes. Denatured protein extracts from \textit{Arabidopsis} 48 HAI germinating seedlings (left) and 32-day-old rosettes (right) were resolved by 12\% SDS–PAGE (lower panel). Proteins were transferred to nitrocellulose membranes and immunodetected with anti-\textit{AthPEPCK1} antiserum (upper panel), as described in the Materials and methods. FW, amount of fresh weight tissue loaded in the gel. (B) Time course of \textit{AthPEPCK1} proteolysis during \textit{Arabidopsis} germination. Western blot performed with anti-\textit{AthPEPCK1} antiserum (upper panel) and load control with anti-\textit{GAPDH} antiserum (lower panel) of denatured protein extracts from \textit{Arabidopsis} seedlings, obtained as described in the Materials and methods. HAI, hours after imbibition; LD, long-day condition (16~h light and 8~h dark); SK, skotomorphogenesis (total darkness). The gel was loaded with 1.3 mg of FW tissue. (C) \textit{PEPCK} activity in \textit{Arabidopsis} germinating seedlings. \textit{PEPCK} carboxylase activity was measured in crude extracts from \textit{Arabidopsis} seedlings, as described in the Materials and methods. Data are the mean ±SE of four biological replicates. Means that are significantly different (P<0.05) using one-way ANOVA test with a 95\% confidence level were grouped with Fisher LSD post-hoc test. In the lower panel, a blot of the measured samples is shown. The gel was loaded with 3 mg of protein. In all the blots, the arrow indicates the full-length \textit{AthPEPCK1} and the asterisk its truncated forms.}
\end{figure}
Based on our results (Fig. 1A, B; Supplementary Fig. S2) and the reports of PEPCK degradation in cucumber cotyledons (Walker et al., 1995), we optimized a protocol for the extraction of native proteins to assess PEPCK activity in germinating seedlings. Extraction was performed with a buffer at pH 9.0 (which diminished PEPCK proteolysis in protein extracts from cucumber; Walker and Leegood, 1995) supplemented with protease inhibitors, to avoid further degradation during the assays. We found that AthPEPCK1 remained stable for at least 4 h after the extraction (Supplementary Fig. S3) and PEPCK activity in crude extracts from Arabidopsis seedlings was in the range described by Malone et al. (2007). As shown in Fig. 1C, AthPEPCK1 activity was highest at 72 HAI, while the peak of protein expression was observed at 24 HAI. The maximum level of AthPEPCK1 protein coincided with the onset of proteolysis, at ~24–72 HAI (Fig. 1B, C).

**Kinetic and regulatory effects on AthPEPCK1 triggered by AthMC9 cleavage**

The multiple AthPEPCK1 bands observed in crude extracts suggested partial proteolysis by a protease (Fig. 1A–C; Supplementary Figs S2, S3). It has been previously reported that AthPEPCK1 was cleaved at the N-terminus by AthMC9 (Tsiatsiani et al., 2013). To test if AthPEPCK1 was cleaved by AthMC9, we incubated recombinant AthPEPCK1 for 60 min with crude extracts from germinating seedlings harvested at 48 HAI at different pH values, as AthMC9 was inactive at alkaline pH (Vercammen et al., 2004). We found that the cleavage of AthPEPCK1 occurred at pH 5.5 and 7.0, whereas it was prevented at pH 9.0 (Fig. 2A).

To analyze the cleavage effect on AthPEPCK1 activity, we cloned the gene coding for AthMC9 from Arabidopsis seedlings and expressed the recombinant protein in E. coli. Recombinant AthMC9 was expressed as a 37.1 kDa zymogen, which is auto-proteolyzed to produce the p10 (15.4 kDa) and p20 (21.73 kDa) subunits that made up the active AthMC9 (Supplementary Fig. S4; Vercammen et al., 2004). Incubation of AthPEPCK1 with AthMC9 for 1 h completely proteolyzed the former (Fig. 2B). The bands observed in Fig. 2B corresponded to the theoretical fragments predicted according to the AthMC9 recognition sites (K19 and R101; Fig. 2C) (Tsiatsiani et al., 2013). The activity of proteolyzed AthPEPCK1 was slightly higher than that of the full-length protein, although the change was not statistically significant (data not shown).

To further study the effects of AthMC9 cleavage on AthPEPCK1 kinetics, we constructed two N-terminal truncated forms of AthPEPCK1, Δ19 and Δ101. We expressed, purified, and characterized these mutants (Table 1; Supplementary Figs S5, S6). In the carboxylation reaction, the kcat values of the Δ19 and Δ101 truncated enzymes were 1.5- and 1.7-fold higher, respectively, than that of the WT; conversely, all enzymes had similar Km values in the reaction of decarboxylation (Table 1). Based on Km values, both mutants had higher apparent affinities for nucleotides (ATP and ADP) and OAA than for PEP, like the WT enzyme (Table 1). The Δ19 mutant showed 2- and 6-fold higher apparent affinity for PEP and OAA, respectively, than the WT. The Δ101 mutant showed 2-fold lower apparent affinity for PEP and 5-fold higher apparent affinity for OAA than the WT. Size exclusion chromatography revealed that both truncated forms were hexamers (Supplementary Fig. S7), like the WT enzyme (Rojas et al., 2019).

Plant PEPCKs are allosterically regulated by metabolites (Hatch and Mau, 1977; Leegood and Ap Rees, 1978; Burnell, 1986; Martín et al., 2011; Rojas et al., 2019). Interestingly, the
response of the Δ19 and Δ101 mutants to different metabolites was altered compared with the full-length form. Both truncated mutants were more sensitive to PPI than the WT (Fig. 3). Sensitivity to shikimate was slightly increased in the Δ19 mutant compared with the WT (although not statistically significant), while the Δ101 mutant was almost insensitive to this metabolite (Fig. 3). Glc6P inhibited the WT enzyme and the Δ19 truncated form but, surprisingly, the Δ101 truncated form was 2-fold activated by the same metabolite (Fig. 3). Malate activated by 2- and 1.5-fold the WT enzyme and the Δ19 truncated form, respectively, while the Δ101 truncated form was insensitive to this metabolite (Fig. 3). To test whether malate still binds to the Δ101 mutant, we performed thermal shift assays (Rojas et al., 2019). We found that malate produced a similar shift in the melting temperature (ΔTm) of the WT, Δ19, and Δ101 truncated forms (Fig. 4; Supplementary Fig. S8), suggesting that this metabolite binds to all enzyme forms.

**Discussion**

The regulation of enzymes by proteolysis is an emergent issue in plant biochemistry. The Arabidopsis genome codes for >800 proteases with distinct temporal and tissue expression profiles (van der Hoorn, 2008; Tsatsianii et al., 2012), but our knowledge on plant proteolytic cascades is still fragmentary (Paulus and Van der Hoorn, 2019). Understanding the regulation of the gluconeogenic pathway during seed germination is of critical importance, as seedling establishment has a direct impact on plant fitness and productivity (Graham, 2008). Based on this, we focused our studies on the proteolytic regulation of AthPEPCK1, a key regulatory enzyme of plant gluconeogenesis (Penfield et al., 2004, 2012; Eastmond et al., 2015).

Some authors have observed that plant enzymes usually contain N- and C-terminal extensions compared with their bacterial or cyanobacterial counterparts. Such extensions are generally susceptible to post-translational modifications (Lepiniec et al., 1993; Ocheretina et al., 1993; Walker and Leegood, 1995; Furumoto et al., 1999). These extensions might represent regulation modules acquired during evolution to accomplish complex regulations, as plants must respond to ever-changing environmental conditions. The first observation of a discrete proteolytic cleavage at the N-terminus of a plant PEPCK was reported by Walker et al. (1995). These authors demonstrated that PEPCK was proteolyzed in crude extracts obtained at neutral pH, which could be prevented by making extractions at alkaline pH (Walker et al., 1995). We found similar results in Arabidopsis, as native extractions at alkaline pH prevented further PEPCK degradation (Fig. 2A; Supplementary Fig. S3). Cleavage of the cucumber PEPCK did not significantly alter its activity (Walker and Leegood, 1995). Therefore, the authors hypothesized that the N-terminal extension would confer unique regulatory properties to the enzyme, not located in the smaller bacterial versions. This model was supported by the fact that plant PEPCK is phosphorylated near the N-terminus, which in turn inhibits the activity of the enzyme (Walker and Leegood, 1995; Leegood and Walker, 1996, 2003; Walker et al., 1997, 2002; Bailey et al., 2007; Chao et al., 2014). The work performed by Shen et al. (2017) described a complex activation and inhibition mechanism on AthPEPCK1, depending on the phosphorylated residue. Thus, it would be important to study in detail the kinetic and regulatory properties, as well as the susceptibility to proteolysis, of the phosphorylated enzyme.

In our studies with Arabidopsis seedlings, we found that AthPEPCK1 is subject to proteolysis around 24–48 HAI,

### Table 1. Kinetic parameters for AthPEPCK1 and truncated mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction direction</th>
<th>Substrate</th>
<th>K_M (µM)</th>
<th>K_cat (s⁻¹)</th>
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<td>AthPEPCK1 WT</td>
<td>Carboxylation</td>
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<td></td>
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<td>ADP</td>
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<td>OAA</td>
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<td>ATP</td>
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<td>AthPEPCK1 Δ19</td>
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</table>

Reactions in both directions of the reaction were performed as described in the Materials and methods. Kinetic constants were calculated by fitting experimental data (Supplementary Figs S5, S6) to the Michaelis–Menten equation and the reported values correspond to the mean ±SE of the adjusted parameters. Comparisons of the parameters were performed with an extra-sum-of-squares F-test and the differences are marked with * (P<0.05) and ** (P<0.01). Fixed substrate concentrations were as follows: *0.25 mM ADP, *15 mM PEP, *0.75 mM ATP, *0.75 mM OAA, *0.5 mM ADP, *5 mM PEP, *1.0 mM ATP, *0.5 mM OAA, *0.5 mM ADP, *15 mM PEP, *0.5 mM ATP, and *0.5 mM OAA. The parameters of the WT enzyme were taken from Rojas et al. (2019).
when the level of protein reaches a maximum (Fig. 1A–C). To discard the possibility that proteolysis occurs during extraction, we employed ultra-denaturing extraction methods (Supplementary Fig. S2), which confirmed that limited AthPEPCK1 degradation occurs in vivo. These findings are in line with the shorter versions of PEPCK found in A. comosus leaves extracted with 10% (w/v) TCA (Martín et al., 2011). We measured PEPCK carboxylase activity in these samples, but we did not find the peak of the activity described by Malone et al. (2007); instead, we observed a gradual increase of PEPCK activity.
activity (~2-fold), coincident with the peak of protein accumulation (Fig. 1C). These differences might originate from different extraction conditions, which might lead to different enzyme populations.

A large-scale study conducted by Tsiatsiani et al. (2013) identified AthPEPCK1 as a target of AthMC9. Crude extracts of mc9 knockout mutants and MC9-overexpressing lines showed decreased and increased PEPCK carboxylase activity, respectively (Tsiatsiani et al., 2013). It is important to note that proteolysis of AthPEPCK1 also occurred in the mc9 mutant line, probably due to the presence of other metacaspases (such as MC1 and MC4) in this tissue (Tsiatsiani et al., 2013). In this study, the recombinant AthPEPCK1 truncated mutants (Δ19 and Δ101) showed similar decarboxylation activity to the WT enzyme. In comparison, the carboxylation reaction was slightly increased in both truncated forms compared with the WT enzyme (Table 1). The $K_M$ values for the substrates of the truncated mutants were in the same range as those determined for the short version of the pineapple PEPCK (Martín et al., 2011). The $K_M$ for PEP of the Δ19 mutant was 2-fold lower than that of the WT enzyme; similarly, the truncated version of pineapple PEPCK has a 10-fold lower $K_M$ for PEP than the non-proteolyzed enzyme (Daley et al., 1977; Martín et al., 2011).

A key characteristic of the AthPEPCK1 truncated mutants is that allosteric regulation by metabolites differs from that observed for the WT enzyme. In particular, Glc6P is an inhibitor of the WT enzyme, but a weak activator of the Δ101 truncated form, whereas malate activates the WT enzyme and has no effect on the Δ101 truncated form (Fig. 3). These characteristics reinforce the idea that the N-terminal extension confers regulatory properties to plant PEPCK. These results agree with the findings of Furumoto et al. (1999), who treated maize PEPCK with enterokinase under controlled conditions to cleave the N-terminus. The proteolyzed enzyme showed 2-fold higher activity and was inhibited by 3-phosphoglyceric acid, while the full-length protein was only slightly affected by this metabolite (Furumoto et al., 1999). In line with our findings, Martin et al. (2011) showed that the small version of pineapple PEPCK is not affected by Glc6P or L-malate in the decarboxylation direction of the reaction; unfortunately, we do not know if these metabolites have any effect on the full-length form of the pineapple enzyme, as it could not be purified and characterized (Martin et al., 2011).

The proteolytic regulation of AthPEPCK1 might be part of a mechanism to regulate its levels and/or activity during the sink-to-source transition. During germination, when carbon is obtained from lipids and amino acids, the levels of malate increase, thus activating AthPEPCK1 and the flux of carbon into gluconeogenesis (Fig. 5). Once the photosynthetic apparatus is developed, gluconeogenesis is replaced by glycolysis. At this stage, reduced carbon is obtained from the Benson–Calvin–Basham cycle, the levels of hexose-phosphates increase, and PEPCK activity diminishes ~10-fold, from ~0.5 U g FW$^{-1}$ in germinating seedlings to ~0.05 U g FW$^{-1}$ in mature leaves (Fig. 1C; Malone et al., 2007). At this point, AthMC9 might generate the shorter AthPEPCK1 isoforms needed for the new metabolic scenario (Fig. 5). PEPCK is a cataplerotic enzyme that withdraws intermediates of the tricarboxylic acid cycle to maintain their balance under different physiological conditions (Leegood and Walker, 2003), acting in coordination with the anaplerotic enzyme PEP carboxylase (Podestá and Plaxton, 1994; O’Leary et al., 2011). In fact, it has been suggested that the major role played by PEPCK in mammals might be in cataplerosis (Wang and Dong, 2019).

In our experiments, performed with extracts from whole germinating Arabidopsis seedlings, we observed that the majority of AthPEPCK1 remained in the non-proteolyzed form. It is important to note that low-stoichiometry PTM sites could reflect the occurrence of the modification at a specific place and time (Prus et al., 2019). If AthPEPCK1 is differentially regulated in different cell types, then the fraction of proteolyzed isoform would be ‘diluted’ in the whole seedling extract. Indeed, AthPEPCK1 expression is differentially regulated in the embryo, the endosperm, and the seed coat of Arabidopsis germinating seeds (Penfield et al., 2004). Similarly, PEPCK from developing pea seeds is differentially expressed in different cell types, but it is only proteolyzed in the cotyledons and the embryonic axis (Delgado-Alvarado et al., 2007). Alternatively, the truncated AthPEPCK1 poly-peptides might represent intermediate degradation products during the turnover of the protein in germinating seedlings. It has been reported that such intermediates have a short half-life (Prus et al., 2019), which in turn would lead to an imbalanced stoichiometry with the full-length enzyme.
Actually, it has been shown that \textit{AthMC9} generates protein fragments bearing destabilizing residues that could be further processed by the N-end rule pathway (Gibbs \textit{et al.}, 2014, 2016). Experiments to test these hypotheses are currently under way.

**Supplementary data**

The following supplementary data are available at \textit{JXB} online.

- Fig. S1. Comparison of the \textit{AthPEPCK1} and \textit{AthPEPCK2} expression levels.
- Fig. S2. Denaturing protein extraction from Arabidopsis leaves and seedlings.
- Fig. S3. Native extraction of \textit{AthPEPCK1} from germinating seedlings.
- Fig. S4. Purification of \textit{AthMC9} by IDA-Ni\(^{2+}\).
- Fig. S5. Substrate saturation curves for the \textit{AthPEPCK1} \(\Delta19\) mutant.
- Fig. S6. Substrate saturation curves for the \textit{AthPEPCK1} \(\Delta101\) mutant.
- Fig. S7. Size exclusion chromatography for the \textit{AthPEPCK1} truncated mutants.
- Fig. S8. Thermal shift assay coupled to differential scanning fluorometry for the \textit{AthPEPCK1} truncated mutants.

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**Author contributions**

Conceptualization, all authors; formal analysis, all authors; funding acquisition, CMF and AAI; investigation, BER and MDH; writing—original draft, BER and MDH; writing—review and editing, CMF and AAI.

**Data availability**

The data that support the findings of this study are openly available in the Dryad Digital Repository at https://doi.org/10.5061/dryad.6t1gjywww (Rojas \textit{et al.}, 2021).
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