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

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Running title

Regulation of phosphoenolpyruvate carboxykinase-1 by metacaspase-9

Highlight

This paper describes the effects of the N-terminal proteolytic cleavage on the kinetic and regulatory properties of *Arabidopsis thaliana* phosphoenolpyruvate carboxykinase-1.

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 expression of *AthPEPCK1* peaks at 24-48 hours 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 $\Delta 19$ and $\Delta 101$ truncated mutants of *AthPEPCK1* showed similar kinetic parameters and the same quaternary structure than the WT. However, activation by malate and inhibition by glucose 6-phosphate were abolished in the $\Delta 101$ 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.

Abbreviations

AthMC9, *Arabidopsis thaliana* metacaspase-9; *AthPEPCK1*, *Arabidopsis thaliana* phosphoenolpyruvate carboxykinase-1; Glc6P, glucose 6-phosphate; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; PPI, inorganic pyrophosphate.

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, 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: $\text{OAA} + \text{ATP} \rightleftharpoons \text{PEP} + \text{ADP} + \text{CO}_2$. 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 Mn^{2+} ion acts as an essential activating cofactor that promotes OAA decarboxylation and stabilizes the enolate ion during catalysis; while one Mg^{2+} cation forms the metal nucleotide complex that constitutes the active form of the substrate (Goldie and Sanwal, 1980; Burnell, 1986; Matte *et al.*, 1997; Johnson *et al.*, 2016).

ATP-dependent PEPCK has different physiological roles in plants: i) it is part of the CO_2 -concentrating mechanisms operating in C_4 and CAM photosynthesis (Edwards *et al.*, 1971; Reiskind and Bowes, 1991; Martín *et al.*, 2011); ii) it participates in biotic and abiotic stress responses (Saez-Vasquez *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 C_4 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 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 (Martín *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).

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, Glc6P, 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 thrice with sterile, distilled water.

Seeds were soaked in sterile 0.1% (w/v) agar and stratified at 4 °C in the dark for two days. In germinating assays, seeds were sown on a mesh soaked with 0.5 X 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 x 7 cm height pots, one plant per pot. In all cases, plants were grown at 23 °C and 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 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 precooled mortar with liquid nitrogen. For denaturing protein extraction, 20 mg of fresh weight (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 x *g* for 10 min to separate the protein extract from tissue debris. For native protein extraction, 20 mg of FW tissue was extracted with 500 μl of native buffer, consisting in 100 mM Bicine-KOH pH 9.0, 10% (v/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 PMSF, 1X Set III protease cocktail (Merck, 539134), 1 mM NaF, 1 mM Na_2MO_4 , and 1 mM Na_3VO_4 . After adding the extraction buffer, samples were vortexed and incubated on ice for 10 min and then centrifuged at 21,000 x *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* (At5g04200) was cloned using cDNA from Arabidopsis seedlings and inserted in frame with an N-terminal His₆-tag between the *Bam*HI and *Eco*RI sites of the pETDuet-1 vector (Novagen). Protein expression was performed in *E. coli* BL21 (DE3) (Invitrogen) grown in LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ 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 x 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.

Production of antisera.

Polyclonal antibodies against *AthPEPCK1* were raised in rabbits using the purified recombinant protein at the Centro de Medicina Comparada (ICIVET Litoral, CONICET-UNL, Argentina). To further increase the specificity, the antiserum was purified with *AthPEPCK1*, following a previously described protocol (Fang, 2012). Polyclonal antibodies against *Triticum aestivum* NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (*TaeGAPDH*) were produced by Piattoni *et al.* (2017).

Protein methods.

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

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 1/1,000 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 between 2.5 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-*TaeGAPDH* antibodies diluted 1/5,000. All subsequent steps were performed as previously described. In this case, exposition times were between 30 s and 1 min.

Enzyme activity assays.

Recombinant *AthPEPCK1* and the Δ19 and Δ101 truncated mutants were assayed as previously described (Rojas *et al.*, 2019). Carboxykinase activity of PEPCK in crude extracts was measured using 100 mM HEPES-NaOH pH 7.0, 4 mM 2-mercaptoethanol, 0.2 mM

172 NADH, 4 mM MgCl₂, 1 mM MnCl₂, 100 mM KHCO₃, 0.2 mM ADP, 10 mM PEP and 1 U malate
173 dehydrogenase. Assays were performed in 250 µl at 30 °C and were corrected for
174 phosphoenolpyruvate carboxylase activity by omitting ADP from the reaction mixture, as
175 previously done by Martin *et al.* (2007).

176 Recombinant *AthMC9* protein was assayed as described previously by Vercammen *et al.*
177 (2004), with minor modifications. Reactions were done with 50 mM MES-KOH pH 5.5, 150 mM
178 NaCl, 300 mM sucrose, 10 mM DTT, 0.3 µg µl⁻¹ *AthMC9*, and 0.15 µg µl⁻¹ *AthPEPCK1*.
179 Aliquots were taken at different time intervals and analyzed by SDS-PAGE and PEPCK
180 activity.

181 Results

182 *Proteolysis of AthPEPCK1 in germinating seedlings.*

183 Transcriptomic data retrieved from the eFP browser (Winter *et al.*, 2007) shows that the
184 expression of *AthPEPCK1* and *AthPEPCK2* in germinating seedlings and mature leaves are
185 considerably different (Fig. S1). *AthPEPCK1* transcripts are 620-fold higher than *AthPEPCK2*
186 transcripts in germinating seedlings at 48 hours after imbibition (HAI; Fig. S1). *AthPEPCK1*
187 transcripts are 10-fold higher in germinating seedlings at 48 HAI than in mature leaves, while
188 *AthPEPCK2* transcripts are relatively low in germinating seedlings and below the detection
189 limit in mature leaves (Fig. S1). Based on this information, our experiments were focused on
190 *AthPEPCK1*. To analyze the integrity of this protein at different developmental stages, we
191 extracted proteins under denaturing conditions from germinating seedlings harvested at 48 HAI
192 and mature leaves from 32-days-old rosettes (Fig. 1A). We found that *AthPEPCK1* is
193 proteolyzed in germinating seedlings but not in mature leaves, although the amount of
194 *AthPEPCK1* is lower in the latter (Fig. 1A). Some proteases can be active during protein
195 extraction, even with sample buffer containing SDS (Plaxton, 2019). To discard the possibility
196 that *AthPEPCK1* from germinating seedlings is degraded during the extraction, the sample
197 buffer was supplemented with 2 M urea. Fig. S2 shows that proteolysis of *AthPEPCK1* occurs
198 *in vivo* and is not an artifact of the extraction procedure.

199 Polyclonal antiserum raised against recombinant *AthPEPCK1* cross-reacts with
200 *AthPEPCK2* (78.4% sequence identity, data not shown). Considering the differences in the
201 relative abundances of *AthPEPCK1* and *AthPEPCK2* transcripts in germinating seedlings (Fig.

202 S1), we assumed that the main isoform detected in Fig. 1A is *AthPEPCK1*. To test our
 203 hypothesis, we analyzed protein extracts obtained under denaturing conditions from
 204 germinating seedlings of WT plants and the *pck1* knock-out mutant. Seedlings were grown
 205 under long-day conditions and samples were harvested at time intervals from 4 to 72 HAI. Fig.
 206 1B shows that *AthPEPCK1* proteolysis peaked at 24-48 HAI in WT plants, whereas no protein
 207 bands were detected in the *pck1* mutant at any time point. The proteolysis of *AthPEPCK1* was
 208 also observed in WT seedlings grown in total dark (skotomorphogenesis), even though the
 209 amount of *AthPEPCK1* was significantly lower than in seedlings grown under long-day
 210 conditions (Fig. 1B).

211 Based on our results (Figs 1A, 1B, and S2) and the reports of PEPCK degradation in
 212 cucumber cotyledons (Walker *et al.*, 1995), we optimized a protocol for the extraction of native
 213 proteins to assess PEPCK activity in germinating seedlings. Extraction was performed with a
 214 buffer at pH 9.0 (which diminished PEPCK proteolysis in protein extracts from cucumber;
 215 Walker & Leegood, 1995) supplemented with protease inhibitors, to avoid further degradation
 216 during the assays. We found that *AthPEPCK1* remained stable for at least 4 h after the
 217 extraction (Fig. S3) and PEPCK activity in crude extracts from Arabidopsis seedlings was in
 218 the range described by Malone *et al.* (2007). As shown in Fig. 1C, *AthPEPCK1* activity was
 219 highest at 4 days after imbibition (DAI), while the peak of protein expression was observed at 1
 220 DAI. The maximum level of *AthPEPCK1* protein coincided with the onset of proteolysis, around
 221 24-72 HAI (Figs 1B and 1C).

222

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

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

231 To analyze the cleavage effect on *Ath*PEPCK1 activity, we cloned the gene coding for
 232 *Ath*MC9 from Arabidopsis seedlings and expressed the recombinant protein in *E. coli* cells.
 233 Recombinant *Ath*MC9 is expressed as a 37.1 kDa zymogen, which is auto-proteolyzed to
 234 produce the p10 (15.4 kDa) and p20 (21.73 kDa) subunits that conform the active *Ath*MC9
 235 (Fig. S4; Vercammen *et al.*, 2004). Incubation of *Ath*PEPCK1 with *Ath*MC9 for 1 h completely
 236 proteolyzed the former (Fig. 2B). The bands observed in Fig. 2B correspond to the theoretical
 237 fragments predicted according to the *Ath*MC9 recognition sites (K19 and R101; Fig. 2C)
 238 (Tsiatsiani *et al.*, 2013). The activity of proteolyzed *Ath*PEPCK1 was slightly higher than the
 239 full-length protein, although the change was not statistically significant (data not shown).

240 To further study the effects of *Ath*MC9 cleavage on *Ath*PEPCK1 kinetics, we
 241 constructed two N-terminal truncated forms of *Ath*PEPCK1, Δ 19 and Δ 101. We expressed,
 242 purified, and characterized these mutants (Table 1, Figs S5-S6). In the carboxylation reaction,
 243 the k_{cat} of the Δ 19 and Δ 101 truncated enzymes were 1.5- and 1.7-fold higher than the WT,
 244 respectively; conversely, all enzymes had similar k_{cat} values in the reaction of decarboxylation
 245 (Table 1). Based on K_M values, both mutants have higher apparent affinities for nucleotides
 246 (ATP and ADP) and OAA than for PEP, like the WT enzyme (Table 1). The Δ 19 mutant
 247 showed 2- and 6-fold higher apparent affinity for PEP and OAA than the WT, respectively.
 248 Contrarily, the Δ 101 mutant showed 2- and 1.5-fold lower apparent affinity for PEP and OAA
 249 than the WT, respectively. Size exclusion chromatography revealed that both truncated forms
 250 are hexamers (Fig. S7), like the WT enzyme (Rojas *et al.*, 2019).

251 Plant PEPCKs are allosterically regulated by metabolites (Hatch and Mau, 1977;
 252 Leegood and Ap Rees, 1978; Burnell, 1986; Martín *et al.*, 2011; Rojas *et al.*, 2019).
 253 Interestingly, the response of the Δ 19 and Δ 101 mutants to different metabolites was altered
 254 compared with the full-length form. Both truncated mutants were more sensitive to PPi than the
 255 WT (Fig. 3). Sensitivity to shikimate was slightly increased in the Δ 19 mutant compared with
 256 the WT, while the Δ 101 mutant was almost insensitive to this metabolite (Fig. 3). Glc6P
 257 inhibited the WT enzyme and the Δ 19 truncated form but, surprisingly, the Δ 101 truncated form
 258 was 2-fold activated by the same metabolite (Fig. 3). Malate activated by 2- and 1.5-fold the
 259 WT enzyme and the Δ 19 truncated form, respectively, while the Δ 101 truncated form was
 260 insensitive to this metabolite (Fig. 3). To test whether malate still binds to the Δ 101 mutant, we

performed thermal-shift assays (Rojas *et al.*, 2019). We found that malate produced a similar shift in the melting temperature (T_m) of the WT, $\Delta 19$, and $\Delta 101$ truncated forms (Figs 4 and S8), suggesting 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 over 800 proteases with distinct temporal and tissue expression profiles (van der Hoorn, 2008; Tsiatsiani *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*, the pacemaker 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 to 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 (Figs 2A and 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 at 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; Shen *et al.*, 2017).

291 In our studies with *Arabidopsis* seedlings, we found that *AthPEPCK1* is subject to
 292 proteolysis around 24-48 HAI, when the level of protein reaches a maximum (Figs 1A-C). We
 293 measured PEPCK carboxylation activity in these samples, but we did not find the peak of the
 294 activity described by Malone *et al.* (2007); instead, we observed a gradual increase of PEPCK
 295 activity (~2-fold), coincident with the peak of protein accumulation (Fig. 1C). These differences
 296 might be originated by distinct extraction conditions, which might lead to different enzyme
 297 populations. By making denaturing protein extractions, we found that the shorter versions are
 298 present in *Arabidopsis* germinating seedlings, as reported for PEPCK from pineapple leaves
 299 (Martín *et al.*, 2011).

300 A large-scale study conducted by Tsiatsiani *et al.* (2013) identified *AthPEPCK1* as a
 301 target of *AthMC9*. Crude extracts of *mc9* knock-out mutants and MC9 overexpressing lines
 302 showed decreased and increased PEPCK carboxylase activity, respectively (Tsiatsiani *et al.*,
 303 2013). The recombinant *AthPEPCK1* truncated mutants ($\Delta 19$ and $\Delta 101$) showed similar
 304 decarboxylation activity than the WT enzyme. In comparison, the carboxylation reaction was
 305 slightly increased in both truncated forms compared to the WT enzyme (Table 1). The K_M
 306 values for the substrates of the truncated mutants were in the same range as those
 307 determined for the short version of the pineapple PEPCK (Martín *et al.*, 2011). The K_M for PEP
 308 of the $\Delta 19$ mutant was 2-fold lower than the WT enzyme; similarly, the short version of
 309 pineapple PEPCK has a 10-fold lower K_M for PEP than the entire isoform (Daley *et al.*, 1977;
 310 Martín *et al.*, 2011).

311 A key characteristic of the *AthPEPCK1* truncated mutants is that allosteric regulation by
 312 metabolites differs from that observed for the WT enzyme. Particularly, Glc6P is an inhibitor of
 313 the WT enzyme, but a soft activator of the $\Delta 101$ truncated form; whereas malate activates the
 314 WT enzyme and has no effect on the $\Delta 101$ truncated form (Fig. 3). These characteristics
 315 reinforce the idea that the N-terminal extension confers regulatory properties to plant PEPCK.
 316 These results agree with the findings of Furumoto *et al.* (1999), who treated maize PEPCK
 317 with enterokinase under controlled conditions to cleave the N-terminus. The proteolyzed
 318 enzyme showed 2-fold higher activity and was inhibited by 3PGA, while the full-length protein
 319 was only slightly affected by this metabolite (Furumoto *et al.*, 1999). In line with our findings,
 320 Martín *et al.* (Martín *et al.*, 2011) showed that the small version of pineapple PEPCK is not
 321 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 (Martín *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 approximately 10-fold, from $\sim 0.5 \text{ U g FW}^{-1}$ in germinating seedlings to $\sim 0.05 \text{ U g FW}^{-1}$ in mature leaves (Fig. 1C and Malone *et al.*, 2007). At this point, *AthMC9* might generate the shorter *AthPEPCK1* isoforms needed for the new metabolic scenario; i.e., to provide intermediates to the tricarboxylic acid cycle (Fig. 5).

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Figures and tables

Fig. 1. Proteolysis of *AthPEPCK1* in germinating seedlings.

A) Analysis of *AthPEPCK1* in germinating seedlings and mature rosettes.

Denatured protein extracts from Arabidopsis 48-HAI germinating seedlings (left) and 32-days-old rosettes (right) were resolved in a 12% SDS-PAGE (lower panel). Proteins were transferred to nitrocellulose membranes and immunodetected with anti-*AthPEPCK1* antiserum (upper panel), as described in the Materials and methods section. FW; amount of fresh weight tissue loaded in the gel.

B) Time course of *AthPEPCK1* proteolysis during Arabidopsis germination.

Western blot performed with anti-*AthPEPCK1* antiserum (upper panel) and load control with anti-*TaeGAPDH* antiserum (lower panel) of denatured protein extracts from Arabidopsis seedlings, obtained as described in the Materials and methods section. HAI; hours after imbibition. LD; long-day condition (16 h light and 8 h dark). SK; skotomorphogenesis (total darkness).

C) PEPCK activity in Arabidopsis germinating seedlings.

PEPCK carboxykinase activity was measured in crude extracts from Arabidopsis seedlings, as described in the Materials and methods section. Data are the mean $\pm$ standard error of four biological replicates.

Fig. 2. Analysis of *AthPEPCK1* cleavage by *AthMC9*.

A) Cleavage of *AthPEPCK1* by crude extracts from Arabidopsis seedlings.

Recombinant *AthPEPCK1* was incubated with protein extracts from Arabidopsis seedlings in MES-NaOH pH 5.5, HEPES-NaOH pH 7.0, or Tricine-NaOH pH 9.0 (150 mM in all cases). Reactions were terminated by adding denaturing sample buffer, resolved on a 10% SDS-PAGE (upper panel), and immunodetected with anti-*AthPEPCK1* antiserum (lower panel).

B) Proteolysis of *AthPEPCK1* by recombinant *AthMC9*.

Reactions were done as described in the Materials and methods section, and aliquots were taken at the specified time-intervals. Then, samples were resolved in a 10% SDS-PAGE (upper panel), transferred to a nitrocellulose membrane and immunodetected with anti-*AthPEPCK1* antiserum (lower panel).

C) Scheme of the *AthPEPCK1* truncated mutants constructed according to *AthMC9* recognition sites identified by Tisiatsiani *et al.* (2013).

Fig. 3. Analysis of allosteric effectors on *AthPEPCK1* and truncated mutants.

Activity of *AthPEPCK1* WT (left panel), $\Delta 19$ (middle panel) and $\Delta 101$ (right panel) was measured in the direction of decarboxylation, as described in the Materials and methods section, using saturating substrate concentrations (0.75 mM OAA and 0.75 mM ATP for the WT, and 0.5 mM OAA and 0.3 mM ATP for the $\Delta 19$ and $\Delta 101$ mutants). In the case of malate, activity was measured in the carboxylation direction using saturating substrate concentrations (10 mM PEP and 0.13 mM ADP).

Fig. 4. Thermal shift assays of *AthPEPCK1* and truncated mutants.

Experiments were performed with the WT (white), $\Delta 19$ (light grey), and $\Delta 101$ (dark grey) enzymes in the presence of malate at the indicated concentrations. The shift in the melting temperature (T_m) was calculated as described in the Materials and methods section. Data are the mean $\pm$ standard error of three technical replicates. * indicates a P-value < 0.05 and ** indicates a P-value < 0.01 using a t-test for two independent samples with a confidence level of 95%.

Fig. 5. Regulation of *Ath*PEPCK1 during the sink-to-source transition.

During the transition of the seedling from heterotrophic (orange) to autotrophic (green) growth, *Ath*PEPCK1 is cleaved at the N-terminus by *Ath*MC9, leading to shorter enzyme forms, which are catalytic but insensitive to regulation by malate and Glc6P. Blue lines, activation; red lines, inhibition.

Table 1. Kinetic parameters for *Ath*PEPCK1 and truncated mutants.

Reactions in both directions of the reaction were performed as described in the Materials and methods section. Kinetic constants were calculated by fitting experimental data (Figs S5-S6) to the Michaelis-Menten equation. Reported values were provided by the fitting software. Fixed substrate concentrations were as follows: ^a0.25 mM ADP, ^b15 mM PEP, ^c0.75 mM ATP, ^d0.75 mM OAA, ^e0.5 mM ADP, ^f5 mM PEP, ^g1.0 mM ATP, ^h0.5 mM OAA, ⁱ0.5 mM ADP, ^j15 mM PEP, ^k0.5 mM ATP, and ^l0.5 mM OAA. The parameters of the WT enzyme were taken from Rojas *et al.* (2019).

Enzyme	Reaction direction	Substrate	K_M (μ M)	K_{cat} (s^{-1})
<i>Ath</i> PEPCK1 WT	Carboxylation	PEP ^a	3700 $\pm$ 500	1.76 $\pm$ 0.04
		ADP ^b	79 $\pm$ 1	
	Decarboxylation	OAA ^c	230 $\pm$ 20	3.2 $\pm$ 0.3
		ATP ^d	72 $\pm$ 2	
<i>Ath</i> PEPCK1 Δ 19	Carboxylation	PEP ^e	1900 $\pm$ 400	2.64 $\pm$ 0.06
		ADP ^f	40 $\pm$ 1	
	Decarboxylation	OAA ^g	36 $\pm$ 9	3.38 $\pm$ 0.06
		ATP ^h	29 $\pm$ 6	
<i>Ath</i> PEPCK1 Δ 101	Carboxylation	PEP ⁱ	7300 $\pm$ 700	3.01 $\pm$ 0.08
		ADP ^j	57 $\pm$ 6	
	Decarboxylation	OAA ^k	340 $\pm$ 60	3.9 $\pm$ 0.2
		ATP ^l	80 $\pm$ 10	