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Viewpoints

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SPECIAL ISSUE: Emerging Voices in Botany

Integrating multiple regulations on enzyme activity: the case of phospho*enol*pyruvate carboxykinases

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Abstract. Data on protein post-translational modifications (PTMs) increased exponentially in the last years due to the refinement of mass spectrometry techniques and the development of databases to store and share datasets. Nevertheless, these data per se do not create comprehensive biochemical knowledge. Complementary studies on protein biochemistry are necessary to fully understand the function of these PTMs at the molecular level and beyond, for example, designing rational metabolic engineering strategies to improve crops. Phospho*enol*pyruvate carboxykinases (PEPCKs) are critical enzymes for plant metabolism with diverse roles in plant development and growth. Multiple lines of evidence showed the complex regulation of PEPCKs, including PTMs. Herein, we present PEPCKs as an example of the integration of combined mechanisms modulating enzyme activity and metabolic pathways. PEPCK studies strongly advanced after the production of the recombinant enzyme and the establishment of standardized biochemical assays. Finally, we discuss emerging open questions for future research and the challenges in integrating all available data into functional biochemical models.

Keywords: Allosteric regulation; carbon metabolism; enzyme regulation; gluconeogenesis; metabolic control; photosynthesis; phosphoenolpyruvate carboxykinases; post-translational modification.

Introduction

Plant biochemistry focuses on understanding the kinetics, structure and regulatory mechanisms that govern enzymes and metabolic pathways in plants (Hartman *et al.*, 2023). This historical approach is essential to understand biological processes in detail and is the foundation of rational strategies in metabolic engineering and synthetic biology for crop improvement (Wurtzel *et al.* 2019). Over the past two decades, the development of mass spectrometry techniques coupled with specialized databases for data storage and sharing has resulted in an explosion of plant protein post-translational modifications (PTMs; Smith and Kelleher 2013; Aebersold *et al.* 2018; Willems *et al.* 2019). Furthermore, the refinement of mass spectrometry protocols allowed the detection of low-stoichiometry PTMs, which were previously considered unimportant but are now being revisited with renewed interest (Prus *et al.* 2019). Having plenty of data/information does not translate into meaningful and practical knowledge/understanding (Ackoff 1999; Dammann 2019). A combined array of genetic and biochemical techniques is necessary to truly comprehend the function of PTMs and their integration with our knowledge of enzyme regulation (Stitt and Gibon 2014). This integrated approach is exemplified by the study of phospho*enol*pyruvate carboxykinases (PEPCKs), which are critical in central metabolism and physiological processes (Fig. 1). The biological relevance of PEPCK is associated with its complex regulation, recently evidenced by combining genetic, biochemical and systems biology approaches. Finally, we discuss open questions, which determine future lines of research.

Plant PEPCK characteristics

PEPCKs catalyse the decarboxylation of oxaloacetate (OAA) to PEP through an *enol* intermediate that is phosphorylated using adenosine triphosphate (ATP) (EC 4.1.1.49), guanosine triphosphate (GTP) (EC 4.1.1.32) or inorganic pyrophosphate (PPi) (EC 4.1.1.38), following a nucleophilic substitution of the S_N2 type (Matte *et al.* 1997; Holyoak *et al.* 2006; Johnson *et al.* 2016). Although the reaction is fully reversible *in vitro*, at least in plants, it mainly courses into the OAA decarboxylation direction *in vivo*. PEPCKs need two metal ions for optimal activity, one that complexes with the nucleotide substrate and the other that acts as a cofactor and might bind the enzyme in the absence of the substrate (Lee *et al.* 1981; Matte *et al.* 1997; Holyoak *et al.* 2006; Rojas *et al.* 2019). The nucleotide is activated by Mg^{2+} and Mn^{2+}, while a transition metal like Mn^{2+}, Co^{2+} or Ca^{2+} acts as the cofactor. The cofactor promotes the decarboxylation of OAA through the formation of an intermediary complex that stabilizes the

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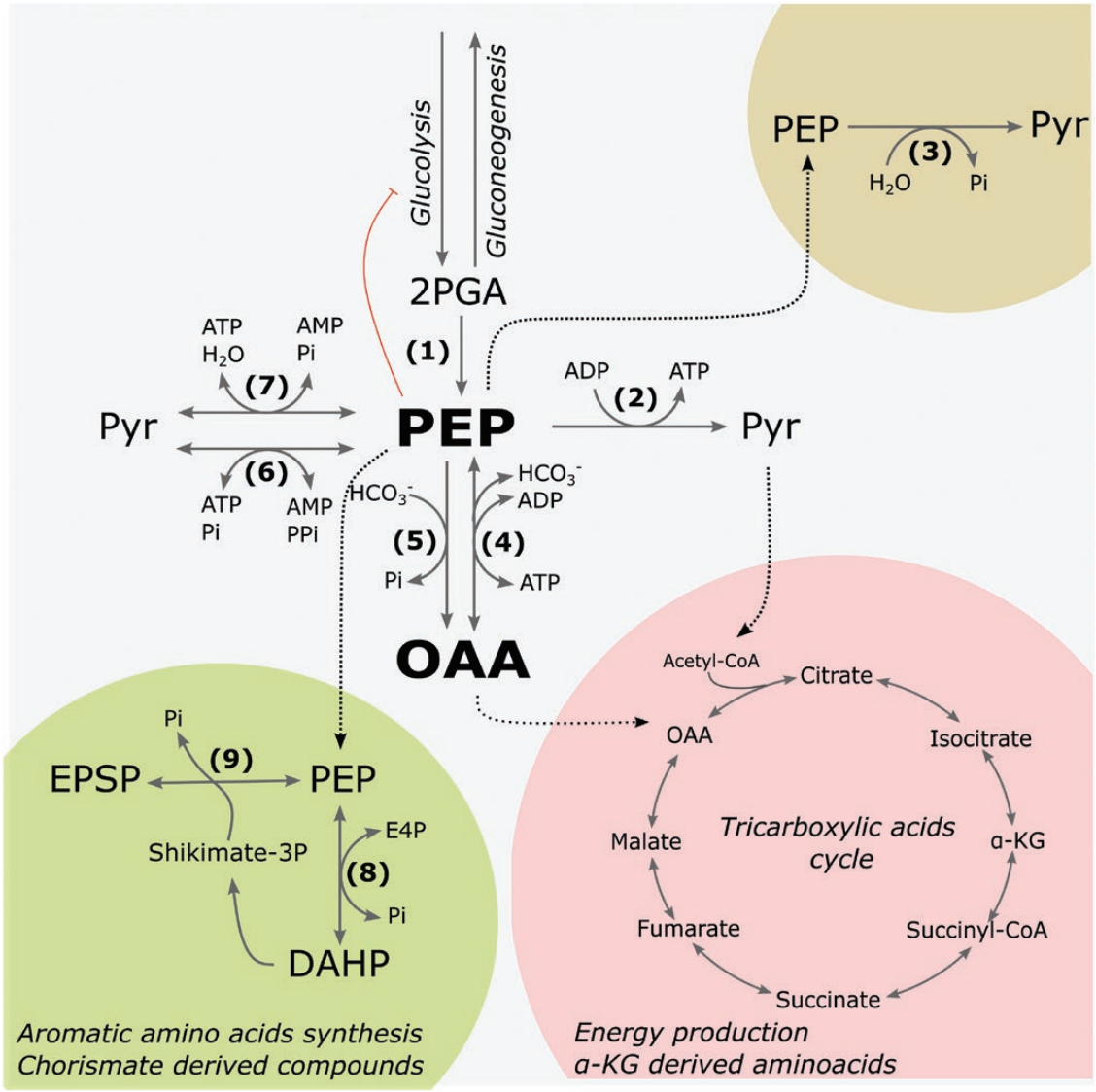

Figure 1. General overview of the metabolic pathways and enzymes that converge on PEP in plants. (1) Enolase (EC 4.2.1.11), (2) Pyr kinase (EC 2.7.1.40), (3) PEP phosphatase (3.1.3.60), (4) ATP-dependent PEPCK (EC 4.1.1.49), (5) PEP carboxylase (EC 4.1.1.31), (6) Pyr PPi dikinase (EC 2.7.9.1), (7) PEP synthase (EC 2.7.9.2), (8) DAHP synthase (EC 2.5.1.54), (9) 3-phosphoshikimate 1-carboxyviniltransferase (EC 2.5.1.19). DAHP, 3-deoxy-7-phosphoheptunolate; E4P, erythrose-4-phosphate, ESPS, 5-5-*enol*pyruvylshikimate 3-phosphate.

enolate ion during catalysis (O´Leary 1992; Matte *et al.* 1997; Johnson *et al.* 2016).

ATP-dependent PEPCKs were found in bacteria, yeasts and plants, while those using GTP are present in mammals, archaea and a small group of bacteria (Fukuda *et al.* 2004; Aich and Delbaere 2007; Chiba *et al.* 2015; Rojas *et al.* 2019). The distribution of PPi-dependent PEPCKs is less clear; this activity was discovered in crude extracts of *Propionibacterium shermanii*, and the coding gene was later identified in *Entamoeba histolytica* (Siu *et al.* 1961; Chiba *et al.* 2015). PEPCKs differ in their quaternary structure, being the GTP-dependent monomeric (Matte *et al.* 1997), the PPi-dependent homodimeric (Chiba *et al.* 2019) and the ATP-dependent homomultimeric, conformed either by four or six subunits (Burnell 1986; Walker and Leegood 1995; Martín *et al.* 2011; Rojas *et al.* 2019; Toressi *et al.* 2023).

The evolutionary trajectory of PEPCKs is a matter of controversy. Some authors have suggested that there is no homology between ATP- and GTP-dependent PEPCKs, but their primary sequence has similar motifs for substrate and metal binding (Matte *et al.* 1997; Fukuda *et al.* 2004; Aich and Delbaere 2007). PPi-dependent PEPCKs lack the catalytic domains described for the ATP- and GTP-dependent PEPCKs, so the evolutionary distance might be higher (Chiba *et al.* 2015). On the contrary, other authors have concluded that all PEPCK forms likely arose from a common ancestor (Walker and Chen 2002, 2021). Supporting this view, the tridimensional protein structures are similar, reinforcing that different PEPCKs are homologous (Chiba *et al.* 2019).

ATP-dependent PEPCKs have cytosolic localization in higher plants (Reiskind and Bowes 1991; Ito *et al.* 2011; Tsiatsiani *et al.* 2013). Other photosynthetic organisms, like the diatom *Skeletonema costatum* and the algae *Laminaria setchellii* contain a chloroplastic PEPCK (Cabello-Pasini *et al.* 2001). The characteristics of the reaction catalysed by PEPCKs attach them to critical energetic nodes in metabolism, independently of the organism. Let's analyse the particularities of the physiological role of PEPCKs in plants.

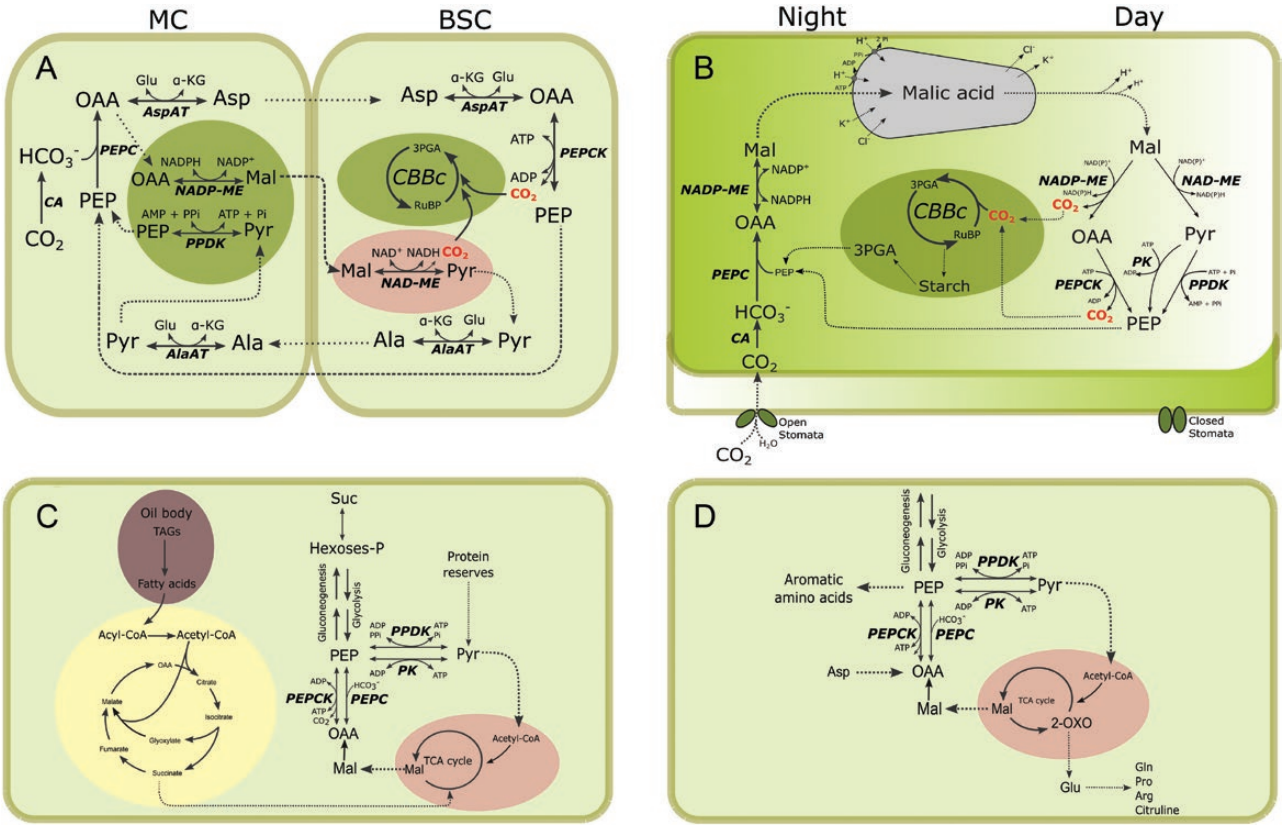

Figure 2. Summary of the physiological roles of PEPCKs. (A) The CO_2-concentrating mechanisms operating in C_4 plants. PEPCKs decarboxylate, in the cytosol, the OAA resulting from Asp decarboxylation. Then, the resulting PEP returns to the MC to restart the C_4 cycle. MC; mesophyll cells, BSC; bundle sheath cells. (B) Mal cycling in CAM plants. CAM plants open their stomata during cold humid nights to incorporate CO_2, reducing H_2O loss. The CO_2 is incorporated into Mal, which accumulates inside the vacuole. During hot dry days, CAM plants keep their stomata closed, and Mal is released from the vacuole to be decarboxylated and the resulting CO_2 fixed into the CBBc. (C) Gluconeogenesis during oilseeds germination. TAGs are degraded in a process distributed between different pathways and organelles. PEPCKs and PPDKs represent the first step of the gluconeogenic pathway. (D) Amino and organic acid metabolism. PEPCKs localize at a metabolic node between the amino acids derived from PEP and those derived from intermediates of the TCA cycle. AspAT, Asp aminotransferase; AlaAT, Ala aminotransferase; CA, carbonic anhydrase; NAD-ME, NAD-dependent malic enzyme; NADP-ME, NADP-dependent malic enzyme; PEPC, PEP carboxylase; PEPCK, ATP-dependent phosphoenolpyruvate carboxykinase; PK, Pyr kinase; PPDK, Pyr PPi dikinase.

Physiological roles

Decarboxylation of C_4 acids in C_4 and crassulacean acid metabolism photosynthesis. In C_3 photosynthesis, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) mediates CO_2 fixation into ribulose-1,5-bisphosphate, to produce two molecules of 3-phosphoglycerate (3PGA, three-carbon). This reaction is the initial step of the Calvin–Benson–Bassham cycle (CBBc), a chloroplast carbon cycle that sustains all life on earth (Sage 2004; Rojas *et al.* 2021a). The oxygenase activity of RuBisCO can fix O_2 instead of CO_2, generating 3PGA and 2-phosphoglycolate. As this latter two-carbon intermediate is toxic to the plant, it has to be recycled through photorespiration, which leads to energy, carbon and nitrogen losses, decreasing plant productivity (Portis 2001; Trípodi *et al.* 2021; Rojas *et al.* 2021a).

C_4 and crassulacean acid metabolism (CAM) photosynthesis evolved from C_3 ancestors to mitigate the detrimental effects of photorespiration and water loss by transpiration, respectively (Sage 2004; John *et al.* 2014; Trípodi *et al.* 2021). The different lineages of C_4 plants developed the Kranz anatomy, differentiation between mesophyll (MC) and bundle sheath cells (BSC), as well as CO_2-concentrating mechanisms. The latter include the initial fixation of CO_2 by phospho*enol*pyruvate carboxylase (PEPC) in mesophyll cells, transport of C_4 acids to BSC and decarboxylation near RuBisCO, enabling efficient carbon fixation via the CBBc. Based on the primary decarboxylase employed in the process, plant lineages were classified as NAD-dependent malic enzyme (NAD-ME, EC 1.1.1.38-39), NADP-dependent malic enzyme (NADP-ME, EC 1.1.1.40) and PEPCK subtypes (Hatch *et al.* 1975; John *et al.* 2014). In the PEPCK lineage, malate (Mal) and Asp are transported to the BSC. Within these cells, Asp is decarboxylated by the consecutive action of Asp aminotransferase (EC 2.6.1.1) and PEPCK in the cytosol, while Mal decarboxylation courses through mitochondrial NAD-ME (Maier *et al.* 2011) (Fig. 2A).

Despite the above-mentioned classification, decarboxylation pathways are flexible and might operate simultaneously in diverse species (Furbank 2011; Maier *et al.* 2011; Wang *et al.* 2014). In maize (NADP-ME subtype), carbon is primarily fixed as Mal, but significant pools (~25% of the fixed carbon) and rapid labelling of Asp have also been observed (Hatch 1971; Meister *et al.* 1996; Wingler *et al.* 1999; Majeran *et al.* 2010; Pick *et al.* 2011; Muhaidat and McKown 2013; Weissmann *et al.* 2015; Arrivault *et al.* 2017). Furthermore, PEPCK is expressed in the BSC of the NADP-ME subtype and supports Asp-dependent photosynthesis in isolated BSC (Chapman *et al.* 1980; Chapman and Hatch 1981; Walker

et al. 1997; Wingler *et al.* 1999; Majeran *et al.* 2010; Pick *et al.* 2011; Washburn *et al.* 2021). It has been suggested that Asp decarboxylation varies with developmental and environmental conditions (Chapman and Hatch 1981; Furbank 2011). Also, in sugarcane (classified as NADP-ME subtype), PEPCK activity is induced during sugar accumulation, drought and shade stress (Sales *et al.* 2018; Cacefo *et al.* 2019; Marquardt *et al.* 2021). However, this is controversial as other authors have shown that sugarcane does not have substantial PEPCK (Walker *et al.* 1997; Sage *et al.* 2013).

In CAM photosynthesis, atmospheric CO_2 fixation also produces a C_4 acid. However, in this case, the initial carbon assimilation by PEPC and the following incorporation into the CBBc are temporary. CAM plants have a high water-use efficiency, which allows them to inhabit desertic environments. They open their stomata to capture CO_2 during cold and humid nights and keep them closed on hot and dry days, reducing transpiration to a minimal level (Trípodi *et al.* 2021). During the night, CO_2 is fixed by the enzymatic tandem carbonic anhydrase (EC 4.2.1.1), PEPC and Mal dehydrogenase, and the product Mal is then stored in the vacuole. During the day, the hydroxy acid undergoes decarboxylation by NAD(P)-ME or PEPCK, releasing CO_2 near RuBisCO to feed the CBBc (Dittrich *et al.*, 1973) (Fig. 2A). CAM plants were classified into two groups, one that decarboxylates Mal via NAD(P)-ME (with undetectable PEPCK) and another that uses PEPCK (having low NAD(P)-ME activity) (Dittrich *et al.* 1973; Holtum and Osmond 1981; Borland *et al.* 1998) (Fig. 2B). However, it is worth noting that this classification is not rigid, and there is evidence of multiple decarboxylase activities (similar to what happens to C_4 plants) present in CAM plants, at similar levels (Black *et al.* 1996; Peckmann *et al.* 2012; Mukundan *et al.* 2023). This would give CAM photosynthesis increased efficiency and plasticity under different physiological and environmental conditions. In *Mesembryanthemum crystallinum*, a species that performs C_3 photosynthesis but can switch to CAM in response to salinity or water-deficit stress, the *PEPCK* gene has a stress-inducible expression that peaks at dawn, indicating a potential role of PEPCK in CAM induction (Lim *et al.* 2019).

Carbon fixation in algae. Giordano *et al.* (2005) reviewed the evidence for the existence of a C_4-like metabolism in algae, although the collected evidence is inconclusive. In the marine alga *Udotea flabellum*, which exhibits C_4-like photosynthesis without cellular compartmentalization, the carboxylase activity of PEPCK was found to be equivalent to that of RuBisCO (Reiskind and Bowes 1991). These authors also reported that 3-mercaptopiconilic acid (3-MPA), a specific inhibitor of PEPCK (Leegood and Ap Rees 1978*a*, *b*), reduced photosynthesis by 70%, highlighting the enzyme's role in carbon fixation in this organism (Reiskind and Bowes 1991). In the diatom *Thalassiosiva weissflogii*, which has a C_4-like metabolism, PEPCK may also decarboxylate C_4 acids (Reinfelder *et al.* 2004). In the diatoms *S. costatum* and *L. setchellii*, the chloroplastic localization of PEPCK coincides with its role as a decarboxylase that delivers CO_2 near RuBisCO (Cabello-Pasini *et al.* 2001). In *Phaeodactylum tricornutum*, PEPCK localizes in the mitochondria, and knockdown of the gene encoding this enzyme reduces growth and photosynthesis while increasing TAG accumulation under nitrogen-limiting conditions (Yang *et al.* 2016). The PEPCK role in algae not only could be related to carbon fixation, but also to gluconeogenesis and organic acids and nitrogen metabolisms (Toressi *et al.* 2023).

Gluconeogenesis in oleaginous seeds. The work of Leegood and Ap Rees (1978*a*, *b*) provided important supportive data for the role of PEPCK in plant gluconeogenesis. These authors studied PEPCK activity in cucumber cotyledons and purified the enzyme from this source. They also demonstrated that treatments with 3-MPA halted the gluconeogenic flux in this system (Leegood and Ap Rees 1978*a*, *b*). The accumulation of PEPCK coincided with those of other gluconeogenic enzymes, such as isocitrate lyase and Mal synthase, in the cotyledons of *Cucurbita pepo* and *Cucumis sativus* (Kim and Smith 1994), *Arabidopsis thaliana* (Rylott *et al.* 2001) and *Ricinus communis* (Martín *et al.* 2007), a few days after the imbibition of seeds. Later, genetic studies in *A. thaliana* furthered the understanding of the physiological functions of PEPCKs. Arabidopsis has two genes coding for ATP-dependent PEPCKs: *PEPCK1* (At4g37870) and *PEPCK2* (At5g65690). The *PEPCK1* gene is predominantly expressed during seed germination, while *PEPCK2* expression is less abundant and restricted to specific plant tissues (Malone *et al.* 2007; Rojas *et al.* 2021b).

The study of knock-out mutants and silenced lines in *PEPCK1* provided further insight into the critical involvement of PEPCK in plant gluconeogenesis (Rylott *et al.* 2003; Penfield *et al.* 2004). These mutants exhibited growth impairment when cultivated on basal media without an external carbon source, but their growth was normal when the media was supplemented with sucrose (Rylott *et al.* 2003; Penfield *et al.* 2004). Once the seedlings developed their photosynthetic apparatus, their growth was similar to that of wild-type (WT) plants. Additionally, these mutants did not alter the catabolism rate of reserve lipids, but showed reduced levels of soluble sugars. However, under suboptimal illumination or dark conditions, their growth was further compromised, resulting in reduced hypocotyl elongation compared to WT plants. This reduction in hypocotyl elongation was also observed when the endosperm, the tissue where oleaginous plants store reserve lipids, was eliminated. Hence, these phenotypes could be attributed to a decrease in the sucrose supply received by the embryo from the endosperm (Penfield *et al.* 2004).

As *pepck1* mutants can develop their photosynthetic apparatus and mature into adult plants, the step catalysed by PEPCK, although critical, is not the only path through which carbon could flow to gluconeogenesis (Rylott *et al.* 2003; Penfield *et al.* 2004). Eastmond *et al.* (2015) discovered that Arabidopsis employs two pathways to channel carbon from reserves to gluconeogenesis: one via PEPCK, which channels carbon from lipids degradation, and the other via PPDK, which channels carbon from protein reserves (Fig. 2C). In Arabidopsis, *PPDK* has an expression pattern like *PEPCK1*, peaking approximately 2 days after imbibition. Seedling establishment is impaired in *ppdk* mutants, and the double-mutant *ppdk/pepck1* displays a severe starvation response (Eastmond *et al.* 2015). This two-enzyme model for carbon channelling during Arabidopsis seeds germination was subsequently confirmed by Henninger *et al.* (2022).

The contribution of PEPCK and PPDK to gluconeogenesis during seed germination may vary across plant species. For instance, germinating cucumber cotyledons exhibit abundant PEPCK activity but undetectable levels of PPDK (Walker *et al.* 2021). Therefore, it is important to investigate the prevalence

of both enzymes in gluconeogenesis during germination across different plants, considering factors such as species, tissue type, developmental stage and environmental conditions like nitrogen supply (Walker *et al.* 2021). Moreover, distinct signalling cascades may regulate the involvement of each enzyme in gluconeogenesis. For example, in Arabidopsis seedling establishment, the SnRK1 kinase serves as a critical regulator of reserve mobilization. SnRK1 phosphorylates the transcription factor BASIC LEUCINE ZIPPER63 which binds and activates the *PPDK* promoter. Notably, SnRK1 does not appear to regulate *PEPCK1* (Henninger *et al.* 2022), suggesting that gluconeogenesis may be controlled through complex interactions of multiple signalling pathways.

Gluconeogenesis during fruit maturation. Studies have demonstrated that gluconeogenesis occurs in several fruits (Sweetman *et al.* 2009; Walker *et al.* 2016; extensively reviewed in Walker *et al.* 2021) with PEPCK (rather than PPDK) being the primary pathway utilized, except possibly in tomato (Famiani *et al.* 2014, 2016). Gluconeogenesis from Mal and citrate can occur in certain fruits even when there is no net breakdown of these organic acids. It seems that gluconeogenesis in fruits is associated with a transient release of Mal and/or citrate from the vacuole (Walker *et al.* 2021). In tomato fruits, silencing the *PEPCK* gene by interfering RNA led to a decrease in sugar concentration and increased levels of Mal (Osorio *et al.* 2013; Huang *et al.* 2015*a*). Conversely, overexpressing the same gene using a constitutive (*35SCAMV*) or fruit-specific promoters (fruit-ripening-specific *E8* promoter; Huang *et al.* 2015*b*) resulted in the opposite phenotype, along with faster germination and seedling growth.

Stress response. In Arabidopsis, *PEPCK1* is expressed in biotic stress-response structures like hydathodes, trichomes and guard cells (Chen *et al.* 2000, 2002; Penfield *et al.* 2012). In *Capsicum annum*, a *PEPCK* gene was isolated from a cDNA library constructed from leaves infected with an avirulent *Xanthomonas* strain (Choi *et al.* 2015). Following infection, PEPCK expression and activity increased, suggesting its involvement in biotic stress response. Additionally, silencing this gene in pepper plants rendered them more susceptible to infection, while overexpression increased resistance (Choi *et al.* 2015). The authors of this work also observed that drought, cold and stress-related hormones (such as salicylic acid and abscisic acid, ethylene and methyl-jasmonate) triggered the induction of the *PEPCK* gene.

Arabidopsis *pepck1* mutants are susceptible to drought due to stomatal malfunction resulting in increased transpiration (Daloso *et al.* 2017). Mal metabolism in guard cells plays a crucial role in the opening and closure of stomata (Daloso *et al.* 2017; Robaina-Estévez *et al.* 2017). In *pepck1* mutants, anomalies in Mal metabolism within these cells may explain the malfunctioning of this mechanism, as ABA signalling is not affected (Penfield *et al.* 2012). Furthermore, the induction of the *PEPCK* gene has been observed in tomato plants exposed to saline stress (Saito *et al.* 2008) and in *Brassica napus* leaves subjected to cold stress (Saez-Vasquez *et al.* 1995). In sugarcane, PEPCK activity is induced during drought stress and shade conditions (Sales *et al.* 2018; Cacefo *et al.* 2019).

Cataplerotic reactions, amino acid metabolism and cytosolic pH regulation. The removal of intermediates from the tricarboxylic acid (TCA) cycle is crucial to prevent their accumulation in specific metabolic situations. These enzymatic steps, known as cataplerotic reactions, are critical to cellular homeostasis (Owen *et al.* 2002). In the case of PEPCK, it converts OAA into PEP, which can undergo gluconeogenesis or generate Pyr (Leegood and Walker 2003) (Fig. 2D). In plants, cataplerotic reactions are important during certain physiological conditions, such as castor oil seeds germination (Stewart and Beevers 1967), amino acid respiration in *Pisum sativum* (Larson and Beevers 1965) or the use of glutamate as a respiratory substrate in sugar beet phloem (Lohaus *et al.* 1994). However, the precise role of PEPCK in these plant processes requires further investigation.

The PEPCK reaction is localized between the OAA/Asp family of amino acids (Asn, Lys, Thr, Met and Ile), those derived from PEP (Phe, Tyr and Trp), and Ala derived from Pyr (Lea *et al.* 2001) (Fig. 2D). In grape seeds, PEPCK activity is induced by nitrogenous compounds (Asp, NH_4^+ and Gln), suggesting a potential role in their metabolism and the regulation of cytosolic pH through Mal formation and dissimilation (Walker *et al.* 1999; Lea *et al.* 2001; Chen *et al.* 2004; Delgado-Alvarado *et al.* 2007). Decarboxylases of C_4 acids are localized in the mid-vein of Arabidopsis, and their mutation affects the abundance of amino acids derived from Pyr and PEP. By feeding the xylem stream of the Arabidopsis *pepck1* mutant with ^{14}C-labelled bicarbonate and Mal, it was observed that the levels of Ala (derived from PEP) were reduced, while Asp (produced from OAA) increased compared to WT plants (Brown *et al.* 2010).

Regulatory mechanisms

Allosteric regulation. Over the years, and under certain assay conditions, several metabolites have been shown to affect PEPCK activity *in vitro*. The enzymes from *Urochloa panicoides*, *Chloris gayana* and *Panicum maximum* (C_4 plants) are inhibited by the glycolytic intermediates 3PGA, Fru6P, Fru1,6bisP and DHAP under certain conditions (Hatch and Mau 1977; Burnell 1986). For the maize (also performing C_4 photosynthesis) PEPCK, sensitivity to 3PGA inhibition is only evident in the enzyme's N-terminal proteolyzed form (Furumoto *et al.* 1999). A detailed study on the allosteric regulation of a short version of the enzyme from *Ananas comosus* (a CAM species) revealed differential regulation between its carboxylase and decarboxylase activities (Martín *et al.* 2011). The decarboxylase activity was inhibited by Fru2,6bisP, 3PGA, Asp and Pro, while succinate activated it. The carboxylase activity was inhibited by Fru6P, Fru1,6bisP, 3PGA, citrate, Mal and UDPGlc (Martín *et al.* 2011). However, the authors of this study were unable to purify the full-length form of the enzyme to make a comparative assessment of differences in allosteric regulation. *Chlamydomonas reinhardtii* has two PEPCKs; a full-length *Chlre*PEPCK1 and a shorter *Chlre*PEPCK2 that lacks 55 amino acids at the N-terminus. *Chlre*PEPCK1 and *Chlre*PEPCK2 carboxylase activity is inhibited by citrate and phenylalanine. *Chlre*PEPCK2 carboxylase activity is also inhibited by glutamine. The decarboxylase activity of *Chlre*PEPCK1 is activated by phenylalanine and malate, while *Chlre*PEPCK2 decarboxylase activity does not show any effect by these metabolites and is inhibited by glutamine (Toressi *et al.* 2023).

It is important to note that PEPCK activity was typically assayed using relatively high concentrations of Mn^{2+} (0.5–5

mM), which allow for maximal activity. However, the concentration of this metal in plant cells remains within the micromolar range (Quiquampoix *et al.* 1993; Pittman 2005). Improvements to the *in vivo* assay of PEPCK allowed the enzyme's activity to be measured at more physiological concentrations of metal ions and so, physiologically relevant conclusions could be obtained from the *in vitro* assays (Chen *et al.* 2002; Rojas *et al.* 2019). In the case of Arabidopsis (a C_3 plant), there are differences in the regulation of *Ath*PEPCK1 and *Ath*PEPCK2 (Rojas *et al.* 2019). Glc6P, Fru6P and Glc1P inhibit *Ath*PEPCK1 but not *Ath*PEPCK2, while Fru1,6bisP inhibits both enzymes. Glc6P is the primary inhibitor of *Ath*PEPCK1, followed by Glc1P, Fru6P and Fru1,6bisP. Mal activates *Ath*PEPCK1 but not *Ath*PEPCK2. The regulation by Mal may be critical in stimulating the flux of carbon released through lipid degradation into gluconeogenesis during seed germination. Once the photosynthetic apparatus develops, increased levels of triose- and hexose-phosphates may inhibit PEPCK, thereby inhibiting gluconeogenesis. *Ath*PEPCK1 is also inhibited by shikimate, a precursor of aromatic amino acids and defence compounds, thus regulating the synthesis of PEP, the initial substrate of the shikimate pathway (Rojas *et al.* 2019). The regulation of PEPCK by Mal and Glc6P is opposite to that of PEPC, which may be an important mode of regulation for two enzymes catalysing opposite reactions and present in the plant cytosol simultaneously. This regulation could be key in preventing a futile carboxylation/decarboxylation cycle that would deplete cytosolic ATP (Leegood and Walker 2003; Martín *et al.* 2011; Rojas *et al.* 2019).

Regulation by dipeptides. Identifying novel protein–metabolite interactions is crucial for discovering new regulatory elements in plant metabolism. For this, PROMIS (*PROtein Metabolite Interactions using Size separation*) proved to be a valuable tool for mining novel small molecule regulators (Veyel *et al.* 2017, 2018). *Ath*PEPCK1 co-elutes with a series of hydrophobic/polar dipeptides: Ile-Gln, Ala-Ile, Phe-Gln, Leu-Thr, Ser-Tyr, and Ser-Val. Enzymatic assays demonstrated that these dipeptides inhibit recombinant *Ath*PEPCK1 with $I_{0.5}$ values ranging from 52 to 828 µM. They can also inhibit PEPCK activity in crude extracts, and this inhibition is not observed with the individual amino acids (Moreno *et al.* 2021). The inhibition of *Ath*PEPCK1 by dipeptides is more potent than that of sugar phosphates (Rojas *et al.* 2019; Moreno *et al.* 2021). The origin and roles of these regulatory dipeptides are still not fully understood. Additionally, the dipeptide's *in vivo* concentration range remains unknown, which is important for determining if they can affect PEPCK activity *in vivo*. It is possible that they participate in signalling during abiotic stress in Arabidopsis (Doppler *et al.* 2019; Thirumalaikumar *et al.* 2021). Moreover, dipeptides have been detected in Arabidopsis root exudates, downstream of the MPK3 and MPK6 signalling cascade, suggesting their involvement in plant–microbe and plant–plant communication systems (Strehmel *et al.* 2017).

Arabidopsis mutants in components of the autophagy pathway, including (*atg18* and *nbr1-2*) simple mutants as well as [*atg4(4a/4b)* and *nbr1-2/atg5*] double mutants exhibited reduced dipeptides levels upon heat stress when compared to the WT (Thirumalaikumar *et al.* 2021). Autophagy plays a critical role in macromolecule recycling (Kenny *et al.* 1976) and seedling germination (Avin-Wittenberg *et al.* 2015). Arabidopsis mutants in genes involved in the autophagic response are sensitive to carbon and nitrogen starvation, which can be alleviated by supplying sucrose to the seedlings (Bassham 2009). Also, these mutants exhibit decreased levels of free amino acids and increased protein content, indicating impaired mobilization of reserve proteins and lipids (Avin-Wittenberg *et al.* 2015). Notably, one of the increased proteins was CRUCIFERIN3, a major reserve protein in Arabidopsis seeds (Herman and Larkins 1999; Wan *et al.* 2007).

It would be intriguing to explore whether the inhibition of *Ath*PEPCK1 by H-P dipeptides regulates the channelling of carbon released through protein degradation during seed germination via PPDK, which does not interact with H-P dipeptides (Veyel *et al.* 2018). Arabidopsis seeds overexpressing the sunflower WRKY10 transcription factor exhibited increased gluconeogenesis, enhanced lipid utilization, reduced protein consumption and a higher flux through PEPCK during germination (Raineri *et al.* 2016).

Proteolytic regulation. Plant PEPCKs undergo proteolysis at the N-terminus *in vivo*. The proteolysis is affected by the pH (diminishing at pH 9–10), cannot be avoided by protease inhibitors and does not alter the quaternary structure of the enzyme (Walker and Leegood 1995; Walker *et al.* 1995; Rojas *et al.* (2021b)). In Arabidopsis, *Ath*PEPCK1 is a target of the cysteine-protease METACASPASE9 (*Ath*MC9) (Tsiatsiani *et al.* 2013). *Ath*MC9 is found in the nucleus, cytosol and apoplast (Vercammen *et al.* 2006; Kwon and Hwang 2013; Tsiatsiani *et al.* 2013), and participates in regulating cellular death in various physiological contexts, such as the immune response (Kim *et al.* 2013; Shen *et al.* 2019) and vascular tissue development (Escamez *et al.* 2016, 2019). Proteolysis of the N-terminal domain of PEPCK appears to activate the enzyme, as crude extracts from the Arabidopsis *mc9* mutant exhibit decreased enzyme activity, while *35S:MC9* overexpressing lines show increased PEPCK activity (Tsiatsiani *et al.* 2013). The level of *Ath*PEPCK1 level peaks 24-48 h post-imbibition, and proteolytic forms of the protein are generated during this germination stage. Shorter PEPCK versions are also present in *Anana comosus* and *C. reinhardtii*, but in these cases, a transcriptional event leading to these shorted versions cannot be excluded (Martín *et al.* 2011; Torresi *et al.* 2023).

In plants, the abundance of the proteolyzed form is low compared to the non-proteolized form (Walker and Leegood 1995; Walker *et al.* 1995; Rojas *et al.* (2021b)). Some authors argue that low-stoichiometry PTMs may be physiologically irrelevant. Nevertheless, studies have shown that low-stoichiometry PTMs can reflect specific modifications occurring at a particular time and location (Prus *et al.* 2019). For instance, if a modification is specific to a group of cells, the modified protein will be diluted in a protein extract, leading to lower abundance (Orsburn *et al.* 2022). This may be the case of PEPCK, as proteolysis has been demonstrated to occur only in the cotyledons and embryonic axis of pea seedlings (Delgado-Alvarado *et al.* 2007).

Truncated mutants of *Ath*PEPCK1 on the putative cleavage sites of *Ath*MC9 (Δ19 and Δ101 mutants) generated protein forms with similar kinetic parameters and quaternary structure compared to the WT enzyme. However, the activation by Mal and inhibition by Glc6P were abolished in the Δ101 mutant (Rojas *et al.* 2021b). Proteolysis during germination may serve as a mechanism to regulate enzyme levels and,

consequently, modulate the gluconeogenic flux. Furthermore, the shorter versions of *Ath*PEPCK1 may fulfil different roles during the transition of seedlings from an autotrophic to a heterotrophic state. Interestingly, the proteolysis of PEPCK cleaved the N-terminal portion of the protein, which has been predicted to contain an intrinsically disordered motif and a major phosphorylation site. Intrinsically disordered motifs evolved to have a disordered structure under physiological conditions (Peterson *et al.* 2017). These motifs exhibit rapid conformational fluctuations and are involved in signalling cascades, protein–protein interaction modules, allosteric regulations and auto-inhibitory domains, and are usual targets of PTMs (Wright and Dyson 2014).

Phosphorylation. In the leaves of C_4 and CAM plants, phosphorylation of PEPCK in its N-terminal domain has been observed during the dark period (Walker and Leegood 1995; Leegood and Walker 1996). This phosphorylation was recreated *in vitro* by PEPC kinase and cAMP-dependent protein kinase (Walker and Leegood 1995). In crude extracts from the fodder *Megathyrsus maximus*, lower PEPCK activity was detected during the night, suggesting an inhibitory effect of phosphorylation (Walker *et al.* 2002). Nevertheless, purification of PEPCK from day and night samples resulted in preparations with different substrate affinities but no differences in specific activity when measured using *in vivo* assay conditions that assess maximum activity (Walker and Chen 2002). Only the enzyme from night samples exhibited increased K_M for OAA and ATP. Therefore, PEPC and PEPCK are regulated through phosphorylation to prevent futile phosphorylation–dephosphorylation cycles (Leegood and Walker 2003; Bailey *et al.* 2007). During the day, in C_4 and CAM plant leaves, dephosphorylation of both enzymes leads to PEPC inhibition and PEPCK activation. Conversely, during the night, when both enzymes are phosphorylated, the opposite activity conditions occur.

The recent development and optimization of mass spectrometry protocols led to unprecedented amounts of phosphoproteomic data (Smith and Kelleher 2013; Aebersold *et al.* 2018; Willems *et al.* 2019). This technological progress has also enabled the detection and analysis of low-stoichiometry PTMs that were previously considered irrelevant (Prus *et al.* 2019). *Ath*PEPCK1 and *Ath*PEPCK2 undergo phosphorylation at multiple sites. Phosphomimetic mutants at Ser-62 exhibited increased enzyme activity, while mutants at Thr-56 decreased activity. The phosphomimetic mutant at Thr-66 displayed no difference compared to the WT enzyme (Shen *et al.* 2017). This information further supports the notion that *Ath*PEPCK1 is intricately regulated in a complex manner by different signals mediated by effectors and PTMs.

In *Zea mays*, mass spectrometry profiling of PEPCK revealed that light conditions altered its phosphorylation status (Chao *et al.* 2014). In Arabidopsis, treatment with flg22, a 22-amino acid peptide derived from *Pseudomonas syringae* flagellin, resulted in increased phosphorylation of Thr-22 and decreased phosphorylation of Ser-62 and Thr-66 (Rayapuram *et al.* 2014, 2018). These findings suggest that phosphorylation may transduce different environmental signals. Although unequivocal evidence of the protein kinase(s) responsible for PEPCKs phosphorylation is lacking, studies have implicated SnRK2.2/SnRK2.3/SnRK2.6, MPK6, GSK3 and TOR (de la Fuente van Bentem *et al.* 2008; Wang *et al.* 2013; Rayapuram *et al.* 2018; van Leene *et al.* 2019).

Open questions and future lines of research

Although significant research has been performed on PEPCKs' function and regulation, many questions await answers: (i) How do different PTMs regulate enzyme activity, and how do they integrate with allosteric regulation? (ii) What are the biological outcomes of the different PTMs?; (iii) What are the modifying enzymes and signalling cascades acting on PEPCKs? (iv) Are dipeptides physiologically important modulators of PEPCK *in vivo*? and (v) Does PEPCK have an important role in non-PEPCK C4 and CAM plants, in particular, under stress situations? More research is needed to answer these questions but results will likely come from a combination of *in vitro* and *in vivo* biochemical approaches. The pioneer studies of Richard Leegood and Robert P. Walker with the enzyme purified from plant sources were key to studying the biochemical properties of plant PEPCKs (Leegood and Ap Rees 1978*a*, *b*; Leegood *et al.* 1996, 2003; Walker and Chen 2002; Walker and Leegood 1995; Walker *et al.* 1995, 1997, 2002). Nevertheless, having a recombinant system to produce high amounts of the enzyme and mutant versions will be important in searching for the biochemical effects of PTMs on the enzyme activity in detail.

In the era of Big Data in biology, vast amounts of transcriptomic, metabolomics and proteomic data are added each day to public databases. Some examples of useful tools are BRENDA (Chang *et al.* 2021), Plant PTM viewer (Willems *et al.* 2019), PhosPhat (Durek *et al.* 2010), BAR (Winter *et al.* 2007), and TAIR (Huala *et al.* 2001). This gives plant biochemists enormous potential to explore novel modes of metabolic regulation in combination with other biochemical techniques (Hartman *et al.* 2023). A pipeline to translate the biochemical knowledge to metabolic engineering research would start with pure preparations of the enzymes and mutant versions that mimic the PTMs to clearly understand their regulation. Enzymes could also be engineered to introduce new regulatory modes or abolish the existing ones (Erb *et al.* 2017). Then, the promising mutants could be expressed *in vivo*, followed by the analysis of the resulting plants' phenotypic and metabolic effects. The PEPCK example is similar to what happens with other highly regulated enzymes (e.g. glyceraldehyde-3-phosphate dehydrogenases, ADP-glucose pyrophosphorylases, sucrose synthases, Glc6P dehydrogenases, among other interesting examples). The current challenge is integrating data from multiple regulations on enzyme activity, generated *in vitro* and *in vivo*, to develop general models and reduce the existing gap between both approaches (for some good examples, we suggest reading the work of Liebermeister *et al.* 2006; Blätke *et al.* 2019; Treves *et al.* 2022). This would lead to more biochemical knowledge and understanding that would certainly speed up agrobiotechnology innovations.

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Contributions by the Authors

All authors discussed the outline of the manuscript and selected the relevant bibliography. B.E.R. wrote the initial manuscript and prepared the figures. B.E.R. and A.A.I. answered reviewers' comments and revised the manuscript to its final version. All authors revised and approved the final version.

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Data Availability

No new data were generated in support of this research.

Conflict of Interest Statement

None declared.

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