REVIEW ARTICLE
The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological functions and post-translational controls of non-photosynthetic PEPCs

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INTRODUCTION

PEPC [PEP (phosphoenolpyruvate) carboxylase] is a tightly controlled enzyme located at the core of plant C-metabolism that catalyses the irreversible β-carboxylation of PEP to form oxaloacetate and P₇. The critical role of PEPC in assimilating atmospheric CO₂ during C₄ and Crassulacean acid metabolism photosynthesis has been studied extensively. PEPC also fulfils a broad spectrum of non-photosynthetic functions, particularly the anaplerotic replenishment of tricarboxylic acid cycle intermediates consumed during biosynthesis and nitrogen assimilation. An impressive array of strategies has evolved to co-ordinate in vivo PEPC activity with cellular demands for C₄-C₆ carboxylic acids. To achieve its diverse roles and complex regulation, PEPC belongs to a small multigene family encoding several closely related PTPCs (plant-type PEPCs), along with a distantly related BTPC (bacterial-type PEPC). PTPC genes encode ∼110-kDa polypeptides containing conserved serine-phosphorylation and lysine-mono-ubiquitination sites, and typically exist as homotetrameric Class-1 PEPCs. In contrast, BTPC genes encode larger ∼117-kDa polypeptides owing to a unique intrinsically disordered domain that mediates BTPC’s tight interaction with co-expressed PTPC subunits. This association results in the formation of unusual ∼900-kDa Class-2 PEPC hetero-octameric complexes that are desensitized to allosteric effectors. BTPC is a catalytic and regulatory subunit of Class-2 PEPC that is subject to multi-site regulatory phosphorylation in vivo. The interaction between divergent PEPC polypeptides within Class-2 PEPCs adds another layer of complexity to the evolution, physiological functions and metabolic control of this essential CO₂-fixing plant enzyme. The present review summarizes exciting developments concerning the functions, post-translational controls and subcellular location of plant PTPC and BTPC isoenzymes.

Key words: 14-3-3 protein, mono-ubiquitination, phosphoenolpyruvate carboxylase, protein phosphorylation, protein–protein interaction.
by a specific Ca\(^{2+}\)-independent serine/threonine kinase known as PPCK (PEPC protein kinase), and dephosphorylation by a PP2A (protein phosphatase 2A) \[8,13,15,16\]. Phosphorylation at the conserved N-terminal serine residue activates the enzyme by decreasing inhibition by malate while increasing activation by glucose 6-phosphate. A number of excellent reviews and book chapters concerning various aspects of the evolution, molecular and biochemical characteristics, and physiological functions and regulation of photosynthetic and non-photosynthetic PEPCs and their corresponding PPCKs have appeared over the last 15 years \[8–10,12,13,15–21\]. Development of a more complete account of PEPC’s diverse and ubiquitous involvement in plant metabolism has recently made considerable progress owing to the use of non-photosynthetic model plant systems such as developing seeds and heterotrophic suspension cell cultures, together with increased access to state-of-the-art genomic, bioinformatic and proteomic tools. The first part of the present review summarizes established and proposed metabolic roles for non-photosynthetic PEPCs. The second section outlines recent advancements in our understanding of the post-translational control, protein–protein interactions and subcellular location of non-photosynthetic PEPCs.

**DIVERSE METABOLIC FUNCTIONS FOR NON-PHOTOSYNTHETIC PLANT PEPC**

**PEPC and primary carbon metabolism**

Besides its fundamental role in the initial fixation of atmospheric CO\(_2\) during C\(_4\) and CAM photosynthesis, PEPC has a wide range of non-photosynthetic roles including supporting carbon–nitrogen interactions, seed formation and germination, fruit ripening, guard cell metabolism during stomatal opening and provision of malate as a respiratory substrate for symbiotic N\(_2\)-fixing bacteroids of legume root nodules (Figure 1). The traditional view of non-photosynthetic PEPC function is its ubiquitous anaplerotic carboxylation of PEP needed to replenish TCA (tricarboxylic acid) cycle carbon skeletons that are withdrawn to support anabolism. Although certainly valid, this oversimplifies the contribution of PEPC to primary metabolism for several reasons. First, the TCA ‘cycle’ does not always operate as a continuous cycle in certain plant cells. Flux through one part of the pathway may be negligible or disproportionately low (e.g. as occurs to various extents in illuminated and senescing leaves and developing seed embryos) \[22–25\]. The partial TCA ‘cycle’ thus resembles a linear pathway with PEPC as a component enzyme, and the notion of anaplerosis is unnecessary as the maintenance of OAA concentrations is of no more importance than any other metabolite \[22\]. Also, when combined with MDH (malate dehydrogenase) and NAD-ME (NAD\(^{+}\)-dependent malic enzyme), PEPC forms an alternative flux mode which can function in place of PK\(_{c}\) (cytosolic pyruvate kinase) to generate pyruvate (Figure 2A) \[22,26,27\]. This endows plants with the ability to respire OAA generated from PEPC, again not an anaplerotic function. Secondly, as discussed below, the organic acids generated by PEPC have diverse roles outside the TCA cycle \[28\]. Thirdly, when coupled with the cytosolic MDH reaction, the metabolism of PEP to malate and other organic acids has important implications in the redox balance of NAD(P)H/NAD(P)\(^{+}\) \[29\]. Malate transported from the cytosol and oxidized to pyruvate within ‘sink’ organelles (e.g. mitochondria or plastids) by ME isoenzymes functions as both a NAD(P)H shuttle and a carbon source (Figure 2).

A distinguishing feature of the anaplerotic PEPC reaction is that it represents a highly flexible aspect of primary plant metabolism. Several studies have quantified in vivo flux changes through primary metabolic pathways in isolated plant cells acclimatized to environmental perturbations such as a change in oxygen concentrations \[30\], temperature \[31,32\], glucose availability \[33\] or nitrogen source \[24\]. As the overall rates of flux and biosynthesis changed, the ratio of carbon flux through most nodes within primary metabolism was relatively rigid, whereas anaplerotic fluxes were relatively plastic. In other words, changes in carbon partitioning at the PEP branchpoint (Figure 2A) are
Functional control of non-photosynthetic plant PEPC

PEPC plays a central role in the control of plant glycolysis and respiration

In animals, primary control of glycolytic flux of hexose phosphates to pyruvate is mediated by ATP-dependent PFK (phosphofructokinase), with secondary control at PK (pyruvate kinase). Activation of PFK increases the level of its product, fructose 1,6-bisphosphate, which is a potent feed-forward allosteric activator of many non-plant PKs [27]. In contrast, quantification of changes in levels of glycolytic intermediates that occur immediately following stimulation of respiration in a wide variety of plant systems (including green algae, ripening fruit, aged storage root slices, germinating seeds and suspension cell cultures) consistently demonstrated that plant glycolysis is controlled from the ‘bottom up’ with primary and secondary regulation exerted at the levels of PEP and fructose 6-phosphate utilization respectively (Figure 2A) [27]. These findings are compatible with the observation that plant ATP-PFKs demonstrate potent allosteric feedback inhibition by PEP. It follows that any enhancement in the activity of PEPC or PK, will relieve the PEP inhibition of ATP-PFK, thereby elevating glycolytic flux from hexose phosphate (Figure 2A). Reduced cytosolic PEP levels also cause elevated levels of the signal metabolite fructose 2,6-bisphosphate (and thus activation of the PP-dependent PFK) since PEP is a potent inhibitor of plant 6-phosphofructo-2-kinase [27]. A possible advantage of bottom-up regulation of glycolysis is that it permits plants to control net glycolytic flux independent of related plant-specific metabolic processes such as the Calvin–Benson cycle and sucrose–starch interconversion. The hypothesis that plant glycolysis is regulated from the bottom up was corroborated by the application of metabolic control analysis to assess the distribution of respiratory flux control in tubers of transgenic potato plants expressing different amounts of E. coli ATP-PFK [43]. It was determined that ATP-PFK exerted a low flux control coefficient over both glycolysis and respiration, whereas far more flux control was exerted in the metabolism of PEP. The relatively low flux control coefficient of ATP-PFK was explained as a consequence of its potent inhibition by PEP. In this way, PEPC and PK, play a central role in the overall regulation of plant respiration, since the control of their activities in vivo will ultimately dictate the rate of mobilization of sucrose or starch for respiration, while simultaneously controlling the provision of: (i) respiratory substrates (e.g. pyruvate and/or malate) for ATP production via oxidative phosphorylation, and (ii) TCA cycle carbon skeletons needed for nitrogen assimilation or as biosynthetic precursors. It is obvious that PEPC and PK, represent promising targets for metabolic engineering of plant respiration and photosynthetic partitioning. For example, transgenic bean plants were generated that expressed a bacterial malate-insensitive PEPC in a seed-specific manner [44].
of the transgenic seeds indicated enhanced PEP partitioning through the PEPC anaplerotic pathway, resulting in a shift of photosyntheate partitioning from sugars/starch into organic acids and amino acids. Consequently, the transgenic seeds accumulated up to 20% more storage protein [44].

PEPC produces malate as an alternative respiratory substrate for plant mitochondria

Malate has been frequently proposed to be an important respiratory substrate for plant mitochondria, such that a significant fraction of pyruvate enters the TCA cycle via the combined reactions of PEPC, MDH and NAD-ME, rather than PK. (Figure 2A). However, in vivo flux studies of plant cells incubated with 13C-labelled substrates demonstrated that the flux of malate to pyruvate via mitochondrial NAD-ME is usually very low, relative to the flux of PEP to pyruvate via PK, [22,27]. Thus a marked reduction in mitochondrial NAD-ME in transgenic potatoes and Arabidopsis had little impact on respiratory metabolism [45,46]. It was concluded that the import of PEPC-derived malate into the mitochondria generally serves an anaplerotic role to support biosynthesis and nitrogen assimilation, whereas pyruvate derived from the PK reaction is the most significant substrate for respiration [45,46]. Nevertheless, there are specific situations, such as prolonged P starvation [47] or the light-enhanced dark respiration peak that occurs immediately after C3 leaf darkening [48], when the oxidation of malate as a respiratory substrate for mitochondrial ATP production appears to be of particular significance. Similarly, overexpression of PEPC in several plant species resulted in a stimulation of respiration when illuminated leaves were darkened, and an increase in extractable NAD-ME and NADP-ME activities [19,49–51]. Future studies are required to determine the full suite of physiological and developmental conditions that stimulate intramitochondrial pyruvate production via the NAD-ME-dependent oxidative decarboxylation of malate (Figure 2A).

Carbon–nitrogen interactions

PEPC plays a crucial role regulating respiratory carbon flux in vascular plant tissues and green algae that are actively assimilating nitrogen [27,52–54]. The organic acids supplied by PEPC have several distinct roles within nitrogen metabolism. α-Oxo acids, especially OAA and 2-OG (2-oxoglutarate), are obligate carbon skeletons on to which NH4+ is assimilated and exported to other tissues (Figure 2A). In nodules, malate is also the predominant respiratory substrate supplied to symbiotic rhizobia bacteria to support the huge energy demand of reducing N2 into NH4+ [55,56]. Malate and citrate may also act as counterions to replace nitrate and maintain cytosolic pH [57]. The tight integration and critical importance of PEPC at the interface of carbon and nitrogen metabolism has been continuously elaborated by numerous studies of green algae and vascular plants. Legume species express a specific nodule-enhanced PTPC isoenzyme that is up-regulated and activated by in vivo phosphorylation during active nitrogen assimilation by N2 fixing root nodules [39,55,58–60], and whose suppression results in greatly diminished rates of nitrogen assimilation [55,56]. In leaves and non-nodular root tissues, PTPC transcripts, activity and phosphorylation status are also up-regulated when metabolism is switched to a nitrogen-assimilating state following the addition of NO3− or NH4+ [42,53,61–66]. PEPC is also up-regulated during nitrogen remobilization from senescing leaves [25].

A study compared metabolic fluxes of autotrophic canola (Brassica napus) embryos supplied with organic or inorganic nitrogen (amino acids compared with NH4+) [24]. The differences in primary metabolism were mainly a restructuring of flow through the TCA cycle caused by the reciprocal changes in PEPC compared with mitochondrial NAD-ME flux. The dynamic response of PEPC to different nitrogen sources has been particularly well studied in chemostats of unicellular green algae where nitrogen availability can be precisely manipulated [52]. NO3− or NH4+ resupply to N-limited green algae leads to an immediate and massive stimulation of: (i) respiratory carbon flux in order to generate TCA carbon skeletons required by GS (glutamine synthetase)/GOGAT (glutamate 2-OG aminotransferase), and (ii) dark CO2 fixation catalysed by PEPC (Figure 2A) [52]. During transient nitrogen assimilation, over 50% of net plant carbon may be committed to the production of carbon skeletons and energy (ATP and reductant) required for the GS/GOGAT system [27,52]. Green algal (Chlamydomonas reinhardtii) PTPC and BTPC transcript and protein levels are both up-regulated upon nitrogen starvation, presumably to facilitate rapid provision of OAA and 2-OG when a nitrogen source becomes available [5,67]. As discussed below, the control of green algal and vascular plant PEPCs by allosteric effectors, particularly glutamate and aspartate, has clearly evolved to be in tune with carbon–nitrogen interactions [68], and to co-operate with other enzymes involved in nitrogen assimilation, particularly PK+, nitrate reductase and GS (Figure 2A) [53,62,69].

Regulatory phosphorylation represents a second level of post-translational PEPC control in plant cells engaged in active nitrogen assimilation [27,54]. Nodule-specific PPCKs have been reported for several legume species whose activity mirrors PEPC’s phosphorylation status and the presence of photosynthetic supply [6,39,40,70,71]. PEPC phosphorylation in roots and leaves also responds to the carbon/nitrogen balance due to the action of PPCK [16,37,42,66,72]. The importance of post-translational PEPC control in mediating carbon–nitrogen interactions has been elegantly demonstrated by a series of metabolic engineering efforts. Various forms of PEPC with diminished feedback inhibition have been expressed in transgenic potatoes, Arabidopsis and Vicia narbonensis (vetch) seeds [44,51,73,74]. These alterations increased carbon flux into organic acids and amino acids, and boosted the overall organic nitrogen content at the expense of starch and soluble sugars. The mutant potato plants had diminished carbon flux and tuber yield, whereas the PEPC-overexpressing Arabidopsis plants suffered severe stunting owing to reduced PEP levels that limited aromatic amino acid production via the shikimate pathway [51]. Conversely, overexpression of PEPC without diminished feedback inhibition has little impact on overall metabolism, even when large increases in extractable PEPC specific activity were achieved [19,49–51]. As a further tier of integration into nitrogen metabolism, PEPC has been shown to be a major target of the plant-specific DoF1 transcription factor which is implicated in organic acid metabolism [75]. Increased expression of DoF1 in transgenic Arabidopsis caused an increase in nitrogen content and improved growth under low nitrogen that was correlated with increased PEPC and PK, expression and activity [75].

PEPC participates in photosyntheate partitioning in developing seeds and organic acid metabolism of germinating seeds

Developing seeds

Developing seeds are crucial metabolic sinks that import large amounts of sugars and amino acids from leaves which must be
efficiently metabolized into starch, triacylglycerols and storage proteins. Owing to the agronomic significance of these storage reserves, a detailed understanding of developing seed metabolism has obvious practical applications in crop breeding and metabolic engineering. PEP metabolism via PEPC, PK, and PKC (plasticid pyruvate kinase) plays a prominent role in partitioning imported photosynthate towards leucoplast fatty acid biosynthesis compared with the mitochondrial production of carbon skeletons and ATP needed for amino acid interconversion in support of storage protein biosynthesis. PEPC may also aid in refixing respired CO₂ to improve overall seed carbon economy [76–80]. Increased PEPC and PPCK activity and PTPC phosphorylation status during seed development coincides with the main stages of storage oil and protein biosynthesis [35,78,79,81–84], and have been shown to be controlled by photosynthesize in developing COS (sunflower) seeds showed that, relative to other precursors, exogenous malate supported maximal rates of fatty acid synthesis [88,89]. As a consequence of malate’s oxidation into acetyl-CoA via the plastidal isoenzymes of NADP-ME and PDH (pyruvate dehydrogenase complex), all of the reductant required for carbon incorporation into fatty acids is produced (Figure 2B). Both PEPC and plastidal NADP-ME are abundant in developing COS endosperm [81,90,91], and a specific malate/Pantiporter exists in the COS and maize embryo leucoplast envelope [92,93]. These results are compatible with the hypothesis that PEPC has an important role in control sucrose partitioning at the level of the cytostolic PEP branchpoint for generation of carbon skeletons and reducing power needed for fatty acid biosynthesis by developing oil seeds such as COS (Figure 2B). Consistent with this view are the high rates of dark CO₂ fixation exhibited by intact pods of developing COS [94].

Metabolic flux studies of developing oil seeds have reported a spectrum of values regarding anaerobic PEPC carboxylation to support biosynthetic pathways [24,26,32,95–99]. The flux from malate to plastidial acetyl-CoA appears to be negligible in developing green oil seeds such as canola [97], despite the strong correlation that exists between PEPC expression and rates of oil synthesis by developing canola embryos [83]. However, fatty acid synthesis by green oilseed rape/canola and soya bean embryos is light-dependent [86,100,101], since most of the reducing power [NAD(P)H] they need to assemble fatty acids from acetyl-CoA is generated via the light reactions of leucoplast photosynthesis. In the non-green developing maize and sunflower embryos, however, 30% and 10% of carbon flux into fatty acids was derived from PEPC-generated malate respectively [95,96]. One caveat of metabolic flux analyses is that they require artificially static conditions, such as constant light and sucrose supply, and non-physiological levels of oxygen [102]. Conversely, the activities of the enzymes surrounding the PEPC node and their support of fatty acid synthesis are likely to be tightly controlled to be in tune with changing environmental conditions, including the diurnal cycle.

## Germinating seeds

Research with starch-storing cereal seeds such as barley and wheat, and oil-storing dicotyledon seeds such as COS have consistently implied an important anaplerotic function for PEPC during germination [36,81,103–107]. Cristina Echevarría and colleagues have performed pioneering studies with PEPC of germinated cereal seeds such as wheat and barley [105,106], in which the aleurone layer synthesizes and secretes various acid hydrolases needed to mobilize starch and protein reserves in the underlying endosperm. Aleurone PEPC is thought to play an important role in regulating the production of organic acids, chiefly malic acid, that are excreted for the acidification of the starchy endosperm, or transported to the growing embryo to replenish pools of TCA cycle intermediates consumed during biosynthesis and transamination reactions. Although barley and wheat seed PTPC becomes progressively activated by phosphorylation following imbibition, PPCK was already present in the dry seeds and was apparently not up-regulated during germination [36,105]. The phytohormone abscisic acid appears to play a role in triggering PPCK accumulation during the desiccation stage of maturing barely seeds [36]. Also, the protein synthesis inhibitor cycloheximide, as well as other pharmacological agents known to block the signal-induced phosphorylation of PEPC in C₄ leaf mesophyll cells, had no influence on PEPC’s phosphorylation in germinated cereal seeds, suggesting that their PTPC may be subject to a post-translational control mechanism [36,105].

The carbon metabolism of germinating oil seeds such as COS is dominated by the mobilization of storage lipid and protein reserves to support the needs of the growing hypocotyls and roots. In germinating COS endosperm, this process includes the massive conversion of reserve triacylglycerols into sucrose, which is absorbed by the cotyledons of the growing seedling [108,109]. PEPC has been suggested to fulfill a crucial function very early in the germination process to build up cellular pools of C₄ acids needed to trigger subsequent TCA and glyoxylate cycle activity [81]. An ensuing role for PEPC in germinating oil seeds is to replenish dicarboxylic acids required as substrates for the substantial transamination reactions that follow storage protein hydrolysis [108], as well as for GS’s reassimilation of NH₄⁺ released during the oxidative deamination of glutamate to 2-OG via glutamate dehydrogenase [110]. The labelling of metabolic intermediates to isotopic steady state and the modelling of the TCA cycle in germinating lettuce seeds indicated that 70% of glycolytic flux from carbohydrate oxidation enters the TCA cycle via the PEPC reaction [111]. At the same time, PEPC carboxykinase catalyses the initial step in the gluconeogenic conversion of storage lipids into sucrose, as it uses ATP to decarboxylate and phosphorylate OAA derived from the glyoxylate cycle into PEPC in C₄ leaf mesophyll cells, had no influence on PEPC’s phosphorylation in germinated cereal seeds, suggesting that their PTPC may be subject to a post-translational control mechanism [36,105].
of glycolysis is about one-tenth that of gluconeogenesis and that the pools of glycolytic and gluconeogenic intermediates probably occur in separate intracellular regions and appear to be regulated independently. Several studies have provided evidence for glycolytic or gluconeogenic multi-enzyme complexes (metabolons) in the plant cytosol [27], including the cytosolic isoenzymes of fructose-1,6-bisphosphate aldolase and fructose-1,6-bisphosphatase in germinated COS [113]. It will therefore be important to establish whether PEPC mono-ubiquitination (see below) possibly contributes to formation of a glycolytic metabolon while minimizing its futile cycling with PEP carboxykinase in the cytosol of the germinating oil seeds such as COS.

PEPC mediates organic acid accumulation by developing fruit and expanding cells

Many developing fruits accumulate substantial concentrations of malate and citrate (>300 mM) in their vacuoles. This stockpile of organic acids is believed to sustain respiration and act as carbon source during ripening when sugars are accumulated [114,115]. A fruit-specific PTPC isozyme has been identified in tomato whose transcripts and activity increase throughout development, simultaneous with enhanced cellular malate and citrate levels [115,116], PEPC from tomato and Citrus sinensis (orange) fruits displayed a significantly decreased sensitivity to malate inhibition relative to many other PTPCs [116,117]. Whether this is the result of specific isoenzyme properties or control by a PTPC (post-translational modification) such as phosphorylation remains unknown. Adding to this uncertainty, a PPCK transcript steadily increases in tomato fruit from early development until ripening, but does not correlate with PEPC activity in this tissue [118]. A Class-1 PEPC has also been purified and characterized from Musa cavendishii (banana) fruit [119]. The PEPC of ripening bananas is subject to regulatory phosphorylation, and was suggested to play an anaplerotic role to replenish carbon skeletons consumed during nitrogen assimilation and/or transamination reactions [120].

The tomato fruit PTPC is located predominantly in large or expanding cells of the pericarp, where malate production via PEPC and MDH has been suggested to sustain osmotic potential to allow rapid cell expansion [116]. A similar role for malate has been suggested for other expanding cells as well. For example, malate accumulation in the guard cell vacuole is involved in stomatal opening. Several studies analysing the genetic or pharmacological alteration of PEPC activity in guard cells have correlated stomatal opening with PEPC activity and PTPC phosphorylation status [19,121,122]. Cotton fibres are hair-like single cells that undergo a stage of rapid elongation to several centimetres in anthesis. PEPC activity and PTPC transcripts, and the accumulation of malate were all correlated with the rate, extent and developmental profile of fibre elongation [123]. Treatment of fibres with LiCl, an inhibitor of PPCK activity, reduced fibre length and implicated regulatory PTPC phosphorylation in this tissue as well. Aside from acting as an osmolyte to increase turgor pressure, the malate generated by cotton fibre PEPC may also support fatty acid synthesis (Figure 2B) needed for the rapid extension of the plasma membrane and tonoplast [123].

PEPC helps plants to acclimatize to abiotic and biotic stresses

PEPC has been widely implicated in plant stress tolerance in two main ways. First, as discussed above, PEPC provides metabolic flexibility that allows PEP metabolism to adjust to environmental conditions. Generally, this involves either increased anaplerosis to support specifically up-regulated biosynthetic processes and/or a glycolytic bypass of PK, as an adjustment to differing P, or reductant levels. Secondly, some roots excrete huge amounts of organic acids into the soil (rhizosphere) to acidify the soil and chelate cations as a response to nutrient deprivation and/or toxic metal stress. PEPC up-regulation, as documented by increased transcripts, protein, extractable activity and phosphorylation status appears to be an integral component of these stress responses.

Drought, salt and ozone stress

An up-regulated PEPC and PPCK response to salinity or drought stress has been well documented in C., C, and CAM plants [11,84,124–133]. Consistent with these reports, RNAi (RNA interference) knockdown of a canola PTPC isozyme resulted in increased sensitivity to poly(ethylene glycol)-induced osmotic stress [134]. The adaptive value of increased PEPC activity upon salt or drought stress requires further clarification, but could potentially improve carbon metabolism during periods of reduced stomatal conductance by reissimilating respired CO2 and/or increasing rates of CO2 fixation at night when stomata are open, as in CAM photosynthesis [126,129,132]. PEPC may also support the biosynthesis of biocompatible osmolytes such as proline that rapidly accumulate upon drought or salinity stress in many plant species [134].

Exposure to elevated ozone causes oxidative stress and leads to a major rearrangement in primary carbon metabolism involving a suppression of photosynthesis and an increase in respiration [135]. Specifically, a major up-regulation of PEPC and concomitant down-regulation of ribulose-1,5-bisphosphate carboxylase is typical of this response [135,136]. The up-regulation of PEPC along with ME is hypothesized to contribute to the increased production of NAD(P)H necessary to detoxify reactive oxygen species, as well as the increased anaplerotic flux to support protein synthesis for defence and repair processes [137].

Nutritional phosphate and iron deficiency

Phosphorus is an essential element for growth and metabolism that plays a central role in virtually all metabolic processes. Plants preferentially absorb phosphorus from the soil in its fully oxidized anionic form, PO43-. Despite its importance, P is one of the least available macronutrients in many terrestrial and aquatic environments. In soil, P is frequently complexed with Al3+ or Ca2+ cations and thus exists as insoluble mineral forms that render it unavailable for plant uptake. PEPC was suggested to provide a metabolic bypass (with MDH and NAD-ME) to the ADP-limited PK, to facilitate continued pyruvate supply to the TCA cycle, while concurrently recycling the PEPC by-product P, for its reassimilation into the metabolism of the P-deficient cells [27]. PEPC induction and its regulatory phosphorylation during P stress has also been correlated with the synthesis and consequent excretion of large amounts of malic and citric acids by roots [27,38,138,139]. This increases P availability to the roots by acidifying the rhizosphere to: (i) solubilize otherwise inaccessible sources of mineralized soil P [138], while (ii) making phosphorus esters more accessible to hydrolysis by secreted acid phosphatases [140]. An excellent correlation of organic acid excretion and PEPC up-regulation with enhanced rates of dark CO2 fixation occurs in proteoid (cluster) roots of P-deficient Lupinus albus (white lupin) [138]. Recent developments have clarified the molecular basis of the PEPC response to P stress in Arabidopsis cell cultures and seedlings [37,38,141,142],
genes when AtPPCK1 and is consistent with the observation that the PPCK-encoding genes AtPPCK1 and AtPPCK2 are among the most strongly induced genes when Arabidopsis is subjected to nutritional P, deprivation [141,144,145].

Iron deficiency also up-regulates PEPC activity [up to 60-fold in Beta vulgaris (sugar beet)], specifically in the cortical cells of root apical meristem [146]. This was hypothesized to support organic acid excretion to the soil for increased iron uptake through PEPC, MDH and NAD-ME as a glycolytic bypass [148].

Heavy metal toxicity

The exudation of PEPC-derived organic acids also functions to chelate metals in the rhizosphere to alleviate heavy metal toxicity by preventing their uptake into the cell [138,151]. Al³⁺ toxicity is the most prevalent form of plant heavy metal stress. Although elevated soil Al³⁺ levels result in root efflux of organic acids, there appears to be no corresponding induction of root PEPC [152,153]. However, a recent study demonstrated that overexpression of a C₄ PTPC isoenzyme in rice led to increased oxalate exudation and Al³⁺ tolerance [154]. In contrast with Al³⁺, Cd²⁺ toxicity leads to PEPC up-regulation [155,156]. This is believed to support the synthesis of the phytochelatins, intracellular metal chelators that rapidly accumulate during heavy metal stress. Phytochelatins are polymers of the tripeptide glutathione whose synthesis requires de novo amino acid synthesis and therefore increased anaplerotic PEP carboxylation via PEPC.

Biotic stress

PEPC is also up-regulated during infection of tobacco (Nicotiana tabacum) plants by the potato virus [157,158]. The up-regulation may be a response to: (i) closed stomata and the ensuing alteration of metabolism, and/or (ii) increased anaplerotic flux needed to sustain the rapid synthesis of viral defence proteins.

THE MULTIFACETED POST-TRANSLATIONAL CONTROL OF PLANT PEPC

Allosteric effectors and regulatory phosphorylation: new tricks for old dogs

Allosteric effectors

The basic features of post-translational control of plant Class-1 PEPCs have been known since the early 1980s. Most Class-1 PEPCs show allosteric inhibition by malate and activation by glucose 6-phosphate. However, the enzyme’s specific kinetic and allosteric properties are quite variable and appear to be well adapted to the physiological role(s) of specific PEPC isoenzymes and the nature of their cellular environment. A good example is the increased Kₐ (PEP) and IC₅₀ (malate) values of C₄ Class-1 PEPC relative to typical C₅ Class-1 PEPCs [10]. The C₄ Class-1 PEPCs from developing fruit, the chloroplastic Class-1 PEPC isoenzyme of rice leaves and the Class-2 PEPC heteromeric complexes composed of tightly associated PTPC and BTPC subunits (discussed below) also display distinctive kinetic properties [116,117,159,160]. Furthermore, aspartate and glutamate are important feedback effectors of Class-1 PEPC and PK, isoenzymes in green algae and plant tissues active in nitrogen assimilation and/or transamination reactions. This provides a regulatory link between nitrogen metabolism and the control of respiratory carbon metabolism (Figure 2A). Feedback inhibition of specific Class-1 PEPC and PK, isoenzymes by glutamate provides a rationale for the known activation of the two enzymes that occurs in vivo during periods of enhanced nitrogen assimilation when intracellular glutamate concentrations become transiently reduced [27,52]. In contrast with PEPC, aspartate functions as an allosteric activator of various plant PK,ₜ by effectively relieving the enzyme’s inhibition by glutamate (Figure 2A) [27,69,161–164]. Reciprocal control of Class-1 PEPC and PK, by aspartate has been documented in a wide range of plant cells and tissues involved in nitrogen assimilation and/or transamination reactions including: green algae, Spinacia oleracea (spinach) and castor oil plant leaves, ripening banana fruit, canola cell cultures and endosperm of developing COS [52,69,82,161–164]. This was suggested to provide a mechanism for decreasing flux from PEP to aspartate (via PEPC and aspartate aminotransferase) while promoting PK, activity whenever cytosolic aspartate levels become elevated. This may occur when the cell’s demands for nitrogen are satisfied, and the overall rate of protein synthesis becomes more dependent on ATP availability, rather than the supply of amino acids. In this instance, respiration may assume a more significant role in terms of satisfying a large ATP demand, rather than the anaplerotic generation of biosynthetic precursors.

PA (phosphatidic acid) is a lipid second messenger that transiently accumulates in plant cells within minutes of applying a wide array of stress conditions [165]. Several C₅ and C₁ PTPC isoenzymes were recently identified as PA-binding proteins [166,167]. Moreover, a low concentration (50 μM) of PA and other anionic phospholipids resulted in significant inhibition (>50%) of C₁ Class-1 PEPC activity [167]. Little or no effect was observed with neutral, zwitterionic or positively charged phospholipids, ruling out a non-specific effect of the fatty acyl chains (which activate E. coli PEPC). Interestingly, hypo-osmotic treatment of Arabidopsis cells increased PEPC’s affinity for PA through an unknown mechanism [166]. Although most of the C₁ PTPC of Sorghum bicolor (sorghum) leaves was in the soluble fraction, a subtraction was membrane-associated [167]. Anionic phospholipids represent novel regulators that may inhibit the activity and/or mediate membrane association of certain PEPCs. How PEPC activity and/or cellular location is influenced by dynamic changes in anionic membrane phospholipids such as PA will be an interesting area for further research.

Regulatory phosphorylation

In the early 1980s, Hugh Nimmo (University of Glasgow) and Raymond Chollet (University of Nebraska) respectively documented the regulatory phosphorylation of CAM and C₅ photosynthetic Class-1 PEPC isoenzymes at their single highly conserved serine residue close to the N-terminus of the protein. Since their seminal discoveries, this PTM has been implicated in the in vivo control of a wide array of non-photosynthetic PTPCs [34,36,38,42,106,120,168–172]. This research area has been elaborated in several excellent reviews that have appeared within the last decade [8,16,17]. In brief, Class-1 PEPC phosphorylation uniformly results in enzyme activation at physiological pH.
Figure 3  Amino acid sequence alignment of Arabidopsis and castor oil plant PEPC isoenzymes

Experimentally verified in vivo phosphorylation sites of castor oil plant and Arabidopsis PTPCs (RcPPC3 and AtPPC1 respectively) and castor oil plant BTPC (RcPPC4) are highlighted in green [34,38,191,201], whereas RcPPC3’s conserved Lys628 mono-ubiquitination site is marked with a red font [103]. BTPC’s highly divergent ∼10-kDa domain that was predicted to exist largely as an intrinsically disordered region [191] is enclosed in a red rectangle. The proteolytically susceptible peptide bond (Lys 446–Ile447) within RcPPC4’s disordered region [4] is marked with an X. Boxes I–III denote conserved subdomains essential for PEPC catalysis [9]. The predicted pI, molecular mass and sequence identity (%) of the various PEPCs are shown. The deduced PEPC sequences were aligned using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Semi-colons and asterisks indicate identical and conserved amino acids respectively.

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by decreasing PEPC’s sensitivity to allosteric inhibitors while increasing its affinity for PEP and sensitivity to allosteric activators. Hence, much attention has also been directed to the Ca\(^{2+}\)-independent PPCK and, to a far lesser extent, PP2A, that mediate Class-1 PEPC’s reversible phosphorylation in \textit{vivo}. Detailed PPCK studies, including its cloning from CAM, C\(_4\) and C\(_3\) plants, continues to provide one of the best understood examples of regulatory enzyme phosphorylation in the plant kingdom [16,17]. There is also compelling evidence from several systems that Class-1 PEPC phosphorylation correlates with increased flux through the enzyme in \textit{vivo} [16]. With molecular masses of 31–33 kDa, the PPCKs are the smallest protein kinases yet reported, consisting of only a core kinase domain [8,17]. Class-1 PEPC phosphorylation status appears to be generally controlled by changes in rates of PPCK synthesis compared with degradation (with the notable exception of germinating cereal seeds; see above) [8,16]. This is a novel means of control, since most protein kinases are post-translationally controlled by second messengers such as Ca\(^{2+}\) ions, intracellular signals such as AMP, and/or phosphorylation by other protein kinases.

PPCK is up-regulated in response to a range of different signals, including light in C\(_4\) leaves, a circadian rhythm in CAM leaves, light and nitrogen supply in C\(_3\) leaves, nutritional P deprivation in C\(_4\) plants and cell cultures, and photosynthetic supply in legume root nodules and developing seeds [16,17,35–37,72,127,173,174]. Endogenous PPCK activity and PTPC subunit phosphorylation of developing COS and soya bean root nodules were both eliminated following prolonged darkness of intact plants [6,34,35,40,70]. Both effects were fully reversed following reillumination of darkened plants. These results imply a direct relationship between the up-regulation of COS or soya bean nodule PPCK and PTPC phosphorylation during the recommencement of photosynthetic delivery from illuminated leaves to these non-photosynthetic sink tissues. The ability of plant cells to sense sugars plays a crucial role in carbon partitioning and allocation in source and sink tissues. These processes are modulated as a consequence of the plant’s sugar status, and sugar signals function both at the transcriptional and translational levels in tight co-ordination with light and other environmental stimuli [84]. PPCK of non-green sink tissues such as soya bean root nodules or developing oil seeds appears to be one of the many proteins whose expression is markedly influenced by sucrose supply. Developing seed and legume nodule gene expression, respiration, storage product synthesis and carbon–nitrogen interactions are highly dependent upon the supply of recent photosynthetic [85]. A key area for future studies will be to establish signalling pathway(s) that link sucrose supply to enhanced PPCK expression and PTPC phosphorylation in heterotrophic sinks such as legume root nodules or developing seeds.

A variety of post-translational PPCK controls have also been proposed including: (i) malate inhibition of PPCK activity, possibly by interacting with PEPC [35,120,175], (ii) thiol–disulfide interconversion [35,176,177], and (iii) a proteasome inhibitor [178]. The mechanisms and \textit{in vivo} relevance of these controls requires further evaluation. Functional genomic studies have evaluated the impact of \textit{in vivo} PTPC phosphorylation on the growth and development of transgenic \textit{C\(_4\)} (\textit{Flaveria bidentis}) and \textit{C\(_3\)} (\textit{Arabidopsis}) plants in which PPCK expression and thus \textit{in vivo} PEPC phosphorylation was disrupted [171,179]. Although perturbation of growth and/or metabolite levels were observed, the results of both studies led to the surprising conclusion that inhibition of PTPC phosphorylation has little impact on metabolic flux through PEPC (albeit, in non-stressed plants).

In \textit{Arabidopsis}, however, several pathways were perturbed, and it was hypothesized that leaf PPCK may have several targets in addition to PEPC [171]. The physiological roles of PTPC phosphorylation, and the substrate specificity of PPCK require further clarification. A PP2A appears to dephosphorylate photosynthetic and non-photosynthetic PTPCs [8,15,16,20]. Additional research is needed to assess the possibility that the activity of PP2A catalytic subunits are themselves controlled by regulatory subunits with which they might be associated.

Protein phosphorylation can not only control enzymatic activity directly, but also generate specific docking sites for other proteins, particularly the 14-3-3 proteins, a family of highly conserved and abundant proteins that play a central regulatory role in eukaryotic cells [27]. The 14-3-3 homodimers bind to specific phospho-sites on diverse target proteins, thereby forcing conformational changes or influencing interactions between their targets and other molecules. In these ways, 14-3-3s ‘finish the job’ when phosphorylation alone lacks the power to drive changes in the activities of intracellular enzymes. For example, the phosphorylated forms of plant nitrate reductase and sucrose-phosphate synthase are both potently inhibited by 14-3-3 binding [27]. Proteomic profiling of tandem-affinity-purified 14-3-3 protein complexes from transgenic \textit{Arabidopsis} seedlings recently identified the PTPC isoform \textit{AtPPC1} as being a 14-3-3-binding protein [180]. However, plant PEPCs, including \textit{AtPPC1}, do not contain any established 14-3-3-binding motifs, and recent attempts to demonstrate 14-3-3 binding by the purified phosphorylated or dephosphorylated forms of native \textit{AtPPC1} [8] using 14-3-3 far-Western overlay assays have proven unsuccessful (C. MacKintosh and W.C. Plaxton, unpublished work). It is possible that \textit{AtPPC1} binds to (i) 14-3-3 proteins, but does not perform well in the overlay assay which requires protein refolding, or (ii) another 14-3-3-interacting phosphoprotein and thus appeared in the tandem-affinity-purified 14-3-3 complexes. It would be of interest to employ 14-3-3 pull-down assays coupled with PEPC immunoblotting to further assess putative 14-3-3 binding by PTPCs, and/or whether 14-3-3s exert any influence on the kinetic and regulatory properties of \textit{AtPPC1} or other plant PEPCs.

\textbf{PEPC mono-ubiquitination: the new kid in town}

Ubiquitin is a highly conserved globular protein of eukaryotic cells that modifies target proteins via its covalent attachment through an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue on a target protein. A multi-enzyme system consisting of activating (E1), conjugating (E2) and ligating (E3) enzymes attach ubiquitin to cellular proteins. Polyubiquitination is a well-known PTM that tags many proteins for their proteolytic elimination by the 26S proteasome. Indeed, degradation of both the PTPC and PPCK by the polyubiquitin–proteasome pathway has been reported [181,182]. However, protein mono-ubiquitination has been demonstrated recently to play a variety of crucial non-destructive functions in yeast and mammalian cells [183,184]. Mono-ubiquitination is a reversible PTM that mediates protein–protein interactions (by recruiting ubiquitin-binding domain client proteins) and localization to help control processes such as endocytosis, DNA repair, transcription and translation, and signal transduction [183,184]. Ubiquitin-related pathways are believed to be of widespread importance in the plant kingdom. Genomic analyses indicated that the ubiquitin-related pathway alone comprises over 6% of the \textit{Arabidopsis} or rice proteomes with thousands of different proteins being probable targets [185].
Recent studies on Class-1 PEPC from germinated COS endosperm provided the first example of regulatory mono-ubiquitination of a metabolic enzyme [103]. Extracts of fully mature and germinating COS endosperm had been shown to contain an immunoreactive PTPC polypeptide that co-migrated with the 107-kDa subunit of the developing COS Class-1 PEPC homotetramer [4,77,81]. However, an additional immunoreactive PTPC polypeptide of approximately 110 kDa appeared immediately following COS imbibition and persisted throughout germination at an equivalent ratio with the 107-kDa subunit. In order to clarify the molecular basis for this observation, a 440-kDa Class-1 PEPC heterotetramer composed of an equivalent ratio of non-phosphorylated 110- and 107-kDa subunits was purified from 3-day-old germinated COS endosperm [103]. N-terminal microsequencing, MS and immunoblotting established that both subunits arise from the same PTPC gene (RePpc3) (Figure 3) encoding the phosphorylated 410-kDa Class-1 PEPC homotetramer of developing COS, but that the 110-kDa subunit is a mono-ubiquitinated form of the 107-kDa subunit. Tandem MS sequencing of tryptic peptides identified Lys628 as PEPC’s mono-ubiquitination site [103]. Lys628 is absolutely conserved in all PTPCs and BTPCs, and is proximal to a PEP-binding/catalytic domain (Figure 3). Incubation of the purified germinated COS Class-1 PEPC with a recombinant human deubiquitinating enzyme [USP-2 (ubiquitin-specific protease-2) core] cleaved ubiquitin from its 110-kDa subunits (Figure 4) while significantly reducing the enzyme’s K_M (PEP) and sensitivity to allosteric activators (hexose phosphates, glycerol 3-phosphate) and inhibitors (malate, aspartate) [103]. It is also notable that elimination of photosynthetic supply to developing COS by detaching intact developing COS pods from the plant caused the 107-kDa PTPC subunits of the endosperm and cotyledon to become dephosphorylated [34,35], and then subsequently mono-ubiquitinated in vivo (B. O’Leary and W. C. Plaxton, unpublished work), concomitant with the disappearance of PPCK activity. Because dephosphorylation abolishes photosynthetic delivery to COS, it is conceivable that the metabolism of the depodded developing endosperm is rearranged to mimic that of the gluconeogenic germinating COS. PTPC mono-ubiquitination has since been demonstrated in Lilium longiflorum (lily) pollen [7] and Arabidopsis seedling (B. O’Leary and W. C. Plaxton, unpublished work). An immunoreactive PTPC ‘doublet’ highly reminiscent of the mono-ubiquitinated Class-1 PEPC of germinated COS has been frequently observed on PTPC immunoblots of clarified extracts from a broad variety of plants, including Hydrilla verticillata (hydrilla) leaves [186], Vicia faba (broad bean) stomata [187], Cucumis sativus (cucumber) roots [188], germinating seeds of wheat, barley and sorghum [104,105,107], and ripening banana fruit [119]. In contrast with the COS system, the Class-1 PEPC of cereal seeds such as barley and wheat is phosphorylated during germination, whereas that of fully mature maize seeds appears to be maximally phosphorylated before germination [36,106,168]. We recently completed a survey of tissue-specific PTMs of PTPC polypeptides in various photosynthetic and non-photosynthetic tissues of the castor oil plant. Immunoblotting using PTPC (anti-RcPpC3) and phospho-site-specific PTPC (anti-pS11) antibodies following pre-incubation of clarified extracts with USP-2 demonstrated that PTPC mono-ubiquitination rather than phosphorylation appears to be the predominant PTM of Class-1 PEPCs that occurs in non-stressed castor oil plants (B. O’Leary and W. C. Plaxton, unpublished work). Moreover, the distinctive developmental patterns of PTPC phosphorylation compared with mono-ubiquitination in the castor oil plant indicated that these PTMs may be mutually exclusive. As discussed above, Class-1 PEPC phosphorylation was mainly observed in plant tissues in which a high and tightly controlled flux of PEP to malate has an obvious metabolic role; e.g. as occurs during atmospheric CO2 assimilation in C3 or CAM leaves, photosynthesize partitioning to storage end-products by developing COS, rapid nitrogen assimilation following NH4+ or NO3− resupply to nitrogen-limited cells, or during nutritional P starvation [16,17,34,38,42,54,169]. Clearly, additional research is warranted to assess the interplay between and metabolic functions of PTPC phosphorylation and mono-ubiquitination. Future research also needs to characterize the: (i) ubiquitin-binding domain proteins that might interact with the ubiquitin-‘docking site’ of mono-ubiquitinated PEPCs, as well as the possible influence of this PTM on their subcellular location, and (ii) signalling pathways and specific E3 ligase that mediate tissue-specific PTPC mono-ubiquitination. High-throughput proteomic screens have identified numerous ubiquitinated metabolic enzymes in Arabidopsis, including PTPC [189,190]. However, it was not determined whether the various targets were poly- or mono-ubiquitinated. This discrimination is crucial for future studies of the plant ubiquitome since polyubiquitination and mono-ubiquitination are destructive and non-destructive PTMs respectively that mediate entirely different effects on target protein function [183,184].

**BTPC functions as a catalytic and regulatory subunit of Class-2 PEPC complexes of vascular plants and green algae**

BTPC genes are unique and specifically expressed

In 2003, interrogation of Arabidopsis and rice PEPC gene families led to the surprising discovery that, alongside their various PTPC genes, both genomes also encode and express an enigmatic BTPC gene whose deduced amino acid sequence shares a slightly higher similarity with PEPCs from proteobacteria than with other plant PEPCs (Figure 5) [3]. These novel PEPC isoenzymes were thus named BTPCs, whereas all remaining C3, C4 and CAM PEPCs were classified as PTPCs. All plant genomes sequenced to date, including that of ancestral green algae, contain at least one BTPC gene. The BTPCs constitute a monophyletic group, separate from either the PTPCs or bacterial and archaeal PEPCs, and appear to have evolved in green algae (Figure 5). BTPC genes have a highly conserved genomic structure composed of approximately ten exons, whereas BTPC genes have a very different and more complex structure with approximately 20 exons [8]. Deduced BTPC polypeptides of vascular plants range from ~116 to 118 kDa (or approximately 130 kDa in green algae).
Figure 5  Phylogenetic analysis of vascular plant, green algal, bacterial and archaeal PEPCs using a neighbour-joining consensus tree

Protein accession numbers from GenBank®, Phytome and Maize sequence databases were used to construct the phylogenetic tree and are shown in square brackets. Bootstrap analysis was carried out with 100 replicates. Numbers at the branches correspond to percentage bootstrap frequencies for each branch. Only values >50 are shown.
compared with ~105–110 kDa for the corresponding PTPCs [8].

All BTPC sequences deduced contain residues critical for PEPC catalysis (Figure 3), and heterologous expression of green algal (C. reinhardtii) and castor oil plant BTPCs yielded active PEPCs [5,160,191]. Deduced PEPC polypeptides are readily classified as a BTPC or PTPC by three main criteria: (i) their C-terminal tetrapeptide is either (R/K)NTG for BTPCs or QNTG for PTPCs, (ii) BTPCs lack the distinctive N-terminal serine phosphorylation motif [acid-base-XXSIDAQLR] characteristic of PTPCs [3–5], and (iii) all BTPCs contain a unique and highly divergent insertion of approximately 10 kDa (corresponding to residues 325–467 of the COS BTPC, RcPPC4) that was predicted to exist in a largely unstructured and highly flexible conformation, known as an intrinsically disordered region (Figure 3) [191]. Disordered-region-containing proteins are ubiquitous in all organisms. Their disordered region typically exists as a flexible linker that connects two globular domains to mediate protein–protein interactions. The flexible linker allows the connected domains to freely twist and rotate through space to recruit their binding partners. Linker sequences can vary greatly in length and amino acid sequence, but tend to be susceptible to proteolytic cleavage while exhibiting a low content of bulky hydrophobic amino acids and a higher proportion of polar and electrically charged amino acids [192]. Indeed, a recent imaging study of transiently expressed wild-type and truncated mutants of castor oil plant BTPC (RcPPC4) and PTPC (RcPPC3) fluorescent fusion proteins in tobacco BY2 suspension cells demonstrated that RcPPC4’s disordered region mediates its in vivo interaction with RcPPC3 (e.g. as a Class-2 PEPC complex, see below) [193].

The tissue-specific expression of BTPC transcripts has been investigated in several species. Although expression of BTPC transcripts tends to be quite low relative to PTPC transcripts, and is dependent upon the developmental stage or metabolic status of the tissue being examined, no obvious pattern of expression has emerged [3,4,6,7,11,194]. Arabidopsis BTPC transcripts were initially detected at low levels in siliques and flowers [3], but were shown subsequently to be specifically expressed in the late stages of pollen development [7] and induced in roots by salt and drought stress [11]. BTPC transcripts and polypeptides are co-ordinately and highly expressed during COS development; their developmental profile coincided with stages of rapid endosperm growth and oil accumulation [4]. Nevertheless, our current understanding of plant/algal BTPC biochemistry was initially built upon a foundation of integrating classical enzyme biochemistry with modern tools of MS and associated bioinformatic databases.

Native BTPCs interact with PTPCs as subunits of heteromeric Class-2 PEPC complexes

The study of plant BTPC biochemistry began unknowingly in the 1990s with a series of papers describing the purification and characterization of two distinct PEPC classes from unicellular green algae (Selenastrum minutum and C. reinhardtii) that exhibited highly dissimilar physical and kinetic properties, but that shared an identical PTPC subunit [195–199]. From these preparations, typical 400-kDa PEPC homotetramers composed solely of PTPC subunits were classified as a Class-1 PEPC, whereas high-molecular-mass complexes of PTPC and an immunologically unrelated 130-kDa subunit (subsequently shown to be a BTPC) were termed Class-2 PEPCs. Relative to the Class-1 PEPC, the algal Class-2 PEPCs displayed enhanced thermal stability, a broader pH-activity profile, biphasic PEP saturation kinetics and a markedly reduced sensitivity to allosteric effectors. This work was corroborated and significantly extended by Raymond Chollet and co-workers who cloned and analysed the expression of C. reinhardtii PTPC and BTPC genes [5,67]. The PTPC cDNA expressed as an active ~110-kDa subunit of both Class-1 PEPC and Class-2 PEPC, whereas the BTPC cDNA expressed as an active ~130-kDa subunit found only in Class-2 PEPC. At the level of gene and protein expression, both algal PEPC subunits responded to inorganic nitrogen levels. However, the effect was more pronounced with the PTPC subunits, consistent with their proposed anaplerotic function during transient nitrogen assimilation [5,67].

Low- and high-molecular-mass PEPC isoforms were subsequently purified and characterized from the triacylglycerol-rich endosperm of developing COS whose respective physical and kinetic/regulatory properties were remarkably analogous to the highly distinctive Class-1 and Class-2 PEPCs of unicellular green algae [82]. Thus native COS Class-1 PEPC is a classic 410-kDa homotetramer of 107-kDa PTPC subunits. In contrast, the allosterically desensitized COS Class-2 PEPC 910-kDa hetero-octameric complex consists of the same Class-1 PEPC homotetrameric core tightly associated with four 118-kDa BTPC subunits (Figures 6 and 7) [4,82]. The COS Class-2 PEPC complex has been documented by additional in vitro techniques including co-IP (co-immunopurification) and non-denaturing PAGE of clarified extracts coupled with in-gel PEPC activity staining and parallel immunoblotting using BTPC- and PTPC-specific antibodies [4,34,77]. Both approaches were recently employed to demonstrate comparable Class-1 and Class-2 PEPC isoforms in extracts of developing lily pollen [7].

Upon extraction, the BTPC subunits of green algal and vascular plant Class-2 PEPCs are extremely susceptible to rapid in vitro proteolytic cleavage at a specific site within their disordered region (e.g. see Figure 3) by an endogenous thiol endopeptidase [4,196,197]. This in vitro proteolysis has prevented purification of native COS Class-2 PEPC containing non-truncated BTPC subunits, even in the presence of a wide array of protease inhibitors and cocktails. However, limited protection, suitable for BTPC immunoblotting and co-IP from clarified extracts, was afforded by a combination of PMSF and the ProteCEASE 100 Cocktail marketed by G-Biosciences [4,77]. Two additional techniques were central in allowing further characterization of non-proteolytically degraded native BTPC from developing COS. First, co-IP of highly enriched native BTPC from developing COS endosperm was achieved using an anti-(castor oil plant PTPC IgG) immunoaffinity column [77]. Relatively large quantities (>5 mg) of purified non-proteolysed 118-kDa BTPC subunits were eluted from this column using Pierce’s Gentle Ag/Ab Elution Buffer and used for detailed MS characterization of its in vivo phosphorylation sites [77,191]. Secondly, E. coli lysates containing heterologously expressed wild-type and mutant versions of castor oil plant BTPC (RcPPC4) were mixed with a recombinant Arabidopsis PTPC (AtPPC3) to create intact stable chimaeric Class-2 PEPCs in vitro, which were then purified and characterized [160,191]. Both PTPC and BTPC subunits were catalytically active and consequently the chimaeric Class-2 PEPC displayed biphasic PEP saturation kinetics [160], as documented previously for the purified non-proteolysed native Class-2 PEPC from the green alga S. minutum [197]. BTPC is a low-affinity allosterically desensitized Class-2 PEPC subunit that exhibits $K_m$ (PEP) and $IC_{50}$ (malate) values approximately 10- and 15-fold higher respectively than those of the PTPC subunits [160,191]. The BTPC subunits appear to have an additional regulatory role because the PTPC subunits of Class-2 PEPC were far less sensitive to allosteric inhibitors compared with the same subunits within a Class-1 PEPC (Figure 7) [160,191]. It is also notable that the
Figure 6  Model illustrating the biochemical complexity of castor bean PEPC

In developing COS endosperm, the PTPE RC PPC exists: (i) as a typical Class-1 PEPC homotetramer (PEPC1) which is activated in vivo by sucrose-dependent phosphorylation of its 107-kDa subunit (p107) at Ser11 [34,35], and (ii) tightly associated with 118-kDa BTPC (RC PPC4) subunits (p118) to form the allosterically desensitized Class-2 PEPC hetero-octameric complex (PEPC2). The RC PPC4 subunits are subject to in vivo multi-site phosphorylation at Thr4, Ser425 and Ser451 [77,191,201]. COS maturation is accompanied by disappearance of the Class-2 PEPC complex and RC PPC4 polypeptides and transcripts, a marked reduction in the amount of Class-1 PEPC coupled with dephosphorylation of its p107-PTPC subunits [4,34,82]. COS imbition and germination is accompanied by increased RC PPC3 gene expression, PEPC activity and amount, and ubiquitination of 50% of RC PPC3’s subunits at Lys628 to form the mono-ubiquitinated Class-1 PEPC heterotetramer. UB, ubiquitin. Figure modified from [103] with permission.

Figure 7  Differential influence of glucose 6-phosphate and malate on the activity of native Class-1 and Class-2 PEPC (PEPC1 and PEPC2 respectively) isoforms purified from developing castor beans

AIC50 and IC50 denote the concentration of glucose 6-phosphate (Glc-6-P/G6P) and malate required for half maximal activation and inhibition respectively. Assays were conducted at pH 7.3 with subsaturating PEP concentrations (0.2 mM). All values represent the means (±S.E.M.) of three separate determinations. Figure modified from [82] with permission.

recombinant COS BTPC readily formed insoluble aggregates when extracted from E. coli cells in which it was overexpressed. However, when mixed with native or recombinant Class-1 PEPCs from various plant sources, the recombinant COS BTPC subunits spontaneously rearranged to form stable soluble Class-2 PEPC complexes [160]. Although plant and algal BTPCs exhibit PEPC activity, several additional lines of evidence strongly suggest that PTPC subunits are essential binding partners for BTPC subunits: (i) native BTPC subunits have only been observed in association with PTPC subunits as a Class-2 PEPC [4,67,82,196,197], (ii) the BTPC subunits tightly associate with the PTPC subunits during purification [77,82], and (iii) FP (fluorescent protein)-tagged castor oil plant PTPC and BTPC subunits interact in vivo during their transient co-expression in tobacco BY2 cells (see below) [193]. BTPCs have thus been hypothesized to be compulsory BTPC-binding partners that play an indispensable role in maintaining BTPCs in their proper structural and functional state in Class-2 PEPCs [160].

Significant levels of Class-2 PEPC have been observed in developing COS endosperm and lily pollen. Both are reproductive sink tissues in which mitosis has been completed and the cells are rapidly expanding while simultaneously metabolizing large amounts of imported photosynthate into storage lipids and proteins [7,82,160]. The unique kinetic and regulatory properties of Class-2 PEPC have been hypothesized to function as a ‘metabolic overflow’ mechanism capable of sustaining significant flux from PEP to malate under in vivo conditions where the corresponding Class-1 PEPC activity would become largely suppressed by feedback allosteric inhibitors [7,160]. For example, malate levels in developing COS endosperm have been measured at 5 mM, a value ~80-fold higher than the IC50 (malate) of COS Class-1 PEPC at physiological pH (Figure 7), but within the range of the IC50 (malate) of the BTPC subunit of Class-2 PEPC [82,89].

BTPC of developing castor beans is subject to multi-site phosphorylation in vivo

As plant BTPCs lack the conserved N-terminal serine phosphorylation motif characteristic of PTPCs (Figure 3), they were suggested to be non-phosphorylatable [11,37]. However, the use of Pro-Q Diamond phosphoprotein staining, immunoblotting with commercially available phospho-(serine/threonine) Akt substrate-specific antibodies, and P2-affinity PAGE using Phos-TAG acrylamide [200] demonstrated that co-immunopurified native
BTPC from developing COS endosperm was phosphorylated at multiple sites in vivo [77]. Detailed analyses of co-immunopurified COS BTPC using Fourier-transform MS (>93% sequence coverage) identified three novel phosphorylation sites: Thr4 at the N-terminus, and Ser425 and Ser451 within its disordered region (respectively corresponding to acidicophilic, proline-directed and basophilic protein kinase recognition motifs) (Figure 3) [77,201]. The Thr4 and Ser451 phosphorylation sites are conserved in other BTPC orthologues, but the Ser425 site is only partially conserved (e.g. see Figure 3) [191,201]. Phosphomimetic mutagenesis of the Ser425 and Ser451 sites have shown them to be regulatory in nature, with both causing marked inhibition of the BTPC subunits within a Class-2 PEPC by increasing their $K_m$ (PEP) and sensitivity to feedback inhibition by malate and aspartate [191,201]. The developmental patterns of BTPC phosphorylation at Ser425 and Ser451 were examined by immunoblotting clarified COS extracts with the respective phosphosite-specific antibodies, and shown to be very distinct from the in vivo phosphorylation activation of COS Class-1 PEPC’s PTPC subunits at Ser411, implying control by separate protein kinases and signalling pathways [191,201]. In green algae, BTPC phosphorylation: (i) also appears to be inhibitory in nature, and (ii) may be involved in mediating BTPC/PTPC subunit stoichiometry within algal Class-2 PEPC complexes [198]. The function of the N-terminal phosphorylation site of developing COS BTPC (Thr4) is unknown. Kinetic analysis of a T4D phosphomimetic RcPPC4 mutant indicated that it is not regulatory in nature [201]. However, it is intriguing that Thr4 of plant BTPC exists in a conserved FHA (forkhead-associated)-binding domain (pTXXD) (Figure 3) and also corresponds to an acidicophilic protein kinase CK2 motif. As FHA domains have gained considerable prominence as phosphothreonine-dependent protein interaction modules [202], it will be necessary to establish the role that BTPC phosphorylation at Thr4 might play in mediating the interaction of Class-2 PEPCs with FHA domain-containing proteins. Characterization of BTPC phosphorylation from additional vascular plant and algal sources, alongside identification of the responsible protein kinases and related signalling pathways, will also be essential to further validate and extend the role of BTPC phosphorylation in Class-2 PEPCs.

SUBCELLULAR LOCALIZATION AND PROTEIN–PROTEIN INTERACTIONS OF PLANT PEPC

Class-2 PEPC appears to associate with the mitochondrial outer envelope

The subcellular location and in vivo interaction of COS PTPC (RcPPC3) and BTPC (RcPPC4) were assessed recently by imaging FP–PEPC fusion proteins that had been transiently expressed in heterotrophic tobacco suspension cells [193]. FP–PTPC sorted to the diffuse cytosol, whereas NLS (nuclear localization signal)–FP–PTPC sorted to the nucleus [193]. In contrast, BTPC–FP localized to discrete punctate structures, tentatively identified as mitochondria by immunostaining of endogenous mitochondrial cytochrome oxidase. However, when NLS–FP–PTPC or FP–PTPC was co-expressed with BTPC–FP, fluorescent PTPC signals co-localized with BTPC–FP. Transmission electron microscopy of immunogold-labelled developing COS endosperm and cotedyledon using monospecific COS BTPC and PTPC antibodies indicated that both proteins tended to cluster together into discrete regions of the mitochondrial outer envelope. The overall results indicate that: (i) COS BTPC and PTPC interact in vivo as a Class-2 PEPC complex, (ii) BTPC’s unique and divergent intrinsically disordered region mediates its tight interaction with PTPC, (iii) the BTPC-containing Class-2 PEPC may be located on the mitochondrial outer membrane, whereas (iv) the PTPC-containing Class-1 PEPC is uniformly distributed throughout the cytosol. Mitochondrion-associated cytosolic glycolytic isoenzymes have also been reported in several studies. Lee Sweetlove and colleagues showed that seven different glycolytic enzymes formed a metabolon on the mitochondrial surface of Arabidopsis suspension cells during periods of increased respiration so as to channel carbon from cytosolic metabolite pools into the mitochondria while restricting substrate use by competing metabolic pathways [203,204]. Furthermore, FP-tagged aldolase, enolase, ATP-PFK and hexokinase have shown a dual mitochondrial (outer envelope) and cytosolic localization [203]. We therefore need to determine the prevalence, mechanism and metabolic role(s) of mitochondrial-associated Class-2 PEPC complexes. In addition, it will be important to identify any Class-2 PEPC-interacting proteins that may form a metabolon to facilitate respiratory CO2 refixation (e.g. carbonic anhydrase) and/or anaplerotic PEP partitioning to metabolic end-products such as storage lipids and proteins.

Class-1 PEPC may interact with a cytosolic-targeted PDH

Biochemical analyses of several purified plant PTPC isoenzymes indicated that they form a complex with the metabolically sequential MDH and ME in C4 leaves [9], its own PPCK in ripening banana fruit [120], as well as the BTPC leading to the formation of Class-2 PEPC hetero-oligomeric complexes of green algae and vascular plants [4,5,67,82,197]. Multienzyme complexes containing PEPc, MDH and acetyl-CoA carboxylase were also isolated from the unicellular photosynthetic protist Euglena gracilis [205]. A surprising observation of the recent co-IP proteomics study of the Class-1 PEPC interactionome of developing COS endosperm was the identification of the PDH$_c$ (plastidal PDH) as a putative PTPC interactor [77]. PDH$_c$ catalyses the irreversible oxidative decarboxylation of pyruvate into acetyl-CoA (Figure 2). Immunoblotting using monospecific antibodies raised against E1$, E1^\beta$, E2 and E3 subunits of Arabidopsis PDH$_c$ verified the presence of all four PDH$_c$ subunits in eluates from the anti-[COS Class-1 PEPC (RcPPC3)] immunoaffinity column [77]. PDH$_c$ appears to specifically associate with the PTPC subunits of COS Class-1 PEPC, since a parallel co-IP study of the PEPC interactionome of germinating COS endosperm (which contains no detectable BTPC or Class-2 PEPC) also identified PDH$_c$ as a PTPC-interacting protein [193]. Although COS Class-1 PEPC and PDH$_c$ are believed to be localized in different metabolic compartments, there are only two enzymatic steps between them (MDH and ME), and PTPC and PDH$_c$ have both been implicated in playing an important role in supporting fatty acid synthesis in developing COS [81,89,206]. Plant cells are unique in containing two different PDH isoenzymes: mitochondrial PDH which links cytosolic glycolysis with the TCA cycle, and PDH$_c$ which produces acetyl-CoA and NADH for plastidic fatty acid synthesis (Figure 2) [206,207]. The structure and post-translational control of PDH$_c$ is very different from that of mitochondrial PDH, and both isoenzymes can be readily discriminated by immunoblotting. Immunoactive PDH$_c$ subunits were markedly enriched when clarified COS extracts were subjected to co-IP on the anti-[COS Class-1 PEPC] immunoaffinity column. In contrast, no immunoactive bands were evident when parallel immunoblots of the same co-IP column eluates were probed with anti-[plant mitochondrial PDH] antibodies [77]. A previous report indicated that PDH$_c$ may not be exclusively plastidic in developing COS.
endosperm [206]. In this study, the plastid marker enzyme acetyl-CoA carboxylase demonstrated 98 % of its total activity to be leucoplast-localized, whereas only 62 % of total PDH activity was in the same fraction. Conversely, 2 % and 38 % of total acetyl-CoA carboxylase and PDH activity were respectively measured in the corresponding cytosolic fraction [206]. Further studies are required to establish PDH’s distribution in the plastid compared with cytosol of non-green plant cells, such as developing and germinating COS endosperm. Nevertheless, the aforementioned findings support the hypothesis that a specific PEPC–PDH interaction might exist in developing COS. This interaction could facilitate CO2 recycling from PDH to PEPC and/or occur as part of a metabolon that channels PEP to cytosolic acetyl-CoA required for the biosynthesis of isoprenoids, flavonoids and malonated derivatives, in addition to the elongation of C16 and C18 fatty acids. Cytosolic acetyl-CoA is believed to be generated from citrate and CoA by ATP-citrate lyase [208]. However, ATP-citrate lyase activity was barely detectable in developing COS extracts [208]. PDHc could provide an alternative metabolic route for acetyl-CoA production within the COS cytosol. It will be interesting to determine whether the observed in vitro interaction between a Class-1 PEPC and PDHc exists in vivo, and, if so, the role that it plays in carbohydrate partitioning and CO2 recycling in developing seeds.

**The chloroplast Class-1 PEPC isoenzyme of rice leaves**

Until recently, PEPC had been described as being exclusively cytosolic, so it was a major surprise when a chloroplastic PTPC isoenzyme was identified in rice leaves [159]. This PTPC isoenzyme contains a transit peptide and its expression was largely confined to leaf mesophyll cells where it was estimated to account for a third of the total PEPC protein. It exhibited a lower affinity for both PEP and allosteric effectors compared with typical C3 PTPC isoenzymes, and gene knockouts produced a phenotype which implied its involvement in leaf nitrogen assimilation [159]. Earlier immunogold imaging studies reported that PTPC may associate with the chloroplast outer envelope in C3, C4 and CAM leaves [209,210], whereas PTPC partially pelleted with the total chloroplast fraction from sorghum leaves [167]. However, this is the first well-documented instance of a plant PEPC being targeted to the plastid stroma. It is unknown whether plastidic PEPC isoenzymes exist in other genera, as plant PEPC being targeted to the plastid stroma. It is unknown whether plastidic PEPC isoenzymes exist in other genera, as orthologous genes were not detected. Nevertheless, the existence of a chloroplastic PTPC isoenzyme endows rice mesophyll cells with an alternative route of organic acid metabolism to support nitrogen assimilation that had not previously been considered in plant primary metabolism [159].

**CONCLUDING REMARKS**

Although the central importance of PEPC in plant metabolism and physiology has been appreciated for many years, recent research has provided numerous insights into the complex biochemical and molecular mechanisms that underpin the control of PEPC activity, and the influence that PEPC and PPCK exert on a wide array of cellular processes. Integrative analyses at the genomic, transcriptomic, enzymological/biochemical and cellular levels have revealed PEPC’s biochemical complexity (e.g. see Figure 6), whereas the application of metabolic flux analysis and functional genomic tools have consistently indicated the importance of post-translational PEPC control in ensuring the optimal regulation and plasticity of anaplerotic PEP metabolism. Owing to its multiple functions and location at a pivotal branchpoint of plant primary metabolism (Figures 1 and 2A), an impressive array of strategies has evolved to post-translationally control the activity of plant PEPCs. Regulatory phosphorylation and mono-ubiquitination, 14-3-3 proteins, as well as changes in intracellular pH and allosteric effector levels have all been described as mechanisms to control Class-1 PEPC activity in different plant tissues under various physiological conditions. The discovery of allosterically desensitized Class-2 PEPC hetero-octameric complexes in green algae and vascular plants that arise from a tight physical association between a Class-1 PEPC with co-expressed BTPC subunits adds another layer of complexity to plant PEPC functions and control. A major challenge will be to link the multifaceted post-translational controls and associated PTMs of Class-1 and Class-2 PEPCs with the cell-specific partitioning of PEP into discrete metabolic pathways.

Many metabolic functions have been attributed to malate and other TCA cycle intermediates within different cell types [28], and the versatility of these metabolites is undoubtedly linked to the evolved diversity of plant PEPC (Figure 1). A comprehensive understanding of the linkages between the expression of individual PEPC isoenzymes, their specific physiological/metabolic functions, and the synchronous regulation of PEPC with other key enzymes of the PEAP branchpoint (e.g. PKc, PEP carboxykinase, pyruvate orthophosphate dikinase, the shikimate pathway) represents a major challenge. Only with the concerted efforts of plant physiologists, biochemists and enzymologists and molecular biologists, will the cell-specific functions and control of plant PEPC be fully comprehended. It is anticipated that the further characterization of green algal and vascular plant Class-1 and Class-2 PEPC, their in vivo PTMs (including the requisite interconverting enzymes such as PPCK or E3 ubiquitin ligases, and relevant signalling pathways), subcellular localization and protein–protein interactions will remain a fruitful research area for the foreseeable future. It will also be important to establish a structural model for plant and algal Class-2 PEPC subunit architecture. Lastly, patterns of Class-1 compared with Class-2 PEPC expression need to be re-evaluated together with the continued application of functional genomic tools to assess the impact of altered PTPC and BTPC expression on anaplerotic PEP-partitioning to storage end-products in developing seeds, as well as plant carbon–nitrogen interactions and acclimatization to environmental stress.

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