REVIEW PAPER

Functional evolution of C$_4$ pyruvate,orthophosphate dikinase

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Abstract

Pyruvate,orthophosphate dikinase (PPDK) plays a controlling role in the PEP-regeneration phase of the C$_4$ photosynthetic pathway. Earlier studies have fully documented its biochemical properties and its post-translational regulation by the PPDK regulatory protein (PDRP). However, the question of its evolution into the C$_4$ pathway has, until recently, received little attention. One assumption concerning this evolution is that changes in catalytic and regulatory properties of PPDK were necessary for the enzyme to fulfil its role in the C$_4$ pathway. In this study, the functional evolution of PPDK from its ancient origins in the Archaea to its ascension as a photosynthetic enzyme in modern C$_4$ angiosperms is reviewed. This analysis is accompanied by a comparative investigation into key catalytic and regulatory properties of a C$_3$ PPDK isoform from *Arabidopsis* and the C$_4$ PPDK isoform from *Zea mays*. From these analyses, it is proposed that PPDK first became functionally seated in C$_3$ plants as an ancillary glycolytic enzyme and that its transition into a C$_4$ pathway enzyme involved only minor changes in enzyme properties per se.

Key words: C$_3$ plants, C$_4$ evolution, C$_4$ photosynthesis, C$_4$ plants, pyruvate, orthophosphate dikinase.

Introduction and review

Evolutionary origin and phylogenetic distribution of PPDK

Pyruvate,phosphate dikinase (PPDK, EC 2.7.9.1) was discovered independently in C$_4$ grasses (Hatch and Slack, 1968) and in the parasitic amoeba, *Entamoeba histolytica* (Reeves, 1968). The enzyme from all organisms catalyses the freely reversible reaction:

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \leftrightarrow \text{PEP} + \text{AMP} + \text{PPi}$$
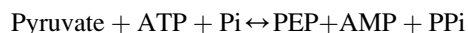
Most of what is known concerning the structure function properties of the enzyme has been derived from studies of PPDK from the bacterium *Clostridium symbiosum* (Herzberg *et al.*, 1996; Lim *et al.*, 2007). The detail provided from this model PPDK has revealed structural features unique to the enzyme mechanism that allow its gene to be reliably identified in genome databases. These show that PPDK has a phylogenetic distribution restricted to select groups of the Archaea, bacteria, protozoa, lower fungi, and green plants (Liapounova *et al.*, 2006; Slamovits and Keeling, 2006) (Table 1). A PPDK phylogenetic tree

analysis assembled from these groups indicates the enzyme originated in the Archaea, but its evolution into prokaryotic and eukaryote taxons is inconsistent with its predicted vertical transmission (Liapounova *et al.*, 2006; Slamovits and Keeling, 2006). For example, PPDKs from eukaryotic protists do not cluster uniformly into a clade, but are isolated into various bacterial subgroups. Because of this irregular transmission, it is proposed that the gene for PPDK has been horizontally transferred among distantly related (unicellular) species. The gene is notably absent in cyanobacteria indicating that plants did not inherit the PPDK gene from the chloroplast endosymbiont ancestor.

PPDK function in non-plant organisms

In most non-plant organisms studied to date (the Archaea, eubacteria, and protists), PPDK is either a primary or secondary enzyme of glycolysis. Its natural selection into these typically metabolically challenged organisms (i.e. anaerobic habitats) was probably promoted by the enzyme's freely reversible catalytic mechanism. This feature allows

the enzyme to fulfil alternate roles in anaerobic metabolism such as gluconeogenesis [operating in the phospho*enol*pyruvate (PEP) forming direction] or for glycolytic ATP synthesis [operating in the pyruvate (Pyr) forming direction]. Bacteria that use PPDK for ATP synthesis are typically anaerobic and lack pyruvate kinase (PK) (Reeves *et al.*, 1968; Pocalyko *et al.*, 1990). Likewise, protozoa that rely on PPDK for ATP synthesis dwell in anaerobic or low oxygen environments and lack mitochondria (Bringaud *et al.*, 1998; Varela-Gomez *et al.*, 2004; Feng *et al.*, 2008). The utility for using PPDK to generate glycolytically derived ATP is that the enzyme, as operating in the Pyr direction, can harvest the high bond energy inherent in pyrophosphate (Huang *et al.*, 2008). In doing so, the yield of glycolytically derived ATP is boosted from two ATPs per glucose oxidized as in conventional PK-dependent glycolysis, to five ATPs per glucose oxidized (Fig. 1). In certain bacterial species, PPDK is used exclusively in the PEP-forming direction for gluconeogenesis (Benziman and Eizen, 1971; Østeras *et al.*, 1997; Eisaki *et al.*, 1999). Other bacteria typically initiate gluconeogenesis with PEP synthetase (PEPS). The preference for PPDK in this step may be due to the ability to drive the reaction by hydrolysis of by-product PPi. Only a single study on the role of PPDK in an Archaea species is available (Tjaden *et al.*, 2006). This investigation linked PPDK expression to alternate roles in gluconeogenesis and glycolytic conversion of PEP to Pyr. Interestingly, the competing PK and PEPS activities also present in this organism are proposed to be co-integrated with PPDK in a carbon-source responsive regulatory scheme.

PPDK function in C_3 plants

PPDK is ubiquitous in cells and tissues of C_3 plants, albeit as a very low abundance enzyme (Chastain and Chollet, 2003). However, in specific C_3 plant tissues, C_3 PPDK has been documented to be an abundantly expressed protein. Examples include developing cereal seeds (Kang *et al.*, 2005; Chastain *et al.*, 2006; Mechin *et al.*, 2007; Hennen-Bierwagen *et al.*, 2009), senescing leaves (Taylor *et al.*, 2010), sperm cells of maturing *Arabidopsis* pollen (Hruz *et al.*, 2008), and water-saturated roots of *Oryza sativa* (rice) (Moons *et al.*, 1998). In all plants, PPDK is located in both cytoplasmic and plastid compartments (Chastain and Chollet, 2003). In eudicots, PPDK is encoded by a single-gene locus (Huang *et al.*, 2008), but contains two promoters in the 5'-region to produce cytoplasmic or plastid targeted transcripts (Parsley and Hibberd, 2006). In monocot species, the gene is encoded at two loci (Sheen, 1991; Huang *et al.*, 2008), one of which expresses an isoform exclusive to the cytoplasm and the other loci configured as a two-promoter gene to generate plastid and cytoplasmic targeted polypeptides as in eudicots. The mature cytoplasmic and chloroplast targeted PPDK polypeptides from this gene are identical. In plants with PPDK genes at two loci, the encoded polypeptides are highly homologous (>90%). With the exception of this report, there is little detailed

Table 1. Taxonomic distribution of InterPro Protein sequence entries for PPDK and PDRP/DUF 299 protein

Data are from the European Bioinformatics Institute-InterPro database pages for IPR010121 (PPDK, http://www.ebi.ac.uk/interpro/IEntry?ac=IPR010121) and IPR005177 (PDRP/DUF 299, http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR005177).

Taxonomic group	Database entries for PPDK	Database entries for PDRP and DUF 299 protein
Archaea	24	0
Eubacteria[a]	832	1360
(Cyanobacteria	0	0)
Eukarya		
Plants[b]	65	31
Diatoms	2	1
Protists	20	0

[a] Both gram-positive and gram-negative species are represented in tally.
[b] Tally includes species of green algae, non-vascular and vascular plants.
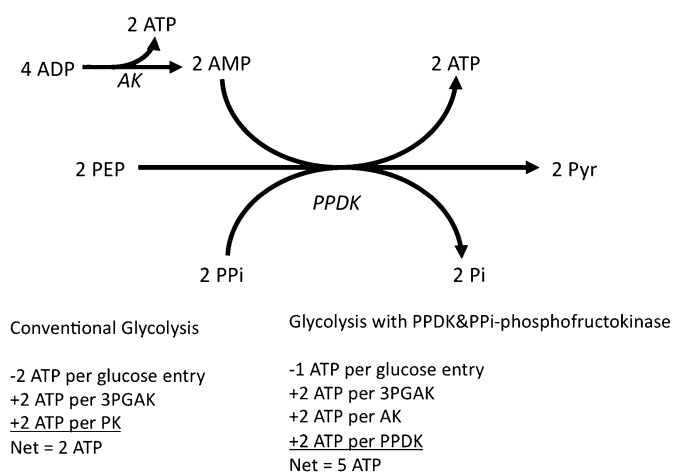
Fig. 1. Glycolytic ATP production for PPDK/PPi-dependent glycolysis versus conventional PK-based glycolysis. As illustrated, the yield of glycolytically derived ATP is boosted by the synergistic activities of PPDK and adenylate kinase (AK) versus conventional pyruvate kinase (PK) based glycolysis.

information concerning the biochemical properties of C_3 PPDK isoforms.

Ascribing a role to PPDK in those C_3 cells and tissues where it is present in very low amounts is problematic. The low level of activity prevents tracking the turnover of PPDK substrates and products using conventional ^{14}C-labelling techniques. Furthermore, competing Pyr/PEP converting activities of PK and PEP carboxykinase coexist alongside PPDK, obscuring the metabolic effect of PPDK on the overall pool of these metabolites. *Arabidopsis* PPDK knockout lines have been isolated but these do not appear to differ markedly from the wild type when grown under optimal conditions (personal communication, K Parsley and JM Hibberd, Department of Plant Science, Cambridge University) and thus do not offer an insight into the low

abundance PPDK functions. Progress in establishing a function for C$_3$ PPDK have been made from studies focusing on regions of the plant where the enzyme is abundantly expressed. These point to a principal role for C$_3$ PPDK as an ancillary glycolytic enzyme. An example is PPDKs role in developing rice and *Zea mays* (maize) seeds, where the enzyme is massively expressed in the cytoplasm of the endosperm (Kang *et al.*, 2005; Chastain *et al.*, 2006; Mechin *et al.*, 2007; Prioul *et al.*, 2008; Hennen-Bierwagen *et al.*, 2009). Its appearance in this part of the developing seed coincides with the onset of starch, protein, fatty acid, and amino acid biosynthesis. Each of these biosynthetic activities is dependent on glycolytically supplied carbon. PPDK, via its freely reversible catalysis, is proposed to balance the flow of this carbon into each respective biosynthetic activity depending on the maturation stage of the seed. In addition, PPDK may supply ATP to hypoxic regions of the developing endosperm (Chastain *et al.*, 2006). In senescing leaves of *Arabidopsis*, cytosolic PPDK has been shown to be extensively up-regulated to facilitate remobilization of nitrogen into glutamine (Taylor *et al.*, 2010). It is proposed that Pyr, originating from amino acid deamination, is converted by PPDK into PEP for subsequent glutamine biosynthesis. Notably, the proposed metabolic schemes for each of these functions require the enzyme to be competent in both forward and reverse catalysis. However, assuming a cytoplasmic pH of 6.8 and constitutively high levels of cytosolic pyrophosphate (PPi) (Stitt, 1990; ap Rees *et al.*, 1991), the direction of catalysis is likely to be restricted to the Pyr forming direction. According to an investigation into the effect of pH on C$_4$ PPDK from maize, the competency of the enzyme in catalysing its PEP-forming reaction at pH 7.0 is dramatically reduced, having only 6% of the rate at pH 8.3 (Jenkins and Hatch, 1985). Furthermore PPi, with cytosolic concentrations estimated to be ~0.3 mM (Dennis and Blakeley, 2000), is a non-competitive inhibitor of the PEP-forming reaction (K_i ~0.3 mM; Jenkins and Hatch, 1985).

As mentioned above, PPDK is also localized in the chloroplasts of C$_3$ plants. A clear function for PPDK in this organelle has yet to be established although it is never abundantly expressed in C$_3$ plant tissues and cells under any circumstances of development or age (Chastain and Chollet, 2003).

PPDK function in C$_4$ plants

In the C$_4$ pathway, PPDK catalyses the regeneration of the primary CO$_2$ acceptor PEP in the stroma of leaf-mesophyll cell chloroplasts. It is hugely abundant in C$_4$ leaves, comprising up to 10% of the soluble protein fraction (Edwards *et al.*, 1985; Chastain, 2011). It is maximally active as a homotetramer of ~95 kDa subunits and is inactive in the dimeric and monomeric forms. It requires Mg^{2+} for oligomerization and NH$_4^+$ as a cofactor for optimal catalysis. The C$_4$ enzyme is highly homologous to C$_3$ plant PPDK (Agarie *et al.*, 1997). Residues and regions involved in catalysis are strictly conserved among C$_4$ plant,

C$_3$ plant, and non-plant PPDKs (Fisslthaler *et al.*, 1995). An important driver for its evolution as a C$_4$ enzyme was the modification of the PPDK gene promoter to confer leaf mesophyll cell specific expression (Sheen, 1991).

Regulation of PPDK in plants and bacteria by the PPDK regulatory protein and DUF 299 proteins

In the C$_4$ pathway, PPDK activity is strictly regulated in an up/down manner by the level of incident light. The PPDK regulatory protein (PDRP), a unique bifunctional enzyme, catalyses this light-dependent regulation by reversible phosphorylation of an active-site Thr residue (Thr456 in maize) (Fig. 2) (Burnell and Hatch, 1985; Roeske and Chollet, 1987; Burnell and Chastain, 2006; for a recent review, see Chastain, 2011). PDRP plays a similar role in the light-regulation of non-photosynthetic PPDK in chloroplasts of C$_3$ plants (Chastain *et al.*, 2002, 2008; Chastain and Chollet, 2003). *Arabidopsis* and other eudicots (e.g. *Cleome* species) encode a second PDRP gene, *At*RP2, which is localized in the cytoplasm (Chastain *et al.*, 2008). This isoform differs from the C$_4$-like plastid isoform by lacking a Pi-dependent, PPi-forming PPDK phosphotransferase activity. In monocots, only a single PDRP gene (i.e. plastid localized PDRP) has been observed in draft and published genomes. Bacterial genomic databases show that homologues of PDRP, referred to as domain of unknown function (DUF) 299 genes, are present in all PPDK-containing bacteria (Burnell, 2010) (Table 1). However, PDRP homologues are also present in bacterial species that lack PPDK (Burnell, 2010). In these cases, the DUF 299 proteins most probably function in the phospho-regulation of PEPS. A recent study demonstrated how the PDRP homologue gene in *E. coli*, which lacks PPDK, regulates the on/off activity of its PEPS via reversible phosphorylation of the PEPS active-site Thr (Burnell, 2010). In the annotated genomes of PPDK containing Archaea and protozoa, PDRP homologues are absent suggesting that PDRP first evolved in bacteria for the purpose of regulating the terminal steps of glycolysis via on/off regulation of PPDK and/or PEPS (Burnell, 2010).
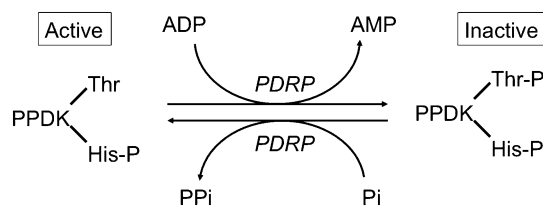
Fig. 2. Reversible phosphorylation of PPDK by PDRP. Inactivation of PPDK by PDRP proceeds by phosphorylation of an active-site Thr residue (Thr 456 in maize). Only the E-His-P intermediate enzyme form, as indicated by the His-P residue (His-458 in maize), is amenable to PDRP phosphorylation. Reactivation of PPDK is catalysed by Pi dependent dephosphorylation of this same.

Summary

Based on the functional evolutionary history of the enzyme, it is hypothesized that plants inherited a PPDK from a lower organism ancestor that was largely adapted for a role in glycolysis. The functional continuity of PPDK in plants with its glycolysis related role is evident in C_3 angiosperms where PPDK is principally seated as an ancillary glycolytic enzyme. As PPDK evolved into the C_4 pathway, it assumed a radically new function that possibly challenged the enzyme with new catalytic and regulatory requirements. In the present study, an attempt was made to uncover any changes the enzyme may have undergone as it evolved from its C_3-cytoplasmic role into its photosynthetic role in C_4 chloroplasts. Our approach consisted of contrasting key catalytic and regulatory properties of the C_3 PPDK isoform from *Arabidopsis* with the C_4 PPDK isoform from maize primarily in the context of the cytoplasmic compartment. In so doing, it was possible to demonstrate only minor differences between the PPDK from these representative C_3 and C_4 plants. It is therefore concluded that C_3 PPDK was inherently pre-adapted for its role in C_4 photosynthesis and this probably facilitated its repeated selection as the PEP-regenerating enzyme for the C_4 cycle.

Materials and methods

Expression and purification of recombinant enzyme

All plasmids used for generating recombinant enzymes have been described previously (Burnell and Chastain, 2006; Chastain *et al.*, 2008). Maize C_4 PPDK, *Arabidopsis* PPDK, and *Arabidopsis* PDRPs were expressed from a pET 28a plasmid (EMD4Biosciences). Recombinant maize PDRP was expressed from the *E. coli* protein expression vector pROExC (Invitrogen). Host *E. coli* BL21 DE3 cells were transformed with the respective plasmid and used for large-scale culture as described before (Chastain *et al.*, 2008). Subsequent extraction and affinity purification of recombinant enzyme was accomplished using a previously described procedure with the following modifications. Cell pellets (harvested from one-litre cultures) were lysed using 20 ml of the 1× BugBuster™+1× Lysonase™ enzyme mix (EMD4Biosciences) in buffer A (50 mM KPi, pH 8.0, 300 mM KCl, 2.5 mM MgSO₄, 5 mM 2-mercaptoethanol, 1× protease inhibitor cocktail VII™; RPI Corporation) and clarified of insoluble matter by high-speed centrifugation (20 min, 74 000 *g*). The clarified lysate was combined with 1 g of Prepease™ high-yield Ni²⁺-silica beads (Affymetrix) for batch-binding of 6 His-tagged recombinant proteins at 4 °C for 4 h. The batch-bound slurry was subsequently decanted into a 1.5 cm diameter Bio-Rad Econo column for washing the beads with 20 ml of buffer A + 5 mM imidizole. Recombinant 6 His-bound protein was eluted from the beads with buffer A + 300 mM imidizole (pH 8.0). The protein eluate was brought to 70% ammonium sulphate saturation and placed on wet ice overnight to maximize protein precipitation. The precipitate was brought to a final 20 mM 2-mercaptoethanol, dispensed into 1 ml aliquots for subsequent centrifugation (20 min, 32 000 *g*). The well-drained protein pellets were sealed under N₂ and stored at –80 °C until use. For recombinant PDRP, no further purification was used. Coomassie-stained gels of recombinant PDRP indicated an approximate 60% purity for each preparation. Recombinant PPDK was further purified using an FPLC-based anion-exchange chromatography procedure (Chastain *et al.*, 1996). PPDK purified in this manner was >95% as indicated by Coomassie-stained gels.

PPDK enzyme assays

PPDK activity was assayed using a previously described spectrophotometric-based procedure and adapted for use with a 96-well plate reader format (Jenkins and Hatch, 1985; Ashton *et al.*, 1990). For assays in the Pyr forming direction, the assay buffer consisted of 25 mM HEPES (pH 7.0 or pH 8.0), 6 mM MgSO₄, 25 mM NH₄Cl, 5 mM DTT, 1 mM PEP, 1 mM PPi, 0.5 mM AMP, 0.25 mM NADH, and 2 U ml⁻¹ lactic acid dehydrogenase (LDH). Assays were initiated by the addition of desalted recombinant PPDK to a final concentration of 25 ng ml⁻¹ at 30 °C. Substrate K_m for Pyr and PEP were calculated from double-reciprocal plots of the respective PEP- or Pyr-forming PPDK assays.

PDRP assays

PDRP phosphorylation activity was assayed using a previously established procedure (Chastain *et al.*, 2008) but modified for use at pH 7.0. Assays were initiated by the addition of partially purified PDRP to a final concentration of 0.26 µg ml⁻¹into a reaction mixture consisting of 0.35 mg ml⁻¹of affinity-purified recombinant *Arabidopsis* or maize PPDK, each as a fully activated, non-phospho form, in 50 mM HEPES, pH 7.0, 10 mM MgCl₂, 5mM DTT, 1 mg ml⁻¹BSA, 1 mM ADP, 1 mM PEP, 0.2 mM Ap5A, and 20 mM NH₄Cl. The reaction mixture was incubated at 30 °C. At specified time intervals, 10 µl aliquots of the reaction were removed for assay of PPDK activity in the PEP to Pyr direction (pH 7.0) as described above. When other nucleotide phosphates were used in the assay in place of ADP, these were present at 1 mM. For the immuno-based PDRP assays, aliquots of the assay were combined with an equal volume of SDS-PAGE sample buffer and subjected to Western blot analysis for phospho-PPDK as previously described (Chastain *et al.*, 2000, 2008). PDRP dephosphorylation activity was assayed using an immuno-based procedure as previously described (Chastain *et al.*, 2008).

Results

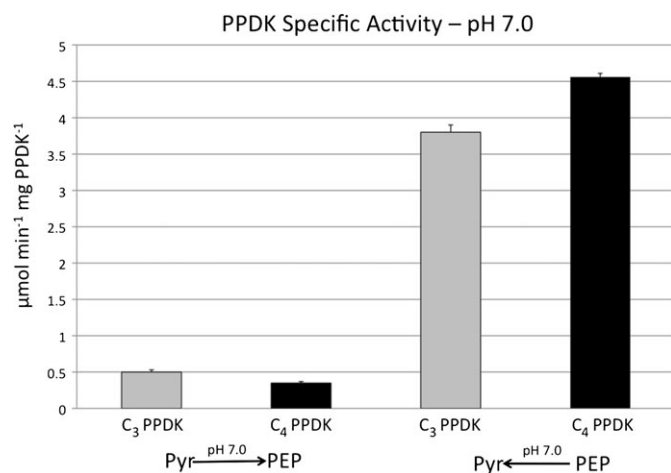*Influence of pH on C_3 versus C_4 PPDK specific activities*

Nearly all previously published analyses of *in vitro* plant PPDK properties have been carried out at pH >8.0 for purposes of simulating the behaviour of the C_4 enzyme in the stroma of illuminated chloroplasts. The choice was made to contrast the biochemical properties of C_3 and C_4 PPDKs primarily at a pH of 7.0, the approximate pH of the cytoplasmic compartment. This allowed us to gauge the differences in the properties of the enzyme not with respect to C_4 photosynthesis, but to the enzyme's competency for glycolytic function. The rationale for this approach was to reveal changes the enzyme may have acquired since it evolved from its ancestral C_3 function. Using recombinant enzyme forms for each species, specific activities for both catalytic directions were obtained (Fig. 3). In agreement with previous studies (Jenkins and Hatch, 1985), these show how the Pyr-forming reaction is strongly favoured at pH 7.0, with catalysis in the PEP-forming direction only 13% (C_3 PPDK) and 8% (C_4 PPDK) of the Pyr-forming direction. Interestingly, specific activities of the C_3- and C_4-PPDK in the Pyr-forming reaction were highly similar. Our earlier comparison of these enzymes at pH 8.3 in the PEP-forming direction also showed a nearly equivalent specific activity (4.2 versus 4.4 µmol min⁻¹ mg⁻¹ PPDK for C_3- and C_4-isoforms, respectively) (Chastain *et al.*, 2008).

C₃ and C₄ PPDK substrate affinities for Pyr and PEP

The sole function of PPDK in the C$_4$-cycle is to catalyse regeneration of the primary CO$_2$ acceptor PEP from Pyr. Alternatively, it is proposed that C$_3$ PPDK synthesizes both Pyr from PEP and PEP from Pyr (Moons *et al.*, 1998; Kang *et al.*, 2005; Chastain *et al.*, 2006; Mechin *et al.*, 2007; Hennen-Bierwagen *et al.*, 2009, Taylor *et al.*, 2010). It was therefore surmised that C$_3$ and C$_4$ PPDK would have different affinities related to the respective roles of the enzymes. This was examined by comparing the K_m for Pyr and PEP at pH 7.0 (Table 2). In agreement with this conjecture, *Arabidopsis* C$_3$ PPDK was found to have a 10-fold lower K_m for Pyr (17 μM) compared with the maize C$_4$ enzyme (178 μM). By contrast, the maize enzyme was found to have a significantly higher affinity for PEP (K_m=194 μM) than the C$_3$ enzyme (K_m=294 μM).

Effect of PPi on Pyr to PEP catalysis at pH 7.0

PPDK is proposed to catalyse PEP formation in the cytoplasm of C$_3$ plants (Moons *et al.*, 1998; Kang *et al.*, 2005; Huang *et al.*, 2008; Taylor *et al.*, 2010). At pH 7.0, this reaction should be highly antagonized by the high cytosolic levels of PPi present in plant cells (Dennis and Blakeley, 2000). To examine the potential effect of intracellular PPi on this reaction, initial activities of C$_3$- and C$_4$-PPDK were determined for the Pyr to PEP reaction in the presence of 0.1 mM and 1 mM PPi, at pH 7.0. In

addition, two levels of substrate Pyr were used (0.1 mM and 1.0 mM) with the lower concentration representing a physiological concentration of cytosolic Pyr (Roeske and Chollet, 1989). When the reaction is assayed in the presence of 1 mM PPi, not only did we fail to observe a predicted inhibition but, on the contrary, a marginal increase was observed compared with the no PPi controls (Fig. 4). At lower levels of Pyr (0.1 mM), the reaction rates declined to 65% and 45% of the rates of saturating Pyr levels for C$_3$ and C$_4$ PPDK, respectively. The greater Pyr-dependent decline for the maize C$_4$ enzyme is consistent with this enzymes higher K_m for Pyr (Table 2). Notably, *Arabidopsis* C$_3$ PPDK exhibited higher specific activities for the Pyr to PEP reaction in the presence of 0.1 mM and 1 mM PPi (pH 7.0) than maize C$_4$ PPDK.

Regulation of C₃- and C₄-PPDK by their respective PDRP isoforms at pH 7.0

The *Arabidopsis* cytosolic PDRP isoform (*At*RP2) is functionally different from its C$_4$-like chloroplast counterpart (*At*RP1) in that it lacks Pi-dependent PPDK-Thr-P protein phosphotransferase activity. As a monofunctional PDRP, it is capable of regulatory phosphorylation (inactivation) of cytoplasmic PPDK but is unable to reactivate the enzyme by reversible dephosphorylation of the regulatory PThr residue. A plausible reason that may account for the absence of this function is that its properties were originally characterized at a pH more typical of illuminated chloroplast stroma (e.g. pH 8.3) versus at the lower pH of the cytoplasmic compartment (e.g. pH 6.8). This was not the case when *At*RP2 was assayed at a cytosolic-equivalent pH of 6.8, as no dephosphorylation of substrate phospho-PPDK could be detected (Fig. 5).

The phosphoryl-donor nucleotide specificity of *At*RP2 was also examined at pH 6.8 (Fig. 6). This shows that this

Fig. 3. Specific activities of *Arabidopsis* C$_3$ and maize C$_4$ PPDK at pH 7.0 in the PEP- and Pyr-forming directions. Shown are the means ±SE (*n*=5).

Table 2. PPDK substrate affinity constants for Pyr and PEP determined at pH 7.0

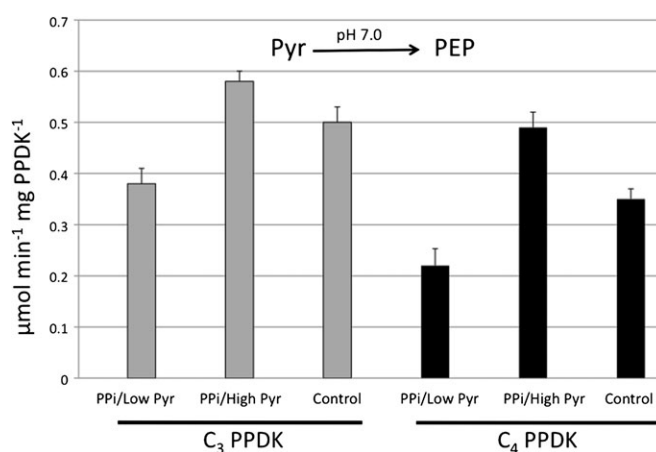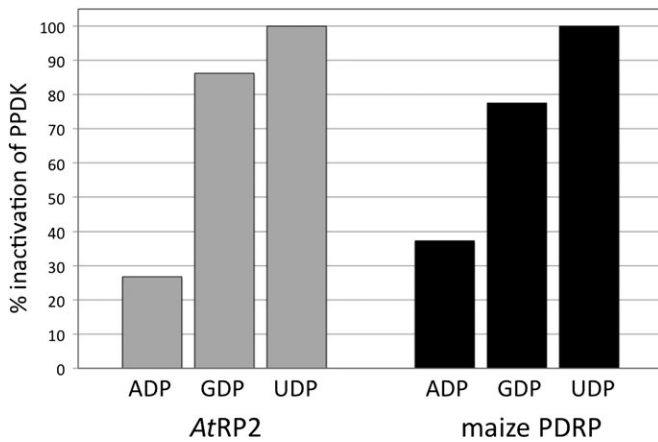Values were calculated using double-reciprocal plots of five independent assays.

K_m Pyr		K_m PEP	
C$_3$ PPDK	C$_4$ PPDK	C$_3$ PPDK	C$_4$ PPDK
17 μM	178 μM	292 μM	194 μM

Fig. 4. Influence of exogenous PPi on initial rates of C$_3$ and C$_4$ PPDK activity in the PEP-forming direction at pH 7.0. PPDK was assayed as before in the presence of 1 mM PPi at two concentrations of substrate Pyr (Low Pyr=0.1 mM, High Pyr=1.0 mM). Control assays excluded PPi and included Pyr at 1 mM). Values shown are the means ±SE (*n*=5).
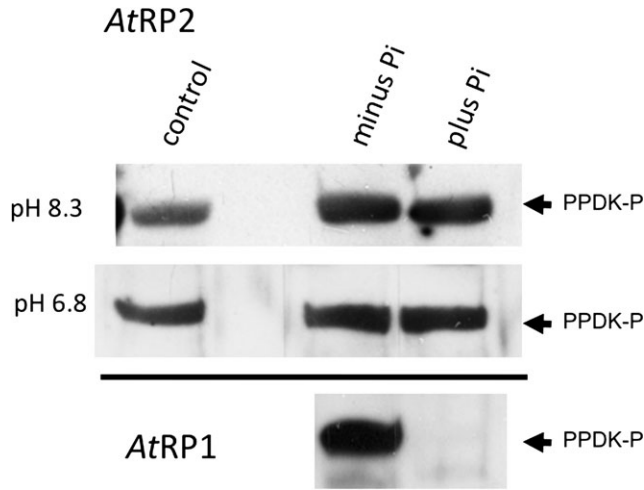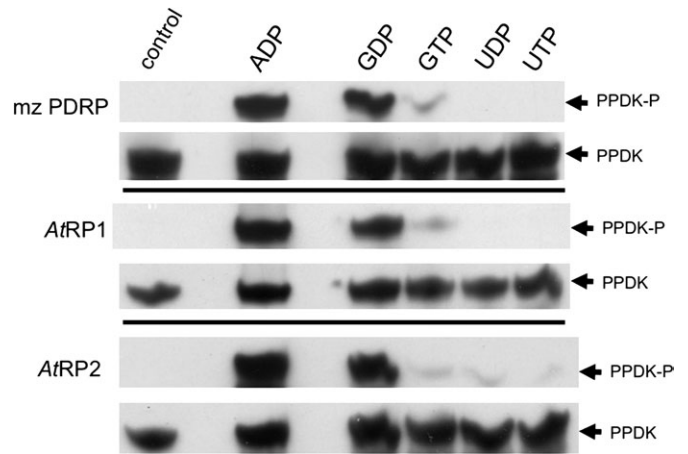
Fig. 5. Immuno-based phospho-PPDK dephosphorylation assays conducted at pH 8.3 and pH 6.8. Shown are representative denaturing Western blots of 20 µl terminated assay reaction aliquots probed with anti-PPDK-ThrP as described in the Materials and methods. Assays were initiated with the addition of 2.5 mM KPi and *At*RP2 or *At*RP1 (pH 8.3 only) and quenched at 30 min with SDS-PAGE sample buffer. Control lanes correspond to a 15 µl aliquot of substrate phospho-PPDK used in the dephosphorylation reaction that was generated by the first-stage AtRP1 or AtRP2 catalysed ADP-dependent phosphorylation of PPDK as described in the Materials and methods. Arrowheads indicate the position of the bands corresponding to the ~94 kDa *Arabidopsis* PPDK monomer as estimated by molecular mass standards on the same blot.

cytosolic isoform shares similar substrate specificities with maize C_4 PDRP: ADP as the high specificity substrate and GDP as a lower affinity substrate. Furthermore, in this spectrophotometric-based PPDK-phosphorylation assay, no PDRP phosphorylating activity could be found when UDP, an abundant nucleotide in the cytosol of sucrose synthesizing tissues (Dennis and Blakeley, 2000), was used as a substrate for this reaction. A more sensitive immuno-based assay was used to compare nucleotide specificity at pH 7.0 for *At*RP2, maize C_4 PDRP and *At*RP1, the C_4-like chloroplast isoform of *Arabidopsis* (Chastain *et al.*, 2008) (Fig. 7). This assay indicated a similar trend in nucleotide specificity for all three PDRPs, with ADP showing the highest specificity followed by a lesser affinity for GDP. A faint signal for phosphorylated PPDK is evident for the GTP substrate lane but this is probably due to traces of GDP known to contaminate commercial preparations of GTP. Interestingly, a low amount of phosphorylated PPDK was detected when UTP and UDP were used as substrates for *At*RP2, but not for *At*RP1 and maize PDRP. However, at this low level of PPDK phosphorylation, it is unlikely that these nucleotides are true *in vivo* substrates for *At*RP2. The *At*RP2 affinity for its most specific nucleotide substrate, ADP (K_m=26 µM, pH 6.8) was also measured. The high affinity for ADP exhibited by this PDRP isoform is similar

Fig. 6. Nucleotide specificity of maize PDRP and *Arabidopsis* *At*RP2 for *in vitro* phosphorylation/inactivation of PPDK. Shown are the results of a spectrophotometric-based PDRP catalysed PPDK-inactivation assay and plotted as a percentage of PPDK inactivation (versus controls) after 15 min incubation with PDRP. Maize C_4- and *Arabidopsis* C_3-PPDK were used as substrates for the respective *At*RP2 and C_4 PDRP catalysed inactivation assays. Values shown are the mean of three independent determinations with associated SEs ≤5%.

Fig. 7. Immuno-based PPDK phosphorylation assays of PDRP with alternate nucleotide phosphoryl-donor substrates. The upper panels are representative Western blots of terminated 15 µl reaction aliquots probed with anti-PPDK-ThrP with the lower panels representing the same blots stripped and reprobed with anti-PPDK polyclonal antibodies as described in the Materials and methods. Assays were initiated with the addition of the PDRP as designated (Mz PDRP, maize C_4 PDRP; *Arabidopsis* C_4-like PDRP, *At*RP1; *Arabidopsis* cytosolic PDRP, *At*RP2) and terminated after 20 min with SDS-PAGE sample buffer. All nucleotide phosphates utilized in the assays as noted above each lane were used at a concentration of 1 mM. Control assays were the same as for plus ADP-reactions but without the addition of PDRP. Arrowheads indicate position of bands corresponding to the ~94 kDa *Arabidopsis* PPDK monomer as estimated by molecular mass standards on the same blot.

Discussion

How PPDK originated in microorganisms to catalyse specific glycolytic reactions has been reviewed here. This same function is evident in C_3 plants where PPDK is principally an ancillary enzyme of the glycolytic pathway. It was therefore hypothesized that, prior to the recent evolution of the C_4 pathway, plants possessed a PPDK that was functionally adapted for glycolytic metabolism in the cytoplasmic compartment. It was assumed that functional evolution of a glycolytic PPDK into a C_4 pathway role would translate into modifications of its catalytic and regulatory properties. However, despite the radically different metabolic roles that each respective isoform fulfils, our analyses of C_3- and C_4-PPDK isoforms revealed very few differences in these properties. The most significant difference was the 10-fold higher affinity for Pyr exhibited by the C_3 isoform. This observation is highly relevant with regard to how C_3-PPDK is proposed to carry out its cytoplasmic-based glycolytic function(s) (Kang *et al.*, 2005; Chastain *et al.*, 2006; Mechin *et al.*, 2007; Hennen-Bierwagen *et al.*, 2009, Taylor, *et al.*, 2010). Accordingly, PPDK must be able to function in reverse catalysis for generating products in both the PEP- or Pyr-forming direction. This stipulation is contradicted by the demonstrated *in vitro* pH sensitivity of the enzyme's reversible reactions (Jenkins and Hatch, 1985) and the reported high cytoplasmic concentrations of PPi (Stitt, 1990; ap Rees *et al.*, 1991; Dennis and Blakeley, 2000). Taking these factors into account, the direction of catalysis is predicted to be restricted to the Pyr-forming reaction. However, with higher affinity for Pyr, it is reasonable to assume that significant catalysis in the PEP-forming direction may take place in this compartment, particularly in light of the lack of inhibition when 1 mM PPi was included in the reaction. Furthermore, it is noteworthy that C_3 PPDK is always abundantly expressed in cells and tissues where it assumes its versatile glycolytic functions. Therefore, the lower rate of catalysis in the PEP-forming direction as dictated by the lower pH of the cytoplasmic compartment is possibly compensated for by (i) the high substrate affinity for Pyr and (ii) the brute force increase in PPDK activity facilitated by the high level expression of the cytosolic isoform in these respective tissues.

As discussed above, PPDK activity is regulated by reversible phosphorylation of an active-site Thr (Thr-456 in maize). This PDRP catalysed regulatory phosphorylation cycle has been extensively documented for the C_4 PPDK isoform (Burnell and Hatch, 1985; Roeske and Chollet, 1987; Ashton *et al.*, 1990; Smith *et al.*, 1994; Chastain *et al.*, 2000). Very little information is available concerning the regulation of C_3 cytosolic PPDK by its respective C_3 PDRP isoform, *At*RP2. In our comparisons of the PPDK-phosphorylating functions of three PDRP isoforms (C_3-cytosolic *At*RP2, C_4-like chloroplastic *At*RP1, C_4 maize

to maize PDRP measured at pH 8.0 (~50 μM) (Burnell and Hatch, 1985; Roeske and Chollet, 1987).

PDRP) no significant C_3- and C_4-related differences were evident. All three PDRP isoforms showed the same inherent nucleotide substrate specificity and similar rates of regulatory phosphorylation of PPDK. Previous work had established that the C_3-cytosolic PDRP isoform from *Arabidopsis*, *At*RP2, lacked Pi-dependent PPDK-Thr-P phosphotransferase activity (Chastain *et al.*, 2008). It was conjectured that this dephosphorylation function for *At*RP2 was not detected in the earlier study because its properties were assessed at pH 8.3 rather than at the pH of its native cytoplasmic compartment. However, this was not the case as it was shown that this activity was lacking at pH 6.8, despite a corresponding robust activity of its PPDK phosphorylating function at this same pH. Why *At*RP2 is a monofunctional regulatory protein and C_4 PDRP is bifunctional is at present unknown. Regulation of the bidirectional activities of the latter is proposed to be the consequence of light/dark-mediated changes in stromal ADP level via its action as a potent competitive inhibitor of the PDRP phospho-PPDK dephosphorylation function (Burnell and Hatch, 1985; Chastain, 2011). For a cytoplasmic localized PDRP, similar regulation by ADP is untenable since the overall adenylate pool, including ADP, is relatively invariant in this compartment (Stitt *et al.*, 1982). Alternatively, the high level of GTP hydrolysing processes in this compartment may cause significant variations in the GDP pool, depending on the cell-type and developmental state. In this regard, it is interesting that GDP can also serve as a substrate for the PPDK regulatory phosphorylation reaction. When this observation was documented earlier for maize C_4 PDRP (Burnell and Hatch, 1985), no significance was attached to it because of C_4 PDRP's functional location in the chloroplast stroma. However, for the PPDK isoform operating in the cytoplasmic compartment, an alternative regulation scheme may include GDP as a contributor to the regulatory inactivation of PPDK.

Concluding remarks

Our investigation of the *in vitro* properties of recombinantly produced *Arabidopsis* C_3- and maize C_4-PPDK revealed few function-specific differences. It is therefore concluded that the evolutionary transition of C_3 PPDK into the C_4 pathway involved only minor changes in enzyme properties per se. This may account for the preferential selection of PPDK into the C_4 pathway as opposed to other PEP-generating enzymes (e.g. PEP carboxykinase). The most significant adaptation for the enzyme to be utilized in C_4 photosynthesis had already occurred well before the emergence of the pathway in modern angiosperms. The moment PPDK became a resident enzyme of the chloroplast stroma, its inherent pH sensitivity would have allowed the enzyme to respond to light induced alkalinization of this compartment in a manner highly favourable for PEP-synthesis. Further facilitating the immediate functional C_4-transition of PPDK is the high amount of stromal pyrophosphatase and adenylate kinase found in chloroplasts of all plants.

Finally, light responsive regulation of chloroplast PPDK by PDRP was also long established in C_3 plant chloroplasts (Chastain *et al.*, 2002). The remaining enigma for understanding the C_4 transition of PPDK is defining its role in C_3 chloroplasts, where it is in very low abundance. Whatever this function, the co-localization of PPDK along with its companion regulatory protein into chloroplasts had been established by the time the unicellular green algal ancestors of plants emerged (Burnell and Chastain, 2006).

Acknowledgements

References

Agarie S, Kai M, Takatsuji H, Ueno O. 1997. Expression of C_3 and C_4 photosynthetic characteristics in the amphibious plant *Eleocharis vivipara*: structure and analysis of the expression of isogenes for pyruvate, orthophosphate dikinase. *Plant Molecular Biology* **34,** 363–369.

ap Rees T, Entwistle TG, Dancer JE. 1991. Interconversion of C-6 and C-3 sugar phosphates in non-photosynthetic cells of plants. In: Emes MJ, ed. *Compartmentation of plant metabolism in non-photosynthetic tissues*. Cambridge, UK: Cambridge University Press, 95–110.

Ashton AR, Burnell JN, Furbank RT, Jenkins CLD, Hatch MD. 1990. Enzymes of C_4 photosynthesis. In: Lea PJ, ed. *Methods in plant biochemistry*, Vol. 3. San Diego: Academic Press, 39–72.

Benziman M, Eizen N. 1971. Pyruvate-phosphate dikinase and the control of gluconeogenesis in *Acetobacter xylinum*. *Journal of Biological Chemistry* **246,** 57–61.

Bringaud F, Baltz D, Baltz T. 1998. Functional and molecular characterization of a glycosomal PPi dependent enzyme in trypanosomatids: pyruvate, phosphate dikinase. *Proceedings of the National Academy of Sciences, USA* **95,** 7963–7968.

Burnell JN. 2010. Cloning and characterization of *Escherichia coli* DUF 299: a bifunctional ADP-dependent kinase- P_i-dependent pyrophosphorylase from bacteria. *BMC Biochemistry* **11,** 1.

Burnell JN, Chastain CJ. 2006. Cloning and expression of maize-leaf pyruvate, Pi dikinase regulatory protein gene. *Biochemistry and Biophysics Research Communications* **345,** 675–680.

Burnell JN, Hatch MD. 1985. Regulation of C_4 photosynthesis: purification and properties of the protein catalyzing ADP-mediated inactivation and Pi-mediated activation of pyruvate, Pi dikinase. *Archives of Biochemistry and Biophysics* **237,** 490–503.

Chastain CJ. 2011. Structure, function, and post-translational regulation of C_4 pyruvate orthophosphate dikinase. In: Raghavendra AS, Sage RF, eds. *Advances in photosynthesis and respiration. C_4 photosynthesis and related CO_2 concentrating mechanisms*, Vol. 32. Dordrecht, The Netherlands: Springer, 301–315.

Chastain CJ, Botschner M, Harrington GS, Thompson BJ, Mills SE, Sarath G, Chollet R. 2000. Further analysis of maize C_4-pyruvate, orthophosphate dikinase phosphorylation by its bifunctional regulatory protein using selective substitutions of the regulatory Thr-456 and catalytic His-458 residues. *Archives of Biochemistry and Biophysics* **375,** 165–170.

Chastain CJ, Chollet R. 2003. Regulation of pyruvate, orthophosphate dikinase by ADP/Pi-dependent reversible phosphorylation in C_3 and C_4 plants. *Plant Physiology and Biochemistry* **41,** 523–532.

Chastain CJ, Fries JP, Vogel J, et al. 2002. Pyruvate, orthophosphate dikinase in leaves and chloroplasts of C_3 plants undergoes light/dark-Induced reversible phosphorylation. *Plant Physiology* **128,** 1368–1378.

Chastain CJ, Heck JW, Colquhoun TA, Voge DG, Gu XY. 2006. Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. *Planta* **224,** 924–934.

Chastain CJ, Thompson BJ, Chollet R. 1996. Maize recombinant C_4-pyruvate, orthophosphate dikinase: expression in *Escherichia coli*, partial purification, and characterization of the phosphorylatable protein. *Photosynthesis Research* **49,** 83–89.

Chastain CJ, Xu W, Parsley K, Sarath G, Hibberd JM, Chollet R. 2008. The pyruvate, orthophosphate dikinase regulatory proteins of *Arabidopsis* possess a novel, unprecedented Ser/Thr protein kinase primary structure. *The Plant Journal* **53,** 854–863.

Dennis DT, Blakeley SD. 2000. Carbohydrate metabolism. In: Buchanan BB, Gruissem W, Jones RL, eds. *Biochemistry and molecular biology of plants*. Rockville: American Society of Plant Physiologists, 630–675.

Edwards GE, Nakamoto H, Burnell JN, Hatch MD. 1985. Pyruvate Pi dikinase and NADP-malate dehydrogenase in C_4 photosynthesis. Properties and mechanism of light/dark regulation. *Annual Reviews in Plant Physiology* **36,** 255–286.

Eisaki N, Tatsumi H, Murakami S, Horiuchi T. 1999. Pyruvate phosphate dikinase from a thermophilic actinomyces *Microbispora rosea* subsp. *aerata*: purification, characterization and molecular cloning of the gene. *Biochimica et Biophysica Acta* **1431,** 363–373.

Feng X-M, Cao L-J, Adam RD, Zhang X-C, Lu S- Q. 2008. The catalyzing role of PPDK in *Giardia lamblia*. *Biochemical and Biophysical Research Communications* **367,** 394–398.

Fisslthaler B, Meyer G, Bohnert HJ, Schmitt JM. 1995. Age-dependent induction of pyruvate orthophosphate dikinase in *Mesembryanthemum crystallinum* L. *Planta* **196,** 492–500.

Hatch MD, Slack CR. 1968. A new enzyme for the interconversion of pyruvate and phosphopyruvate and its role in the C_4 dicarboxylic acid pathway of photosynthesis. *Biochemical Journal* **106,** 141–146.

Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers AM. 2009. Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts. *Plant Physiology* **149,** 1541–1559.

Herzberg O, Chen CCH, Kapadia G, McGuire M, Carroll LJ, Noh SJ, Dunaway-Mariano D. 1996. Swiveling-domain mechanism for enzymatic phosphotransfer between remote reaction sites. *Proceedings of the National Academy of Sciences, USA* **93,** 2652–2657.

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P. 2008. Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics* 420747.

Huang S, Colmer TD, Millar AH. 2008. Does anoxia tolerance involve altering the energy currency towards PPi? *Trends in Plant Science* **13,** 221–227.

Jenkins CLD, Hatch MD. 1985. Properties and reaction mechanism of C$_4$ leaf pyruvate, Pi dikinase. *Archives of Biochemistry and Biophysics* **239,** 53–62.

Kang HG, Park S, Matsuoka M, An G. 2005. White-core endosperm floury endosperm-4 in rice is generated by knockout mutations in the C-type pyruvate orthophosphate dikinase gene (*OsPPDKB*). *The Plant Journal* **42,** 901–911.

Liapounova NA, Hampl V, Gordon PMK, Sensen CW, Gedamu L, Dacks JB. 2006. Reconstructing the mosaic glycolytic pathway of the anaerobic eukaryote monocercomonoides. *Eukaryotic Cell* **12,** 2138–2146.

Lim K, Read RJ, Chen CC, Tempczyk A, Wei M, Ye D, Wu C, Dunaway-Mariano D, Herzberg O. 2007. Swiveling domain mechanism in pyruvate phosphate dikinase. *Biochemistry* **46,** 14845–14853.

Méchin V, Thévenot C, Le Guilloux M, Prioul J-L, Damerval C. 2007. Developmental analysis of maize endosperm proteome suggests a pivotal role for pyruvate orthophosphate dikinase. *Plant Physiology* **143,** 1203–1219.

Moons A, Valcke R, Van Montagu M. 1998. Low-oxygen stress and water deficit induce cytosolic pyruvate orthophosphate dikinase (PPDK) expression in roots of rice, a C$_3$ plant. *The Plant Journal* **15,** 89–98.

Østeras M, Driscoll BT, Finan TM. 1997. Increased pyruvate orthophosphate dikinase activity results in an alternative gluconeogenic pathway in *Rhizobium (Sinorhizobium) meliloti*, *Microbiology* **143,** 1639–1648.

Parsley K, Hibberd JM. 2006. The *Arabidopsis* PPDK gene is transcribed from two promoters to produce differentially expressed transcripts responsible for cytosolic and plastidic proteins. *Plant Molecular Biology* **62,** 339–349.

Pocalyko DJ, Carroll LJ, Martin BM, Babbitt PC, Dunaway-Mariano D. 1990. Analysis of sequence homologies in plant and bacterial pyruvate phosphate dikinase enzyme I of the bacterial phosphoenolpyruvate: sugar phosphotransferase system and other PEP-utilizing enzymes. Identification of potential catalytic and regulatory motifs. *Biochemistry* **29,** 10757–10765.

Prioul J-L, Méchin V, Damerval C. 2008. Molecular and biochemical mechanisms in maize endosperm development: the role of pyruvate-Pi-dikinase and Opaque-2 in the control of C/N ratio. *Comptes Rendus Biologies* **331,** 772–779.

Reeves RE. 1968. A new enzyme with the glycolytic function of pyruvate kinase. *Journal of Biological Chemistry* **243,** 3203–3204.

Reeves RE, Menzies RE, Hu D. 1968. The pyruvate phosphate dikinase reaction. *Journal of Biological Chemistry* **243,** 5486–5491.

Roeske CA, Chollet R. 1987. Chemical modification of the bifunctional regulatory protein of maize leaf pyruvate, orthophosphate dikinase: evidence for two distinct active sites. *Journal of Biological Chemistry* **262,** 12575–12582.

Roeske CA, Chollet R. 1989. Role of metabolites in the reversible light activation of pyruvate, orthophosphate dikinase in *Zea mays* mesophyll cells *in vivo*. *Plant Physiology* **90,** 330–337.

Sheen J. 1991. Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. *The Plant Cell* **3,** 225–245.

Slamovits CH, Keeling PJ. 2006. Pyruvate-phosphate dikinase of oxymonads and parabasalia and the evolution of pyrophosphate-dependent glycolysis in anaerobic eukaryotes. *Eukaryotic Cell* **5,** 148–154.

Smith CM, Duff SMG, Chollet R. 1994. Partial purification and characterization of maize-leaf pyruvate, orthophosphate dikinase regulatory protein: a low-abundance, mesophyll-chloroplast stromal protein. *Archives of Biochemistry and Biophysics* **308,** 200–206.

Stitt M. 1990. Fructose-2,6-bisphosphate as a regulatory molecule in plants. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **41,** 153–185.

Stitt M, Lilley RM, Heldt HW. 1982. Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. *Plant Physiology* **70,** 971–977.

Taylor L, Nunes-Nesi A, Parsley K, Leiss A, Leach G, Coates S, Wingler A, Fernie AR, Hibberd JM. 2010. Cytosolic pyruvate, orthophosphate dikinase functions in nitrogen remobilization during leaf senescence and limits individual seed growth and nitrogen content. *The Plant Journal* **62,** 641–652.

Tjaden B, Plagens A, Dörr C, Siebers B, Hensel R. 2006. Phosphoenolpyruvate synthetase and pyruvate, phosphate dikinase of *Thermoproteus tenax*: key pieces in the puzzle of archaeal carbohydrate metabolism. *Molecular Microbiology* **60,** 287–298.

Varela-Gomez M, Moreno-Sanchez R, Pardo JP, Perez-Montfort R. 2004. Kinetic mechanism and metabolic role of pyruvate phosphate dikinase from Entamoeba histolytica. *Journal of Biological Chemistry* **279,** 54124–54130.