



Kinetic properties of recombinant phosphomimic mutant of *Zea mays* phosphoenolpyruvate carboxylase (*ZmPEPC*^{S15D})

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Abstract Phosphoenolpyruvate carboxylase (PEPC) is the key enzyme for fixing atmospheric carbon dioxide in C₄ and CAM plants, and in anaplerotic metabolism in most plants. It is regulated by reversible phosphorylation, and allosteric activation or inhibition. The regulatory role of phosphorylation at N-terminal serine residue (S15) is documented earlier. In this study PEPC was cloned from maize and engineered to generate phosphomimic mutant enzyme by conversion of the codon for Serine 15 to aspartate (PEPC^{S15D}). In vitro study with recombinant wild-type PEPC and phosphomimic PEPC^{S15D} showed that both the enzymes are active catalytically. Kinetics analysis showed that PEPC^{S15D} enzyme has comparatively lower K_m value over WT PEPC, thus implying higher affinity towards its substrate PEP than the wild type PEPC. However, both WT PEPC and mutant PEPC^{S15D} showed similar regulation with the allosteric activator (Glycine) and allosteric inhibitors (L-Aspartate or L-Malate). Thus, the engineered phosphomimic mutant PEPC^{S15D} with high affinity for PEP will be useful to engineer rice and other C₃ plants for enhancing photosynthesis and minimizing respiratory CO₂ loss.

Keywords Phosphoenolpyruvate carboxylase · S15D mutation · Allosteric activation · Substrate affinity

Introduction

Phosphoenolpyruvate carboxylase (EC 4.1.1.31, PEPC) is a ubiquitous enzyme present in diverse organisms including plants. It utilizes HCO₃⁻ to catalyze irreversible β -carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate and orthophosphate (O’Leary 1982; Izui et al. 2004). In higher plants PEPC supplies oxaloacetate to replenish the intermediates of citric acid cycle, precursor for amino acid biosynthesis (Jeanneau et al. 2002), and stomatal opening (Britto and Kronzucker 2005). PEPC also plays a crucial role in plant acclimation to nutritional Pi deficiency (O’Leary et al. 2011; Plaxton and Tran 2011). In C₄ and Crassulacean Acid Metabolism (CAM) plants, PEPC is the key photosynthetic enzyme which fixes CO₂ in cytosol of mesophyll cells to form Oxaloacetate (OAA), which is then converted malate/aspartate and exported to bundle sheath cells to supply CO₂ to the Rubisco. PEPC exists in multiple isoforms with different catalytic and regulatory properties with specific isoforms for each metabolic function.

The typical class I type PEPC is a homotetramer with subunits of ~ 110 kDa each with a conserved N terminal phosphorylation domain and C terminal tetrapeptide QNTG in plants (Izui et al. 2004). PEPC is a highly regulated enzyme. Its activity is regulated by metabolite effectors (allosteric regulation) and post-translational modification (phosphorylation-dephosphorylation). PEPC is activated by hexose phosphate (Wong and Davies 1973) in dicots, while PEPCs from monocot plants are activated by glycine and alanine (Bandarian et al. 1992; Tovar-Méndez et al. 2000). PEPC is inhibited by L-malate or Aspartate (Huber and Edwards 1975; Tovar-Méndez et al. 2000). Post translational regulation of PEPC is brought about by reversible phosphorylation of conserved serine

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residue at N-terminus by a calcium-independent Ser-Thr protein kinase known as PEPC kinase (PEPC-PK) (Vidal and Chollet 1997; Nimmo 2000; Taybi et al. 2000; Tsuchida et al. 2001) and dephosphorylation is catalysed by protein phosphatase 2A (Dong et al. 2001). C4 PEPC shows light dependent phosphorylation of N terminal serine residue. In maize, phosphorylation of Serine 15 activates PEPC and reduces the affinity of PEPC towards its inhibitors such as malate and aspartate (Echevarria et al. 1994) and increases the affinity towards its substrate (PEP) (Duff et al. 1995) or Mg-PEP (Tovar-Méndez et al. 1998). The C-terminal residue mainly G970 and N968 essentially play a well-defined role in catalysis and in allosteric regulation of the maize PEPC enzyme. R647, K835, R894, and N968 residues in PEPC enzymes are involved in the binding of allosteric inhibitor aspartate (Izui et al. 2004). The site-directed mutagenesis and crystallographic data confirmed the role of Serine 100 in activation of PEPC by glycine. This Serine 100 affects not only Glycine binding but also regulates substrate binding (PEP), and allosteric effectors binding like Glucose-6-phosphate and malate (González-Segura et al. 2018).

Several efforts are being made to engineer rice with C4 photosynthetic pathway (Ermakova et al. 2019). Overexpression of C4 PEPC in rice showed differential results, i.e., in some studies the photosynthesis was enhanced (Ku et al. 1999; Lian et al. 2014) but often no increase in photosynthesis was observed (Giuliani et al. 2019). In photosynthetic mesophyll cells of C4 plant, the key C4 enzyme PEPC is regulated by phosphorylation under light. One of the reasons for low photosynthesis in rice is due to the fact that in leaves of transgenic rice most of the maize PEPC remained in its dephosphorylated and less active form in the light (Matsuoka et al. 2001). Therefore, the non-phosphorylated form of PEPC may function at lower rates due to feedback inhibition and low affinity to its substrate PEP (Jeanneau et al. 2002). Thus, overexpression of PEPC-PK independent constitutively active PEPC (phosphomimic mutant of PEPC^{S15D}) may help enhance photosynthesis in rice.

Under normal conditions the nocturnal respiration is about 20–25% of the photosynthetic carbon fixation. Under high night temperature, nocturnal respiration increase up to 30–40%. If this CO₂ is recaptured during night, this will increase the carbon availability for grain development and yield. The night temperature increase appears to be more detrimental to yield in rice than the day temperature rise (Shah et al. 2011). An analysis of impact of night temperature on rice yield at the International Rice Research Institute Farm from 1979 to 2003 revealed that grain yield of rice declined by 10% for each 1 °C increase in growing-season minimum temperature (Peng et al. 2004). Increase in night temperature from 22 to 26 °C resulted in about

11% grain yield decrease in rice (Zhang et al. 2013). High night time temperature was found to increase night respiration, which was associated with a decrease in yield (Mohammed and Tarpley 2009). High night temperatures increase vegetative respiration rates (Loka and Oosterhuis 2010) and decrease the assimilate supply to the spikelet (Hirai et al. 2003). Increase in night respiration was attributed as one of most important factor that causes yield reduction under high night temperature (Mohammed et al. 2013). Thus, overexpression of constitutively active PEPC (phosphomimic mutant of PEPC^{S15D}) may help reduce CO₂ loss during the night and may enhance the biomass in rice.

In this report, effort was made to engineer phosphomimic mutant PEPC^{S15D}, and analyze its kinetics. Both the WT PEPC and mutant PEPC^{S15D} were overexpressed in *Escherichia coli* and the recombinant enzymes were studied in vitro with respect to their kinetics, allosteric activation (Glycine), allosteric inhibition (L-Malate or L-Aspartate). PEPC isolated from *Zea mays* was also taken as a control and both L-Malate and L-Aspartate allosteric inhibition and Glycine allosteric activation were analyzed. The in vitro study showed that PEPC^{S15D} mutant is functional, with similar response to its allosteric regulators by comparatively higher affinity towards its substrate PEP as compared with WT PEPC enzyme.

Materials and methods

Cloning and mutagenesis of PEPC

RNA was isolated from seedlings of *Zea mays* inbred line ZI72-221 (Source: Division of Genetics, IARI, New Delhi) using TRIZOL reagent (Thermo Fischer Scientific, Massachusetts, USA) and treated with RNase-free DNase. Total RNA template was used for cDNA synthesis using Superscript III kit (Invitrogen, USA). Using this cDNA as template, the full length CDS of *ZmPEPC* was amplified using KOD DNA polymerase and cloned in TA cloning vector pTZ57R/T (Thermo Fisher Scientific, Massachusetts, USA) and sequenced. Confirmed plasmid was used as a template for cloning PEPC in prokaryotic expression vector pGEX6P2 at *Sma*I and *Xho*I restriction sites using Forward primer 5'-CCCCGGATGGCGTC-GACCAAGG-3', and Reverse primer 5'-CGGCTCGAGCTAGCCAGTGTCT-3' (IDT, Iowa, USA). Polymerase chain reaction was performed with Phusion[®] High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA), with 30 cycles of 98 °C for 20 s, 55 °C for 1 min, and 72 °C for 4 min. PCR amplicon was digested with *Sma*I and *Xho*I and cloned into pGEX6P2.

Site directed mutagenesis of *ZmPEPC* at S15D position was carried out using Invitrogen GENEART site directed mutagenesis system (Thermo Fischer Scientific, Massachusetts, and USA). The primer pair used for the mutagenesis are 1) PEPC-mF 5'-CTGGCGAGAAGCACCA CgaCATCGACGCGCAGCTCCGT-3' and PEPC-mR 5'-ACGGAGCTGCGCGTCGATGtcGTGGTGCTTCTCGCC AG-3'. The mutagenesis was confirmed through DNA sequencing and the confirmed plasmid was used as a template for cloning the mutant PEPC (PEPC^{S15D}) in prokaryotic expression vector pGEX6P2 at *Sma*I and *Xho*I restriction sites. The confirmed pGEX6P2 constructs harbouring wild type *ZmPEPC* (WTPEPC) and mutant PEPC^{S15D} (mPEPC) were transformed into *E. coli* BL21 Rosetta cells.

Heterologous expression and purification of PEPC

A single colony of *E. coli* BL21 Rosetta cells harboring pGEX6P2-WTPEPC or pGEX6P2-mPEPC was inoculated in 2 ml Luria–Bertani (LB) supplemented with Ampicillin (100 µg ml⁻¹) and Chloramphenicol (50 µg ml⁻¹) and grown overnight at 37 °C, 220 rpm. From the overnight grown culture, secondary culture was inoculated by adding 1% of primary culture into LB supplemented with Ampicillin (100 µg ml⁻¹) and Chloramphenicol (50 µg ml⁻¹) and grown at 37 °C at 220 rpm. At O.D₆₀₀-0.5-0.6, cells were separately induced at two temperatures i.e. 16 °C and 37 °C with 1 mM or 2 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, G Biosciences, Noida, India) and grown overnight. After completion of induction, cells were harvested at 5000 g for 5 min at 4 °C. The pellet was resuspended in lysis buffer containing 50 mM Tris–Cl (pH 8.0), 50 mM NaCl, 10% glycerol and 4 mM β-mercaptoethanol). The resuspended cells were lysed by adding lysozyme (0.1 mg/ml) and incubated for 1 h on ice. The lysed cells were sonicated at 20% amplitude with 8 cycles of 10/60 s on/off, and clarified at 13,000 g for 30 min at 4 °C. The supernatant so obtained was loaded onto column containing glutathione beads (G Biosciences, Noida, India). Washing was done with wash buffer containing 50 mM Tris–Cl (pH8.0), 50 mM NaCl, and 10% glycerol. Elution was done with elution buffer containing 50 mM Tris–Cl (pH 8.0), 50 mM NaCl, 10% glycerol, and 20 mM reduced glutathione (G Biosciences, Noida, India). The elution fractions were run on 10% SDS-PAGE.

Enzyme assays

PEPC activity was assayed using coupled spectrophotometric method as described by Endo et al. (2008). All the chemicals used were procured from Sigma. The standard reaction mixture contained 100 mM Hepes pH 7.3, 10 mM

NaHCO₃, 10 mM MgSO₄, 2 mM PEP, 0.1 mM NADH, one IU malate dehydrogenase (Sigma) and 20 units of purified WTPEPC or mPEPC (PEPC^{S15D}). The reaction was initiated by addition of substrate PEP. The decrease in absorbance of NADH was measured at 340 nm in UV–VIS spectrophotometer (Thermo scientific, Evolution 201). One unit of PEPC was defined as 1 µM of NADH converted to NAD per min. For enzyme inhibition assays, standard reaction was carried out in presence of different concentrations of L-Malate or L-Aspartate (2 mM, 4 mM, and 6 mM). Similarly for activation assays, Glycine in the concentration range of 0.5–20 mM was added to the standard reaction mixture and the change in absorbance of NADH was measured at 340 nm. Graphs were prepared in graphpad prism.

Enzyme kinetics

The standard reaction for enzyme kinetics was performed in a final volume of one ml reaction consisting of 100 mM Hepes pH 7.3, 10 mM NaHCO₃, 10 mM MgSO₄, 0.1 mM NADH, one IU malate dehydrogenase (Sigma), varying concentration of PEP from 0.1 to 6.0 mM (0.1, 0.3, 0.5, 1.0, 2.0, 4.0 and 6.0 mM) and 20 units of purified WTPEPC or mPEPC (PEPC^{S15D}). Mean of three replicates at each substrate concentration was plotted on graph and a total of 21 points were analyzed. Graphs were prepared in graphpad prism. Statistical analysis was performed by two-way ANOVA.

Statistical analysis

All data presented are mean of 3 replicates. The SE values are given as error bars. The *t* test was used to compare the means of WTPEPC and PEPC^{S15D} activities.

Results and discussion

Cloning, mutagenesis and heterologous expression

Heterologous overexpression of GST-WTPEPC and GST-mPEPC fusion proteins was induced by IPTG at 37 °C and 16 °C (Fig. 1a, b). Overexpression of GST-mPEPC was more at 16 °C than at 37 °C (Fig. 1b). Most of overexpressed protein was obtained in active soluble fraction. The GST-WTPEPC or GST-mPEPC was purified using glutathione beads (Fig. 2). Similarly, glutathione S-transferase was also purified which served as negative control in enzyme assays (Fig. 2). The purified GST-WTPEPC or GST-mPEPC showed bands corresponding to 133 kDa and purified GST appeared as band of 26 kDa on SDS-PAGE

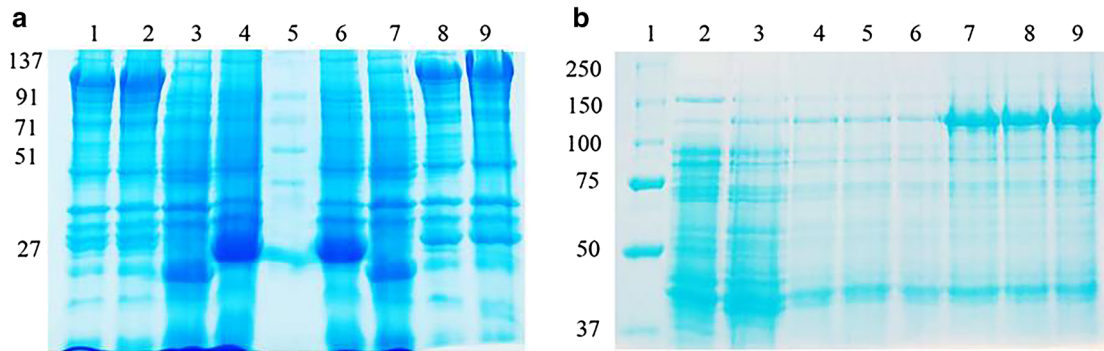


Fig. 1 Heterologous expression of PEPC in *E. coli*. **a** Heterologous expression of GST-WTPEPC. Lanes 1 and 2, Overexpression at 37 °C with 1 mM, and 2 mM IPTG; lane 3, uninduced GST-WTPEPC; lane 4, empty vector control; lane 5, protein molecular weight Marker; lane 6, empty vector control; lane 7, uninduced GST-WTPEPC; lane 8, overexpression of GST-WTPEPC at 16 °C with

1 mM IPTG; lane 9, overexpression of GST-WTPEPC at 16 °C with 2 mM IPTG. **b** Heterologous expression of GST-mPEPC. Lane 1, protein molecular weight Marker; lane 2, empty vector control; lane 3, uninduced GST-mPEPC; lanes 4, 5 and 6—GST-mPEPC at 37 °C with 1, 2, and 3 mM IPTG, respectively. Lanes 7, 8, 9—GST-mPEPC at 16 °C with 1, 2, and 3 mM IPTG, respectively

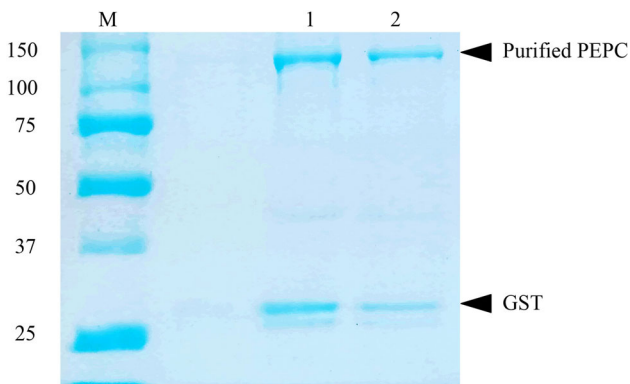


Fig. 2 Affinity purification of WTPEPC and mPEPC (PEPC^{S15D}). The wild type (WT) PEPC (lane 1) and mutant PEPC (PEPC^{S15D}) (Lane 2) were overexpressed in *E. coli* with GST fusion tags. The proteins were purified using glutathione beads and reduced glutathione

(Fig. 2). Thus, the recombinant protein molecular weight is about the expected size along with the GST tag.

In vitro enzyme kinetics of WTPEPC and mPEPC

Since the recombinant proteins were expressed with a 26 kDa GST-tag, to access whether or not the tag has affected the enzymatic activity of the protein, enzymatic assays of the purified WTPEPC and mPEPC was performed. Based on malate dehydrogenase coupled reaction, the activity of in vitro WT PEPC and mPEPC were found to be 22.83 $\mu\text{mol min}^{-1}$ and 19.45 $\mu\text{mol min}^{-1}$ respectively (Fig. 3). Thus both the proteins were found to be functional and neither GST nor S15D mutation affected the enzymatic activity of these proteins.

Saturation kinetics of in vitro WTPEPC and mPEPC was studied as a function of concentration of PEP. The reaction was performed in presence of saturating

concentrations of Mg^{2+} (10 mM) and bicarbonate (10 mM) and the reaction were carried out at pH 7.3 to mimic the cytosolic pH of mesophyll cells. The rate of reaction for WTPEPC or mPEPC was found to increase with increase in concentration of PEP from 0.1 to 2 mM, beyond which saturation was achieved (Fig. 4). The enzymes WTPEPC or mPEPC followed Michaelis–Menten kinetics. The V_{max} for WTPEPC was 22.43 μM with K_{m} value 1.068 mM. The V_{max} value for mPEPC was calculated to be 23.42 μM with K_{m} value of 0.6264 mM (Fig. 4, Table 1). Thus both the enzymes exhibited similar V_{max} values, but significant differences in the K_{m} values. The K_{m} value of mPEPC was lower than that of WTPEPC. This is in consistent with earlier studies where illumination/phosphorylation of PEPC has been shown to significantly increase the affinity to PEP in native maize PEPC (Doncaster and Leegood 1987; Tovar-Méndez et al. 1998) and recombinant sorghum PEPC (Duff et al. 1995). Thus, the engineering of S15D mutation in maize PEPC (PEPC^{S15D}) imparts constitutive negative charge mimic phosphorylation state and enhanced affinity to PEP.

Effect of allosteric inhibitors and activators on WTPEPC and mPEPC activity

Malate and aspartate are allosteric inhibitors of PEPC (Chollet et al. 1996). Enzymatic activity of WTPEPC or mPEPC was analyzed either in presence or absence of malate or aspartate. Malate inhibited WTPEPC in a concentration dependent manner with maximum inhibition attained at 6 mM (Fig. 5a). Fifty percent inhibition of WTPEPC activity was achieved at 1.5 mM of malate ($\text{IC}_{50} = 1.5 \text{ mM}$) (Fig. 5a). Malate inhibited mPEPC with IC_{50} value of 1.25 mM (Fig. 5a). A similar IC_{50} of

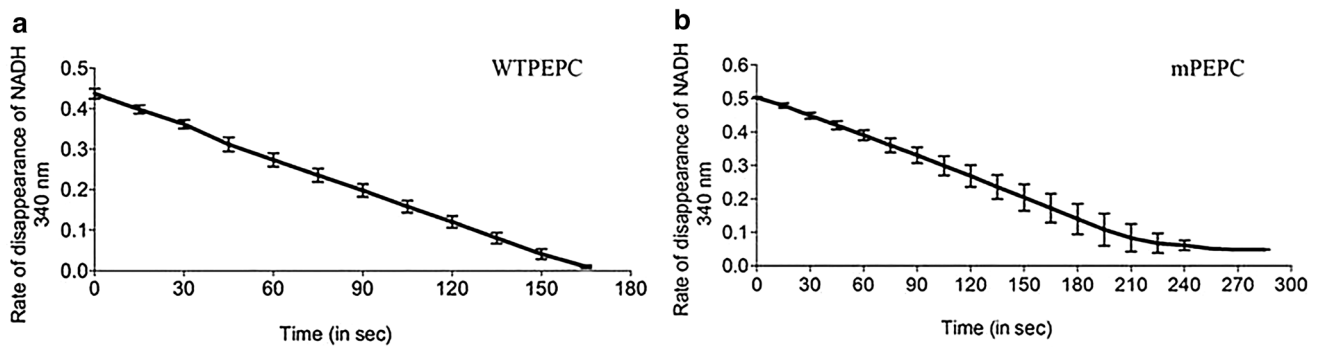


Fig. 3 Enzymatic activity of WTPEPC (a), and mPEPC (b) in *E. coli*. Reaction was carried out at pH 7.3 in presence of 2 mM PEP and saturating concentrations of bicarbonate and Mg^{2+} (10 mM).

Rate of disappearance of NADH was measured at 340 nm. All reactions were carried out in triplicate. Error bars indicate \pm SE (n = 3)

Fig. 4 Kinetics of saturation of WTPEPC (a), and mPEPC (b) as a function of PEP. Concentrations of PEP used in the assays (0.1 to 6 mM). The curve represent the best fit of experimental data in Michaelis-Menton equation. All reactions were carried out in triplicate. Error bars indicate \pm SE (n = 3)

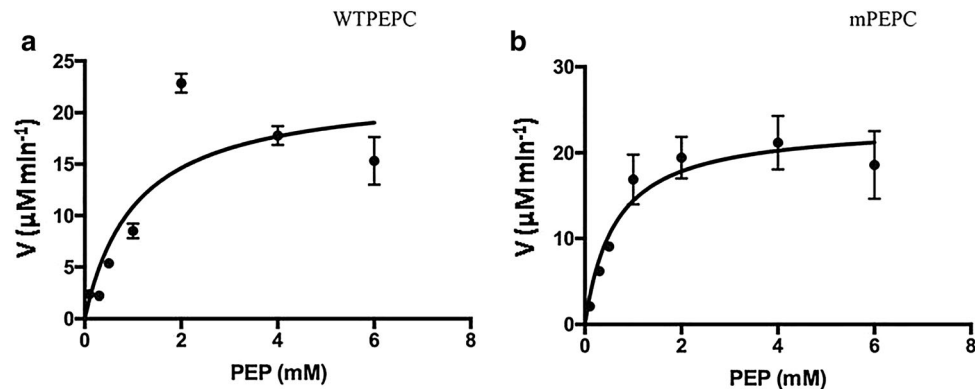


Table 1 V_{max} and K_m values of the WTPEPC and mPEPC overexpressed in *E. coli*

Enzyme	V_{max} (μ M)	K_m (μ M)	IC_{50} (mM)	
			L-Malate	L-Aspartate
WTPEPC	22.43 \pm 0.53 ^a	1.068 \pm 0.008 ^a	1.50	1.10
PEPC ^{S15D}	23.42 \pm 1.41 ^a	0.626 \pm 0.017 ^b	1.25	1.20

Same letter in the superscript indicates no difference between the means, while different letters indicate significant difference between the means ($p < 0.01$)

1.60 mM was earlier reported for WTPEPC (Endo et al. 2008).

The IC_{50} value for aspartate inhibition for WTPEPC and mPEPC was 1.1 mM and 1.2 mM, respectively (Fig. 5b). Though mPEPC shows higher affinity for the substrate but it exhibits the similar sensitivity to malate or aspartate compared to WTPEPC. Malate and aspartate inhibition assays carried out with PEPC extracted from maize leaves also showed similar sensitivity to malate or aspartate observed for recombinant PEPCs (Fig. 6a, b).

Glycine is an activator of PEPC (Chollet et al. 1996). Glycine allosterically activates WTPEPC by binding to serine at 100th position which is away from C-terminal catalytically active site. Glycine activated WTPEPC and mPEPC in a concentration dependent manner. In both the cases saturation of WTPEPC and mPEPC activity by glycine was attained at 5 mM glycine (Fig. 7). Thus S15D mutation did not affect the glycine-mediated activation of mPEPC. Glycine activation assay with PEPC extracted from maize leaves showed similar activation pattern (Fig. 8) as compared with recombinant PEPCs.

Overexpression of three genes viz, ZmPEPC, aspartate aminotransferase (GmAspAT), and glutamine synthetase (NtGS) in *Arabidopsis* enhanced biomass through reduction in photorespiratory loss of C and N (Kaachra et al. 2018). Engineering rice with C4 photosynthetic pathway is often resulted in no increase in photosynthesis (Giuliani et al. 2019), due to the fact that in leaves of transgenic rice most of the maize PEPC remained in its dephosphorylated and less active form in the light (Matsuoka et al. 2001). However, Thus, the engineered PEPC^{S15D} may be useful to reduce respiratory and photorespiratory losses in rice and other C3 crops.

Fig. 5 Allosteric inhibition of WTPEPC and mPEPC by L-Malate (a), L-Aspartate (b). Concentrations of L-Malate or L-Aspartate used in the assays (2 to 8 mM). All reactions were carried out in triplicate

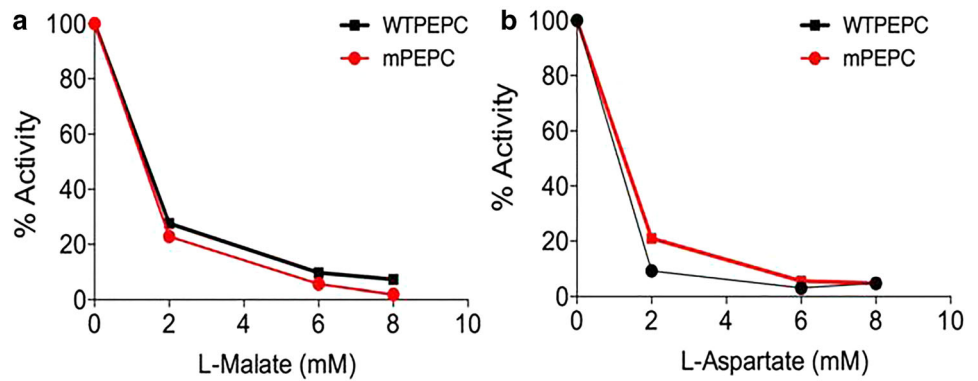


Fig. 6 Allosteric inhibition of ZmPEPC by L-Malate (a), L-Aspartate (b). Concentrations of L-Malate or L-Aspartate used in the assays (2 to 8 mM). All reactions were carried out in triplicate

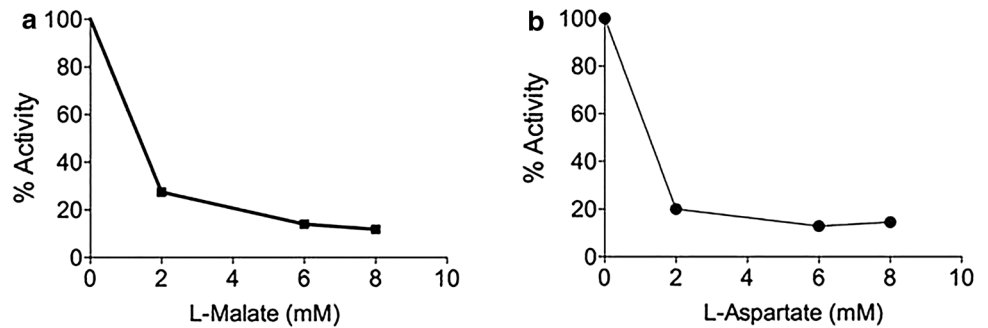


Fig. 7 Activation of WTPEPC (a), or mPEPC (b). Concentrations of glycine used in the assays (0.1 to 20 mM). All reactions were carried out in triplicate. Error bars indicate \pm SE ($n = 3$)

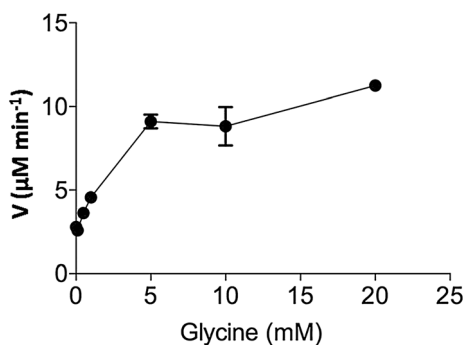
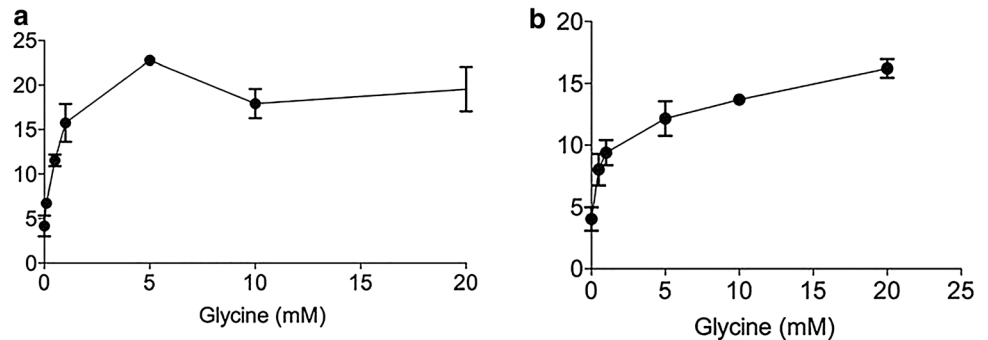


Fig. 8 Activation of ZmPEPC by Glycine. Concentrations of glycine used in the assays (0.1 to 20 mM). All reactions were carried out in triplicate. Error bars indicate \pm SE ($n = 3$)

Conclusion

Under hot, arid and light intensive conditions the C4 plants have significantly higher photosynthetic productivity than that of C3 plants. In this study, a C4 form of PEPC was cloned from maize and engineered to produce phosphomimic mutant PEPC^{S15D}. In vitro kinetic assays with recombinant WT PEPC and phosphomimic mutant PEPC^{S15D} proteins showed that both the proteins are functional and comparable glycine, malate and aspartate sensitivity. Interestingly PEPC^{S15D} enzyme showed significantly higher affinity to its substrate PEP. Since over-expression of WT PEPC from maize plant in rice transgenic often did not enhance photosynthesis due to the fact that the maize PEPC remained in its dephosphorylated and less active form in the light in rice (Matsuoka et al.

2001), the cloned PEPC^{S15D} with kinase/light independent action will be useful for engineering rice plants with enhanced photosynthesis and/or reduced respiratory carbon loss. Since the engineered phosphomimic PEPC^{S15D} has very low K_m , i.e., ~ 50% less concentration of PEP requirement as compared with WTPEPC, PEPC^{S15D} can be employed for CO₂ fixation in tissues where PEP concentration is low.

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