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# Phosphoenolpyruvate carboxylase (PEPC) from a thermophilic cyanobacterium, *Synechococcus vulcanus*: Unusual allosteric properties and its gene expression in *Arabidopsis thaliana*

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## 1. Introduction

The activities of most PEPCs investigated up to now are affected by wide varieties of metabolites depending on their sources. For instance, the activity of PEPCs from most bacteria are strongly dependent on acetyl-CoA and inhibited by Asp or malate (Izui, 1970; Nakamura et al., 1996) while PEPCs purified from green algae are weakly activated by Gln and inhibited by Glu (Rivoal et al., 1998). The PEPCs from higher plants are activated by glucose 6-phosphate (Glc-6-*P*) and often by Gly, and inhibited by L-malate in an allosteric manner (Chollet, 1996). Though it has been reported that PEPC purified from a cyanobacterium, *Coccochloris peniocystis*, resembles the enzyme isolated from C3 plant and functions anaplerotically in the cyanobacterium (Owttrim and Colman, 1986), the role of PEPC in cyanobacterial carbon metabolism and its regulation is still poorly understood. We have been intending to obtain a gene for thermo-stable PEPC from a thermophilic cyanobacterium, *Synechococcus vulcanus*, which can be utilized for engineering of organic acid metabolism in higher plant. Previously, the gene was cloned from this organism and and expressed in *E.coli* (Chen et al., submitted). Here the molecular properties of recombinant PEPC were analyzed and the gene was introduced into *Arabidopsis thaliana* and expressed under the control of CaMV 35S promoter.

## 2. Procedures

2.1 *Prokaryotic expression and purification of recombinant* S. vulcanus *PEPC*
The coding region of *S. vulcanus PEPC* was expressed in *E. coli* BL21 Codonplus (DE3). The purified recombinant fusion PEPC protein from soluble fraction was used for characterization of enzymatic properties.
2.2 *Assay of PEPC activity*
PEPC activity was assayed by a coupled spectrophotometric method (Izui et al., 1981).
2.3 *Expression of* S. vulcanus *PEPC in* Arabidopsis thaliana
The coding region of *S. vulcanus PEPC* was introduced into *Arabidopsis thaliana* (Columbia*)* by *Agrobacterium tumefaciens-mediated* in planta transformatiom under the

control of the cauliflower mosaic virus 35S promoter. Transgenic plants were selected on kanamycin-containing agar plate supplied with 1.0 % sucrose.

## 3. Results and Discussion

### 3.1 *Asp and malate inhibition of SvPEPC*

As seen in Fig. 1A and B, malate inhibition on *Sv*PEPC was almost negligible at pH 7.5 while it was significant at the optimum pH of 9.0. Similarly, the sensitivity to Asp, which is a stronger inhibitor than malate, is higher at pH 9.0 than pH 7.5. The half inhibition concentrations (*K*i) for Asp at pH 9.0 and 7.5 were 0.025mM and 2 mM, respectively. This tendency of pH dependence on inhibitor sensitivity is just oppsite to those observed with PEPCs from *C. peniocystis* (Owttrim and Colman, 1986) and *Zea mays*(Chollet, 1996). Thus at higher pH, *Sv*PEPC was more sensitive to Asp inhibition than lower pH. When Asp concentration was increased to 0.5 mM, the inhibition became constant and showed a saturation tendency, suggesting that Asp inhibition was accomplished in a non-competitive manner.

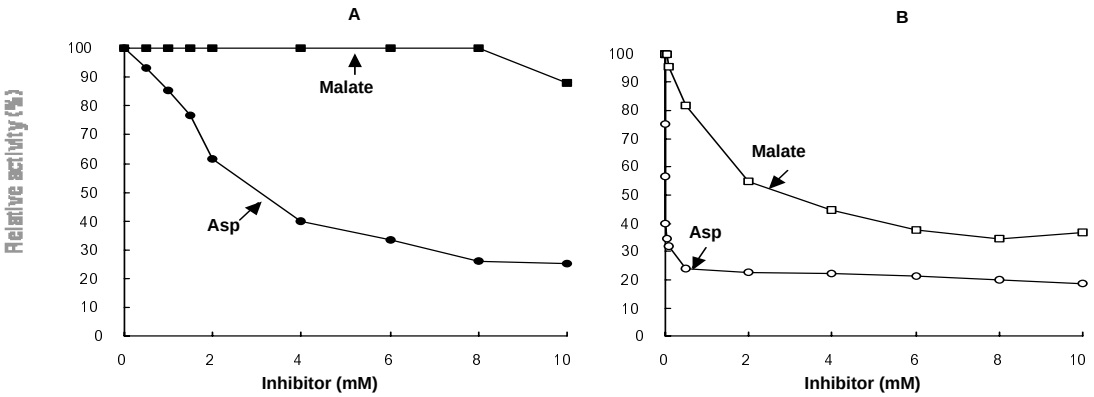


**Figure 1.** Asp and malate inhibition curves of SvPEPC at pH 7.5 (A) and pH 9.0 (B). Assay was carried out in a standard assay mixture using half-saturating concentration of PEP (0.6 mM at both pH) at30 $^{o}$C. The activities in the absence of inhibitor was taken as 100%.

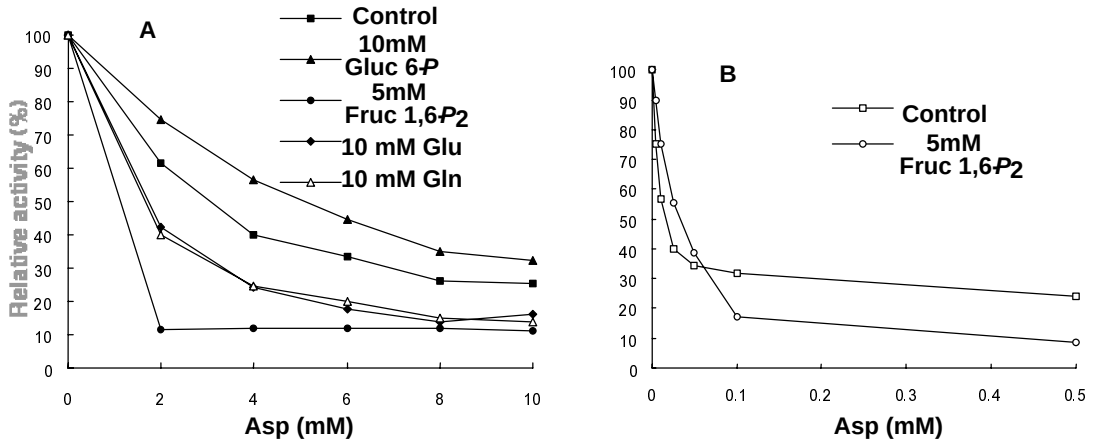


**Figure 2.** Asp inhibition curves of SvPEPC in the presence of activators at pH 7.5 (A) and pH 9.0 (B). Assay was carried out in a standard assay mixture using half-saturating concentration of PEP (0.6 mM) at 30 $^{o}$C. The activities in the absence of effectors were taken as 100% and the inhibition by Asp in the absence of activators was set as control.

3.2 *Effects of Fruc-1,6-$P_2$, Gluc-6-P Glu and Gln on Asp inhibition of SvPEPC*

Since Fruc-1,6-$P_2$, Gluc-6-*P* Glu and Gln are all allosteric activators of SvPEPC in the decreasing order of strength (Chen et al., submitted), their effects on Asp inhibition was investigated. At pH 7.5, only Gluc-6-*P* alleviated the inhibiton by Asp, while Fruc-1,6-$P_2$ or Glu or Gln, on the contrary, enhanced Asp inhibition (Fig. 2A). At pH 9.0, Asp inhibition was also strengthened by the presence of Fruc-1,6-$P_2$ (Fig. 2B). It appears that action mode of Fruc-1,6-$P_2$ Glu or Gln on Asp inhibition was different from that of Gluc-6-*P*, but similar to the effect of pH increase. Upon binding with Fruc-1,6-$P_2$, Glu or Gln, the conformation of SvPEPC seems to change to a state that is much more sensitive to the inhibition by Asp than that in their absence. Such regulatory property has not been reported previously and not readily interpretable according to the theoretical models of allosteric control available at present. Thus, this is the most unique character of *Sv*PEPC and may represent a novel regulatory mechanism.

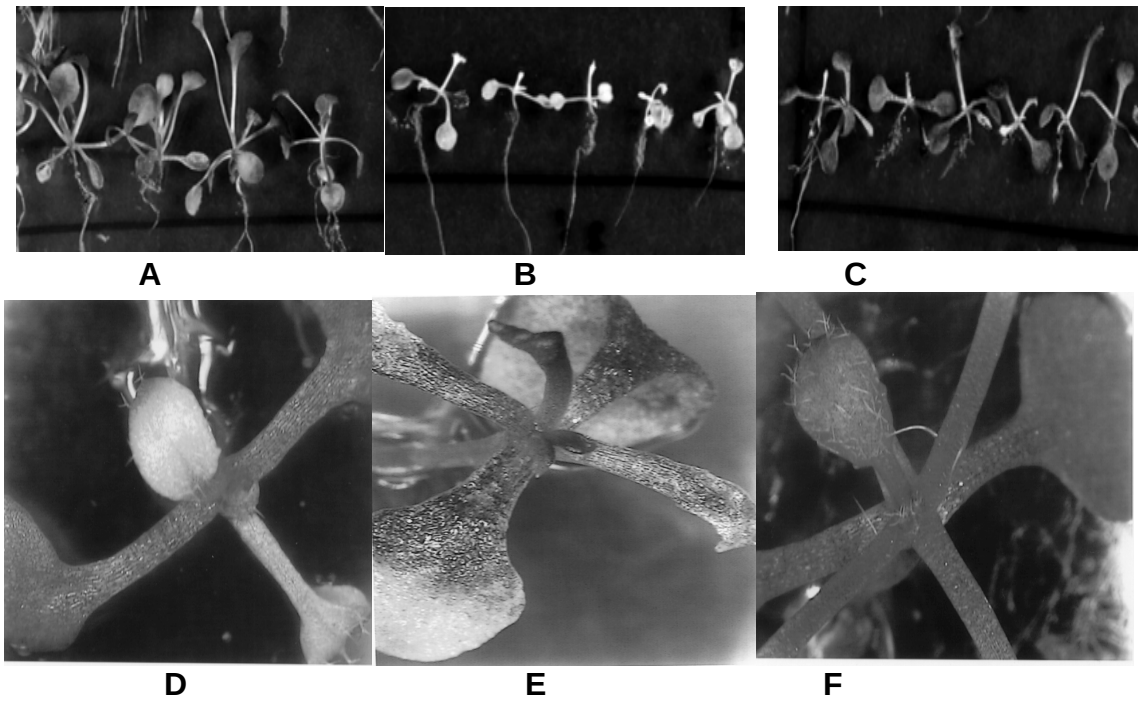


**Figure 3.** 3-week transgenic plants grown on agar in the presence of 1% sucrose.

3.3 *The effect of* SvPEPC *expression in* Arabidopsis thaliana *on plant growth*

Phenotypic characterization of transformants was undertaken with plants from T2 generation. Visible phenotype change was observed in SvPEPC transgenic plants grown on agar plate in the presence of 1% sucrose under constant light at 22 $^{o}$C (Fig. 3 and Table 1). SvPEPC transformants displayed growth hindrance or growth inhibition and leaf or cotyledon bleaching (A, B, D and F in Fig. 3) under the condition employed while nothing happened in control plants (GUS gene transformants, C and F in Fig. 3) and wild type plants (data not shown). Such phenotype change was restored by feeding of aromatic amino acids (B and C in Fig. 4). The evidence from feeding experiment suggests that over- expression of SvPEPC in plants may cause PEP depletion and then induced such pleiotropic effects on the development of *Arabidopsis thaliana*. This result

**Table 1 The characteristics comparision of control and SvPEPC transformant plants grown on agar plate containing 1% sucrose for 3 weeks**

| Characters | control | SvPEPC transformant |
|---|---|---|
| Leaf number of (per seedling) | 6-7 | 0-4 |
| Fresh weight (g. plant-1) | 0.01-0.02 | 0.004-0.008 |
| Leaf colour | greeen | white or green |
| Leaf shape | normal | abnormal or normal |
| Leaf trichome | normal | decrease or disappeare on abnormal shape leaf |
| Lateral root number (per plant) | 15-20 | Decrease in white leaf plants (5-10) |
| Primary bolt (cm) | 0-2 | 0 |

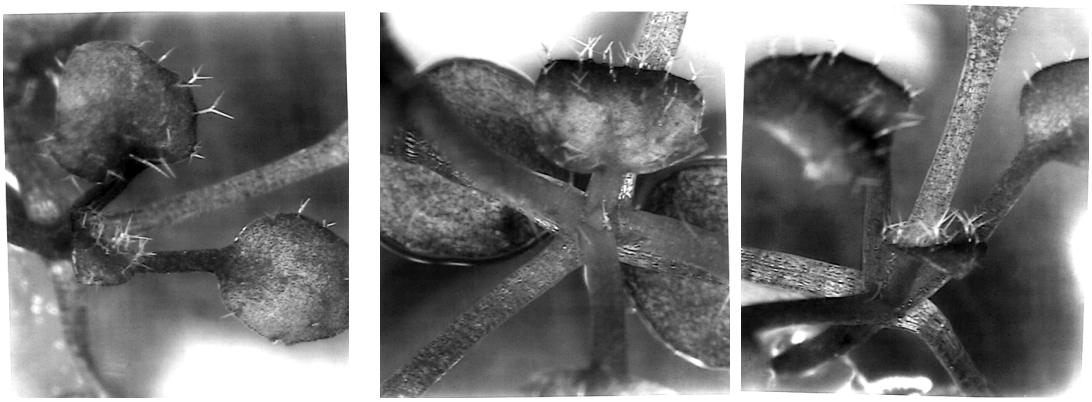


**Figure 4.** Transgenic plants grown on agar in the presence of 1% sucrose supplied with 1 mM Phe and Tyr. A:Control plants; B and C: Transformant plants.

is similar to those observed with a plastid inner membrane PEP translocator mutant (Streatfield et al., 1999) and plastid transketolase antisense tobacco transformants (Henkes et al., 2001). After the seedlings were transplanted to soil, a part of them could restore to normal growth but growth retardation was observed and apical dominance decreased in some adult plants (data not shown). Their life span was extended 2 or 3 weeks than control plants. The growth retardation was caused by vegetative growth delay and led to flowering transition time delayed 2 or 3 weeks than control plants.

## 4. References


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