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Phylogenic and phosphorylation regulation difference of phosphoenolpyruvate carboxykinase of C3 and C4 plants

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ABSTRACT

In C4 plants, phosphoenolpyruvate carboxykinase (PEPCK) plays a key role in the C4 cycle. PEPCK is also involved in gluconeogenesis and is conserved in both lower and higher organisms, including in animals and plants. A phylogenic tree constructed from PEPCK sequences from bacteria to higher plants indicates that the C4 Poaceae PEPCKs are conserved and have diverged from the PEPCKs of C3 plants. The maximum enzymatic activities of wild-type and phosphorylation mimic PEPCK proteins indicate that there is a significant difference between C3 and C4 plant PEPCKs. The conserved PEPCK phosphorylation sites are regulated differently in C3 and C4 plants. These results suggest that the functions of PEPCK have been conserved, but that sequences have diverged and regulation of PEPCK is important in C4 plants, but not in herbaceous and, in particular, woody C3 plants.

1. Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that catalyzes the reversible reactions, decarboxylation of oxaloacetate (OAA) and carboxylation of phosphoenolpyruvate (PEP) (Hatch and Mau, 1973). The decarboxylation of OAA, catalyzed by PEPCK, plays a crucial role in a process fundamental to C4 plants- the C4 cycle of photosynthesis, in which the concentration of CO_2 is increased in bundle sheath (BS) cells, minimizing photosynthesis and increasing the efficiency of photosynthesis. Based on the type of enzyme utilized in the decarboxylation phase of the C4 cycle, C4 plants are classically divided into three subgroups: NADP-malic enzyme (ME)-type, NAD-ME-type, and PEPCK-type (Hatch et al., 1975). In PEPCK-type plants, PEPCK is present in the bundle sheath (BS) cells, where it catalyzes the decarboxylation of OAA and supplies CO_2 to the Calvin cycle (Hatch and Osmond, 1976; Walker et al., 1997). Decarboxylase activity of PEPCK has also been detected in more and more NADP-ME-type and NAD-ME-type species, such as maize, *Flaveria* species, *Cleome gynandra*, and Guinea grass (Christin et al., 2011; Koteyeva et al., 2015; Walker et al., 1997; Wang et al., 2014). As PEPCK activity has been detected in the BS cells in several C4 plants, the PEPCK pathway is thought to be an accessory pathway for the NADP-ME and NAD-ME pathways. It has been hypothesized that combining PEPCK with either NADP-ME or NAD-ME could robustly maintain high photosynthetic efficiency under a broad range of light regimes because of the decreasing need to maintain high concentrations and concentration gradients of transport metabolites (Wang et al., 2014).

The functions of PEPCK are not limited to the C4 cycle. The PEPCK-catalyzed conversion of OAA to PEP is an early and rate-limiting step in the gluconeogenesis pathway in all plants, and PEPCK plays an important role in different stages of plant growth and development. PEPCK may be involved in the conversion of fats to sugars during seed germination (Leegood and ap Rees, 1978; Martín et al., 2007), the accumulation of soluble sugars and dissimilation of organic acids during fruit ripening (Famiani et al., 2016; Huang et al., 2015a,b), and the metabolism of nitrogenous assimilates and the increase in seed protein content (Beihaghi et al., 2015; Delgado-Alvarado et al., 2007; Leegood and Walker, 2003). It has been proven that PEPCK plays an important role in malate metabolism, which is significant during dark-induced stomatal closure (Penfield et al., 2012). In rice leaves, PEPCK is present in the stomata, hydathodes, and parenchyma cells close to the xylem and phloem, where it contributes to nitrogen recycling (Bailey and Leegood, 2016).

PEPCK also plays important roles in species other than plants. In fact, PEPCK is an ancient enzyme present in all known groups of living organisms (Aich and Delbaere, 2007). PEPCKs are traditionally divided into two groups based on nucleotide substrate specificity: ATP-dependent PEPCKs, which are mainly present in

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; OAA, decarboxylation of oxaloacetate; PEP, carboxylation of phosphoenolpyruvate; ME, malic enzyme; BS, bundle sheath.

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bacteria, yeast, and plants, and GTP-specific PEPCKs, which are mostly present in higher eukaryotes, major archaea, and also in some bacteria (Aich and Delbaere, 2007; Fukuda et al., 2004). Interestingly, significant sequence homology is found among the members of each group, whereas there is no statistically remarkable homology between the PEPCKs from the two different classes (Fukuda et al., 2004). Furthermore, based on structural evidence, the oxaloacetate-binding and metal-binding active site residues are highly conserved in both ATP- and GTP-dependent PCKs (Cotelesage et al., 2005; Dunten et al., 2002; Holyoak and Nowak, 2001; Leduc et al., 2005; Matte et al., 1996; Sudom et al., 2001; Trapani et al., 2001). In 2015, PPi-PEPCK was identified for the first time from *Entamoeba histolytica*, a eukaryotic human parasite (Chiba et al., 2015).

The activity of PEPCK is regulated by many kinds of external and internal factors, such as light, biotic and abiotic stress, and the pH value of the system. The phosphorylation of PEPCK has been observed in all CAM leaves and C3-tissues that have been studied to date and also in some C4 leaves (Walker et al., 1997; Walker and Leegood, 1996). In Guinea grass, PEPCK is dephosphorylated in illuminated leaves and phosphorylated in dark-adapted leaves (Walker and Leegood, 1996). PEPCK is also phosphorylated in the cotyledons and endosperm of many germinating seeds. For example, in cucumber (*Cucumis sativus* L.) cotyledons, dephosphorylation of PEPCK is stimulated by illumination (Walker and Leegood, 1995, 1996). The phosphorylation of PEPCK was also detected in the ripening fruit flesh of grapes, tomatoes, cherries and plums (Walker et al., 2016). Using mass spectrometry, four phosphorylated residues (Ser55, Thr58, Thr59, and Thr120) were identified in the maize PEPCK protein, ZmPCK1 (Chao et al., 2014), and the phosphorylation of these four residues was positively regulated by light. Because the decarboxylase activity of PEPCK in leaves is higher in the dark than in the light, it is possible that the depressing effect of light on PEPCK decarboxylation activity might be mediated by reversible phosphorylation (Chao et al., 2014).

The catalytic action of PEPCK is conserved across species but it has also undergone significant divergence, playing different roles in different species. In plants, PEPCK has evolved functions in the C4 cycle, but it is not clear how these functions have arisen. In this study, our goal was to answer two key questions regarding PEPCK evolution. What kinds of changes in PEPCK allowed it to become a key enzyme in C4 photosynthesis, and is the regulation of PEPCK evolutionarily conserved? To answer these questions, we examined the evolutionary relationships between PEPCKs from representative bacteria, fungi, and C3 and C4 plant species by constructing phylogenetic trees, identifying protein sequences that may be responsible for differential regulation of PEPCKs, and comparing the decarboxylase activity of PEPCKs from selected C4 and C3 plants *in vitro*. We found that the maximum enzymatic activities of PEPCKs from C4 plants and the woody C3 plant, *Populus trichocarpa*, were significantly higher than those in C3 herbs. Because we found divergence in the N-terminal region containing known PEPCK phosphorylation sites, we also assayed the decarboxylation activity of specific amino acid mutants of PEPCKs that mimic phosphorylation. Our findings suggest that although plant PEPCKs are ancient enzymes descended from bacteria, there has been considerable divergence in the N-terminal region that has likely led to divergence in the regulation of PEPCK activity by phosphorylation.

2. Materials and methods

2.1. Sequence retrieval and phylogenetic analysis

The PEPCK coding sequences (CDS) and protein sequences used in this study were retrieved from the Phytozome database (v11.0, http://phytozome.jgi.doe.gov) and the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein). The ID and CDS of PEPCKs are listed in Supplementary Table S1.

CDS of PEPCKs were aligned using CLUSTAL-X program (version 1.83) with the default parameters. Only positions with column scores over 15 were included in the following analysis. Statistical confidence for the evolutionary trees was assessed by bootstrap (1000 replicates). The phylogenetic tree was drawn using the MEGA7 program.

2.2. PEPCK *cloning and site-directed mutagenesis*

The full-length *PEPCK* coding sequences were amplified from cDNA obtained from the leaves of wild-type *Z. mays*, *S. bicolor*, *M. maximus*, *O. sativa*, *P. trichocarpa*, and *A. thaliana*.

The constructs for expression of PEPCK in bacteria were generated by cloning the cDNA into the vector pET28a (Novagen). Site-directed mutagenesis was performed using a double-stranded plasmid mutagenesis kit according to the manufacturer's protocol (QuickChange Lightning Site-Directed Mutagenesis Kit; Agilent Technologies). Mutations were verified by two independent sequence analyses of the same DNA strand.

The primers used for cDNA cloning, generating expression constructs, and site-directed mutagenesis are listed in Supplementary Table S2.

2.3. *Protein expression and* in vitro *enzyme assays*

Recombinant PEPCK proteins were expressed in *Escherichia coli* strain BL21 (RIL) and purified using an immobilized metal affinity resin (Profinity IMAC Ni-Charged Resin; Bio-Rad) as previously described (Jiang et al., 2016).

PEPCK decarboxylase activities of the purified recombinant proteins were measured using a previously described method (Chao et al., 2014) with some modifications. The decarboxylase activity reaction mixture (800 μl) contained 40 mM HEPES-KOH (pH 8.0), 0.25 mM ATP, 0.5 mM $MnCl_2$, and 0.1 mM oxaloacetate (Sigma). After incubation for 10 min at 25 °C, reactions were stopped by heating at 100 °C for 2 min. To determine the amount of PEP generated, 200 μl of each reaction solution was mixed with 800 μl of 30 mM K_2HPO_4/20 mM NaH_2PO_4 (pH 7.0), 10 mM $MgCl_2$, 1 mM ADP, 1.5 mM NADH, and 6 U lactate dehydrogenase (Sigma). Each PEP assay was initiated by addition of 0.5 U pyruvate kinase (Sigma; Walker and Leegood, 1995).

Decarboxylase activity was determined spectrophotometrically at 25 °C by following NADH oxidation at 340 nm. One unit of PEPCK activity corresponds to the production of 1 μmol/min NAD^+ at 25 °C. Carboxylase activity was calculated as $Vt \times \Delta A/\Delta t \times \varepsilon \times L \times Vs \times Pc$, where ΔA is the change in absorbance at 340 nm, Δt is the change in time, Vt is the total volume of the reaction mixture, Vs is the volume of the protein extract, Pc is the concentration of total protein in the extract, and L is the cuvette optical path. The extinction coefficient (ε) of NADH is 6220 M^{-1} cm^{-1}. To ensure the accuracy of the results, the enzyme activity of each reaction was measured every 30 s, and denatured crude extracts were used as a control. For the decarboxylation assay, the change in absorbance for the pyruvate kinase assay represents only a quarter (200 μl/800 μl) of each protein extraction sample. Therefore, the total decarboxylase activity was corrected by multiplying the value of the formula given above by four.

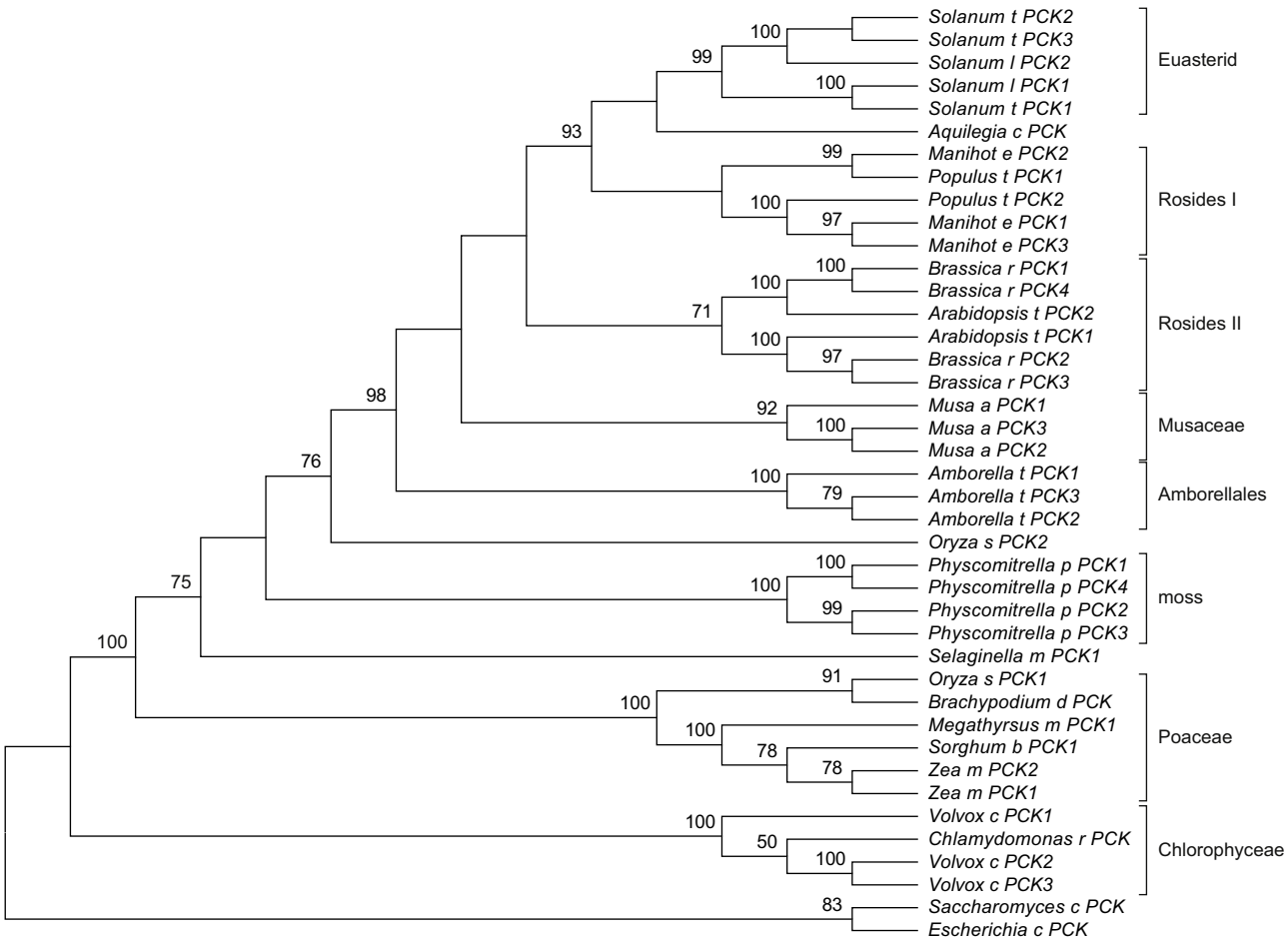

Fig. 1. Molecular phylogenetic analysis using the Neighbor-Joining method. The evolutionary history of the PEPCK family was inferred using the Neighbor-Joining method (Zuckerkandl and Pauling, 1965). The optimal tree with the sum of branch lengths = 3.98151923 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 41 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated; i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 915 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

3. Results

3.1. Phylogenetic analysis

To determine the evolutionary relationships between PEPCKs, a phylogenic tree was constructed using the coding sequences from different representative species, including *E. coli*, yeast, moss, and plant groups including Amborella, Aquilegia, monocots, Eurosid I and II, and Euasterid I (Fig. 1, Table 1). Initial sequence alignments of the *PEPCK* cDNAs from these species were performed using ClustalW. A phylogenetic tree was then constructed using Neighbor-Joining on a distance matrix file in Mega7. This tree revealed that the *PEPCK* genes from *E. coli* and yeast (*Saccharomyces*) are in a clade distinct from plants, and Chlorophyceae and moss *PEPCKs* are also separated into distinct clades. Monocot *PEPCKs* are divided into two clades, Poaceae *PEPCKs* are present in a single clade, except for *Oryza sativa PCK2*, and Musa *PEPCKs* are closely related to dicots. The *PEPCKs* of the C3 Poaceae species, *Oryza sativa* (*O. sativa PCK1*) and *Brachypodium*, are closely related to the *PEPCKs* of the C4 Poaceae species, *Zea mays*, *Sorghum*, and *Megathyrsus*. The PEPCKs from Eurosid I and Euasterid I are conserved and are clearly classified into two groups. This phylogeny suggests that *PEPCKs* from Poaceae are more conserved in dicots and the other monocots. The two maize *PEPCKs* may have arisen from gene duplication in maize.

To examine the differences in protein sequences, ten PEPCKs were selected for multiple sequence alignment, including PEPCKs from three C4 plants with different mechanisms of decarboxylation, *Z. mays* (NADP-ME-type), *S. bicolor* (NAD-ME-type), and *P. maximum* (PEPCK-type), and from three C3 plants, *A. thaliana* (herbaceous dicot), *O. sativa* (herbaceous monocot), and *P. trichocarpa* (woody dicot) (Fig. 2). The C-terminal sequences of these proteins are conserved across species, but the N-terminal sequences are quite different. ZmPCK1 has a unique amino acid sequence, 47-SAPSTPKR-54, not found in the other PEPCKs. Five phosphorylation sites were previously identified in the N-terminal sequence of ZmPCK1, including Ser55, Thr58, Thr59, Ser119, and Thr120, the phosphorylation state of some of which are known to impact PEPCK activity (Chao et al., 2014; Ning et al., 2016). The first three phosphorylation sites, Ser55, Thr58, and Thr59, are conserved in all PEPCKs except for OsPCK2. ZmPCK1-Thr120 is only conserved in the C4 PEPCK sequences, ZmPCK2, SbPCK, and MmPCK (Table 1). ZmPCK1- Thr119 is a unique phosphorylation site that is not present in the other PEPCKs.

3.2. Enzymatic activities of PEPCKs in C4 and C3 plants

To determine the difference in the enzymatic activities of PEPCKs in C4 and C3 plants, the maximum decarboxylase enzymatic activity of ten PEPCK proteins was assayed *in vitro*. These

Fig. 2. Amino sequence alignment of PEPCKs from different plants. Ten PEPCKs from three C4 (*Zea mays*, *Sorghum bicolor*, *Megathyrsus maximus*) and three C3 species (*Populus trichorcarpa*, *Oryza sativa*, *Arabidopsis thaliana*) were aligned. The yellow shading indicates three conserved phosphorylation sites: Ser55, Thr58, and Thr120, and marked as S1 to S3 respectively. The green shading indicates the ZmPCK1-specific phosphorylation site Ser119 and the conserved site Thr120, and marked as S4 and S5. The protein sequences used for this multiple sequence alignment are as follows: ZmPCK1 (*Zea mays*, GRMZM2G001696), ZmPCK2 (*Zea mays*, GRMZM5G870932), SbPCK (*Sorghum bicolor*, Sobic.001G432800.1), MmPCK (*Megathyrsus maximus*, AAQ10076.1), PtPCK1 (*Populus trichocarpa*, Potri.007G011200.1), PtPCK2 (*P. trichocarpa*, Potri.002G107700.1), OsPCK1 (*Oryza sativa*, LOC_Os03g15050.1), OsPCK2 (*O. sativa*, LOC_Os10g13700.2), AtPCK1 (*Arabidopsis thaliana*, AT5G65690.1), and AtPCK2 (*A. thaliana*, AT4G37870.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins included two PEPCKs each from *A. thaliana* (AtPCK1, AtPCK2), *O. sativa* (OsPCK1, OsPCK2), *P. trichocarpa* (PtPCK1, PtPCK2), and *Z. mays* (ZmPCK1, ZmPCK2), and one PEPCK each from *P. maximum* (PmPCK) and *S. bicolor* (SbPCK). For protein expression, the full-length *PEPCK* cDNAs were obtained from each species, cloned into the bacterial expression vector, pET28a, and transformed into *E. coli* BL21-RIL host cells. Next, the soluble proteins were purified and separated on an SDS–PAGE gel. Based on SDS–PAGE analysis, the recombinant proteins were successfully purified and could be used to assay enzymatic activity *in vitro*.

The *in vitro* enzymatic activities of all the PEPCKs, except for PtPCK1, were significantly lower than those of ZmPCK1, a C4 type PEPCK in maize. (Fig. 3). The activities of the two maize PEPCKs were higher than those of the C4 Poaceae PEPCKs, SbPCK and PmPCK, and also significantly higher than the two rice (C3) PEPCKs. Interestingly, the activity of OsPCK2, which has a divergent N-terminal sequence compared with the other Poaceae PEPCKs, was lower than that of OsPCK1. The *in vitro* decarboxylation activities of PEPCKs in monocotyledonous herbs were higher than those in dicotyledonous herbs, but lower than PEPCKs from the woody plant *P. trichocarpa*. In addition, among the monocotyledonous herbs, the *in vitro* decarboxylation activities of PEPCKs in C4 plants were higher than those in C3 plants.

Table 1
Phosphorylated sites conserved with ZmPCK1 and phosphorylation mimic mutations.

ID	S1	S2	S3	S4	S5
ZmPCK1	S55	T58	T59	S119	T120
ZmPCK2	S47	T50	T51	/	T112
SbPCK	S47	T50	T51	/	T112
MmPCK	S43	T46	T47	/	T98
AtPCK1	S62	T56	T66	/	/
AtPCK2	S66	T69	T70	/	/
OsPCK1	S50	T53	T54	/	/
PtPCK2	S66	T69	T70	/	/
PtPCK2	S63	T66	T67	/	/
Phosphorylation mimics	S1D	S2D	S3D	S4D	S5D
	DDD			DD	
	4D (S1–S5 without S4) or 5D (S1–S5)				

3.3. PEPCK activities are partially dependent on phosphorylation site and level of phosphorylation

The five conserved phosphorylation sites were named Site1 (S1), S2–S5 (Fig. 2). The first three PEPCK phosphorylation sites (S1–S3) in C4 and C3 plants are located nearly 60 amino acids upstream of the other two sites, S4 and S5. In a previous study, it was found that the suppressive effect of light on ZmPCK1 decarboxylation activity might be mediated by reversible phosphorylation of Ser55 (Chao et al., 2014). To investigate the relationship between site-specific phosphorylation and PEPCK enzymatic activity in C3 and C4 plants, a series of PEPCK mutations containing Ser/Thr-to-Asp substitutions designed to mimic phosphorylation of PEPCK were made, and the enzymatic activity of the mutant PEPCK proteins (S1D, S2D, S3D, S4D, S5D) was assayed *in vitro*. Because S1–S3 and S4–S5 are located in two separate areas, we also tested the enzyme activities of proteins with mutations in all three S1-S3 sites (DDD), with mutations

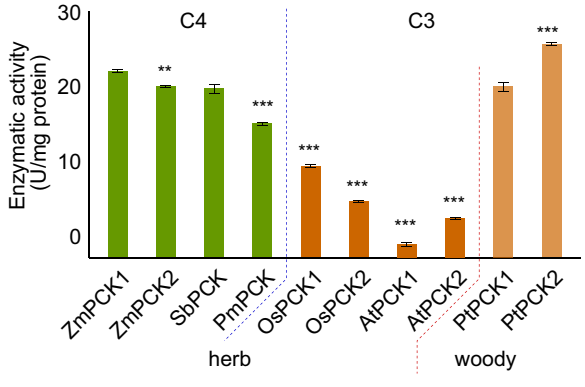

Fig. 3. The *in vitro* maximum enzymatic activity of purified recombinant PEPCKs from selected C3 and C4 plants. Decarboxylation activity of PEPCKs from six species was assayed *in vitro* under the same conditions. Green, orange, and light orange bars show the enzymatic activities of PEPCKs from C4 herbs, C3 herbs, and a C3 woody plant, respectively. Each bar indicates the mean and the standard error for three replicates (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

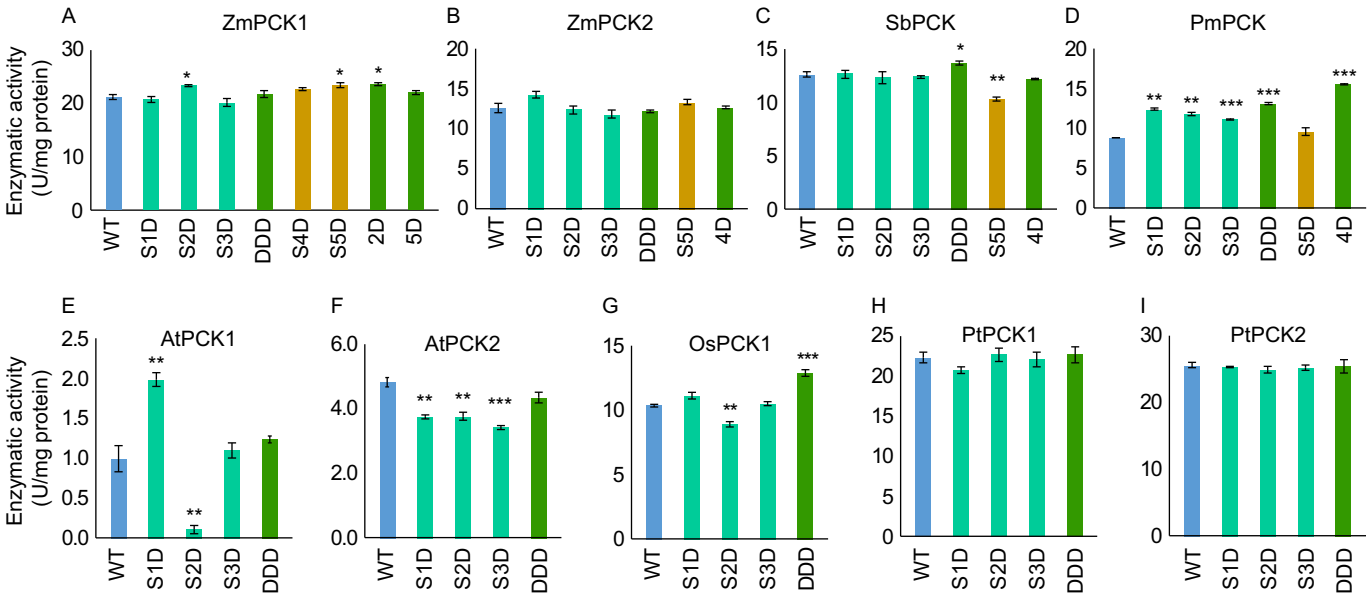

Fig. 4. The *in vitro* enzymatic activity of selected C3 and C4 PEPCKs with phosphorylation mimic mutations. Bar graphs show the maximum decarboxylation enzymatic activity of wild-type and phosphorylation mimic mutants of (A) ZmPCK1, (B) ZmPCK2, (C) SbPCK, (D) PmPCK, (E) AtPCK1, (F) AtPCK2, (G) OsPCK1, (H) PtPCK1, and (I) PtPCK2. Blue bars indicate WT, light green bars indicate single mutations in phosphorylation sites S1, S2, or S3 (S1D, S2D, S3D), orange bars indicate single mutations in S4 or S5 (S4D, S5D), and dark green bars indicate mutations in multiple sites (S1–S3, DDD; S1–S5, 5D). Each bar indicates the mean and standard error of three replicates (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in both the S4 and S5 sites (DD), with mutations in S1–S4 (4D) or with mutations in all five S1–S5 sites (5D) (Table 1).

The *in vitro* maximum enzymatic activities are shown in Fig. 4. The enzymatic activities of ZmPCK1-S2D, ZmPCK1-S5D, and ZmPCK1-DD were all significantly decreased compared with ZmPCK1-WT. All ZmPCK2 phosphorylation mimic mutants were not significantly different from WT. As observed for ZmPCK2, the SbPCK-SD1, −SD2, and −SD3 mutants exhibited WT enzyme activities, but the activity of SbPCK-DDD was higher than WT, and the activity of SbPCK-S5D was significantly decreased compared with WT. In the PEPCK-type plant, *P. maximum*, the enzyme activities of the PmPCK-S1D, −S2D, −S3D, DDD, and −4D proteins were much higher compared to WT. These results suggest that there is more regulation of C4-type PEPCK proteins by phosphorylation.

It is noteworthy that the enzymatic activities of herb dicot PEPCKs were more affected by phosphorylation mimic mutations than monocot PEPCKs. The activity of AtPCK1-S1D was two-fold higher than that of the WT protein, and AtPCK1-S2D decreased enzyme activity to an extremely low level, about 11% of WT. The activity of AtPCK2-S1D, AtPCK2-S2D, and AtPCK2-S3D was significantly decreased compared with WT (by 22%, 22%, and 29%, respectively), but the activity of AtPCK2-DDD showed no apparent change compared with WT. Compared with WT OsPCK, the enzymatic activities of OsPCK1-S2D and OsPCK1-DDD were decreased by 14% and increased 24%, respectively. In total, the changes in the enzymatic activity levels of mutant herb dicot PEPCKs were significantly higher than the changes observed for mutant monocot PEPCKs, indicating that the phosphorylation state of the sites S1-S3, and especially of S2, is essential for the regulation of PEPCK's enzymatic activity.

Interestingly, the activities of PtPCK1 and PtPCK2 phosphorylation mimic mutants were not significantly different from PtPCK1-WT and PtPCK2-WT, respectively. Furthermore, the activities of both PtPCKs were significantly higher than those of the herb C3 plant PEPCKs. This indicates that the regulation of PEPCK in woody plants is quite different than in C4 and herb C3 plants.

4. Discussion

PEPCK is a ubiquitous enzyme expressed in bacteria, yeast, animals, and plants, and catalyzes the conversion of oxaloacetate to PEP and carbon dioxide during gluconeogenesis and the C4 cycle in certain plants (Hatch et al., 1975; Walker et al., 1997). All plant PEPCKs are ATP-dependent, whereas PEPCKs in bacteria and fungi can be ATP- or GTP- dependent (Aich and Delbaere, 2007; Fukuda et al., 2004). The phylogenetic tree (Fig. 1) constructed from representative ATP-dependent PEPCKs is consistent with the tree made in a previous study (Choi et al., 2015), but is more extensive and focused on plants. We have included PCKs from different species, including bacteria, fungi, algae, and the major groups of plants, to explain the evolutionary origin of PCK. The tree reported here also shows that ATP-dependent PEPCKs from moss, Chlorophyceae, and bacteria and yeast are divided into separate clades. PEPCKs diversified, becoming more complicated, during the evolution of the Angiospermae. The sequences of ATP-dependent PEPCKs are not conserved between microorganisms and higher plants, but they are relatively similar between angiosperms. PEPCKs are not conserved between Poaceae and Musaceae, but are highly conserved between C4 and C3 plants. The genomic analysis of protein evolution showed that C4 plants had independent origins.

PEPCK is involved in many aspects of plant metabolism. In certain C4 plants, it acts during the day as a decarboxylating enzyme that provides CO_2 for diurnal RuBisCO-dependent carbon fixation. Phosphorylation of PEPCK near the N-terminal extension and changes in regulatory properties associated with this modification have been reported (Chao et al., 2014; Ning et al., 2016). In Poaceae, the N-terminal sequences of PEPCK, especially the first 150 amino acids, are not conserved among C4 and C3 plants, which include the sites modified by phosphorylation. Phosphorylation and dephosphorylation of these N-terminal sites in *Z. mays and M. maximum* PEPCKs follows the circadian rhythm (Chao et al., 2014; Walker et al., 2002). For example, ZmPCK1-T120 is dephosphorylated during the day and phosphorylated in the dark (Chao et al., 2014). The enzymatic activity of PEPCK also responds to the phosphory-

lation state; ZmPCK1 has higher activity in the dark than during the day (Chao et al., 2014). In our study we found that the activity of the phosphorylation mimic mutant ZmPCK1-pT120 (SD5) was higher than that of the WT protein (Fig. 4). This suggests that regulation of ZmPCK1 activity is complicated and dependent on the phosphorylation state. It was reported that the enzymatic activity of MmPCK is increased after dephosphorylation in dark (Ray and Black, 1976). This is opposite to what we found in our assays: the *in vitro* enzymatic activities of all MmPCK phosphorylation mimic mutants, except for MmPCK-Thr98D (S5D), were nearly two-fold higher compared with MmPCK-WT. This indicates that the enzymatic activity of MmPCK may be weakly regulated by phosphorylation *in vitro*, regulated by substrate concentration, and impacted by the change in activity of malate dehydrogenase (MDH).

The mechanisms used to regulate PEPCK activity are very complicated given that enzyme activity assays performed *in vivo* and *in vitro* yield different and even opposite results. Enzymatic activity assays of three single and triple phosphorylation mimic mutants of C3 PEPCKs showed that PEPCK activity was decreased by phosphorylation. This suggests that the phosphorylation state of the conserved site, Thr58 (S2), in C3 herbs is useful for regulating PEPCK. ZmPCK1-Ser119 was previously found to be phosphorylated, but the enzymatic activity of the ZmPCK1-S119D (ZmPCK1-S4D) phosphorylation mimic mutant protein was similar to that of the WT, suggesting that Ser119 is not the key regulatory site in ZmPCK1.

Recently, the PEPCK-type C4 cycle has been defined as an auxiliary pathway to the NAD-ME- and NADP-ME-type C4 cycles (Wang et al., 2014), and *M. maximus*, a classical PEPCK-type plant, also has an NAD-ME-type cycle (Ray and Black, 1976). It has been shown that Poaceae PEPCKs have a more significant role in C4 photosynthesis than in other C4 species. This, and the division of Poaceae PEPCKs in the phylogenic tree, suggests that Poaceae PEPCKs from C4 plants have different sequences from other PEPCKs from other families.

The enzymatic activities of the two PEPCKs from the woody plant, *P. trichocarpa*, were higher than those of herb PEPCKs, and there was no apparent change in activity of PEPCKs phosphorylation mimic mutants compared to wild type. In C3 plants, especially in woody plants, PEPCK is only involved in gluconeogenesis and providing carbon resources. Maintaining a high level of PtPCK enzymatic activity is useful to growth and development, which requires large numbers of carbon skeletons. However, the enzymatic activity of the PtPCKs is sufficient for poplar growth, and there is no need to improve the activity by phosphorylating N-terminal modification sites.

5. Conclusion

The C4 Poaceae PEPCKs are conserved and have diverged from the PEPCKs of other C4 monocots and of C3 plants. The C3 and C4 PEPCKs are regulated differently by the conserved phosphorylated sites in N-terminal sequences. The maximum enzymatic activities of PEPCKs were impacted differently with mimic phosphorylation state between C3 and C4 plant PEPCKs. The functions of PEPCK have been conserved but sequences have diverged. The regulation of PEPCK is important in C4 plants, but not in herbaceous and, in particular, woody C3 plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.02.008.

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