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Molecular cloning, gene expression and functional expression of a phospho*enol*pyruvate carboxylase *Osppc1* in developing rice seeds: implication of involvement in nitrogen accumulation

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Abstract

We isolated two cDNAs of phospho*enol*pyruvate carboxylase (PEPC) from developing rice seeds, *Osppc1* and *Osppc3*. The deduced amino acid sequences of both cDNAs share several conserved motifs with other non-photosynthetic PEPCases, and these common motifs are known to be functionally important to their regulatory properties. The deduced protein sequence of *Osppc1* was clustered into a monocotyledonous plant-specific clade, and *Osppc3* was clustered into a gramineous plant-specific clade in the phylogenetic tree of plant PEPCases. The mRNA accumulations of *Osppc1* and *Osppc3* were found in developing rice seeds throughout the grain-filling stages, although their expression patterns differed: *Osppc1* was strongly expressed at 7 d after flowering, and *Osppc3* was strongly expressed at 4 d after flowering. The kinetic properties of the Osppc1 recombinant protein were quite similar to those of maize root-type PEPCase, except that the sensitivity for malate at pH 7.3 was weaker. Mining rice microarray data, we observed that *Osppc1* was co-expressed with aspartate aminotransferase and alanine aminotransferase, which are involved in seed nitrogen metabolism. Moreover, reannotation of the co-expressed genes revealed that *Osppc1*, the two aminotransferases and the enolase were mapped on to the consecutive reaction from 2-phosphoglycerate to glutamate and pyruvate in the cytosol. These results imply that *Osppc1* functions cooperatively with the two aminotransferases in the synthesis of amino acids that are used for storage protein synthesis in developing rice seeds.

Keywords: amino acid synthesis, kinetics, nitrogen accumulation, phospho*enol*pyruvate carboxylase, protein, rice, seeds

Introduction

PEPCase (phospho*enol*pyruvate carboxylase) (EC 4.1.1.31) in C_3 plants is recognized as an important enzyme in the anaplerotic provision of carbon skeletons to the citrate acid cycle (O'Leary *et al.*, 2011). This enzyme catalyses the irreversible β-carboxylation of phospho*enol*pyruvate in the presence of HCO_3^- to yield oxaloacetate, which is a precursor of citrate, malate and aspartic acid. The PEPCase enzyme can be allosterically regulated by certain substances, such as malate and glucose-6-phosphate (G6P), in accordance with its phosphorylation status. The physiological role of this enzyme in various plant organs has been investigated by examining its enzymological properties and expression patterns: the relationship between PEPCase and nitrogen metabolism, and the contribution of PEPCase to nitrogen assimilation have been well established by previous studies (Van Quy *et al.*, 1991; Sugiharto and Sugiyama, 1992; Van Quy and Champigny, 1992; Huppe and Turpin, 1994; Podesta and Plaxton, 1994a, b; Suzuki *et al.*, 1994; Duff and Chollet, 1995;

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Zhang *et al.*, 1995). Recent molecular approaches have revealed that C_3 plants contain several PEPCase isoforms that can be classified into plant and bacterial types; the bacterial-type PEPCase isoforms lack the distinctive N-terminal serine-phosphorylation site, which is seen in plant-type PEPCase (Sánchez and Cejudo, 2003; Sullivan *et al.*, 2004; Mamedov *et al.*, 2005; Gennidakis *et al.*, 2007; Igawa *et al.*, 2010).

A physiological role of PEPCase in immature seeds has been investigated and has been speculated to include support of the biosynthesis of storage compounds. In castor oil seeds, two PEPCases were characterized, and their possible functions in storage protein and fatty acid synthesis were discussed (Blonde and Plaxton, 2003). A PEPCase in *Vicia faba* L. seems to be involved in amino acid synthesis in cotyledons (Golombek *et al.*, 1999). We have shown a correlation between nitrogen content and PEPCase activity in some soybean cultivars (Sugimoto *et al.*, 1989). We also observed an increase in PEPCase activity in developing rice seeds in response to nitrogen fertilization in the flowering period, causing simultaneous high nitrogen accumulation in rice seeds (Sugimoto *et al.*, 1997). However, it remains unclear whether PEPCase contributes to nitrogen accumulation in seeds.

Rice is one of the major crops in the world and the nitrogen content in its seeds is a crucial key to its quality (Yan-Lin *et al.*, 2007). Rice seeds are considered to provide 20% of dietary protein intake in all developing countries (International Year of Rice Secretariat, 2004). In contrast, the protein content in rice grains influences the texture of cooked rice (Tamaki *et al.*, 1989; Hamaker and Griffin, 1990; Okadome *et al.*, 1999; Champagne *et al.*, 2001; Martin and Fitzgerald, 2002; Derycke *et al.*, 2005; Xie *et al.*, 2008) and flavour (Onate *et al.*, 1964; Juliano *et al.*, 1965; Park *et al.*, 2001; Champagne *et al.*, 2004, 2007). Low nitrogen content is also a critical factor of brewer's rice seed quality (Furukawa *et al.*, 2006). Although it also increases the nitrogen content in rice seeds (Perez *et al.*, 1996), nitrogen fertilization in the flowering stage is an effective technique to increase the yield.

Six PEPCase isoforms have been found in the rice genome: five plant-type isoforms and a bacterial type having an expression pattern with distinctive features (Sánchez and Cejudo, 2003; Lin *et al.*, 2004; Masumoto *et al.*, 2010). The putative polypeptide length diverges slightly among them, ranging from 92.5 to 103.6 kDa. At the protein level, four PEPCase subunits have been detected in leaves by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Matsuoka and Hata, 1987). Although such molecular-level information has been accumulated for rice PEPCase, there has been no report on PEPCase in developing rice seeds.

In this study, we cloned PEPCase *Osppc1* (MSU ID: LOC_Os02g14770, assigned at the Rice Genome Annotation Project: http://rice.plantbiology.msu.edu/) as a candidate gene working in developing rice seeds. This full-length cDNA was isolated from a cDNA library of developing rice seeds with its isoform *Osppc3*. Sequence analysis revealed that the translation product of *Osppc1* seemed to have PEPCase enzymatic characteristics similar to a C_3-type isoform, and recombinant protein expression analysis confirmed its predicted enzymatic properties. *Osppc1* mRNA in seeds was increased after the flowering stage and peaked in the middle of the grain-filling stage, when the rate of nitrogen accumulation in seeds was rapid. On the other hand, *Osppc3* mRNA was expressed strongly during the early part of the grain-filling stage. *Osppc1* showed a co-expression pattern with the two aminotransferases that are located downstream of the PEPCase reaction in the metabolic pathway. *Osppc1* may work in supporting biosynthesis of amino acids that are consumed in storage protein synthesis in rice seeds, by the provision of oxaloacetate (OAA).

Materials and methods

Plant material

Rice (*Oryza sativa*, L. cv. Nipponbare) plants for DNA and RNA experiments were grown in an experimental greenhouse at Kyoto Prefectural University under normal conditions in 1999. The flowering date was marked for each hull. Developing rice seeds were collected at 4, 7, 10 and 14 d after flowering and immediately frozen in liquid nitrogen. The samples were stored at −80°C until use. Developing rice seeds for protein analysis were grown in a paddy field at Kobe University in 1997 and 1998. Developing rice seeds at approximately 10 d after flowering were collected and frozen in liquid nitrogen. The samples were stored at −20°C until use.

cDNA cloning

We first prepared cDNA probes obtained by reverse transcription-polymerase chain reaction (RT-PCR) to screen PEPCase cDNA from developing rice seed-cDNA libraries. Total RNA of developing rice seeds at 7 d after flowering was extracted as described elsewhere (Masumura *et al.*, 1990), and poly(A)$^+$ RNA was purified from the total RNA using a polyAT tract system (Promega, Madison, Wisconsin, USA). The poly(A)$^+$ RNA was reverse-transcribed using Superscript II (Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions. RT-PCR reactions were performed using r*Taq* polymerase

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(Toyobo, Osaka, Japan). The primers for RT-PCR were designed based on the nucleotide sequences of a maize C_3 PEPCase, a maize C_4 PEPCase and three rice expression sequence tag sequences (Genbank ID: AU069759, C27144 and C72139) showing homology to the PEPCase nucleotide sequences in C_3 plants (Table 1). The RT-PCR parameters for the different primer sets were as follows: (i) for the primer set ZmppcCF and Zmppc4R, initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 7 min; (ii) for Zmppc3F and Zmppc3R, initial denaturation at 94°C for 2 min followed by 5 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 90 s and 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 7 min; (iii) for ZmppcCF and Zmppc3R, initial denaturation at 94°C for 2 min followed by 5 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s and 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 7 min; (iv) for AU069759F and C72139R, initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 7 min. The amplified fragments were collected separately after agarose gel electrophoresis and analysed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI-PRISM 310 Genetic Analyzer (Perkin-Elmer, Heidelberg, Germany) to confirm that their sequences were partial fragments of PEPCase.

Next, we screened PEPC cDNAs from a cDNA library of developing rice seeds at 7 d after flowering using RT-PCR-amplified cDNA probes as probes for the plaque hybridization. This cDNA library was constructed from developing rice seeds at 7 d after flowering using a λZiplox cDNA cloning kit (Life Technologies) following the manufacturer's instructions. The cDNA library was comprised of 1.3×10^6 independent clones. Positive phage clones were isolated from the libraries by plaque hybridization with the probes incorporating [^{32}P]dCTP (^{32}P-labelled deoxycytidine triphosphate) by using a BcaBEST DNA labelling kit (Takara BIO, Otsu, Japan), and their cDNA inserts were separately excised and converted into pZL-1 plasmid clone in DH10B using the λZIPLox system (Life Technologies). The obtained plasmid clones were classified into groups according to the obtained *Eco* RI restriction digestion patterns. A representative clone that seemed to have the longest insert for each group was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI-PRISM 310 Genetic Analyzer. The sequences of the two representative clone inserts were deposited separately in GenBank on 24 May 2000 and 26 January 2001, respectively (GenBank ID Nos. AF271995 and AB055074).

Phylogenetic tree construction

A phylogenetic tree of PEPCase was constructed using GENETYX software (GENETYX, Tokyo, Japan). The polypeptide sequences were corrected based on the results of homology search of Osppc1 and Osppc3 against non-redundant protein database 'nr'. The top 100 sequences for each search were collected from GenBank and integrated into a non-redundant sequence set. After selecting sequences of seven monocotyledonous species (*Sorghum bicolor*, *Zea mays*, *Hordeum vulgare*, *Brachypodium distachyon*, *Saccharum* sp., *Phalaenopsis amabilis*, *Phalaenopsis equestris*, *Echinochloa crus-galli*) and five dicotyledonous species (*Arabidopsis thaliana*, *Glycine max*, *Ricinus communis*, *Medicago truncatula*, *Populus trichocarpa*), the multiple alignment of the selected sequences was carried out with rice PEPCase sequences. The alignment data were used for constructing a phylogenetic tree with the neighbour-joining method. Bootstrap testing was conducted with 1000 resamples (Saitou and Nei, 1987). Because the Osppc-b sequence, which encodes the bacterial type PEPCase in rice, showed quite low conservation with the other rice PEPCase sequences, it was omitted on this tree.

Northern hybridization

The total RNA of developing rice seeds at 4, 7, 10 and 14 d after flowering was extracted by the method described previously (Masumura *et al.*, 1990) and approximately 20 μg of total RNA from rice seeds was subjected to electrophoresis on 1.2% agarose gels containing 1.8% formaldehyde, and transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences, Piscataway, New Jersey, USA) with a solution of 0.1 N NaOH/3 M NaCl. The membrane was baked at 80°C for 2 h to allow linking of the RNA

Table 1. Primer sequences for RT-PCR

Primer name	Sequence	Corresponding sequence
ZmppcCF	ATGTTTGAGCTCTCTATGTGGCG	*Zea mays* C_4 PEPCase (X61489)
Zmppc4R	GTAGCCGATCTGAAGTACTCG	*Zea mays* C_4 PEPCase (X61489)
Zmppc3F	AGCTACTTCCACGAAACAATT	*Zea mays* C_3 PEPCase (X15238)
Zmpppc3R	GCAAGGCGGAAATACTCGAC	*Zea mays* C_3 PEPCase (X15238)
AU069759F	GCTTCTGCAACGTGAATGTCA	Rice EST (AU069759)
C72139R	CTCGTTTCCACCTACCAGTG	Rice EST (C72139)

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to the membrane. [^{32}P]dCTP-labelled cDNA probes were prepared by using a BcaBEST DNA labelling kit. The PCR products for gene-specific regions of the two isolated cDNAs, positions 3061–3272 of the longer cDNA clone insert and positions 742–991 of the other cDNA clone insert, were used as templates. The membrane was prehybridized for 5 h at 42°C in hybridization solution [6 × saline sodium phosphate EDTA buffer (SSPE), 5 × Denhardt's reagent, 50% (v/v) formamide, 0.1% (w/v) SDS, 20 mg ml^{-1} denatured salmon sperm DNA]. Then the membrane was hybridized at 42°C for approximately 16 h in hybridization buffer with the cDNA probe. After hybridization, the membrane was washed twice with wash buffer [0.1 × saline sodium citrate buffer (SSC), 0.1% (w/v) SDS] at 42°C for 15 min, and exposed to X-ray film at −80°C. The expression of *Actin1* was monitored as an internal control.

Heterologous expression of PEPCase protein

The full-length cDNA for Osppc1 was cloned into a *Not* I site of the pET-30b (+) His-tag vector and transformed into *Escherichia coli* JM109 (DE3) pLysS (Novagen, Madison, Wisconsin, USA). The transformed *E. coli* cells were grown in Luria–Bertani (LB) media with 50 μg ml^{-1} of kanamycin at 37°C until its middle growth-phase ($OD_{600} = 0.6$), and production of Osppc1 protein was induced by incubation with 0.6 mM isopropyl-β-D-thiogalactopyranoside at 25°C for 6 h.

Enzyme activity assay

PEPCase activity was assayed by coupling with the malate dehydrogenase reaction according to the method of Echevarria *et al.* (1994) with modifications. This assay was performed in 1 ml of a medium containing 50 mM Tris–HCl, 5 mM $MgSO_4$, 0.15 mM NADH, 5 mM $KHCO_3$, 5 mM PEP (cyclohexylammonium salt), 4 mM dithiothreitol (DTT) and 1.5 U pig heart malate dehydrogenase at 30°C at pH 8.3. The decreasing rate of the absorbance at 340 nm was measured. One unit of enzyme activity was defined as the amount of PEPCase catalysing the production of 1 μmol of oxaloacetate per minute at 25°C. The assay was carried out three times for each sample, and the average of the measured values was adopted. The protein concentration was determined by the method of Bradford (1976).

Purification of recombinant Osppc1 protein

Recombinant protein was extracted from the *E. coli* cells as follows. The bacterial culture was centrifuged to collect the *E. coli* cells. The obtained bacterial pellet was suspended in extraction buffer [50 mM sodium phosphate (pH 7.8), 300 mM NaCl], frozen in liquid nitrogen and defrosted by dipping the centrifuge tube into water. The *E. coli* cells were shaken vigorously. This frozen–defrost–shaking procedure was repeated ten times. After centrifuging the suspension, the supernatant was collected as a crude extract.

Purification of the recombinant PEPCase protein was carried out in the following steps at 4°C by chasing PEPCase activity. At the first step, the crude extract was brought to 20% (w/v) ammonium sulphate, and the supernatant was collected by centrifugation. The supernatant was brought to 50% (w/v) ammonium sulphate, and the precipitate was collected by centrifugation. The protein pellet was suspended in buffer A [50 mM Hepes/KOH, pH 7.8, 10 μM leupeptin, 100 μM phenylmethanesulphonyl fluoride (PMSF) and 20% (w/v) $(NH_4)_2SO_4$] and loaded on to Butyl-TOYOPEARL 650M (Tosoh, Tokyo, Japan) equilibrated in buffer A. The Butyl-TOYOPEARL 650M column was washed with an equal volume of equilibration solution, and the PEPCase protein was eluted with a linear gradient of 100–0% buffer A simultaneously with 0–100% buffer A without $(NH_4)_2SO_4$. The fractions exhibiting PEPCase activity were collected and subjected to diafiltration against buffer B [50 mM sodium phosphate (pH 7.8), 300 mM NaCl] overnight and loaded on to TALON Metal Affinity Resin (Clontech Laboratories, Mountain View, California, USA) equilibrated in buffer B. The resin column was washed with a tenfold volume of buffer B. PEPCase was eluted with buffer B supplemented with 150 mM of imidazole and collected. The TALON Metal Affinity chromatography step was repeated once, and the obtained purified fraction was brought up to 50% (v/v) glycerol and stored at −20°C. SDS-PAGE analysis was performed by the method of Laemmli (1970).

Purification of PEPCase from developing rice seeds

All procedures for PEPCase purification were performed at 4°C. PEPCase was extracted from developing rice seeds at 10 d after flowering. The frozen seeds were ground by a homogenizer in extraction buffer C [100 mM Tris–HCl, pH 7.8, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM L-malate and 20% (w/v) glycerol] with Complete Protease Inhibitor Cocktail (F. Hoffmann-La Roche, Basel, Switzerland). The homogenate was filtered through two layers of gauze and centrifuged to collect the supernatant liquid.

PEPCase protein was purified from the supernatant by four steps of column chromatography. The crude extract was brought to 20% (w/v) ammonium sulphate, and the supernatant was collected by centrifugation. Next, the supernatant was loaded on

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to Butyl-TOYOPEARL 650M equilibrated in buffer D [100 mM Tris–HCl (pH 7.8), 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM L-malate, 1 mM PMSF, 5 μg ml^{-1} chymostatin and 10% (w/v) glycerol] with 20% (w/v) $(NH_4)_2SO_4$. The Butyl-TOYOPEARL 650M column was washed with an equal volume of the equilibration solution, and PEPCase protein was eluted with a linear gradient of 100–0% buffer A with 20% (w/v) $(NH_4)_2SO_4$ and a linear gradient of 0–100% buffer D simultaneously. The fractions including PEPCase activity were collected and subjected to diafiltration against buffer C overnight and loaded on to DEAE-TOYOPEARL 650M equilibrated in buffer C. The DEAE-TOYOPEARL 650M column was washed with an equal volume of the equilibration solution, and PEPCase was eluted with a linear gradient of 100–0% buffer D and a linear gradient of 0–100% buffer D with 0.5 M NaCl simultaneously. The eluted fractions exhibiting PEPCase activity were collected, diafiltered against buffer E [50 mM sodium phosphate (pH 7.8), 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM L-malate, 1 mM PMSF, 5 μg ml^{-1} chymostatin and 10% (w/v) glycerol] and loaded on to a hydroxyapatite column equilibrated with buffer E. The hydroxyapatite column was washed with an equal volume of the equilibration solution, and PEPCase was eluted by a linear gradient of 100–0% buffer E and a linear gradient of 0–100% buffer E with 400 mM NaCl simultaneously. The eluted fractions exhibiting PEPCase activity were collected, diafiltered against buffer D and loaded on to a Mono-Q chromatographic column (Pharmacia Biotech, Piscataway, New Jersey, USA). The same procedure was carried out using a DEAE-TOYOPEARL 650M column chromatography to obtain the purified PEPCase fraction.

Mining of co-expressed genes with Osppc1

We downloaded 624 rice gene expression profiles from a public database, the Gene Expression Omnibus (GEO) monitored by the Affymetrix rice genome array platform GPL2025 (Barrett *et al.*, 2010). The raw data were pre-processed using GCRMA routine in R/BioConductor (http://www.bioconductor.org/) to the $\log_2$-transformed gene expression level (Irizarry, 2003). After excluding the data of indica rice, callus, inoculated samples and seeds, the remaining 38 profiles were used to calculate the Pearson correlation efficiency between *Osppc1* and the other genes (supplementary Table S1, available online). Positive Pearson correlations were statistically validated by a test of non-correlations. Spearman correlation coefficients were calculated to extract reliable co-expression relationships. The functional annotations of the rice genes were obtained from OryzaExpress (Hamada *et al.*, 2011).

Results

cDNA cloning of PEPCase Osppc1 *and* Osppc3

We isolated PEPCase cDNAs from developing rice seeds at 7 d after flowering and obtained 3.3 kb of a full-length cDNA and 1.1 kb of a partial-length cDNA of PEPCase. By searching against the rice whole-genome sequence, we mapped these cDNA sequences into the corresponding genes that were annotated as PEPCase, MSU ID: LOC_Os02g14770 and LOC_Os01g55350, respectively, at the Rice Genome Annotation Project. These genes were described as *Osppc1* and *Osppc3* by Masumoto

Figure 1. Graphical representation of primary structures of *Osppc1* and *Osppc3*. Open reading frames of *Osppc1* and *Osppc3* are shown. The black bar shows the longest open reading frame and the hatched bar represents poly(A). Sequence alignment of the deduced polypeptide of the 5′ region of *Osppc1* and maize root-type PEPCase is shown in the box.

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et al. (2010). According to the genomic sequence of the latter gene, we amplified the corresponding full-length cDNA from developing rice seeds by RT-PCR using a 5′-UTR and 3′-UTR sequence primer set (5′-UTR–GGGAAAAGGAGTCGGCTTTC-, 3′-UTR–CCATAACACACCATCAGAAC-) and cloned it into pGEM-T Easy vector (Promega). The insert sequence was confirmed to be identical to the sequence of the partial cDNA clone and the predicted coding sequence in the rice genome.

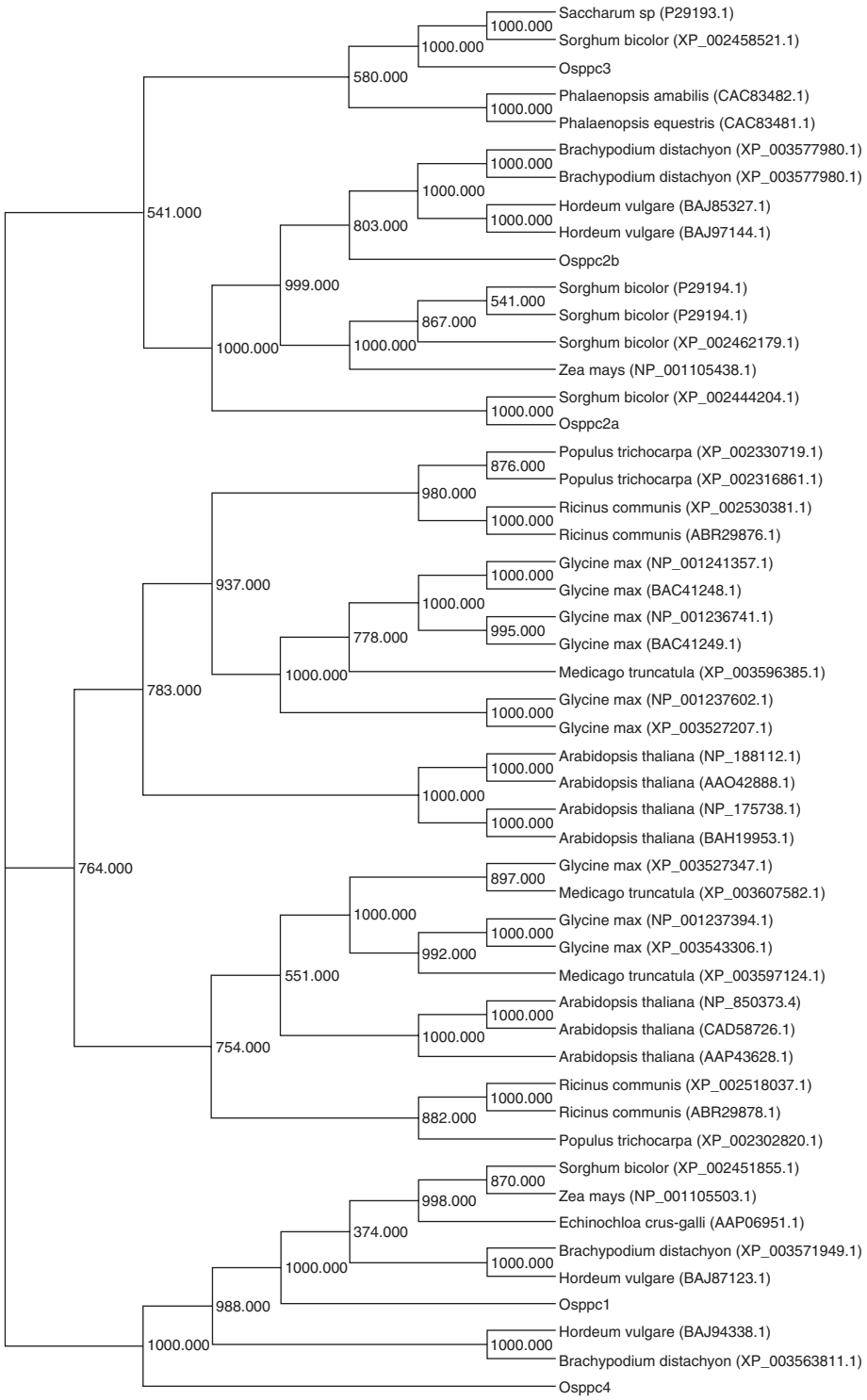

Figure 2. A phylogenetic tree of PEPCases. The tree was constructed from 51 polypeptide sequences including Osppc1, Osppc2a, Osppc2b, Osppc3 and Osppc4.

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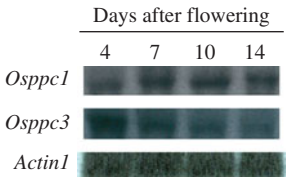

Figure 3. (colour online) Gene expression of *Osppc1* and *Osppc3* in developing rice seeds.

Sequence analysis of PEPCase Osppc1 *and* Osppc3 *cDNA*

The nucleotide sequences of the 3308 bp cDNA insert of *Osppc1* and 2961 bp cDNA insert of *Osppc3* contain the longest open reading frames: 2904 bp in the second frame and 2898 bp in the third frame, respectively (Fig. 1). We noticed that the potential translation initiation codon of *Osppc1* could be assigned to either the first ATG at positions 161–163 or the second ATG at positions 188–190 in frame according to the sequence alignment with the maize PEPCase cDNA sequence (AB012228). We designated the second ATG as the translation initiation codon based on the similarity to the maize gene sequence (Dong *et al.*, 1998). The deduced polypeptide sequences from *Osppc1* and *Osppc3* showed typical conserved features of plant PEPCase (Kai *et al.*, 2003). These sequence regions included the consensus sequences for the PEPCase motifs; phosphorylation site, catalytic bases, G6P binding site, hydrophobic pockets, PEP binding site, tetramer formation, Mg^{2+} binding site, HCO_3^- binding site, PEP and aspartate binding site (see supplementary Fig. S1, available online). Both sequences possess an alanine residue at position 778/775 which is equivalent to position 774 of the C_4 PEPCases of *Flaveria* (Svensson *et al.*, 1997). This implies that *Osppc1* and *Osppc3* encode a PEPCase having substrate affinities similar to those of the non-photosynthetic type PEPCase (Bläsing *et al.*, 2000).

We constructed a phylogenetic tree of Osppc1, Osppc3 and other PEPCases from 51 polypeptide sequences deposited in GenBank to investigate the molecular evolutionary relationship among them (Fig. 2). Osppc1 and Osppc3 were clustered into subfamilies comprising genes of monocotyledonous plant species and gramineous plant species genes, respectively. In our phylogenetic tree, *Osppc1* is likely to be the orthologue of the maize root-type gene (NP_001105503.1), whereas *Osppc3* is likely to be the orthologue of the sugarcane PEPCase (P29193.1) argued as a housekeeping isoform (Albert *et al.*, 1992). The same results were also obtained in the case of phylogenetic analysis by Kimura's 2-parameter model (data not shown). Osppc1 shares significant homology with maize root-type PEPCase (95% identity, 98% similarity), and Osppc3 shares significant homology with the sugarcane PEPCase (92% identity, 98% similarity). These results suggest the following: (1) the gene family including *Osppc1* and *Osppc3* evolved from a common ancestor with monocotyledonous and dicotyledonous plant species before the divergence of C_3, C_4 and CAM plant species; (2) *Osppc1* might encode PEPCase with enzymatic properties similar to those of the maize root-form PEPCase; (3) the gene subfamily including *Osppc3* was evolved specifically in gramineous plant species; and (4) *Osppc3* might encode a housekeeping PEPCase.

Gene expression of Osppc1 *and* Osppc3 *in developing rice seeds*

To monitor the gene expression pattern of *Osppc1* and *Osppc3* in developing rice seeds, we performed Northern blot analysis by using gene-specific probes corresponding to the 3′-UTR sequences. We detected expression of these genes throughout the grain-filling stages (Fig. 3). The expression patterns of *Osppc1* and *Osppc3* were slightly different from each other; the expression of *Osppc1* was highest at 7 d after flowering (DAF) (early–middle stage), and the expression of *Osppc3* was highest at 4 DAF (early stage), although the mRNA accumulation of both genes decreased toward the end of the maturation stage. These decreasing trends are likely to be consistent with the pattern of PEPCase activity in developing rice seeds (Sugimoto *et al.*, 1997).

Enzymatic properties of Osppc1

In order to obtain biochemical information about *Osppc1*, we expressed the recombinant Osppc1 protein

Table 2. Purification of recombinant Osppc1 protein

Step	Protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification (-fold)	Yield (%)
Crude extract	216	106	0.49	1	100
$(NH_4)_2SO_4$	57.6	62.2	1.08	2.2	59
Butyl-TOYOPEARL	19.9	43.5	2.18	4.5	41
TALON Metal (1st)	2.15	21.4	9.97	20.3	20
TALON Metal (2nd)	0.50	10.1	20.3	41.5	10

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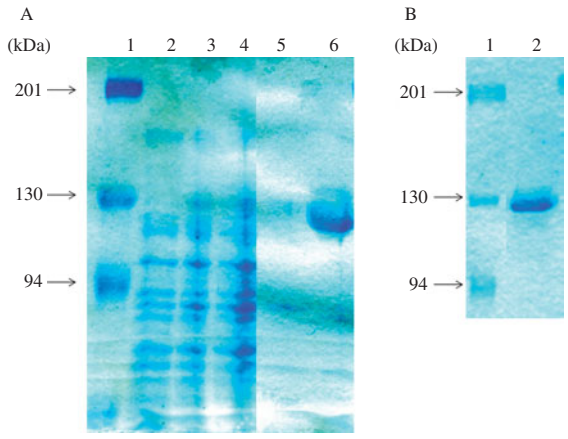

Figure 4. (colour online) SDS-PAGE analysis of recombinant Osppc1 protein. The fraction showing PEPCase activity from each purification step was electrophoresed and stained with Coomassie brilliant blue. The size of molecular mass markers is indicated on the left. (A) Lanes: 1, molecular mass marker; 2, crude extract (no insert); 3, crude extract; 4, $(NH_4)_2SO_4$ fractionation; 5, butyl-TOYOPEARL; 6, TALON Metal (first). (B) Lanes: 1, molecular mass marker; 2, TALON Metal (second).

in *E. coli* in order to characterize its kinetics. The recombinant protein from the cells was purified by monitoring PEPCase activity, as summarized in Table 2. The relative PEPCase activity of the purified recombinant protein was 20.3 units (mg protein)$^{-1}$, which was 41.5-fold that of the crude extract. The detected polypeptide showed mobility similar to a molecular weight marker of 130 kDa, which was consistent with the expected size of the deduced polypeptide comprised of 1028 amino acids, including His-tagged sequences (Fig. 4). We confirmed that a His-tagged protein showing mobility identical to the polypeptide in SDS-PAGE was detected in Western blotting analysis using an anti-His-tag antibody (data not shown).

The enzymatic parameters of the Osppc1 protein were measured and compared with those of other PEPCase proteins characterized previously (Dong *et al.*, 1998; Masumoto *et al.*, 2010). The pH optimum of Osppc1 was determined to be 9.0, and the relative activity at pH 7.0 was decreased to approximately 75% of that at pH 9.0. The fundamental parameters shown in Table 3 are similar to those of the maize root-type PEPCase. Nevertheless, the regulatory properties appeared to be different with respect to the inhibitory effect of malate, which is one of the allosteric effectors of PEPCase (Table 4). Compared to other rice PEPCase enzymes, the I_{50} (malate) of Osppc1 was higher. This suggests that Osppc1 works more actively in an intracellular environment close to pH 7.0 than the other PEPCases listed. The influences of G6P and glycine on the inhibitory effect of malate seem to be similar to those of maize root-type PEPCase.

Detection of the PEPCase protein in developing rice seeds

We prepared a partially purified PEPCase from rice seeds in the middle of the maturation stage. The relative activity was 35.6 U (mg protein)$^{-1}$. In the partially purified PEPCase fraction of developing rice seeds, we detected two major polypeptides in the 97–116 kDa mass range (Fig. 5A). These two polypeptides were also immunologically detected using a polyclonal antibody against a soybean mature seed PEPCase with the same mobility (data not shown). The estimated molecular weights of the polypeptides were 109.5 and 103.5 kDa, respectively. These values were quite close to the molecular weights of the deduced proteins of *Osppc1* (109.2 kDa) and *Osppc3* (105.3 kDa), respectively (see supplementary Table S2, available online). Two polypeptides were also detected in both embryos and endosperms with aleurone layers (Fig. 5B).

Co-expressed genes of Osppc1 *in rice plants*

In order to investigate whether *Osppc1* is co-regulated with any other metabolic gene in amino-acid synthetic pathways, we mined co-expressed genes with *Osppc1* from DNA microarray data of the Affymetrix rice gene chip. Under the threshold of 1% statistical significance for Pearson correlation, we found that 47 putative metabolic genes showed a strong positive correlation with *Osppc1*. This gene list was narrowed down to 28 genes to eliminate apparent false-positive correlations by checking the Spearman correlation efficiency at the threshold of 0.7 (Table 5). Out of the 28 genes, 22 genes were annotated to encode enzymes, including three aminotransferase genes: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and aminotransferase of class IV family. Enolase 2 (ENO2), succinate dehydrogenase flavoprotein subunit (SdhA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cysteine synthase were also listed.

The co-expressed genes that were assigned as the three aminotransferases, the cysteine synthase, the two glycolytic pathway enzymes and the TCA cycle enzyme protein mentioned above were reannotated by evaluating their protein sequences to clarify whether these genes could work coordinately with

Table 3. Fundamental kinetic parameters of Osppc1 protein

	Ospcc1		Maize root type	
	pH 7.3	pH 8.0	pH 7.3	pH 8.0
Vmax (unit mg^{-1})	26.6	31.4	21.8	28.3
Km (PEP) (mM)	0.144	0.082	0.05	0.04
Km (Mg^{2+}) (mM)	0.271	0.075	0.17	0.06
Km (HCO_3^-) (mM)	0.06	0.037	0.06	0.05

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Table 4. Effect of G6P and glycine on $I_{0.5}$* (L-malate)

	pH 7.3			pH 8.0		
Protein	None	G6P	Glycine	None	G6P	Glycine
Ospcc1	4.6	8.07	6.01	34.26	96.23	46.03
Maize root type	0.24	0.57	0.28	–	–	–
Osppc2a	0.06	–	–	–	–	–
Osppc4	0.7	–	–	–	–	–

* $I_{0.5}$ (mM) was determined as the concentration of L-malate in the reaction buffer to reduce PEPCase activity by half. The concentration of both glucose-6-phosphate (G6P) and glycine was 10 mM. Parameters of recombinant maize root-type PEPCase are from Dong *et al.* (1998). Parameters of recombinant Osppc2a and Osppc4 are from Masumoto *et al.* (2010).

Osppc1. LOC_Os01g55540, annotated as AST, was also described as a cytoplasmic AST (Song *et al.*, 1996), the enzyme of which catalyses the reversible reaction between OAA and aspartate. The AST, which was called OsAAT2, was overexpressed in rice, and enhanced seed nitrogen metabolism (Zhou *et al.*, 2009). LOC_Os10g25130, annotated as ALT, seemed to be a cytosolic enzyme because no signal, mitochondrial targeting or chloroplast transit peptides were found in iPSORT prediction (http://ipsort.hgc.jp/). Kikuchi *et al.* (1999) observed that ALT was expressed in the maturing endosperm and suggested the involvement in nitrogen metabolism during the maturation of rice seeds. LOC_Os03g24460, annotated as an aminotransferase of class IV family, was also assigned as a branched-chain amino acid aminotransferase (BCAT) in the UniProt database (http://www.uniprot.org/). BCAT catalyses the synthesis or the degradation of leucine, isoleucine and valine (Diebold *et al.*, 2002). The intracellular localization of this BCAT protein was predicted as a plastid gene by iPSORT. The ENO2 (LOC_Os03g14450) and the cysteine synthase (LOC_Os03g53650) had no signal, mitochondrial targeting or chloroplast transit peptides in iPSORT prediction. In contrast, the SdhA (LOC_Os07g04240) and the GAPDH (LOC_Os02g38920) had mitochondrial targeting signal sequences. Therefore, given the above facts, the ENO2, the AST and the ALT were mapped near the PEPCase reaction on the metabolic pathway (Fig. 6).

Discussion

In the present study, we isolated two full-length cDNAs of PEPCases *Osppc1* and *Osppc3* that were expressed in developing rice seeds. The deduced amino acid sequence of *Osppc1* was quite similar to that of maize root-form PEPCase and shared the typical conserved features of plant PEPCases. Biochemical assay of the recombinant protein of *Osppc1* demonstrated that its kinetic properties were similar to those of the maize PEPCase, except for the inhibition status by malate. We observed that its gene expression was high in the early and the middle stages of grain filling, in which PEPCase activity was also high in parallel (Sugimoto *et al.*, 1997). The corresponding PEPCase protein of *Osppc1* seemed to accumulate in developing rice seeds at these stages. During these stages, nitrogen accumulation and synthesis of storage proteins in rice grains were notable (Yamagata *et al.*, 1982; Iwasaki *et al.*, 1992). Considering all these results together, we propose that *Osppc1* is involved in nitrogen accumulation in developing rice seeds.

Interestingly, co-expression analysis indicated that *Osppc1* was coordinately regulated with the two aminotransferases AST and ALT that seem to be involved in seed nitrogen metabolism. These aminotransferase genes were strongly expressed in developing seed tissues (Kikuchi *et al.*, 1999; Zhou *et al.*, 2009). Therefore, Osppc1, AST and ALT might be involved in the same biological process in immature seeds. On the metabolic map, AST and ALT are located near the PEPCase reaction (Fig. 6). OAA generated from phospho*enol*pyruvate could be used for synthesis of aspartic acid (ASP) and 2-oxoglutarate (2-OG) by AST. 2-OG is a substrate of ALT, which generates glutamic acid (Glu) and pyruvate. Asp, Glu and pyruvate are precursors of other amino acids. So, the function of Osppc1 might be to provide OAA which is consumed in amino acid synthesis. Analysis of mutants or

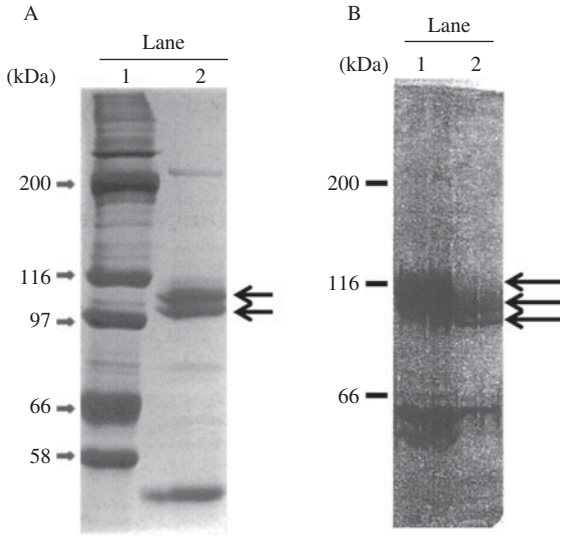

Figure 5. (A) SDS-PAGE analysis of purified PEPCase from developing rice seeds. Lanes: 1, molecular weight marker; 2, PEPCase fraction. (B) Immunological detection of PEPCase polypeptides in developing rice seeds using a polyclonal antibody of soybean seed PEPCase (Sugimoto *et al.*, 1992). Embryos and endosperms were prepared from developing rice seeds at 10 d after flowering. Crude extract proteins from them were separated on SDS-PAGE and analysed by Western blotting. Lanes: 1, embryo; 2, endosperm.

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Table 5. Metabolic genes co-expressed with *Osppc1* in rice vegetative organs

Locus ID	Pearson correlation coefficient	No correlation test (*P* value)	Spearman correlation coefficient	Functional annotation
LOC_Os03g14450	0.777	9.58E−09	0.843	Enolase 2
LOC_Os05g43360	0.812	6.17E−10	0.711	Thioredoxin fold domain containing protein
LOC_Os02g55890	0.822	2.52E−10	0.710	H()-translocating (Pyrophosphate-ENERGIZED) inorganic pyrophosphatase beta-1 polypeptide
LOC_Os03g19890	0.723	2.95E-07	0.704	NADH−ubiquinone oxidoreductase
LOC_Os03g57120	0.796	2.25E−09	0.721	Ferredoxin−NADP reductase
LOC_Os06g51150	0.799	1.75E−09	0.748	Catalase isozyme B
LOC_Os04g56540	0.777	9.92E−09	0.701	ATPase V0 complex, subunit E domain containing protein
LOC_Os02g19140	0.728	2.28E−07	0.728	Ubiquitin ligase SINAT5
LOC_Os01g42430	0.762	2.74E−08	0.702	ATPeVPF, ATP6F, V-type H + -transporting ATPase 21 kDa proteolipid subunit
LOC_Os07g04240	0.714	4.76E−07	0.740	Succinate dehydrogenase flavoprotein subunit, mitochondrial
LOC_Os03g24460	0.760	3.04E−08	0.767	Aminotransferase, class IV family protein
LOC_Os04g39020	0.810	7.18E−10	0.769	Betaine aldehyde dehydrogenase 1
LOC_Os03g01810	0.759	3.44E−08	0.715	Charged multivescicular body protein 3
LOC_Os10g25130	0.775	1.12E−08	0.717	Alanine aminotransferase
LOC_Os01g01369	0.809	8.05E−10	0.717	Sterol-8,7-isomerase
LOC_Os03g16150	0.702	8.95E−07	0.749	Mannose-1-phosphate guanyltransferase
LOC_Os02g04700	0.775	1.14E−08	0.763	Dars-prov protein
LOC_Os04g34630	0.862	3.76E−12	0.740	Peroxidase
LOC_Os02g38920	0.843	3.23E−11	0.773	Glyceraldehyde-3-phosphate dehydrogenase
LOC_Os02g56300	0.768	1.82E−08	0.737	Pyridoxal phosphate-dependent transferase
LOC_Os03g53650	0.786	4.93E−09	0.808	Cysteine synthase
LOC_Os05g25310	0.902	1.08E−14	0.874	AMP-dependent synthetase and ligase domain containing protein
LOC_Os01g40470	0.813	5.49E−10	0.780	Vacuolar ATP synthase subunit d
LOC_Os08g10600	0.718	3.92E−07	0.784	UTP−glucose-1-phosphate uridylyltransferase family protein
LOC_Os11g10510	0.875	6.79E−13	0.743	Alcohol dehydrogenase
LOC_Os01g55540	0.760	3.03E−08	0.811	Aspartate aminotransferase, cytoplasmic
LOC_Os08g33200	0.745	7.91E−08	0.716	Chaperonin Cpn60/TCP-1 family protein
LOC_Os03g15860	0.814	4.98E−10	0.788	Mitochondrial substrate carrier family protein

transgenic rice lines is expected to verify the physiological role of *Osppc1* in nitrogen metabolism.

PEPCase might be one of the essential enzymes to achieve an increased flow rate of the AST reaction. Sentoku *et al.* (2000) reported that the overexpression of proso millet cytoplasmic AST led to the enhancement of PEPC protein expression in transgenic tobacco. This observation suggests that a metabolic change caused by overexpression of the cytoplasmic AST affects the regulation of the PEPCase gene. Enhancement of the AST reaction would induce the consumption of cytosolic OAA and Glu and the production of cytosolic Asp, 2-OG and the derivatives of Asp and 2-OG. According to Zhou *et al.* (2009), overexpression of cytoplasmic AST enhanced amino acid and protein content in rice seeds, suggesting that free amino acids were richer during maturation in the transgenic rice. Thus, it seems that PEPCase responds to conditions of low C/N ratio for replenishment of OAA. This concept agrees with the report that RNAi knockdown of the chloroplast PEPCase, Osppc4, suppressed ammonium assimilation and amino acid synthesis by reducing levels of organic acids (Masumoto *et al.*, 2010). For production of rice seeds with much higher protein content, *Osppc1* might be useful, together with other key genes such as AST and ALT, in combinational genetic transformation, which can enhance the genes of several consecutive enzymes on a metabolic pathway (Zhu *et al.*, 2008).

The existence of PEPCase isoforms in immature seeds has been described in several plant species. We isolated two cDNAs of PEPCase in a developing soybean seed library (Sugimoto *et al.*, 1992) and confirmed the accumulation of two PEPCase polypeptides in the seeds (unpublished data). In faba bean, the cDNAs of two PEPCase isoforms were isolated, and their expression patterns differed (Golombek *et al.*, 1999). Moreover, Blonde and Plaxton (2003) reported that there were two C_3 PEPCase isoforms that were likely to be involved in supporting the synthesis of storage proteins and fatty

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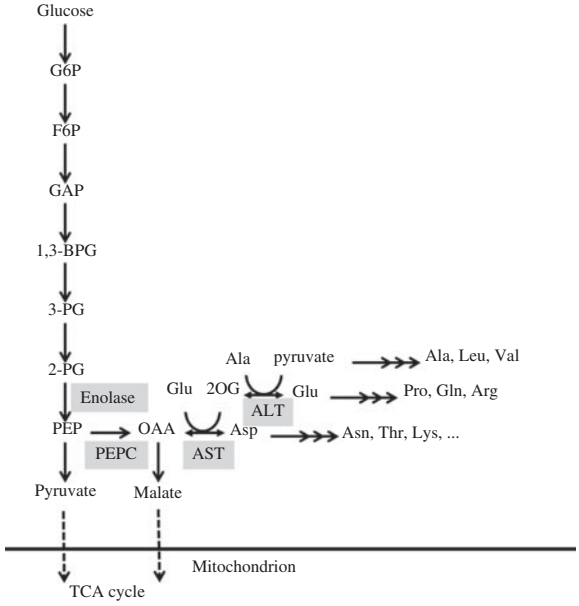

Figure 6. A metabolic pathway map of glycolysis with phospho*enol*pyruvate carboxylase (PEPC), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) reactions. The enolase, PEPC, AST and ALT were mapped on the consecutive reactions from 2-phosphoglyceric acid (2-PG) to glutamate (Glu) and pyruvate.

acids, respectively, in castor bean seeds. With regard to monocotyledonous species, González *et al.* (1998) reported that two main PEPCase polypeptides with apparent molecular masses of 108 and 103 kDa were detected in developing wheat grains. Consistent with these findings, in the present work we isolated cDNAs of two PEPCase isoforms that were expressed in developing rice seeds, and detected two PEPCase polypeptides with apparent molecular masses of 109.5 and 103.5 kDa that seemed to correspond to the cDNAs. As a preliminary result, we observed that two main PEPCase polypeptides were accumulated in rice embryos and endosperms with aleurone layers. In a public gene expression database from microarray and RNAseq (Sato *et al.*, 2013; the Rice Genome Annotation Project: http://rice.plantbiology.msu.edu/), we observed that *Osppc1* was strongly expressed in endosperm and embryo. On the contrary, we observed that *Osppc2b* was expressed moderately in embryo. The expression levels of the other PEPCase isoforms genes were low. Further analysis of the localization of each PEPCase polypeptide would help to clarify the functional partitioning of the isoforms in developing rice seeds.

Our phylogenetic analysis revealed that *Osppc1* was in the same gene family as maize root-form PEPCase. The fundamental enzymatic properties of both proteins were similar to each other, as shown by the biochemical results (Table 3). However, the regulatory properties of malate at pH 7.3 diverged (Table 4). The differing regulatory properties might be due to the evolutionary changes among PEPCases at the amino acid sequence level. Among the sequence differences between Osppc1 and maize root-form PEPCase, we found conservation of ten sites between Osppc1 and a PEPCase isoform in *Hordeum vulgare* (BAJ94338.1), which is in the nearest location in our phylogenetic tree (see supplementary Fig. S2, available online). With regard to eight of these sites, the amino acid changes yielded different characteristics in polarity or charge. Further studies, such as site-directed mutagenesis analyses, will be needed to investigate factors determining malate inhibition.

A possible physiological explanation for the kinetic property of Osppc1 in malate inhibition is that it is a countermeasure against hypoxia. It is well known that in plant root cells under oxygen deprivation the cytosol is acidified (Roberts *et al.*, 1992; Xia and Saglio, 1992; Gout *et al.*, 2001; Tournaire-Roux *et al.*, 2003). Paddy rice plants are occasionally grown under a low oxygen environment. Roberts *et al.* (1992) showed that the concentration of malate in root cells was immediately increased for alanine synthesis when rice plants were exposed to hypoxia. Rolletschek *et al.* (2005) reported a marked degree of oxygen starvation in developing soybean seeds because of the consumption of ATP for the synthesis of storage compounds such as lipids and proteins. A decrease of pH of barley starchy endosperm during the maturation period has also been observed with an increase in the malate pool (Macnicol and Jacobsen, 1992).

We are still conducting our studies on *Osppc3*. Its deduced amino acid sequence is relatively similar to those of the isoforms *Osppc2a* and *Osppc2b*, but its expression seems to be restricted to spikelets (Masumoto *et al.*, 2010). Notably, *SCPEPCD1*, an orthologous gene of *Osppc3* in sugarcane, was expressed in several organs, especially stems (Albert *et al.*, 1992). In order to investigate the biochemical properties of Osppc3, we have established a construct to express the recombinant protein in *E. coli*. Our *in silico* analysis using Affymetrix gene chip data in rice might also provide an opportunity to determine the function of *Osppc3* in developing rice seeds.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0960258513000354.

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Conflicts of interest

None.

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