The metabolism of phospho*enol*pyruvate in thermogenic spadices of skunk cabbage, *Symplocarpus renifolius*

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Chapter 1

Thesis Introduction

Chapter 1

Metabolic pathways are paramount important for carbon and energy metabolism in plants. The complexity of plant metabolic pathways are need to be overcome to understand intermingled points of carbon and energy metabolism focusing on recent technological development (Farré et al., 2014). Most of the metabolic pathways are interconnected to each other and major challenges are to be uncovered the regulatory mechanism of metabolic networks (Lunn, 2007). Photosynthesis and respiration are the fundamental pathways of carbon and energy metabolism in plants. Photosynthesis utilizes CO_2, H_2O and light to synthesis carbohydrates with the concomitant release of O_2, whilst respiration uses these carbohydrates accomplishing to support growth, development and physiological regulation through primary metabolism associated with release of energy as ATP (Vanlerberghe, 2013). All of these, glycolysis is a central pathway of carbohydrate metabolism among living organisms from bacteria to plants and humans (Fig.1) (Scrutton and Utter, 1968; Plaxton, 1996). In the glycolytic pathway, phospho*enol*pyruvate (PEP) is an important intermediate, as it occupies the highest position on the thermodynamic scale of known phosphorylated metabolites (Davies, 1979). In the thermodynamic scale, the Gibbs free energy change of PEP is -61.9 KJ/mole (Table 1) (Frey and Arabshahi, 1995; Nelson and Cox, 1996), which is very important to determine whether direction a biochemical reaction or pathway (Noor et al., 2014; Fromm et al., 2016). In

animal cells, PEP is predominantly catalyzed by pyruvate kinase (PK; EC 2.7.1.40) with the concomitant phosphorylation of ADP to ATP (Scrutton and Utter, 1968). However, in addition to PK, PEP carboxylase (PEPC; EC 4.1.1.31) and PEP phosphatase (PEPtase; EC 3.1.3.60) play roles in the catabolism of PEP and overall regulation of mitochondrial respiration in plant cells (Plaxton, 1996; O'Leary et al., 2011). In contrast, PEP carboxykinase (PEPCK; EC 4.1.1.49) is an enzyme involved in gluconeogenesis (Scrutton and Utter, 1968). Thermogenesis is uncommon phenomena in plant which increased temperature due to endogenous heat-production instead of energy and several species belongs to the family of Araceae have been characterized as thermogenic plants (Nagy et al., 1972; Meeuse and Raskin, 1988; Ito et al., 2003; Seymour et al., 2003; Seymour et al., 2004). The glycolytic pathway is important in thermogenic plants such as skunk cabbage (*Symplocarpus renifolius*), which utilize carbohydrates as a major respiratory substrate for their metabolic heat-production (Fig. 1) (Seymour and Blaylock, 1999). During the metabolic heat-production alternative respiratory pathway which is called cyanide-insensitive electron transport chain pathway has been activated due to activity of alternative oxidase (AOX) (Meeuse, 1975; Meeuse and Raskin, 1988). AOX is located inner mitochondrial membrane and catalyzes the oxidation of ubiquinol producing water by reducing oxygen through complex I and II (Fig. 2) (Sunderhaus et al., 2006; Bar-Even et al., 2012). In the case of *S. renifolius*, organ-specific thermogenesis occurs in the inflorescence known as the spadix, and maintains a temperature of approximately 23°C during

flowering, even when ambient temperatures drop below freezing (Knutson, 1974; Onda et al., 2008; Seymour et al., 2009). Because the mitochondrial cyanide-insensitive AOX allows for a dramatic decrease in free energy between ubiquinol and oxygen (Moore and Bonner, 1982; Moore et al., 2013), *SrAOX* identified in *S. renifolius* is likely to play a role in thermogenesis in this plant (Onda et al., 2007; Onda et al., 2008; Kakizaki et al., 2012). Previous study showed that sustained thermogenesis in spadices were associated with the import of carbohydrates including sucrose, glucose, and fructose from roots (Onda and Ito, 2005). Although these results suggest that glycolysis and subsequent AOX-mediated mitochondrial respiration plays a crucial role in organ-specific thermogenesis in *S. renifolius*, the mechanisms of carbohydrate metabolism in this plant remain poorly understood. To summarize the above literature review, the carbohydrate metabolism is very important to provide metabolic substrate to the electron transport chain through respiratory complex I and II to the AOX for producing endogenous heat in the thermogenic tissues (Fig. 2). As PEP comprises final steps of glycolytic pathways, can be branched into either PK and PEPtase or PEPC and PEPCK mediated pathway to form pyruvate or oxaloacetic acid (OAA), respectively (Fig. 1). Hence, identification of PEP metabolism pathway could be interesting tools to pave the thermoregulatory mechanisms in thermogenic spadices of *S. renifolius*. To unveil the PEP metabolic pathway in the thermogenic spadices of *S. renifolius*, I have conducted three studies that are to be presented in this thesis in three different chapters. I have sequenced

four full length cDNAs of PK, PEPtase, PEPC and PEPCK which are involved in PEP metabolism in *S. renifolius*. Thereafter, I have investigated tissue specific gene expression level by quantitative reverse transcription polymerase chain reaction (qRT-PCR) to identify the major gene in this pathway. Finally, I confirmed that PEPC expression is higher in florets, petal and pistil than other genes and tissues which are presented in the Chapter 2. In chapter 3, I extracted crude enzymes from the thermogenic florets of *S. renifolius* from different samples to assay enzymes activities. The study of enzymes assay revealed that PEPC activity is higher than PK, PEPtase and PEPCK in thermogenic florets. This study suggest that PEP metabolism follows PEPC mediated pathway. The following chapter, I discussed about the data of mitochondrial respiration analysis. In this experiment, I have purified intact mitochondria from the thermogenic florets and determined different substrates dependent oxygen-consumption to know the respiratory substrate for electron transport chain. These data suggested that pyruvate is not a metabolic substrate for AOX respiration, whilst malate significantly increased AOX respiration which strongly recommended that PEP metabolism follows PEPC mediated pathway and intra-mitochondral produced pyruvate stimulate AOX respiration. These results have been documented in the chapter 4. Finally, I have summarized the above findings and presented in the chapter 5. I discussed about the metabolic pathways of PEP and its significance for the heat-production during thermogenesis in this chapter. I have also recommended future perspective role of PEPC for thermoregulatory

mechanism and how they recycle CO_2 produced inside the tricarboxylic acid (TCA) cycle by respiratory complexes. Based on my findings, future research interests have been suggested for the understanding the most significant bottlenecks to be overcome to unlock the thermoregulatory mechanism in the thermogenic spadices in *S. renifolius*.

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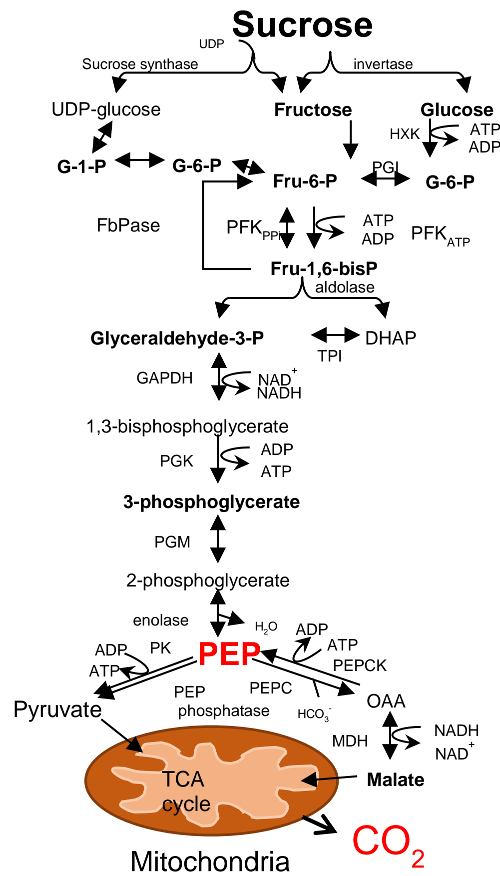

Figure 1. Carbohydrate metabolism in thermogenic cells of *S. renifolius.* Sucrose, glucose and fructose are the main substrates for the glycolysis. PEP is the end product and yielded by glycolytic pathway is predominantly catabolized by either PEPC to yield oxaloacetic acid (OAA) or pyruvate (Pyr) by PK and PEPtase. OAA is either directly oxidized in the mitochondria or converted to malate (Mal) by cytosolic malate dehydrogenase (MDH) or converted to PEP by catabolized by PEPCK. Abbreviations: UDP, Uridine diphosphate, HXK, Hexokinase; PGI, Phosphoglucose isomerase; G-1-P, Glucose-1-phosphate; G-6-P, Glucose-6-phosphate; Fru-6-P, Fructose-6-phosphate; FbPase, Fru-1,6-bisP, Fructose 1,6-bisphosphate; Phosphofructo bisphosphatase; PFK, Phosphofructokinase; TPI, Triose-phosphate isomerase; DHAP, Dihydroxyacetone phosphate; GAPDH, Glyceraldehyde 3- phosphate dehydrogenase; PGK, Phosphoglycerate kinase; PGM, Phosphoglycerate mutase; NAD, Nicotinamide adenine dinucleotide; ADP, Adenosine diphosphate, ATP, Adenosine triphosphate; PK, Pyruvate kinase; PEP, Phospho*enol*pyruvate; PEPCK, Phospho*enol*pyruvate carboxykinase; PEPtase, PEP phosphatase.

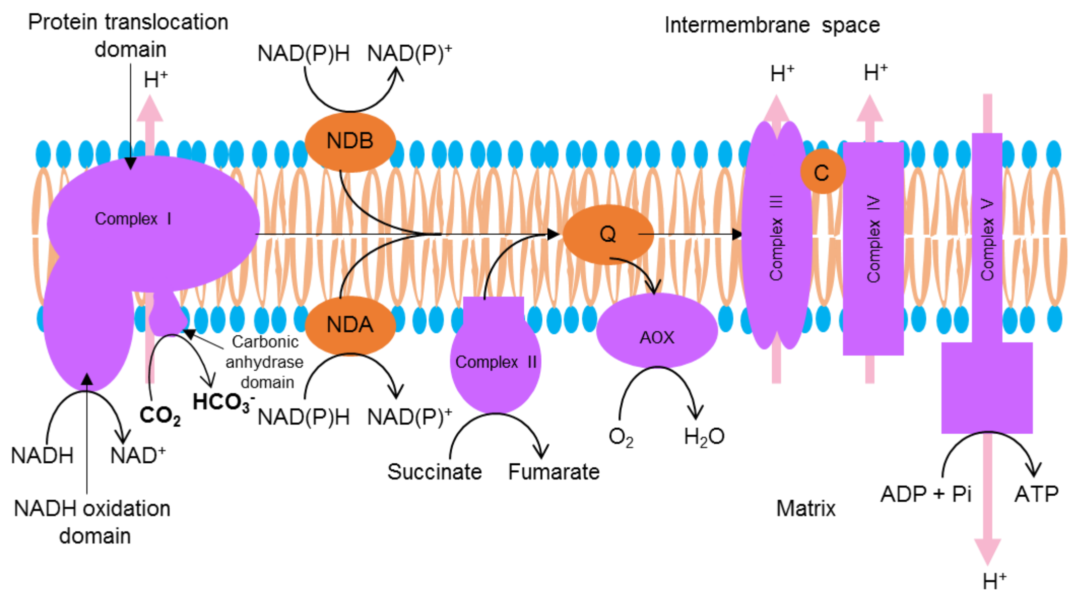

Figure 2. Schematic representation of respiratory electron transport chain in the thermogenic spadix of *S. renifolius.* Abbreviations: NDA and NDB, Type II or alternative NAD(P)H dehydrogenase on the internal and external surfaces of the membrane, respectively; AOX, Alternative oxidase.

Table 1. Energy rich biomolecules with their free energy changes (Neloson, DL and Cox, MM, 2008; Frey, PA and Arabshahi, 1995).

Biomolecules	Energy, $\Delta G'^{o}$ (KJ/mol)
Phospho*enol*pyurvate	-61.9
1,3-bisphosphoglycerate	-49.3
Adenosine triphosphate (→AMP+PPi)	-45.6
Phosphocreatine	-43.0
Adenosinediphosphate (→AMP+Pi)	-32.8
Acetyl-CoA	-31.4
Adenosine triphosphate(→ADP+Pi)	-30.5
Glucose 3-phosphate	-20.9
Pyrophosphate	-19.2
Fructose 6-phosphate	-15.9
Adenosine monophosphate (→adenosine +Pi)	-14.2
Glucose 6-phosphate	-13.8
Glycerol 3-phosphate	-9.2

Chapter 2

cDNA cloning and tissue specific expression analysis of PEPC, PK, PEPtase and PEPCK transcripts in skunk cabbage, *Symplocarpus renifolius*

Chapter 2

Summary

Skunk cabbage (*Symplocarpus renifolius*) blooms in early spring and its inflorescence, called the spadix, is capable of producing enough heat to melt the snow. Here, I isolated cDNAs encoding PEPC, PK, PEPCK and PEPtase, all of which are associated with PEP metabolism in glycolytic pathway. qRT-PCR analyses revealed that *SrPEPC* is abundantly co-expressed with transcripts of mitochondrial *SrAOX* in the florets of thermogenic spadices. Interestingly, petal and pistil in the florets showed a considerably higher expression of *SrPEPC* and *SrAOX* than those of *SrPK*, *SrPEPCK* and *SrPEPtase* transcripts.

Introduction

Thermogenic activities have been described in the floral parts of some species in *Araceae* (Nagy et al., 1972; Knutson, 1974; Seymour and Blaylock, 1999; Ito et al., 2004). These plants rapidly increases respiratory rates, resulting in sufficient heat production in spadices (Knutson, 1974). The mechanisms of heating are quite different between species depending on various climatic factors whilst they showed similar degrees of thermoregulatory principle (Gibernau et al., 2004; Umekawa et al., 2016). For instance, *S. renfolius* and *A. maculatum*, are two thermogenic species showed

different molecular bases underlying mitochondrial respiratory activities (Kakizaki et al., 2012) whereas, both of these species utilized carbohydrate as their respiratory substrate during heat production (ap Rees et al., 1976; ap Rees et al., 1977; Onda and Ito, 2005).

It has been reported that respiratory quotient about 1 and subsequently, sucrose, glucose and fructose concentration were found to be 2-5 mM in xylem sap in thermogenic stage of *S. renifolius* indicated that the carbohydrate is the respiratory substrate (Seymour and Blaylock, 1999; Onda and Ito, 2005). Similarly, carbohydrate oxidation predominantly occurred in glycolysis before and during thermogenesis, thereafter, high rate of glycolysis has been observed when starch breakdown experiment was conducted in the thermogenic spadices of *A. maculatum* still attached with the plant (ap Rees et al., 1976; ap Rees et al., 1977). Considering the above information, it can be assumed that glycolytic pathway plays a central role to supply the metabolic intermediates into the TCA cycle during floral thermogenesis, where PEP metabolism plays a key role for controlling the plant glycolysis (O'Leary et al., 2011). Intriguingly, PEP metabolism is followed by PEPC, because of extremely high activities PEPC in thermogenic tissues in some species of Araceae including *A. maculatum* (ap Rees et al., 1981). On the other hand, the PEPC activities in thermogenic spadix of *S. renifolius* remains unknown.

Biological functions of PEPC would be interesting subject to pave the way to unlock the thermoregulatory mechanism.

Generally, PEP metabolism can be carried out by either one or both PK and PEPtase to produce pyruvate. Alternatively, PEP can be converted into OAA by PEPC and OAA can be converted into PEP by PEPCK. Interestingly, OAA is the metabolic product of PEPC rapidly decarboxylated and produced malate by malate dehydrogenase (MDH). All of these information suggested the first hand idea to identify the possible metabolic pathway of PEP metabolism which will help for understanding the thermoregulatory phenomena in *S. renifolius,* because there is no data regarding the PEP metabolism in this plant. To elucidate the PEP metabolism, I isolated and sequenced the full length cDNAs of PK, PEPtase, PEPC and PEPCK which are involved in PEP metabolism. I also determined the tissue specific expression level of PK, PEPtase, PEPC and PEPCK transcripts.

Materials and methods

Plant materials

All plant materials were sampled from wild *S. renifolius* grown outdoors. For thermal imaging, *S. renifolius* plants that were transplanted from Hakuba (Nagano prefecture, Japan) to Iwate University campus (Iwate

prefecture, Japan) in April 2005 were used (Onda et al., 2008). For preparation of total RNAs, fresh spathe, leaf, floret and pith were collected from *S. renifolius* at Fujine (Iwate prefecture, Japan) on April 3, 2012 and at Omori (Akita prefecture, Japan) on April 1, 2014.

Temperature measurements

Temperature of the spadices were measured by automatic temperature recording thermometer attached to an electronic thermocouple (TR-5106; T & D) punched on the surface of spadix of female flower about 30 mm in depth at 1 minute interval. The ambient temperature was also measured by the same automatic thermometer 30 cm above the ground (Ito et al., 2003; Onda et al., 2008).

Thermal imaging

Thermal images were obtained using an infrared thermal camera as described previously (Onda et al., 2008).

Total RNA extraction, cDNA amplification, and isolation of full-length cDNAs encoding *SrPK*, *SrPEPtase*, *SrPEPC*, and *SrPEPCK*

Total RNAs were extracted from thermogenic florets using either an RNeasy Plant mini kit (Qiagen, Hilden, Germany) or a FastPure RNA kit

(Takara Bio, Shiga, Japan). First-strand cDNA synthesis was performed using PrimeScript™ 1st strand cDNA synthesis kits (Takara Bio) with oligo dT primers provided by the manufacturer.

Partial fragments of cDNAs encoding *PK*, *PEPtase*, *PEPC*, and *PEPCK* were initially amplified using first strand cDNA and Taq DNA polymerase (*Takara Ex Taq®*, Takara Bio). Primers were designed to target conserved *PK*, *PEPtase*, *PEPC*, and *PEPCK* gene sequences shared by *Arabidopsis thaliana*, *Oryza sativa*, *Solanum tuberosum*, *Zea mays*, and *Musa acuminata subsp. Malaccensis*, and are denoted PKF1-PKR1, PEPtaseF1-PEPtaseR1, PEPCF1-PEPCR1, and PEPCKF1-PEPCKR1 (Table 1), respectively. PCR amplification (My Cycler™ Thermal Cycler, BioRAD) was performed under the following conditions: 1 cycle at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 50°C (for *PEPC* and *PEPCK*) or 58°C (for *PK*) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. PCR amplification of *PEPtase* was performed under the following conditions: 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 68°C for 2 min with a final extension at 68°C for 5 min. PCR products were electrophoresed in 1% agarose gels and DNA bands of expected sizes were excised and purified using Nucleospin® Gel and PCR Clean-Up (Takara Bio). Purified DNA fragments were then ligated into T-Vector (pMD19, Takara Bio, or pCR2.1, Thermo Fisher Scientific) using DNA Ligation Kit <Mighty Mix> (Takara Bio) and were transformed into *E.coli* cells. Expected colonies contain *PK*,

PEPtase, *PEPC*, and *PEPCK* were identified and plasmids were purified by LaboPass^{TM} Mini Plasmid DNA Purification Kit (Hokkaido System Science) for sequencing. Gene specific primers (Table 2) were then designed for 5'- and 3'-Rapid Amplification of cDNA Ends (RACE) using SMARTer^{TM} RACE cDNA Amplification Kit (Takara Bio). SrPKF2, SrPKF3, SrPKF4 and SrPKR2, SrPKR3, SrPKR4 (Sr indicates *Symplocarpus renifolius*) were used to amplify targeted genes with Universal Primer Mix (UPM) for 3'- and 5'-RACE of *SrPK*, respectively. PCR amplification was performed under the following conditions: 5 cycles at 94°C for 30 s and 72°C for 3 min followed by 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, and finally 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. All PCR products for 5' and 3'-RACE were then mixed separately and applied to nested PCR analyses using SrPKR2 for 5'-RACE and SrPKF2 for 3'-RACE with Nested Universal Primer A (NUP), respectively. PCR amplification was performed under the following conditions: 1 cycle at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min.

SrPEPtaseF2 and SrPEPtaseR2 primers (Table 2) were used with UPM to amplify the gene for *SrPEPtase* in 3'- and 5'-RACE analyses, respectively. PCR reactions were performed under the following conditions: 5 cycles at 94°C for 30 s and 72°C for 3 min, followed by 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, and, finally, 25 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min. PCR products of 3'- and 5'-RACE were

used with NUP for nested PCR using SrPEPtaseF3 for 3'-RACE and SrPEPtaseR2 for 5'-RACE. PCR amplification was performed under the following conditions: 1 cycle at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min.

SrPEPCF2 and SrPEPCR2 primers (Table 2) were used for 3'- and 5'-RACE of *SrPEPC*, respectively. PCR amplification was performed under the following conditions: 5 cycles at 94°C for 30 s and 72°C for 3 min, followed by 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, and, finally, 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min.

SrPEPCKF2 and SrPEPCKR2 primers (Table 2) were used with UPM to amplify for 3'- and 5'-RACE of *SrPEPCK*, respectively, followed by nested PCR using SrPEPCKR2 and SrPEPCKF3 with NUP for 5'- and 3'-RACE of *SrPEPCK*, respectively. PCR amplification was performed under the following conditions: 5 cycles at 94°C for 30 s and 72°C for 3 min, followed by 5 cycles at 94°C for 30 s, 70°C for 30 s and 72°C for 3 min, and finally 25 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 3 min. The PCR products of 3'- and 5'-RACE were used for nested PCR using SrPEPCKF3 for 3'-RACE and SrPEPCKR2 for 5'-RACE with NUP. PCR amplification was performed under

the following conditions: 1 cycle at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 1 min with a final extension at 72°C for 7 min.

PCR products of 3'- and 5'-RACE of *SrPK*, *SrPEPtase*, *SrPEPC* and *SrPEPCK* were purified by QuickStep^{TM}2 PCR Purification Kit (Edge Bio) and then cloned into pMD19 with DNA Ligation Kit <Mighty Mix>. After transformation into *E.coli*, at least 10 colonies of 3'- RACE and 5'-RACE of all these genes were identified by colony PCR, and each plasmid was purified by LaboPass^{TM} Mini Plasmid DNA Purification Kit for sequencing.

To isolate full-length cDNAs, final PCR amplifications were performed with KOD polymerase (KOD -Plus-, Toyobo) using the following gene-specific primers (Table 3): SrPKF5-SrPKR5 for *SrPK*; SrPEPCF3-SrPEPCR3 for *SrPEPC*, and SrPEPCKF4-SrPEPCKR3 for *SrPEPCK*. PCR amplification was performed under the following conditions: 1 cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 2 min, 55°C for 30 s, and 68°C for 2 min. The primers SrPEPtaseF4-SrPEPtaseR3 for *SrPEPtase* were used under the following PCR conditions: 1 cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 60°C for 30 s, and 68°C for 2 min. Amplified PCR products were then subjected to 10x A-attachment mix (Toyobo), and incubated at 60°C for 10

min. Products were purified using a QuickStep™2 PCR Purification Kit. Purified fragments were then cloned into pMD19 using DNA Ligation Kit <Mighty Mix>. After transformation into *E.coli*, colonies of *SrPK*, *SrPEPtase*, *SrPEPC* and *SrPEPCK* were identified by colony PCR, and each plasmid was purified by LaboPass™ Mini Plasmid DNA Purification Kit for sequencing in both directions. Full-length sequences were determined by isolating at least four clones, including at least two clones with identical inserts. DNA sequences were analyzed using GENETYX software (Genetyx, Tokyo, Japan). Complete cDNAs encoding *SrPEPC*, *SrPK*, *SrPEPCK*, and *SrPEPtase* were deposited in the DNA Data Bank of Japan with Accession numbers LC155943, LC155944, LC155945, and LC155946, respectively.

Expression analyses of genes encoding *SrPK*, *SrPEPtase*, *SrPEPC*, and *SrPEPCK*

For expression analysis of *SrPK*, *SrPEPtase*, *SrPEPC* and *SrPEPCK*, total RNA was extracted from different tissues of *S. renifolius* like spathe, leaf, florets, pith, petal, stamen and pistil by RNeasy Plant mini kit (Qiagen). cDNA were synthesized using ReverTra Ace® qPCR RT kit (Toyobo) and quantitative real-time PCR was performed with SYBR Green Real-time PCR Master Mix-Plus (Toyobo) using a Thermal Cycler Dice (TP800; Takara Bio) instrument as described previously (Ito et al., 2011; Kakizaki et al., 2012). Gene-specific primers (Table 4) were designed from identified cDNAs and the

housekeeping gene *EF1α* was used as a normalization control (Kakizaki et al., 2012). qRT-PCR was performed under the following conditions: an initial denaturing step at 95^{0}C for for 30 seconds; 40 amplification cycles of 95^{0}C for 5 seconds and 60^{0}C for 30 seconds and a dissociation curve analysis at 95^{0}C for 15 seconds, 60^{0}C for 15 seconds and 95^{0}C for 30 seconds.

Phylogenetic tree analyses of PEPC proteins and sequence alignments

A phylogenetic tree was constructed using the neighbor-joining method with ClustalW (Thompson et al., 1994) for 22 PEPC proteins gathered from GeneBank®. A bootstrap consensus tree was inferred from 1000 replicates to represent the evolutionary history of the present taxa. Branches corresponding to partitions that were reproduced in less than 50% of bootstrap replicates were collapsed. Multiple sequence alignments were performed using GENETYX software.

Statistical Analysis

All data were compared using one-way factorial ANOVA (SPSS, IBM, Armonk, NY, USA). Tukey's honest significance posthoc tests were used to identify significantly different means. Significant differences between means were calculated at P = 0.05.

Results

Temperature measurements

I measured the temperatures of spadices of *S. renifolius* and ambient, respectively for 5 days continuously (details in materials and methods) in the field. These data shows that spadix temperature remained almost constant around 23°C, whereas ambient temperature fluctuated even went down to below freezing (Fig. 1). These data revealed that thermogenic stage of spadices sustained 2-3 days and produced sufficient heat to maintain their body temperature during heavy cold.

cDNA cloning and sequence analysis

I cloned partial cDNA of *SrPK*, *SrPEPtase*, *SrPEPC* and *SrPEPCK* about 903, 200, 723 and 776 bp, respectively in order to get full length cDNA of those genes (Fig. 2a-5a). 5′- and 3′-RACE were performed designing primer form the partial sequence to get at least four identical clone (Fig 2 b, c-5 b, c). Again primers were designed from the 5′- and 3′-RACE cDNA to amplify the full length cDNA of all these genes. These full length cDNAs were used to search the NCBI data base of non-redundant sequences, all of these four cDNAs showed higher homology with others non-reductant cDNAs of PK, PEPtase, PEPC and PEPCK. Start and stop codons were marked out to obtained the deduced amino acid sequences for open reading frame (ORF) from the 5′- and 3′-end, respectively (Fig. 2d-5d). Finally, the full length cDNAs of *SrPK*, *SrPEPtase*, *SrPEPC*, and *SrPEPCK* were sequenced about 2040,

1987, 3192 and 2241bp, respectively (Fig. 6-9). Molecular mass was calculated theoretically from the deduced amino acid sequences which are 55.4, 56.22, 110.46, 74.17 kDa of SrPK, SrPEPtase, SrPEPC and SrPEPCK, respectively.

Gene expression analyses for PK, PEPtase, PEPC, and PEPCK in thermogenic and non-thermogenic tissues

For gene expression analysis of identified transcripts of *SrPK*, *SrPEPtase*, *SrPEPC*, and *SrPEPCK*, purified plasmids were diluted serially to make the standard curve by qRT-PCR to check the either level of gene expression works smoothly or not. After running the qRT-PCR, dissociation curve, amplification plot and standard curve of purified plasmids were analyzed (Fig. 10-15) and all three types curve of respective plasmids showed that gene expression analysis works well without showing any trouble. Thereafter, I determined the gene expression using different tissues of *S. renifolius*. Tissue-specific expression patterns of *SrPK*, *SrPEPtase*, *SrPEPC*, and *SrPEPCK* transcripts were determined by qRT-PCR using RNAs from the spathe, leaf, floret, and pith tissues collected from *S. renifolius* plants during thermogenesis (Fig. 16). In these experiments, temperatures of ambient air and thermogenic spadix were shown in the figures for three different plants, respectively (Fig. 17 a-c). Previous study showed that florets are thermogenic, while the spathe, leaf, and pith are non-thermogenic (Onda et al., 2008).

Thermogenic florets contain the stamen, pistil, and petal (Fig. 16). *SrAOX* mRNA expression was used as a thermogenic tissue-specific control to reflect the thermogenic status of the samples. These data clearly showed that expression levels of the *SrPEPC* and *SrAOX* transcripts were significantly higher in florets than those in the non-thermogenic tissues of spathe, leaf, and pith in three different plant samples (Fig. 17 a-c). In contrast, the expression levels of *SrPK*, *SrPEPtase*, and *SrPEPCK* were nearly undetectable in the spathe, leaf, floret, and pith tissues (Fig. 17 a-c). Next, to examine gene expression in more detail, I focused on 3 tissues, the petal, stamen, and pistil, each of which comprises the florets of the spadix (Fig. 16). *SrPEPC* transcripts were again co-expressed with *SrAOX* transcripts in petals and pistils, whereas *SrPK*, *SrPEPtase*, and *SrPEPCK* transcripts were expressed at low levels in all tissues examined from three different preparations (Fig. 18 a-c).

Predicted amino acid sequence of SrPEPC

Phylogenetic analysis of PEPC amino acid sequences from 15 plants, 3 algae, and 4 bacteria clearly characterized SrPEPC as a C3-plant-type PEPC (Fig. 19a). Moreover, amino acid sequences of SrPEPC and the homolog NnPEPC from the thermogenic plant species *Nelumbo nucifera* (Ming et al., 2013) were closely related on the phylogenetic tree (Fig. 19a).

Multiple sequence alignments of the deduced PEPC amino acid

sequence with other protein sequences from C3- and C4-plant PEPCs showed that SrPEPC possesses conserved alanine and arginine residues, characteristic of C3-plant-type PEPCs (Paulus et al., 2013) (Fig. 19b). Moreover, a serine residue with a potential phosphorylation site (O'Leary et al., 2011) was highly conserved across C3- and C4-types of plant PEPCs, including that from *S. renifolius* (Fig. 19c).

Discussion

In this study, I sequenced the full length cDNA for *SrPK*, *SrPEPtase*, *SrPEPC* and *SrPEPCK* in *S. renifolius*. The cDNA of *SrPK* was found encoding 1530 bp open reading frame in which deduced 510 amino acids that is more or less similar with previously characterized PK in non-thermogenic plant (Blakeley et al., 1990; Blakeley et al., 1995; Ambasht and Kayastha, 2002; Qin et al., 2013). Generally, two isozymes of PK have been identified namely plastid PK and cytosolic PK (Blakeley et al., 1990; Ambasht and Kayastha, 2002). Assuredly, I identified cDNA encoding *SrPK* is cytosolic. The full length cDNA encoding *SrPEPtase* showed homology with the acid phosphatase as well as PEPtase with *Allium cepa* (Shinano et al., 2001) and *Phaseolus vulgaris* (Bargaz et al., 2012). Intriguingly, the size of full length cDNA encoding *SrPEPC* was similar with *Arabidopsis thaliana*, *Oryza sativa* and other C3 plants (Uhrig et al., 2008; Masumoto et al., 2010; O'Leary et al., 2011). Different isoforms of PEPC like PEPC1, PEPC2, PEPC3 and PEPC4

have been purified from the green alga (Rivoal et al., 1996). Furthermore, plant, bacterial, C3, C4, algal, protists, archeae type PEPC have been identified and sequenced (O'Leary et al., 2011; Peng et al., 2012). In this study, I identified and sequenced only one cytosolic type of *SrPEPC*. According to phylogenetic tree analysis, I confirmed that identified sequenced *SrPEPC* is C3 plant type and closely related to thermogenic plant type PEPC of *N. nucifera* (Fig. 19a). Although, two isoforms of PEPCK (1 & 2) have been identified in *Arabidopsis thalilana* (Fontaine et al., 2002), I obtained only one cytosolic type of *SrPEPCK* which showed similarity with the previously characterized in terms of size, open reading frame and molecular weight (Laivenieks et al., 1997; Hartwell et al., 1999; Fontaine et al., 2002).

Gene expression study (Fig.17 & 18) further suggested that *SrAOX* mRNA expressed exclusively in thermogenic tissue of *S. renifolius* (Onda et al., 2007). Interestingly, *SrPEPC* and *SrAOX* mRNA co-expressed in florets, petal and pistil which revealed that *SrPEPC* expression significantly high in thermogenic tissue. To the best of my knowledge, this is the first determination of *SrPEPC* mRNA expression high in thermogenic tissues of *S. renifolius.* Generally, PEPC plays a very important role to fix the atmospheric CO_2 in CAM and C4 plant for photosynthesis (Rajagopalan et al., 1994; Chollet et al., 1996), but PEPC in thermogenic plants fixed the CO_2 produced in TCA cycle by the reaction with H_2O as HCO_3^- which is the unique feature for thermogenic

tissues (ap Rees et al., 1981). These gene expression data suggested that *SrPEPC* might play predominant role to recycle the internal CO_2 of TCA cycle. The role of non-photosynthetic PEPC from C3, C4 and CAM plants are also associated with the anaplerosis of TCA cycle to acclimatize to biotic and abiotic stresses (O'Leary et al., 2011).

The results from this study suggested that *SrPEPC* gene identified in *S. renifolius* showed tissue-specific expression and might play a substantial role to replenish TCA cycle intermediates to generate heat. Interestingly, *SrPEPC* and *SrAOX* abanduntly co-expressed in the thermogenic tissues of florets and more specifically expressed in petal and pistil. It is concluded that *SrPEPC* may play a crucial role to supply respiratory substrates to the terminal complex of AOX to produce heat instead of energy in petal and pistil.

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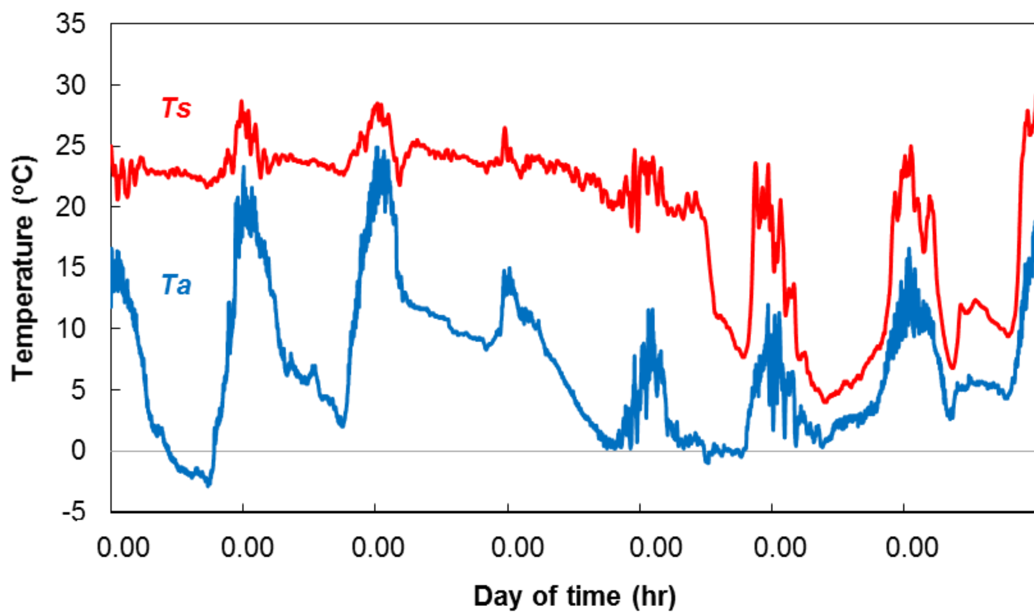

Figure 1. Thermogenic stage-specific homothermic control in the spadix of *S. renifolius.* Changes of spadix temperature (*Ts*) and ambient temperature (*Ta*) for thermogenic and non-thermogenic stages.

Table 1. Sequences of the primer used for partial cDNA cloning in this study.

Primer name	Sequence 5′-3′	Tm
PKF1	AAGGGTCCTGAGATCCGTAC	58.0
PKR1	AGCAGATGACGCAAGAGACTC	59.7
PEPtaseF1	GTGATTTGGGGCAGACATTT	59.8
PEPtaseR1	TCATGATTTCCAGCAGTCCA	60.2
PEPCF1	AGGAGGTCCCTGCTCCAGAA	67.4
PEPCR1	GTGACCCTGAAGAATGGCCACTC	69.9
PEPCKF1	GTCAGCGACAGCTCCCTCAA	68.0
PEPCKR1	GGCGAACACCTCGAAGTTCTTC	68.0

Table 2. Sequences of the primer used for RACE cloning in this study.

Primer name	Sequence 5′-3′	Tm
SrPKF2-3′RACE	GACCGATGTTGCCAATGCTGTTC	71.5
SrPKF3-3′RACE	CGACAGGTTCTTGGGCCTTATGC	71.2
SrPKF4-3′RACE	CCGTCGTCACTGCCACTCAAATG	72.7
SrPKR2-5′RACE	CACAGTCAGTGCCGTCAAGAACA	69.6
SrPKR3-5′RACE	CAGGGAGGACTCTGCCTCGTTAC	69.1
SrPKR4-5′RACE	TCCAGGGAGGACTCTGCCTCGTC	73.9
SrPKF2-3′RACE Nested	GACCGATGTTGCCAATGCTGTTC	71.5
SrPKR2-5′RACE Nested	CACAGTCAGTGCCGTCAAGAACA	69.6
SrPEPtaseF2-3′RACE	ACATGCAGAGTGGAGGACAGGCT	70.4
SrPEPtaseR2-5′RACE	AGCAGTCCACATCCAGGGCTGA	72.4
SrPEPtaseF3-3′RACE Nested	GGTGATCGCTGGGACACTTGGG	73.8
SrPEPtaseR2-5′RACE Nested	AGCAGTCCACATCCAGGGCTGA	72.4
SrPEPCF2-3′RACE	CGCTCAATTGTCTTCCAGGAACC	69.6
SrPEPCR2-5′RACE	CACCAAAGCCAAGCCATACAGGA	69.6
SrPEPCKF2-3′RACE	TAAGCGGCTATACGGCTCTGGTC	68.9
SrPEPCKR2-5′RACE	GTTGCCACAACCGTAACTTCCAC	68.3
SrPEPCKF3-3′RACE Nested	AGATCATCGACGCCATACACGAG	69.2
SrPEPCKR2-5′RACE Nested	GTTGCCACAACCGTAACTTCCAC	68.3
UPM	CTAATACGACTCACTATAGGGC	52.7
NUP	AAGCAGTGGTATCAACGCAGT	65.9

Table 3. Sequences of the primer used for full-length cDNAs cloning in this study.

Primer name	Sequence 5′-3′	Tm
SrPKF5	GGAAAGCGAGTTGACTGG	61.0
SrPKR5	GATCTGACCCGTGCAAAGAT	60.0
SrPEPtaseF4	TGCAGGGAAGGGAGGATTTG	68.3
SrPEPtaseR3	AGTACCCAAAAAGGGCTGCT	63.7
SrPEPCF3	CTTCCTGTGCCTAATCGAGC	60.0
SrPEPCR3	CCAATTCTGCAGCTGATTTG	63.3
SrPEPCKF4	GGGCCTTCTTGTTGTTTGAA	60.0
SrPEPCKR3	CTCATTCCCCAACGTATGCT	59.9

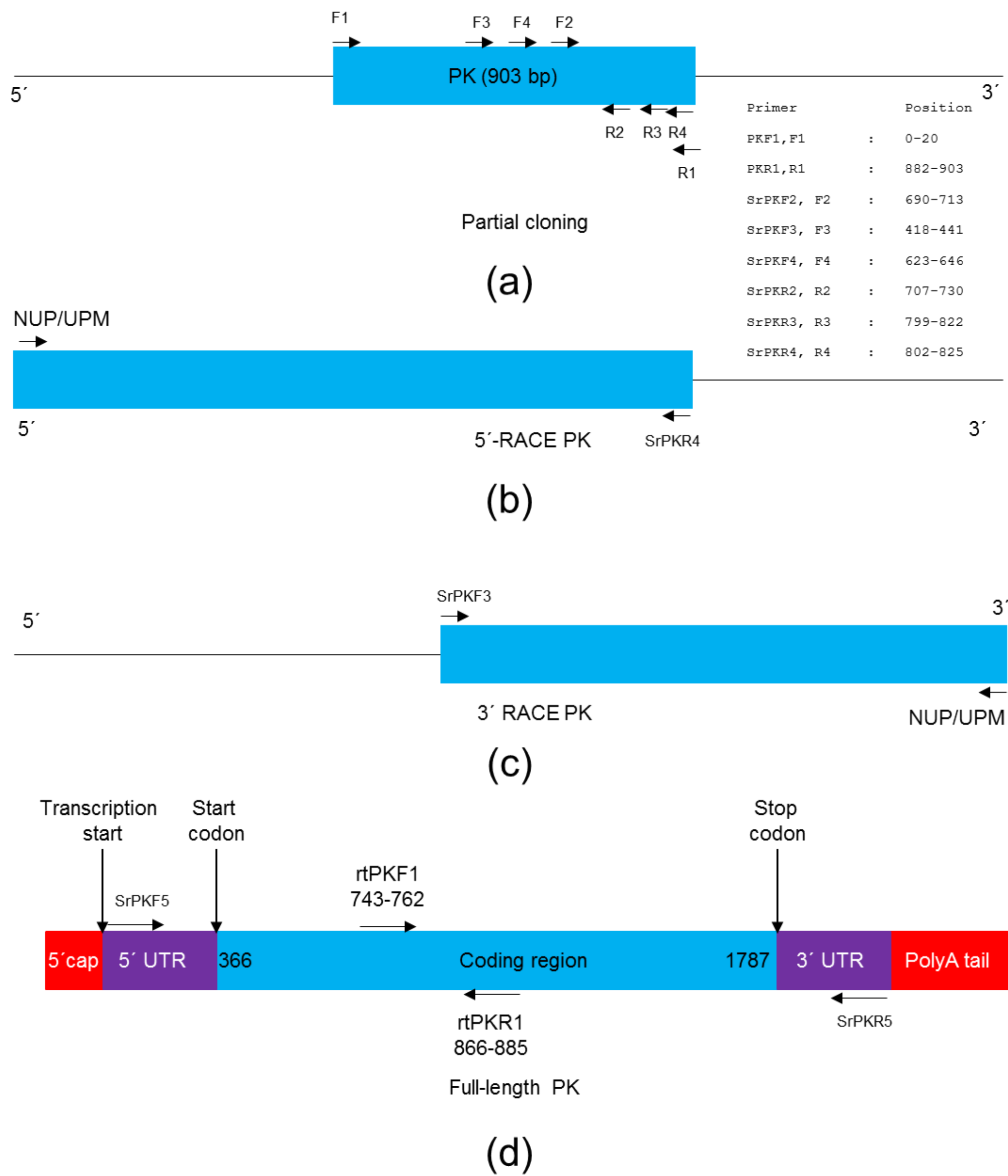

Figure 2. Putative gene map of pyruvate kinase (PK) are shown as (a) partial sequence; (b) 5´-RACE sequence (c) 3´-RACE sequence and (d) full length gene. Primer positions are marked as arrows in each cloning.

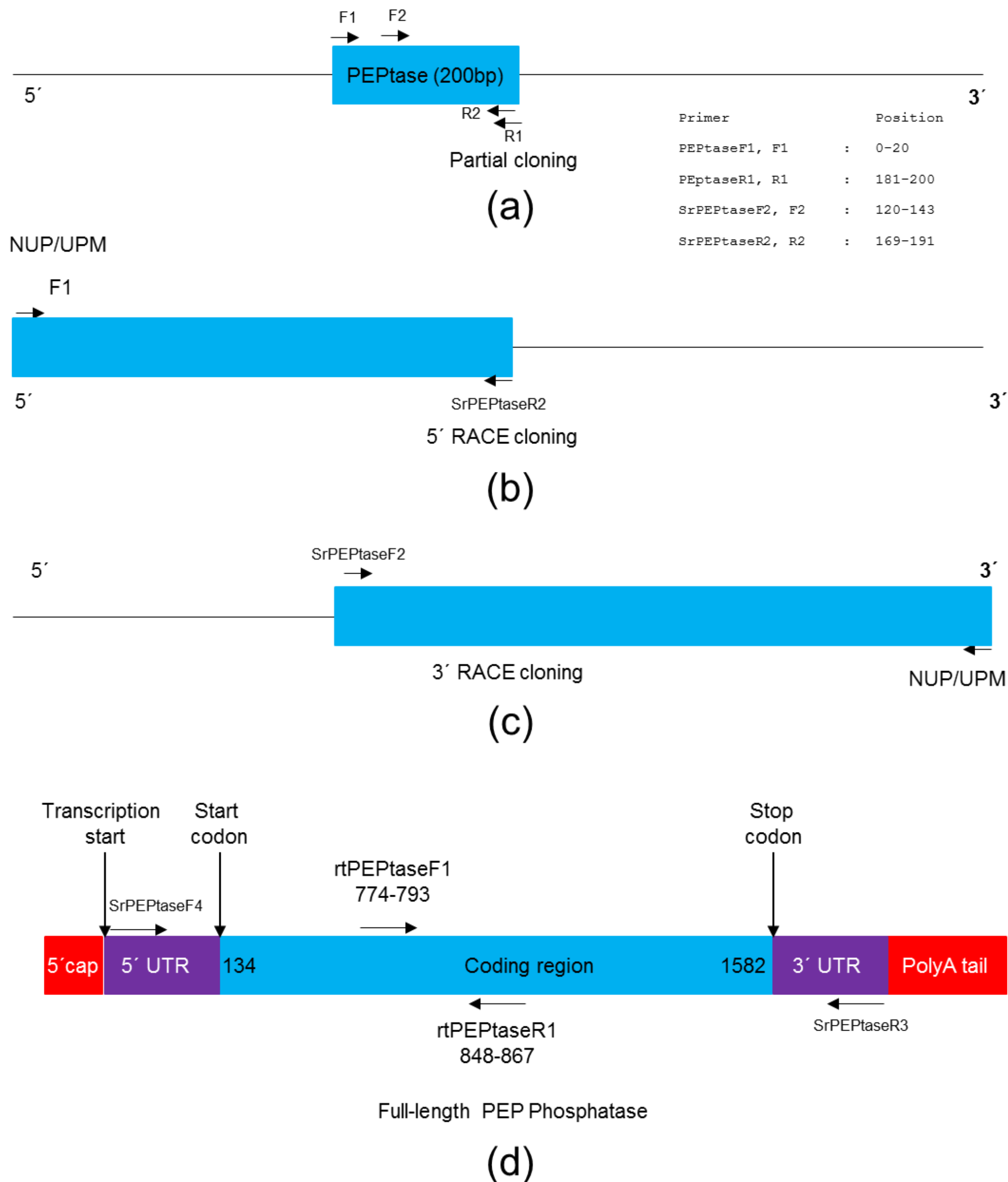

Figure 3. Putative gene map of phospho*enol*pyruvate phosphatase (PEP Phosphatase; PEPtase) are shown as (a) partial sequence; (b) 5´-RACE sequence (c) 3´-RACE sequence and (d) full length gene. Primer positions are marked as arrows in each cloning.

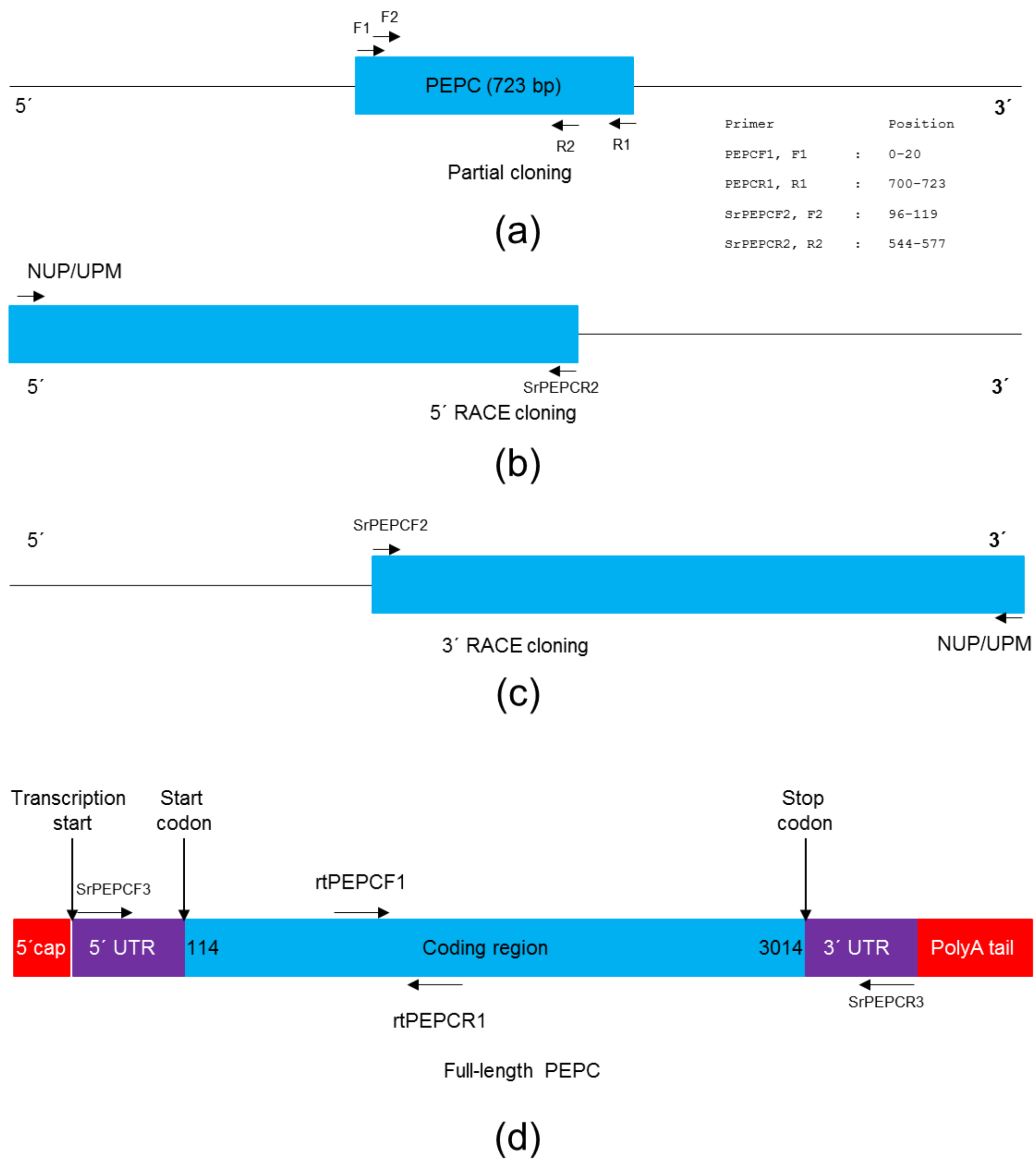

Figure 4. Putative gene map of phospho*enol*pyruvate carboxylase (PEPC) are shown as (a) partial sequence; (b) 5´-RACE sequence (c) 3´-RACE sequence and (d) full length gene. Primer positions are marked as arrows in each cloning.

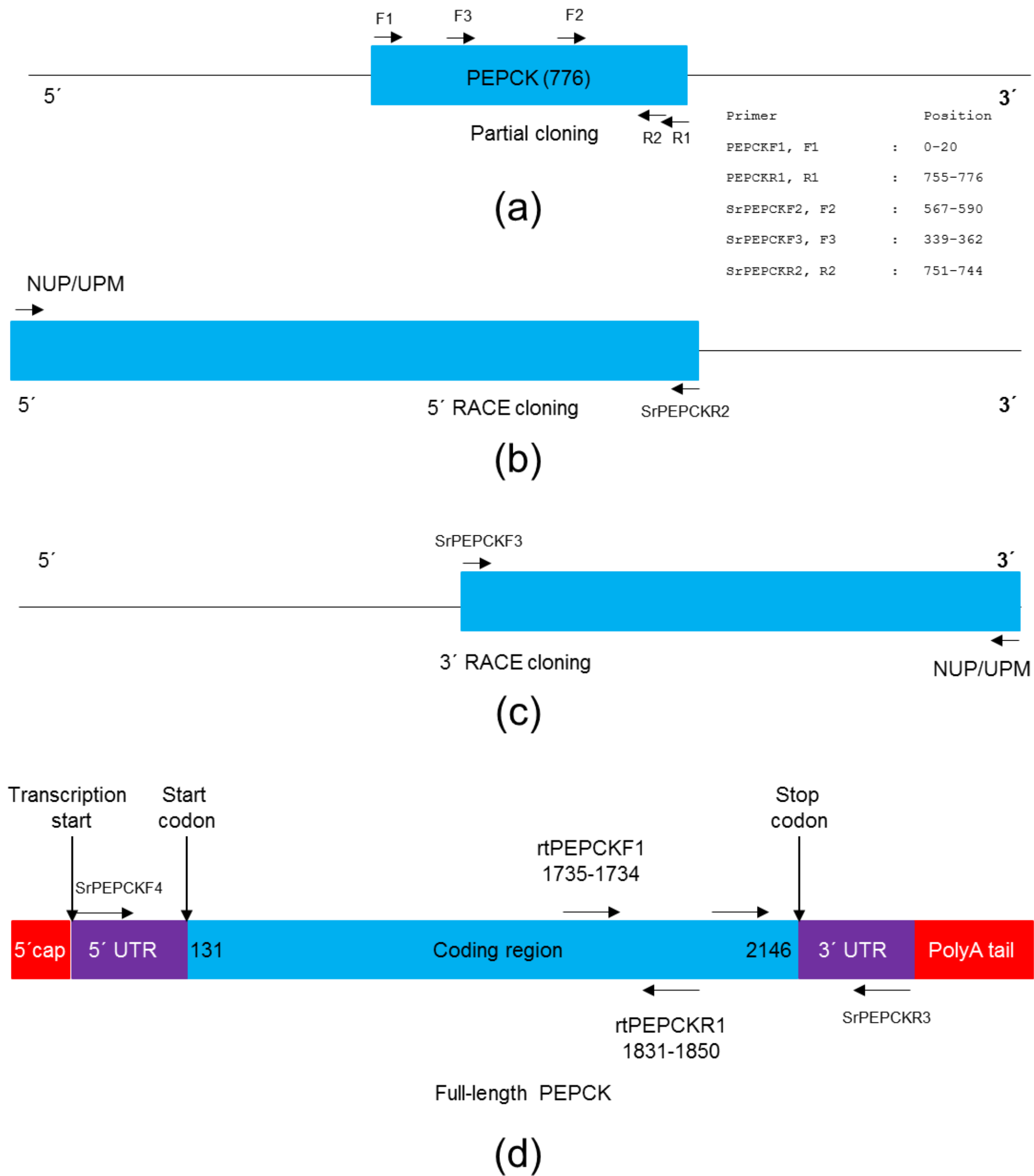

Figure 5. Putative gene map of phospho*enol*pyruvate carboxykinase (PEPCK) are shown as (a) partial sequence; (b) 5´-RACE sequence (c) 3´-RACE sequence and (d) full length gene. Primer positions are marked as arrows in each cloning.

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    1  CGTTCGACGATTGGAAAGCGAGTTGACTGGGAGTTCTCTGGAAAGAAGTGCAGTTGGAGA  61
   61  GAGAGAAAGCAACGGAGTAGTGTCTTTTTATTATTAACATTTTAATTTTTGTTAAATTAA  121
  121  GAAATATACGCGTATCAGAGGGCTTTAAATTTTCTTCTTCCCACCACCCGATCTCGCCGT  181
  181  CGTCAGATCTCCGTTGCATTTTTTTTCCCTCCGATCCCAGCTAGAGAAATCGGGTTTCGT  241
  241  TCCTTGCTGAGGATAGGATGGCGAACATCGACATTGAGGGGATACTGAAGGGGCTGCCGG  301
  301  ACGACGGGCGGGTGCCGAAGACGAAGATCGTGTGCACGCTGGGACCGGCGTCGAGGTCGG  361
  361  TGCCGATGCTGGAGAAGCTGCTGAGGGCGGGCATGAACGTGGCCCGGTTCAATTTCTCCC  421
             M  L  E  K  L  L  R  A  G  M  N  V  A  R  F  N  F  S
  421  ACGGCACCCACGAATACCACCAGGGGACGCTCGACAACCTCCGGATCGCCATGCAGAACA  481
       H  G  T  H  E  Y  H  Q  G  T  L  D  N  L  R  I  A  M  Q  N
  481  CCCAGATCATGTGCGCCGTCATGCTTGATACTAAGGGACCAGAGATCCGTACTGGCTTCT  541
       T  Q  I  M  C  A  V  M  L  D  T  K  G  P  E  I  R  T  G  F
  541  TAAAGGATTCAAATCCTATACAACTGAAGGAGGGACAAGAAATCACTATATCCACAGATT  601
       L  K  D  S  N  P  I  Q  L  K  E  G  Q  E  I  T  I  S  T  D
  601  ACAGTCTCAAGGGAGACGAGAACGTGATTACTATGAGTTATAAAAAGCTTCCGGTAGATT  661
       Y  S  L  K  G  D  E  N  V  I  T  M  S  Y  K  K  L  P  V  D
  661  TAAAGCCCGGGAATACCATCCTGTGTGCAGATGGTACCATATCCTTAAATGTTCTGTCCT  721
       L  K  P  G  N  T  I  L  C  A  D  G  T  I  S  L  N  V  L  S
  721  GTGACCCAGCTGCTGGGACTGTGAGATGCCGGTGCCAAAATACAGCAATGCTAGGTGAGA  781
       C  D  P  A  A  G  T  V  R  C  R  C  Q  N  T  A  M  L  G  E
  781  GAAAGAATGTCAATCTACCAGGTGTTGTCGTGGACCTTCCTACTCTTACTGAGAAGGATA  841
       R  K  N  V  N  L  P  G  V  V  V  D  L  P  T  L  T  E  K  D
  841  AGGAAGACATCTTGGGATGGGGTGTGCCCAACAACATCGACATGATTGCGCTGTCCTTTG  901
       K  E  D  I  L  G  W  G  V  P  N  N  I  D  M  I  A  L  S  F
  901  TGCGCAAGGGGTCTGATCTTGTTAATGTCCGACAGGTTCTTGGGCCTTATGCAAAAAACA  961
       V  R  K  G  S  D  L  V  N  V  R  Q  V  L  G  P  Y  A  K  N
  961  TAAAGTTGATGTCAAAGGTTGAGAACCAGGAGGGAGTGATAAACTTTGATGAAATCCTAA  1021
       I  K  L  M  S  K  V  E  N  Q  E  G  V  I  N  F  D  E  I  L
 1021  GAGAAACGGATTCTTTCATGGTTGCAAGGGGTGATCTTGGAATGGAGATCCCTGTTGAGA  1081
       R  E  T  D  S  F  M  V  A  R  G  D  L  G  M  E  I  P  V  E
 1081  AGATCTTCCTCGCTCAAAAGATGATGATTTACAAGTGCAACCTCGTGGGTAAGCCCGTCG  1141
       K  I  F  L  A  Q  K  M  M  I  Y  K  C  N  L  V  G  K  P  V
 1141  TCACTGCCACTCAAATGCTTGAATCCATGATCAAATCCCCCCGTCCAACACGTGCTGAGG  1201
       V  T  A  T  Q  M  L  E  S  M  I  K  S  P  R  P  T  R  A  E
 1201  CGACCGATGTTGCCAATGCTGTTCTTGACGGCACTGACTGTGTCATGCTGAGTGGAGAGA  1261
       A  T  D  V  A  N  A  V  L  D  G  T  D  C  V  M  L  S  G  E
 1261  GTGCTGCTGGATTATACCCTGAGCTTGCCGTCAAGATCATGGCCCGTATCTGTAACGAGG  1321
       S  A  A  G  L  Y  P  E  L  A  V  K  I  M  A  R  I  C  N  E
 1321  CAGAGTCCTCCCTGGATTATGGTGCCATCTTCAAGGAGATGATAAAATCCACCCCTCTTC  1381
       A  E  S  S  L  D  Y  G  A  I  F  K  E  M  I  K  S  T  P  L
 1381  CCATGAGCCCACTGGAGAGCCTTGCATCATCTGCGGTTCGAACAGCCAACAAGTGCAGGG  1441
       P  M  S  P  L  E  S  L  A  S  S  A  V  R  T  A  N  K  C  R
 1441  CCAAGCTAATTGTTGTTCTGACTCGAGGTGGGACTACTGCCAAGCTGGTTGCCAAGTACC  1501
       A  K  L  I  V  V  L  T  R  G  G  T  T  A  K  L  V  A  K  Y
 1501  GCCCGGAGGTCCCGATCCTGTCGGTGGTAGTCCCCGTGCTCACCACAGATTCCTTCGATT  1561
       R  P  E  V  P  I  L  S  V  V  V  P  V  L  T  T  D  S  F  D
 1561  GGAATGTCAGTGATGAGAGACCGGCGAGGCACAGTCTCATCTACAGGGGCCTGATCCCAC  1621
       W  N  V  S  D  E  R  P  A  R  H  S  L  I  Y  R  G  L  I  P
 1621  TGCTGGCAGAAGGTTCTGCCAAGGCCACCGATGCTGAATCGACAGAGGTTATTTTGGAAG  1681
       L  L  A  E  G  S  A  K  A  T  D  A  E  S  T  E  V  I  L  E
 1681  CTGCTCTCAAGTCTGCGGTACAGAGGCGTCTATGCAAGCCGGGAGACGCAATTGTTGCAC  1741
       A  A  L  K  S  A  V  Q  R  R  L  C  K  P  G  D  A  I  V  A
 1741  TGCACCGCATTGGTGCTGCCTGTGTCATCAAGATCTGCCTCGTAAAATGATCATGGTAAC  1801
       L  H  R  I  G  A  A  C  V  I  K  I  C  L  V  K
 1801  TCTCCGGAGGAAGAGTCACCAGTTTTCGCTTTGCTGCCGCGTTTGTTTCGAGTCTTAGTA  1861
 1861  ACTTCCGGAGGATCTGACATATCATCTATATAATTTTGCAACTCTATCTACAGTTTACAG  1921
 1921  GGTTTTGTAATCTCTGCAGTCTTCGCTTTGCTGCCGCGTTTGTTTCGAGTTTTAGTAAGT  1981
 1981  TCCGGAGGATCTGAGATATCATCTATATAATTTTGCAACTCTTATCTGCAGTTTACAGGG  2040
```

Figure 6. Putative full length gene of pyruvate kinase with translated amino acid.

```
   1  TGCAGGGAAGGGAGGATTTGAACGAAGCAGCAGCAGCAGCCTCATCTGCTGCTTCTGCTT  61
  61  CAGATGTGAGATTTTTCCTCGGCCGCAGCACCACCACGCACCCTCCACATTCCAGGCAAA  121
 121  TCAATTGACAACTATGTTGGAGGTTGTCAGTCCCTGGAGATTCACGTTTCAGCTACTATT  181
                     M  L  E  V  V  S  P  W  R  F  T  F  Q  L  L  L
 181  GGCGTTTCTCATTTTCTTGTGCACCTTGGACAATGTAATTTCTGGGTTCACGAGCTCGTT  241
        A  F  L  I  F  L  C  T  L  D  N  V  I  S  G  F  T  S  S  F
 241  CATCCGATCACAGTGGCCGTCAACGGACATCCCCCTAGACAATGAAGCATTTGCAGTTCC  301
        I  R  S  Q  W  P  S  T  D  I  P  L  D  N  E  A  F  A  V  P
 301  AAAGGGTTACAATGCCCCCCAACAAGTCCATATTACGCAAGGTGACTATGATGGAAAGGC  361
        K  G  Y  N  A  P  Q  Q  V  H  I  T  Q  G  D  Y  D  G  K  A
 361  TGTTATAATATCGTGGGTAACATTTTCAGAACCAGGTTCCAATGAAGTGCAATATGGCAA  421
        V  I  I  S  W  V  T  F  S  E  P  G  S  N  E  V  Q  Y  G  K
 421  ATCGAATCACCAATACGATCACACTGCACATGGACATACCACAAATTATACATTCTACAA  481
        S  N  H  Q  Y  D  H  T  A  H  G  H  T  T  N  Y  T  F  Y  K
 481  ATATAATTCTGGGTACATTCATCACTGTCTTGTGGACGGACTTGAGTATGATACCAAGTA  541
        Y  N  S  G  Y  I  H  H  C  L  V  D  G  L  E  Y  D  T  K  Y
 541  TTACTACAAGATTGGCACGCGTGATTCAGCTCGAGAATTTTGGTTTCAAACCCCACCAAC  601
        Y  Y  K  I  G  T  R  D  S  A  R  E  F  W  F  Q  T  P  P  T
 601  GATTGGTCCAGATTCTACTTACAAATTTGGTATTATAGGTGATTTGGGTCAAACATTTAA  661
        I  G  P  D  S  T  Y  K  F  G  I  I  G  D  L  G  Q  T  F  N
 661  TTCTCTTTCTACTCTCGAGCATTACATGCAGAGTGGAGGACAGGCTGTGTTATATGTTGG  721
        S  L  S  T  L  E  H  Y  M  Q  S  G  G  Q  A  V  L  Y  V  G
 721  TGATCTCTCTTATGCTGATAGATATGAGTACAATGATGGTGATCGCTGGGACACTTGGGG  781
        D  L  S  Y  A  D  R  Y  E  Y  N  D  G  D  R  W  D  T  W  G
 781  CCGTTTTGTTGAGCGTAGTGCTGCAAATCAGCCCTGGATTTGGTCTGCTGGCAATCATGA  841
        R  F  V  E  R  S  A  A  N  Q  P  W  I  W  S  A  G  N  H  E
 841  AATAGAATACAGGCATGATCTGGGGGAAGTAATTTCTTTCAAAGCTTATTTACATAGATA  901
        I  E  Y  R  H  D  L  G  E  V  I  S  F  K  A  Y  L  H  R  Y
 901  CACAACGCCATACATGGCATCCAAGAGCAGCTCTCCTTTGTGGTATGCTATAAGACGTGC  961
        T  T  P  Y  M  A  S  K  S  S  S  P  L  W  Y  A  I  R  R  A
 961  ATCTGCTCACATCATTGTGCTGTCAAGCTATTCGCCATTTGTGAAGTACACCCCTCAATG  1021
        S  A  H  I  I  V  L  S  S  Y  S  P  F  V  K  Y  T  P  Q  W
1021  GACTTGGCTACGGAGTGAGCTTAAGCGAGTGGATAGAGAGAAGACACCTTGGCTTATTAT  1081
        T  W  L  R  S  E  L  K  R  V  D  R  E  K  T  P  W  L  I  I
1081  TCTCATGCATTCTCCACTATACAACAGTAATGAAGCACACTACATGGAGGGTGAAAGCAT  1141
        L  M  H  S  P  L  Y  N  S  N  E  A  H  Y  M  E  G  E  S  M
1141  GAGGGCTGTTTTTGAGAAATGGTTTGTTCGTCGCAAGGTTGACCTTGTCTTTGCTGGCCA  1201
        R  A  V  F  E  K  W  F  V  R  R  K  V  D  L  V  F  A  G  H
1201  TGTTCATGCGTATGAAAGATCGTACCGAATCTCGAACATCAACTACAATATCACAAGTGG  1261
        V  H  A  Y  E  R  S  Y  R  I  S  N  I  N  Y  N  I  T  S  G
1261  CGACCGTTATCCCATGCCCGACAAATCTGCACCCATTTACATAACTGTTGGTGATGGTGG  1321
        D  R  Y  P  M  P  D  K  S  A  P  I  Y  I  T  V  G  D  G  G
1321  AAATCAGGAAGGCCTTGCTGGAAGATTTTACGACCCACAACCAGATTATTCTGCATTCAG  1381
        N  Q  E  G  L  A  G  R  F  Y  D  P  Q  P  D  Y  S  A  F  R
1381  GGAAGCCAGTTATGGTCATTCTACGTTGGAGCTGAAGAACAGAACCCATGCATTCTACAA  1441
        E  A  S  Y  G  H  S  T  L  E  L  K  N  R  T  H  A  F  Y  N
1441  TTGGAATCGAAATGACGATGGAACACGTGTACCAACGGATTCCGTTGTATTTCACAACCA  1501
        W  N  R  N  D  D  G  T  R  V  P  T  D  S  V  V  F  H  N  Q
1501  ATATTGGGGAAGCCATCATCATAGGAGGAGACTGAAGAAGAACCATCAACTCTGGGATGC  1561
        Y  W  G  S  H  H  H  R  R  R  L  K  K  N  H  Q  L  W  D  A
1561  CACTGGCTATACTGCTGCATATTAAAGTGAGAGAGTGGAAGGAGGCTGTGTTCTGAATTA  1621
        T  G  Y  T  A  A  Y
1621  AAAATTTGGGGTGCCTGGGGATTCTGCAATTGATTGGCAATAAATGGGGTATCATATCGA  1681
1681  ATAAATAAAAAAAGGTGCTCTCTTGTTAAGCAGAGCGCAGCAACCGTACCTTCCTGGACA  1741
1741  GTTAATTTCACGATCTCCTAGTTGCTGATTGCTAGCTTCGTGAGGAGAAATACTTAGTTG  1801
1801  AGACGGAAATCACAGACAAAAAGTTGAGACGCAAGGCAAAAGTGGTGTGTGTGTGGGGAC  1861
1861  CCTAAGCATTTCTCTAGTTTCAGTGGGCATTTTTCTATTCAACCTACATTGAAGTGGACG  1921
1921  TGGAATGCTGTAGGTTTTAAAATTTGGCGTGCCATGGTAGTTGCTCCTAATGATGGGTAG  1981
1981  TTCTTTT  1987
```

Figure 7. Putative full length gene of phospho*enol*pyruvate phosphatase (PEP phosphatse; PEPtase) with translated amino acid.

```
   1  CTTCCTGTGCCTAATCGAGCTCTTTTGGTGCGTAGTCATCGTGTTCTGTCCCCATATATA  61
  61  GTGGGGGAGAGCAAGTGATATCGAGACAGTGGGGGTGTGGAAGAGTGAGAGAGATGGCCC  121
                                                            M  A
 121  GGAACGCGAACAATTTGGAGAAGCTGGCTTCGATAGACGCGCAGCTGCGGCTGCTGGCGC  181
      R  N  A  N  N  L  E  K  L  A  S  I  D  A  Q  L  R  L  L  A
 181  CAGGGAAGGTCTCCGAGGACGACAAACTCGTCGAGTACGACGCCCTCCTCTTGGATCGGT  241
      P  G  K  V  S  E  D  D  K  L  V  E  Y  D  A  L  L  L  D  R
 241  TCCTCGACATCCTCCAGGACCTGCATGGCGAGGATCTCAAGGAGACGGTTCAAGAGTGCT  301
      F  L  D  I  L  Q  D  L  H  G  E  D  L  K  E  T  V  Q  E  C
 301  ACGAACTTTCTGCGGAGTATGAGGGGAAGCATGACCCTCAGAAGTTGGAAGAGCTTGGGA  361
      Y  E  L  S  A  E  Y  E  G  K  H  D  P  Q  K  L  E  E  L  G
 361  ATGTTCTCACAAGTTTGGATCCCGGGGACTCCATTGTCATCGCCAGCTCTTTCTCTCACA  421
      N  V  L  T  S  L  D  P  G  D  S  I  V  I  A  S  S  F  S  H
 421  TGCTTAACTTGGCCAACTTGGCTGAGGAGGTCCAGATTGCCTATAGGAGGCGCATCAAGC  481
      M  L  N  L  A  N  L  A  E  E  V  Q  I  A  Y  R  R  R  I  K
 481  TGAAAAAGGGAGATTTTGCTGATGAGAACTCTGCAACCACTGAGTCTGACATTGAAGAGA  541
      L  K  K  G  D  F  A  D  E  N  S  A  T  T  E  S  D  I  E  E
 541  CACTGAAGAGGCTCGTGGTGCAGTTGAAGAAGTCCCCAGAGGAAGTATTCGATGCTCTCA  601
      T  L  K  R  L  V  V  Q  L  K  K  S  P  E  E  V  F  D  A  L
 601  AGAACCAGACTGTGGATCTAGTTTTCACTGCCCATCCAACTCAGTCAGTCCGGAGATCAT  661
      K  N  Q  T  V  D  L  V  F  T  A  H  P  T  Q  S  V  R  R  S
 661  TGCTTCAGAAACATGGAAGGATCCGGAATTGTTTGGCACAATTGTATGCCAAGGACATTA  721
      L  L  Q  K  H  G  R  I  R  N  C  L  A  Q  L  Y  A  K  D  I
 721  CACCTGATGACAAGCAGGAGCTGGACGAAGCTCTCCAGAGGGAGATTCAAGCTGCCTTCC  781
      T  P  D  D  K  Q  E  L  D  E  A  L  Q  R  E  I  Q  A  A  F
 781  GAACTGATGAAATCAGGAGAACTCCTCCCACTCCACAAGATGAAATGCGAGCAGGGATGA  841
      R  T  D  E  I  R  R  T  P  P  T  P  Q  D  E  M  R  A  G  M
 841  GTTATTTTCATGAAACAATCTGGAAAGGAGTTCCAAAATTCTTACGCAGGGTCGACACAG  901
      S  Y  F  H  E  T  I  W  K  G  V  P  K  F  L  R  R  V  D  T
 901  CTTTAAAAAACATTGGGATTAATGAGCGCCTTCCCTACAATGCACCTCTCATCCAGTTCT  961
      A  L  K  N  I  G  I  N  E  R  L  P  Y  N  A  P  L  I  Q  F
 961  CTTCTTGGATGGGTGGGGATCGTGATGGAAATCCTAGAGTTACTCCTGAGGTTACCAGGG  1021
      S  S  W  M  G  G  D  R  D  G  N  P  R  V  T  P  E  V  T  R
1021  ATGTTTGCTTGTTGGCTCGGATGATGGCCGCAAACTTGTATTATTCACAGATAGAGGATC  1081
      D  V  C  L  L  A  R  M  M  A  A  N  L  Y  Y  S  Q  I  E  D
1081  TAATGTTCGAGTTATCTATGTGGCGTTGCAGCGAGGAACTCCGAGTCCGAGCAAATGAAC  1141
      L  M  F  E  L  S  M  W  R  C  S  E  E  L  R  V  R  A  N  E
1141  TACACCGTTCCTCAAGGAAAGATGCAAAACATTACATCGAGTTCTGGAAGAAAGTTCCAA  1201
      L  H  R  S  S  R  K  D  A  K  H  Y  I  E  F  W  K  K  V  P
1201  GTAACGAGCCTTATCGTGTTATACTTGGTGATGTGAGGGATAAGCTATATCATACACGTG  1261
      S  N  E  P  Y  R  V  I  L  G  D  V  R  D  K  L  Y  H  T  R
1261  AGCGTTCTCGTCATTTGTTGGCCAGTGGGATTTCCGACATCCCTGAGGAATCAACTTTTA  1321
      E  R  S  R  H  L  L  A  S  G  I  S  D  I  P  E  E  S  T  F
1321  CGGATGTTGAACAGTTCCTGGAACCTCTTGAACTTTGTTACAGATCACTTTGCGCTTCTG  1381
      T  D  V  E  Q  F  L  E  P  L  E  L  C  Y  R  S  L  C  A  S
1381  GTGATCGAGCAATTGCTGATGGAAGCCTTCTTGATTTCTTACGTCAAGTATCAACCTTCG  1441
      G  D  R  A  I  A  D  G  S  L  L  D  F  L  R  Q  V  S  T  F
1441  GCCTGTCTCTCGTAAGGCTTGATATCAGGCAGGAATCTGATCGTCACACTGATGTAATCG  1501
      G  L  S  L  V  R  L  D  I  R  Q  E  S  D  R  H  T  D  V  I
1501  ATGCTATCACACAACACTTGGGAATCGGATCTTATCGAGATTGGCCTGAGGAAAAACGGC  1561
      D  A  I  T  Q  H  L  G  I  G  S  Y  R  D  W  P  E  E  K  R
1561  AGGAATGGTTGTTGTCTGAACTTAGTGGCAAGCGCCCATTGTTCGGTCCAGACCTCCCTA  1621
      Q  E  W  L  L  S  E  L  S  G  K  R  P  L  F  G  P  D  L  P
1621  AAACTGAAGAGATCTCCGATGTGTTAGAAACATTGCATGTCATTGCTGAACTCCCTTCTG  1681
      K  T  E  E  I  S  D  V  L  E  T  L  H  V  I  A  E  L  P  S
1681  ATAATTTTGAGGCATACATCATCTCAATGGCAACTTCCCCATCTGATGTGCTTGCTGTTG  1741
      D  N  F  E  A  Y  I  I  S  M  A  T  S  P  S  D  V  L  A  V
1741  AGCTCTTGCAGCGTGAATGTCATGTGAAGAAACCATTGAGGGTCGTTCCATTGTTTGAGA  1801
      E  L  L  Q  R  E  C  H  V  K  K  P  L  R  V  V  P  L  F  E
1801  AACTTGCAGATCTAGAGGCAGCCCCTGCAGCTCTTGCTCGTCTATTCTCGATAGATTGGT  1861
      K  L  A  D  L  E  A  A  P  A  A  L  A  R  L  F  S  I  D  W
1861  ACAAGAACAGGATTGATGGGAAGCAGGAAGTCATGATTGGATACTCAGATTCAGGGAAGG  1921
      Y  K  N  R  I  D  G  K  Q  E  V  M  I  G  Y  S  D  S  G  K
1921  ATGCTGGGCGTTTTTCTGCAGCTTGGCAATTATATAAAGCTCAAGAGGAGCTTATAAAAG  1981
      D  A  G  R  F  S  A  A  W  Q  L  Y  K  A  Q  E  E  L  I  K
1981  TTGCCAAACAATATGGAGTGAAGTTGACAATGTTTCATGGACGGGGAGGGACTGTTGGCC  2041
      V  A  K  Q  Y  G  V  K  L  T  M  F  H  G  R  G  G  T  V  G
2041  GAGGAGGTGGTCCTACCCATCTTGCTATCTTGTCTCAGCCTCCAGATACGATTCATGGAT  2101
      R  G  G  G  P  T  H  L  A  I  L  S  Q  P  P  D  T  I  H  G
2101  CTCTTCGTGTGACTGTTCAAGGTGAAGTCATTGAACAATCTTTTGGGGAGGAGCATTTGT  2161
      S  L  R  V  T  V  Q  G  E  V  I  E  Q  S  F  G  E  E  H  L
2161  GCTTCAGAACTCTTCAACGTTTCACGGCTGCTACTCTTGAACATGGGATGCACCCCCCTG  2221
      C  F  R  T  L  Q  R  F  T  A  A  T  L  E  H  G  M  H  P  P
2221  TCTCTCCAAAGCCAGAATGGCGTGCCCTTATGGATGAGATGGCTGTGGTGGCTACTGAGG  2281
      V  S  P  K  P  E  W  R  A  L  M  D  E  M  A  V  V  A  T  E
2281  AATACCGGTCTATTGTCTTCCAGGAACCACGTTTTGTTGAATATTTCCGCCTCGCCACAC  2341
      E  Y  R  S  I  V  F  Q  E  P  R  F  V  E  Y  F  R  L  A  T
2341  CTGAGACAGAATATGGTCGAATGAACATTGGTAGCCGACCATCAAAACGAAAGCCGAGTG  2401
      P  E  T  E  Y  G  R  M  N  I  G  S  R  P  S  K  R  K  P  S
2401  GGGGCATAGAATCACTTCGGGCAATCCCATGGATCTTTGCATGGACCCAAACAAGGTTTC  2461
      G  G  I  E  S  L  R  A  I  P  W  I  F  A  W  T  Q  T  R  F
2461  ACCTTCCTGTATGGCTTGGCTTTGGTGCCGCATTTAAGCATATCCTGGAGAAGGACATCA  2521
      H  L  P  V  W  L  G  F  G  A  A  F  K  H  I  L  E  K  D  I
2521  GGAATTTCCATATGCTTCAGGAGATGTACAATGAATGGCCATTCTTCAGGGTCACAATTG  2581
      R  N  F  H  M  L  Q  E  M  Y  N  E  W  P  F  F  R  V  T  I
2581  ACTTGGTTGAGATGGTTTTTGCCAAGGGAGACCCAGGCATAGCTGCTTTGTATGACAAAT  2641
      D  L  V  E  M  V  F  A  K  G  D  P  G  I  A  A  L  Y  D  K
2641  TGCTAGTATCAGATGATCTGTGGTCATTTGGAGAGCGCCTGAGAACCAACTATGAAGAAA  2701
      L  L  V  S  D  D  L  W  S  F  G  E  R  L  R  T  N  Y  E  E
2701  CAAAACACCTACTCCTCCAGGTTGCTGGCCACAAACATCTTCTGGAAGAAAATCCCTACC  2761
      T  K  H  L  L  L  Q  V  A  G  H  K  H  L  L  E  E  N  P  Y
2761  TAAAACAGAGGCTGCGACTGCGTCACTCTTACATCACAACTCTCAATGTTTGCCAAGCGT  2821
      L  K  Q  R  L  R  L  R  H  S  Y  I  T  T  L  N  V  C  Q  A
2821  GCACGCTGAAGCGGATAAGAGACCCCAGTTTCCATGTAAAAGTGAGACCCCATCTGTCAA  2881
      C  T  L  K  R  I  R  D  P  S  F  H  V  K  V  R  P  H  L  S
2881  AAGAGATAGTAGATTCTAGCAAATCAGCTGCAGAATTGGTGAAGCTCAACCCAACAAGCG  2941
      K  E  I  V  D  S  S  K  S  A  A  E  L  V  K  L  N  P  T  S
2941  AGTATGCTCCTGGGCTGGAGGACACCCTCATCTTGACCATGAAGGGTATTGCTGCCGGCA  3001
      E  Y  A  P  G  L  E  D  T  L  I  L  T  M  K  G  I  A  A  G
3001  TGCAGAACACCGGTTAAAGTGTGTTCATGCATCTGAAGATTTTTCTTCAAATCGTGTGTC  3061
      M  Q  N  T  G
3061  AAGTGCCCTTTTTTCCCCTTATTTACCTATCTAAAGAGCGGTTTGTACATTGGCCAAGGT  3121
3121  ACTTTTTGGCTGCTTGTAATCTCTCTCTCTCTCTCTCTCTCGTTTGCACAAAAAAAAAAAA  3181
3181  AAAAAAAAAAAA  3192
```

Figure 8. Putative full length gene of phospho*enol*pyruvate carboxylase (PEPC) with translated amino acid.

```
   1  ATTGGGCCTTCTTGTTGTTTGAAGAAAATCCCCTGGGTAAGGTGCAGCAACAGCTACCCA  61
  61  CCCCACAAGAAGCGGTGCGAAAGCGGGAGATATCATCTCCAGTCTACTCCCTCCCCCGTC  121
 121  AGGGGACGAGATGGCCGAGAACGGAGAGTTCAGCTTCGCCAACGGTGGTGCCGACGTCAG  181
                 M  A  E  N  G  E  F  S  F  A  N  G  G  A  D  V  S
 181  CGCCGCCAGCGTGGTCGCCGCTCGGACGGGGCTCCCGAGGATCCAGACCCACAAGAAAGA  241
        A  A  S  V  V  A  A  R  T  G  L  P  R  I  Q  T  H  K  K  E
 241  GAACGGGATCTGCCACGACGACAGCGCTCCCCCGGTGAAGGCGCAGACCATCGACGAGCT  301
        N  G  I  C  H  D  D  S  A  P  P  V  K  A  Q  T  I  D  E  L
 301  CCACTCCCTGCAGAAGAAGAGGTCCGCCCCAACCACCCCCATCAAGGGCGCCGCCCAGGG  361
        H  S  L  Q  K  K  R  S  A  P  T  T  P  I  K  G  A  A  Q  G
 361  TGGCGCCTTCGCCCCGCCGCCCGTCTCCGACGAGGAGCGCCAAAAGCAGCAGCTCCAATC  421
        G  A  F  A  P  P  P  V  S  D  E  E  R  Q  K  Q  Q  L  Q  S
 421  CATCAGCGCCTCCCTCGCGTCGCTGACCAGGGAGACGGGTCCGAAGCTGGTGAAGGGAGA  481
        I  S  A  S  L  A  S  L  T  R  E  T  G  P  K  L  V  K  G  D
 481  CCCCGCGAGGAAGGCCGAGACGCCCAGGGTCTCCGCCCACCACCAACACGACTACTTCAC  541
        P  A  R  K  A  E  T  P  R  V  S  A  H  H  Q  H  D  Y  F  T
 541  CCCAACCCTCAGCGTCAGCGACAGCGCCCTCAAGTTCACCCACGTCCTCTACAACCTCTC  601
        P  T  L  S  V  S  D  S  A  L  K  F  T  H  V  L  Y  N  L  S
 601  CCCCGCCGAGCTGTACGAGCAGGCCATCAAGTACGAGCACGGCTCCTTCGTCACGTCGAG  661
        P  A  E  L  Y  E  Q  A  I  K  Y  E  H  G  S  F  V  T  S  S
 661  CGGCGCCCTGGCCACCCTCTCCGGGGCGAAGACGGGTCGATCCCCGCGTGACAAGCGCGT  721
        G  A  L  A  T  L  S  G  A  K  T  G  R  S  P  R  D  K  R  V
 721  CGTGCGGGACGAGACCACCGAGGACGAGCTCTGGTGGGGCAAGGGTTCGCCAAACATTGA  781
        V  R  D  E  T  T  E  D  E  L  W  W  G  K  G  S  P  N  I  E
 781  GATGGACGAGCACACCTTTCTGGTGAACAGGGAGAGGGCCGTCGATTACTTGAATTCGCT  841
        M  D  E  H  T  F  L  V  N  R  E  R  A  V  D  Y  L  N  S  L
 841  GGACAAGGTTTATGTGAACGACCAGTTCCTGAATTGGGACCCCGAAAATCGGATAAAGGT  901
        D  K  V  Y  V  N  D  Q  F  L  N  W  D  P  E  N  R  I  K  V
 901  CCGGATCGTGTCCGCAAGGGCGTACCACTCCTTGTTCATGCACAACATGTGCATCCGACC  961
        R  I  V  S  A  R  A  Y  H  S  L  F  M  H  N  M  C  I  R  P
 961  CACGCCCGAAGAGCTGGAGGATTTTGGTACTCCGGACTTCACAATATACAATGCCGGGCA  1021
        T  P  E  E  L  E  D  F  G  T  P  D  F  T  I  Y  N  A  G  Q
1021  GTTTCCCTGTAATCGTTACACACATTATATGACATCCTCCACTAGCATAGACCTTAACCT  1081
        F  P  C  N  R  Y  T  H  Y  M  T  S  S  T  S  I  D  L  N  L
1081  TGCTAGAAGGGAGATGGTCATCCTCGGCACGCAGTACGCCGGGGAGATGAAGAAGGGTCT  1141
        A  R  R  E  M  V  I  L  G  T  Q  Y  A  G  E  M  K  K  G  L
1141  ATTCAGCGTCATGCACTATCTCATGCCTAAGAGACACATCCTCTCCCTGCACTCTGGCTG  1201
        F  S  V  M  H  Y  L  M  P  K  R  H  I  L  S  L  H  S  G  C
1201  CAATATGGGGAAAGATGGCGATGTTGCCCTCTTCTTTGGACTCTCAGGTACCGGGAAGAC  1261
        N  M  G  K  D  G  D  V  A  L  F  F  G  L  S  G  T  G  K  T
1261  CACTCTGTCTACGGATCATAATAGGTTCCTTATCGGAGACGACGAGCACTGCTGGGGTGA  1321
        T  L  S  T  D  H  N  R  F  L  I  G  D  D  E  H  C  W  G  E
1321  GAATGGTGTTTCGAACATCGAGGGGGGCTGCTATGCGAAGTGCATCGACCTGTCAAGGGA  1381
        N  G  V  S  N  I  E  G  G  C  Y  A  K  C  I  D  L  S  R  E
1381  GAAGGAGCCCGACATCTGGAACGCCATAAAGTTCGGGACTGTGCTGGAGAACGTGGTATT  1441
        K  E  P  D  I  W  N  A  I  K  F  G  T  V  L  E  N  V  V  F
1441  CGAGGAGCACACTCGAGAGGTGGATTACTCGGACAAATCTGTCACAGAGAATACTAGGGC  1501
        E  E  H  T  R  E  V  D  Y  S  D  K  S  V  T  E  N  T  R  A
1501  TTCTTACCCGATCGAGTACATCCCAAATGCGAAGATACCATGTGTCGGCCCCCACCCAAG  1561
        S  Y  P  I  E  Y  I  P  N  A  K  I  P  C  V  G  P  H  P  R
1561  GAACGTCATCCTGCTGGCATGCGATGCTTTTGGCGTGCTCCCTCCCGTGAGCAAGCTGAG  1621
        N  V  I  L  L  A  C  D  A  F  G  V  L  P  P  V  S  K  L  S
1621  CCTGGCGCAGACCATGTACCACTTCATAAGCGGCTATACGGCTCTGGTCGCTGGCACAGA  1681
        L  A  Q  T  M  Y  H  F  I  S  G  Y  T  A  L  V  A  G  T  E
1681  GGATGGCGTGAAGGAGCCGCAGGCGACTTTTTCAGCTTGCTTTGGGGCCGCTTTCATAAT  1741
        D  G  V  K  E  P  Q  A  T  F  S  A  C  F  G  A  A  F  I  M
1741  GCTGCACCCCACAAAGTATGCGGCGATGCTGGCTGAGAAGATGCAGAAGCAGGGAGCCAC  1801
        L  H  P  T  K  Y  A  A  M  L  A  E  K  M  Q  K  Q  G  A  T
1801  GGGATGGCTTGTGAACACCGGCTGGTCTGGTGGAAGTTACGGTTGTGGCAACCGCATCAA  1861
        G  W  L  V  N  T  G  W  S  G  G  S  Y  G  C  G  N  R  I  K
1861  GCTGCCCCACACCCGGAAGATCATCGACGCCATACACGAGGGCAGCCTCCTGAACGCCAA  1921
        L  P  H  T  R  K  I  I  D  A  I  H  E  G  S  L  L  N  A  N
1921  CTACGTCGAGACAGAGGTGTTTGGGCTTGAGATTCCGACGGAAGTCGAAGGCGTGCCCGC  1981
        Y  V  E  T  E  V  F  G  L  E  I  P  T  E  V  E  G  V  P  A
1981  GGAGATCCTGAATCCGGTGAACACTTGGGCGGACAAGGCGGCCTACAAGGAGACTCTACT  2041
        E  I  L  N  P  V  N  T  W  A  D  K  A  A  Y  K  E  T  L  L
2041  GAAGCTGGCTGGTCTCTTCAGGAAGAACTTCGAGGTCTTCGCGAACTACAAGATCGGCAA  2101
        K  L  A  G  L  F  R  K  N  F  E  V  F  A  N  Y  K  I  G  K
2101  GGACAACAAACTTACTGAGGAAATCCTCGCAGCCGGCCCCATCTTCTGATCTGGGTTGGG  2161
        D  N  K  L  T  E  E  I  L  A  A  G  P  I  F
2161  GAGAGGAGGGTACCTTCTTTCTATGTATGGCATCTCGTTGATAATTTAGCATACGTTGGG  2221
2221  GAATGAGAATCGTCGAACGGC  2241
```

Figure 9. Putative full length gene of phospho*enol*pyruvate carboxykinase (PEPCK) with translated amino acid.

Table 4. Sequences of the primer used for qRT-PCR in this study.

Primer name	Sequence 5′-3′	Tm
rtPKF1	GAGATGCCGGTGCAAAATA	53.0
rtPKR1	TCATGTCGATGTTGTTGGGC	52.0
rtPEPtaseF1	CTTGGGGCCGTTTTGTTGAG	68.6
rtPEPtaseR1	TCCCCCAGATCATGCCTGTA	67.6
rtPEPCF1	CATTTGTTGGCCAGTGGGAT	52.0
rtPEPCR1	TCACCAGAAGCGCAAAGTGA	55.0
rtPEPCKF1	CATAATGCTGCACCCCACAA	53.0
rtPEPCKR1	TGCCACAACCGTAACTTCCA	53.0
rtAOXF1	AAGGAGATCGACAACGGGACCATC	68.5
rtAOXR1	CTGGTAATGGATGTCCGAGGCAAAG	68.5
rtEF1αF1	AGCATTGTGGTCATTGG	57.8
rtEF1αR1	CTCTTGTTCATCTCAGCAG	55.9

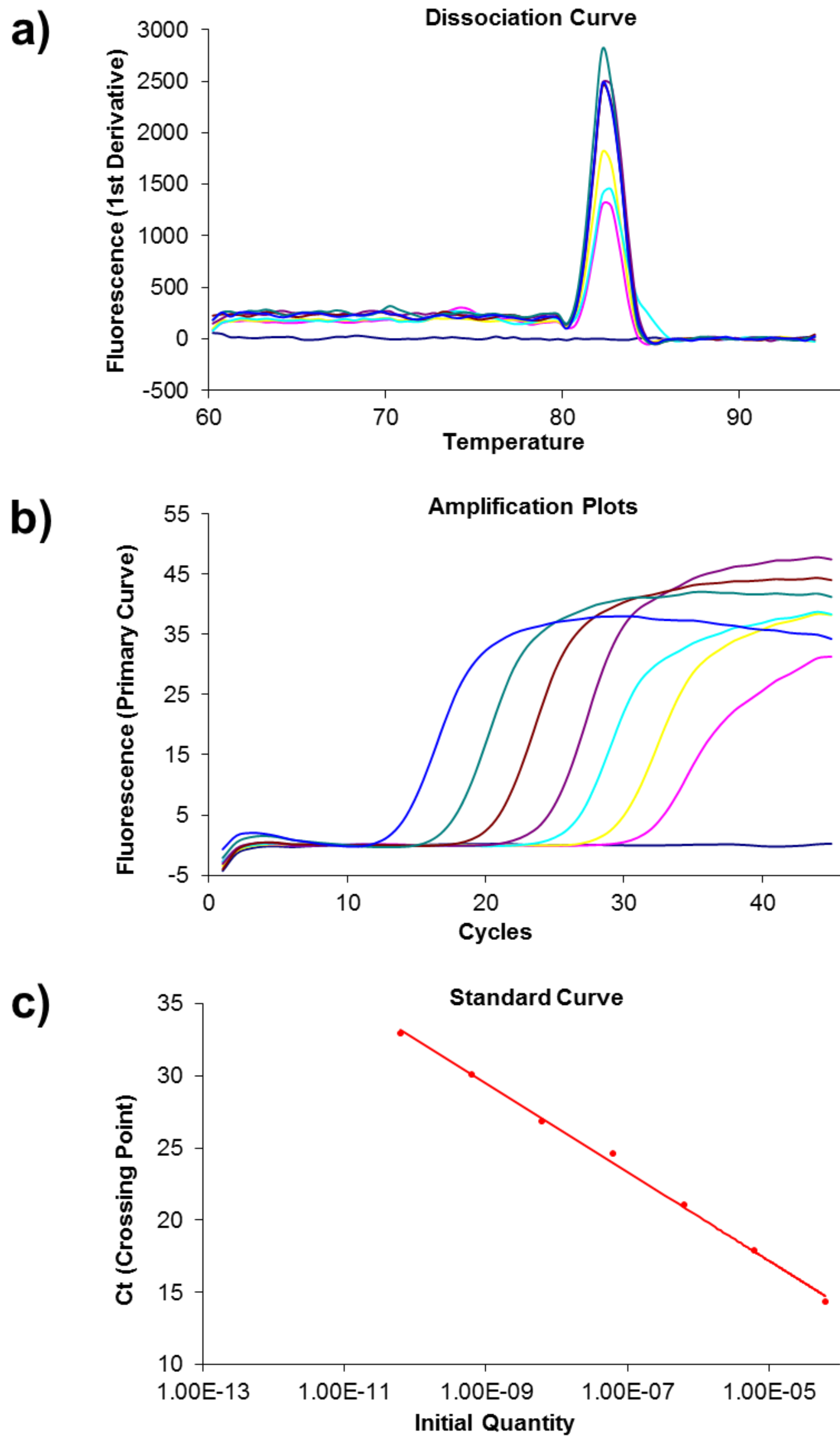

Figure 10. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of elongation factor 1 alpha (EF1α).

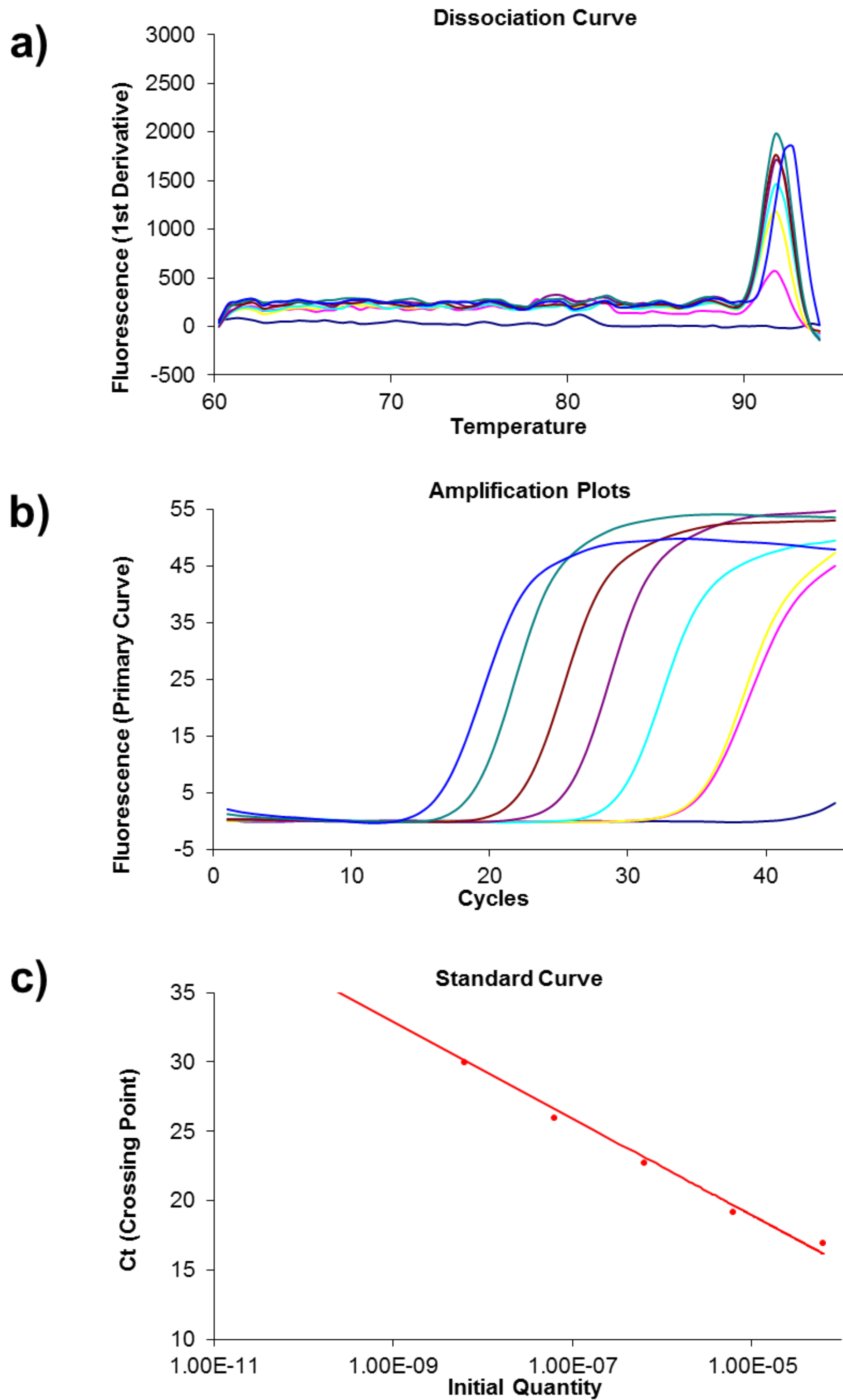

Figure 11. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of alternative oxidase (AOX).

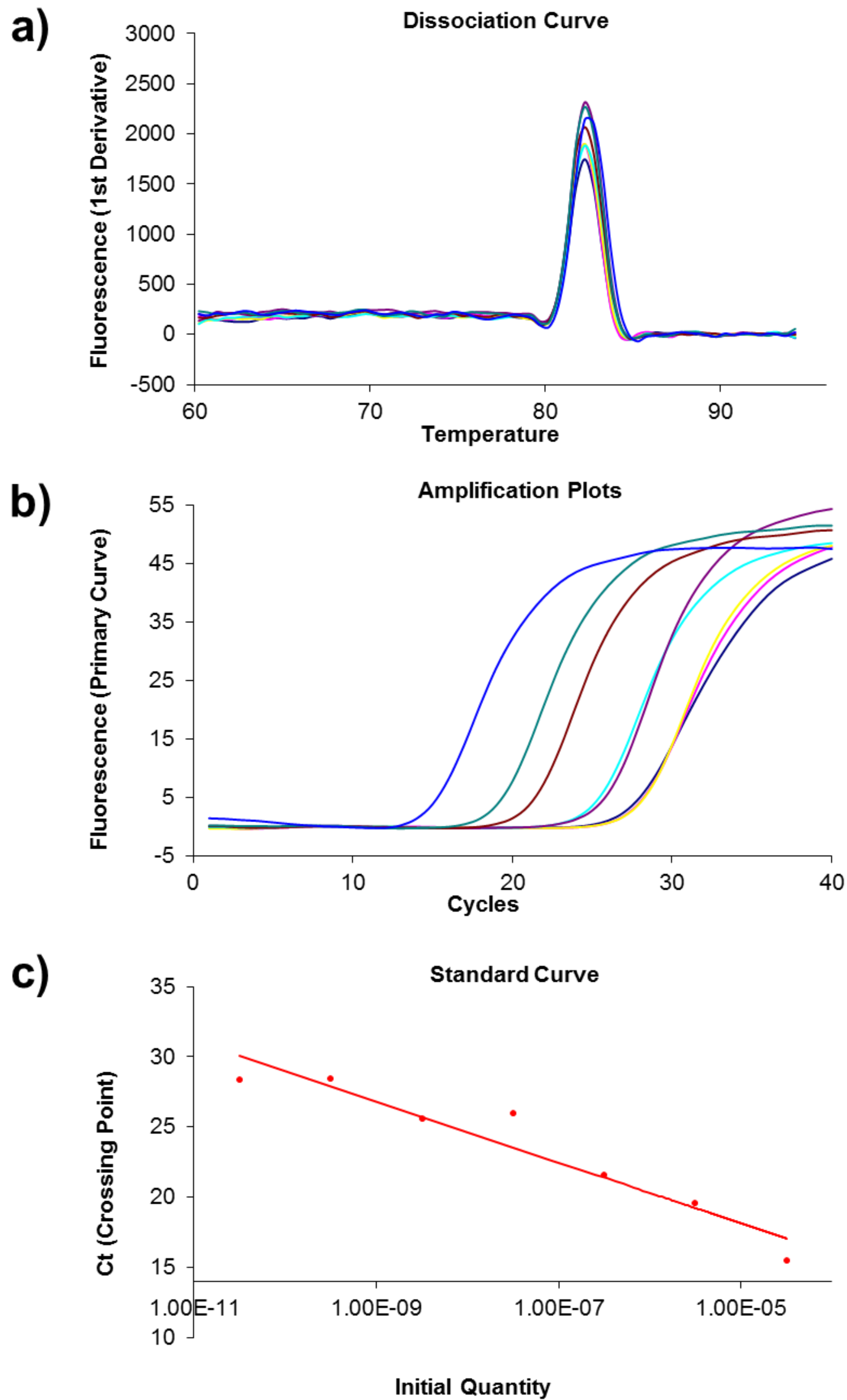

Figure 12. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of pyruvate kinase (PK).

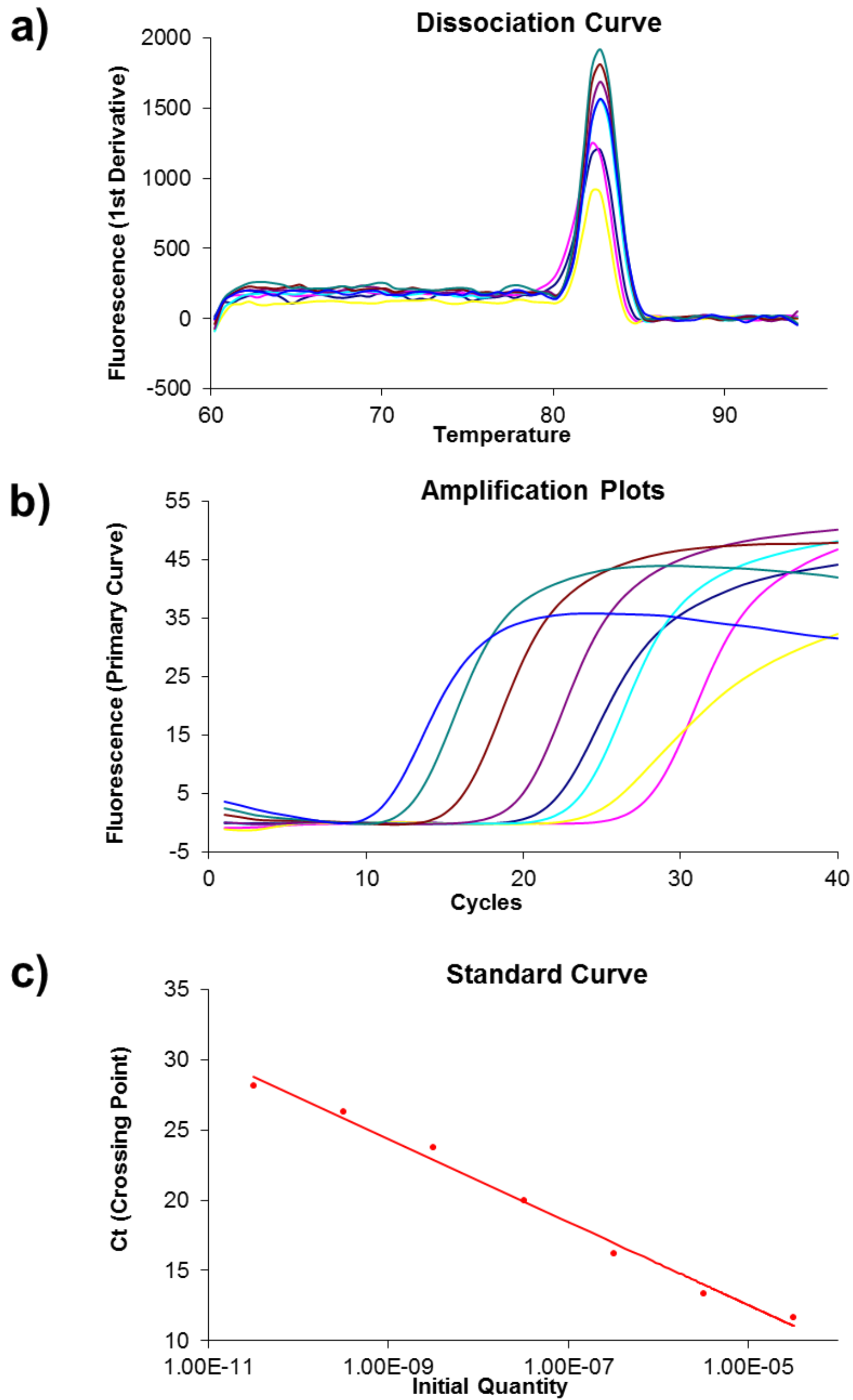

Figure 13. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of phospho*enol*pyruvate phosphatase (PEP Phosphatase; PEPtase).

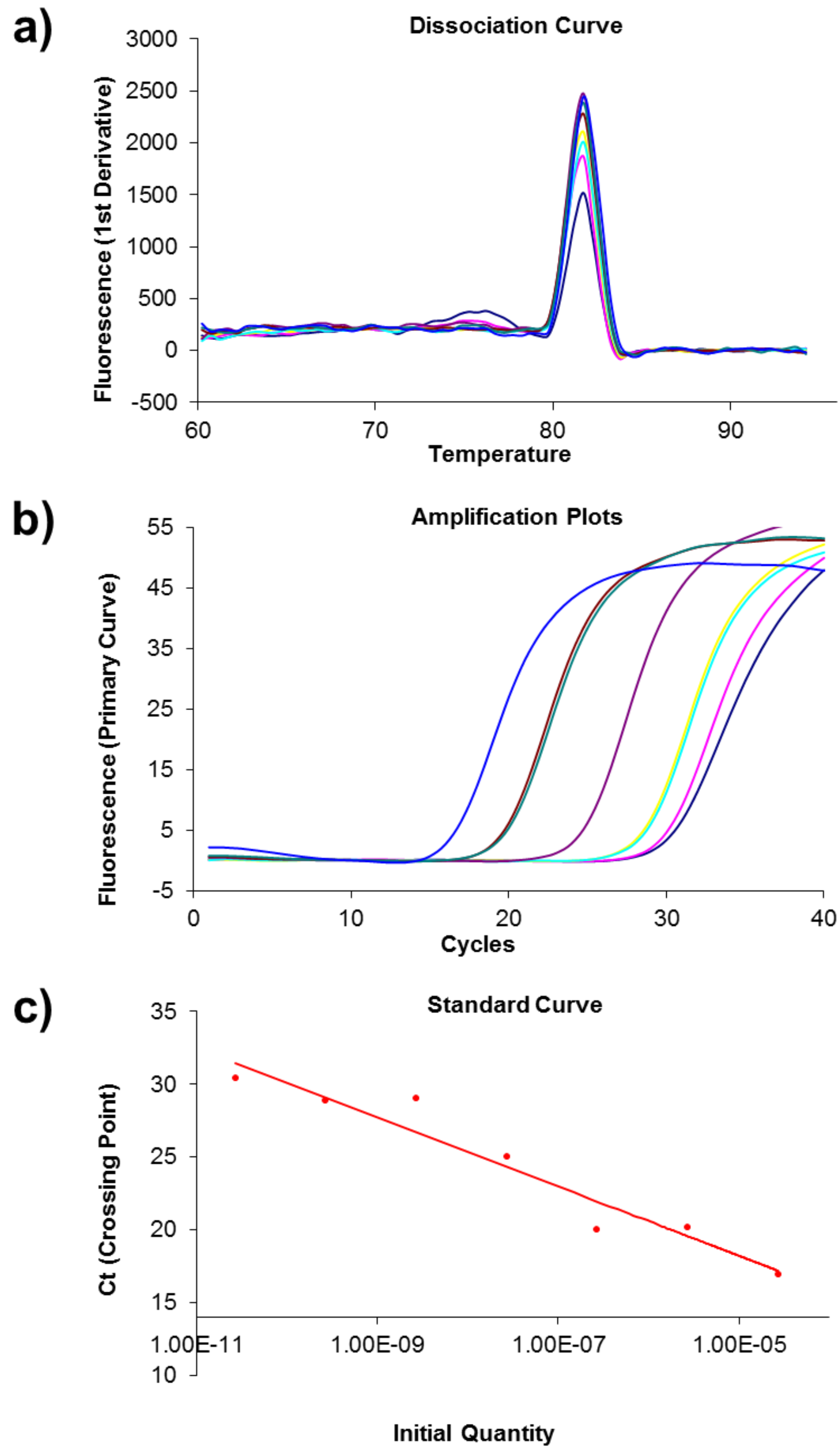

Figure 14. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of phospho*enol*pyruvate carboxylase (PEPC).

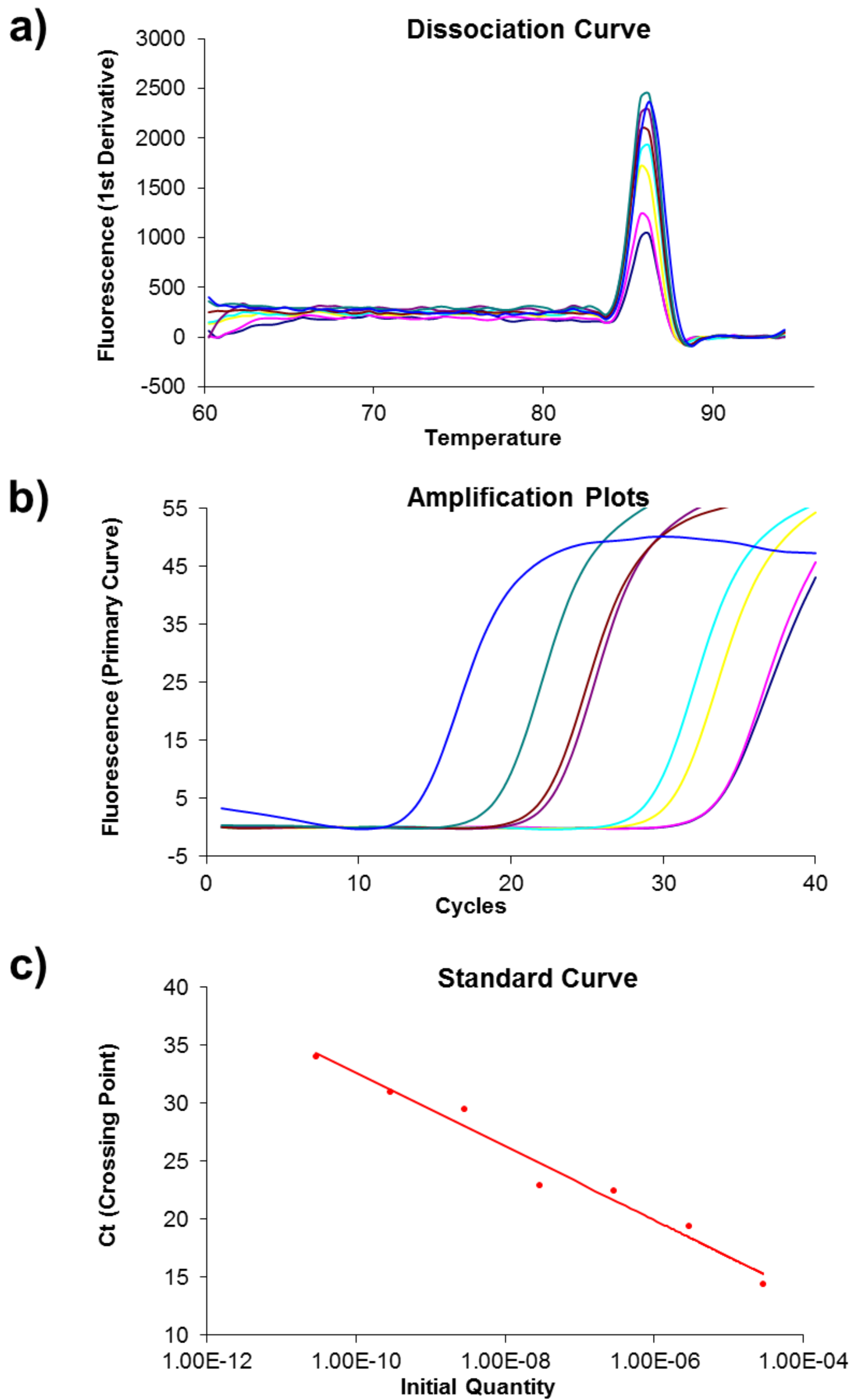

Figure 15. Dissociation curve (a), amplification plot (b) and standard curve (C) obtained from serial dilution of target cDNA of phospho*enol*pyruvate carboxykinase (PEPCK).

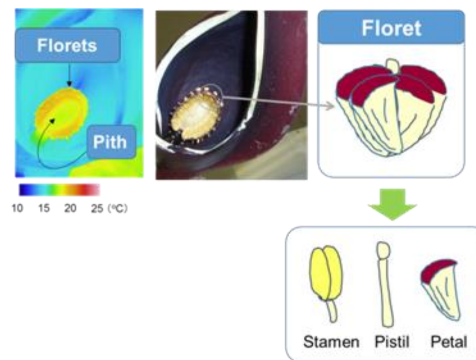

Figure 16. Infrared thermal imaging of longitudinal section of the spadix. The positions of florets, pith, and spathe are shown. Each floret composed of the stamen, pistil, and petal is also illustrated.

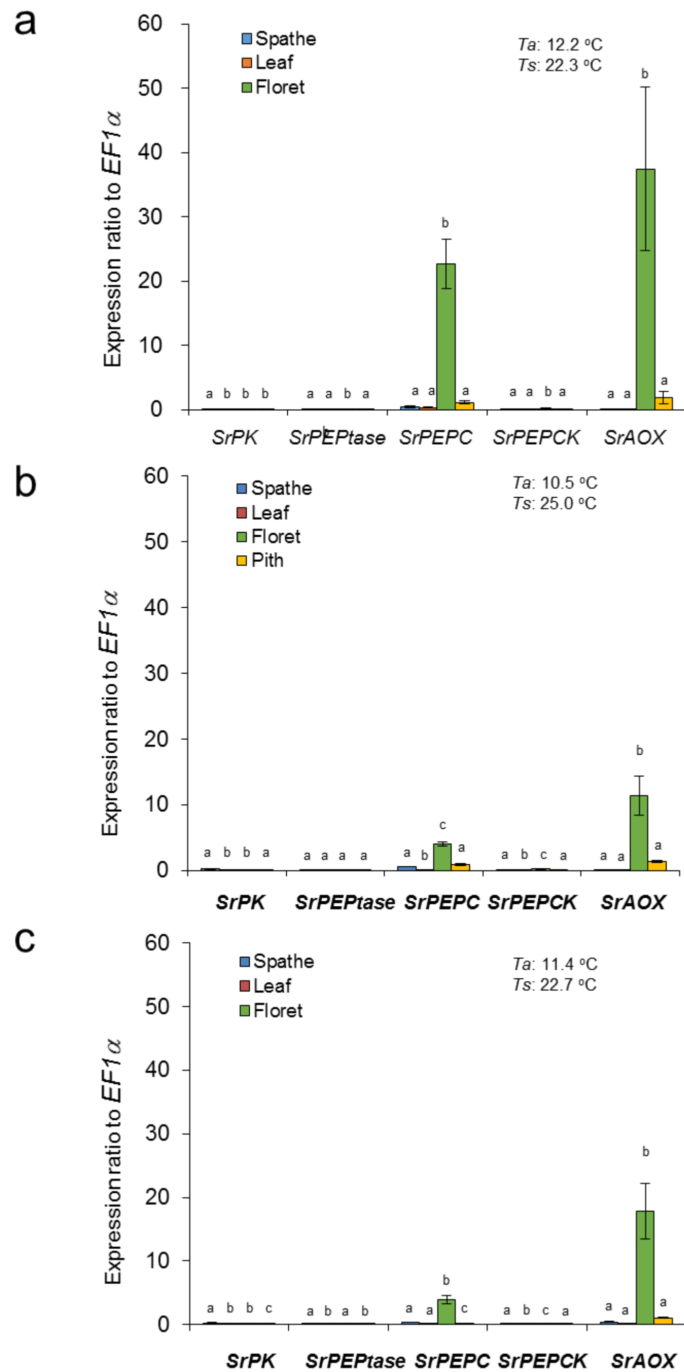

Figure 17. Levels of *SrPK*, *SrPEPtase*, *SrPEPC*, *SrPEPCK*, and *SrAOX* transcripts in various tissues during thermogenesis in *S. renifolius*. Expression profiles of *SrPK*, *SrPEPtase*, *SrPEPC*, *SrPEPCK*, and *SrAOX* transcripts in the spathe, leaf, floret, and pith of three different plant samples (a-c). *SrEF1α* transcripts were used as a normalization control. Experiments were performed in triplicate for each sample. Data are expressed as the mean ± standard deviation. Values with different letters in the graph indicate that they are statistically significantly different (n = 3; P<0.05). AOX, alternative oxidase; PK, pyruvate kinase; PEP, phospho*enol*pyruvate; PEPtase, PEP phosphatase; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase; *Ta*, ambient temperature; *Ts*, Spadix temperature.

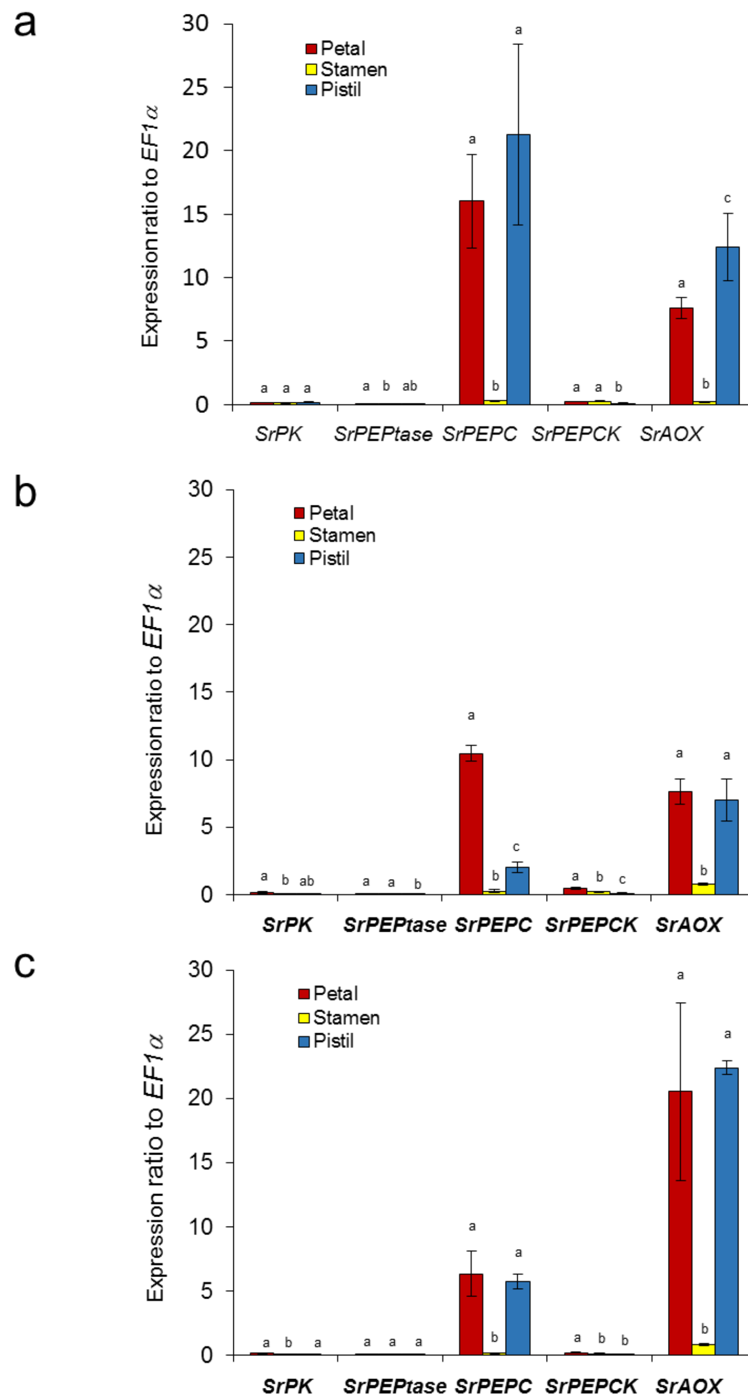

Figure 18. Levels of *SrPK*, *SrPEPtase*, *SrPEPC*, *SrPEPCK*, and *SrAOX* transcripts in various tissues during thermogenesis in *S. renifolius*. Expression profiles of *SrPK*, *SrPEPtase*, *SrPEPC*, *SrPEPCK*, and *SrAOX* transcripts in the petal, stamen and pistil of three different sample preparations (a-c). *SrEF1α* transcripts were used as a normalization control. Experiments were performed in triplicate for each sample. Data are expressed as the mean ± standard deviation. Values with different letters in the graph indicate that they are statistically significantly different (n = 3; P<0.05). AOX, alternative oxidase; PK, pyruvate kinase; PEP, phospho*enol*pyruvate; PEPtase, PEP phosphatase; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.

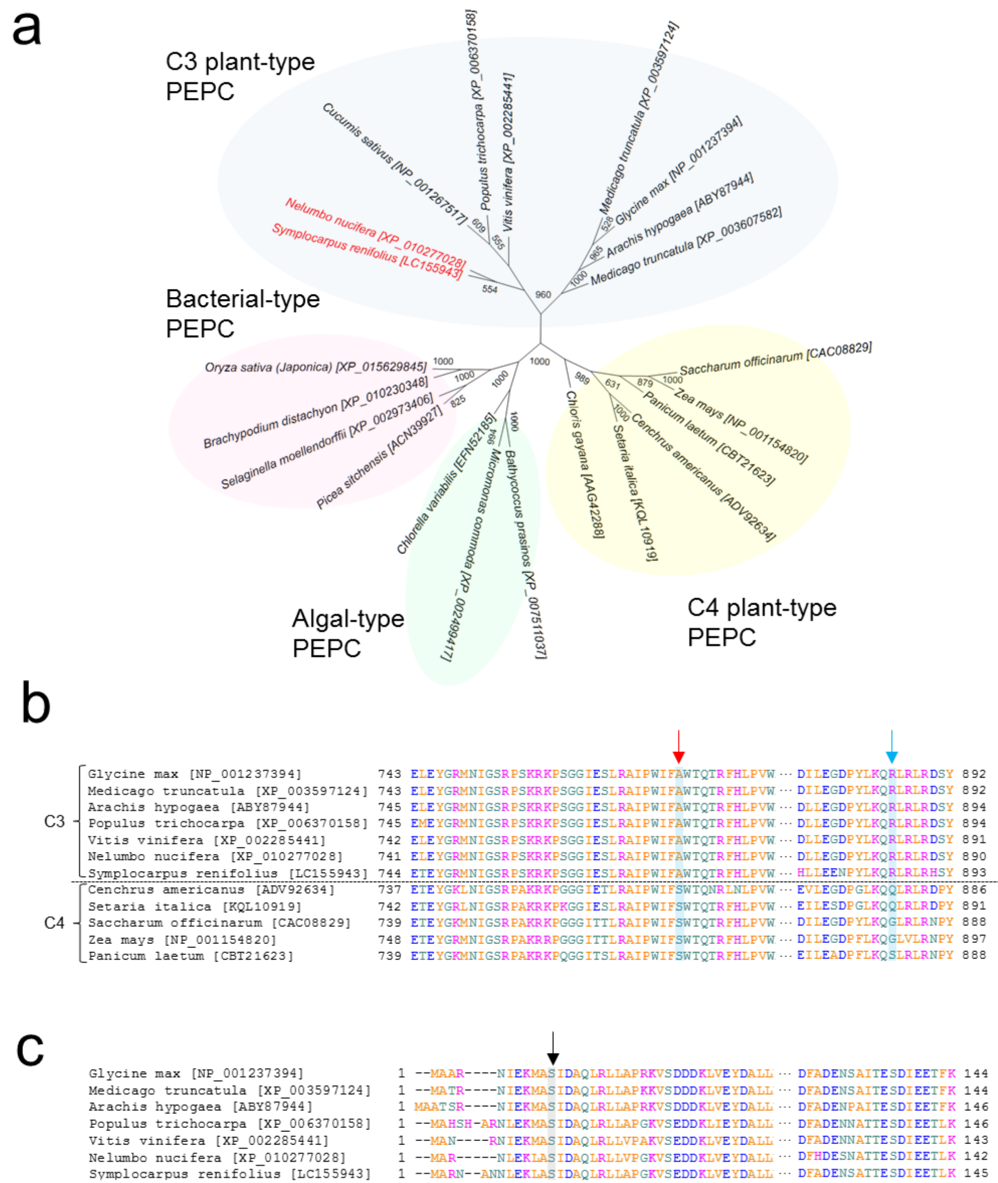

Figure 19. Phylogenetic tree and amino acid alignments of PEPC proteins.
(a) Phylogenetic tree of deduced amino acid sequences of PEPC from *S. renifolius*, other C3- and C4-plants, and bacterial and algal type organisms. (b) Positions of HCO_3^--binding loop (red arrow) and inhibitor binding site (blue arrow). (c) Conserved potential phosphorylation sites (black arrow) at the N-terminal region of PEPC. PEPC, phosphoenolpyruvate carboxylase.

Chapter 3

Measurement of enzymes activity of PEPC, PK, PEPtase and PEPCK in cytosolic fraction of thermogenic florets in skunk cabbage, *Symplocarpus renifolius*

Chapter 3

Summary

Skunk cabbage (*Symplocarpus renifolius*) is a thermogenic plant can be produced enough heat in which carbohydrates are main respiratory substrate for glycolysis. PEP is the branched point of glycolytic pathway plays a predominant role during heat production. Hence, activity of all enzymes involved in PEP metabolism were analyzed to uncover the major pathway. Enzymes assay of PEPC, PK, PEPtase and PEPCK showed a significantly high activity of PEPC in the crude enzyme extracts of thermogenic florets. Collectively, these results suggest that PEPC plays an important role in glycolysis by yielding C_4-dicarboxylic acids.

Introduction

Glycolysis, TCA cycle and mitochondrial electron transport chain play pivotal role for the respiratory pathways of higher organism for carbon metabolism and bioenergetics (Plaxton and Podestá, 2006). PEPC is one of the most important enzymes in glycolytic pathway played central role in plant which is redirected PEP to OAA with HCO_3^- to replenish the TCA cycle with the addition of CO_2 (Davies, 1979; O'Leary et al., 2011). In addition, the high energy compound of PEP in glycolytic pathway can also be catabolized by PK,

PEPtase with the addition of hydrogen (Davies, 1979). PEPCK has reversible function like carboxylation and decarboxylation for the PEP metabolism in plant (Walker et al., 2002). However, PEPC is ubiquitously present in all plants and plays particularly significant role for CO_2 fixation in chloroplast cells of C4 and CAM (Crassulacean acid metabolism) plants called photosynthetic type of PEPC (Leary, 1982; O'Leary et al., 2011). The PEPC of C3 plants is called non-photosynthetic and it can play a crucial role to replenish the TCA cycle for carbon and nitrogen metabolism (O'Leary et al., 2011). Photosynthetic and non-photosynthetic PEPC is called plant type of PEPC and bacterial types of PEPC has been reported which has functions for regulatory mechanism due to post translational modifications (Muramatsu et al., 2015).

Thermogenic spadix of *S. renifolius* produce substantial amount of heat in which carbohydrates are the main respiratory substrates, because glucose, fructose and sucrose found in the xylem sap about 2-5 mM in thermogenic stage and metabolic flows of carbohydrate have been occurred from sucrose to mitochondrial electron transport chain via glycolysis and TCA cycle (Onda and Ito, 2005). The entire carbohydrate metabolism in the spadix of *S. renifolius* is very important to provide biochemical basis to unlock the thermoregulatory mechanism (Umekawa et al., 2016). Because PEPC enzymes shows extremely high activities in the thermogenic tissues of *A. maculatum* wherein CO_2 recycled as HCO_3^- (ap Rees et al., 1981; Chivasa et

al., 1999), clearly denoted that uncovering the metabolic pathway of PEP, an important glycolytic intermediate, can be enhanced our ideas to know the thermoregulatory mechanism in thermogenic spadix of *S. renifolius*. To know the PEP metabolic pathway, I assayed crude enzyme activities of PK, PEPtase, PEPC and PEPCK which are involved in PEP metabolism.

Materials and Methods

Plant materials

All plant materials were sampled from wild *S. renifolius* grown outdoors. For enzyme assays, florets were collected from thermogenic spadices of *S. renifolius* that were sampled in ice box at Kanegasaki on March 15, 2016 and Omori on April 15 & 20, 2016.

Enzyme assays

Enzyme activities of PK, PEPtase, PEPC, and PEPCK were determined in thermogenic florets after extraction in ice-cold extraction buffer containing 0.3 M mannitol, 20 mM MOPS (pH 7.5), 2 mM EDTA, 2 mM pyruvate, 7 mM cysteine and 0.2 % bovine serum albumin. Extracts were filtered through 8 layers of Miracloth (EMD Millipore, Billerica, MA, USA). Filtrates were collected in 50-mL tubes and centrifuged at 120 x g for 10 min at 4°C. Supernatants were collected and centrifuged again at 12,000 x g for 10 min at 4°C, and stored at -80°C until enzymatic analyses (Fig. 1). Enzyme

assays of PK and PEPtase and assays of PEPC and PEPCK for decarboxylation were conducted as previously described (Ireland et al., 1980; Ueno et al., 1997; Walker et al., 2002) with a double beam spectrophotometer (Biospec-1600, Shimadzu, Kyoto, Japan) at 25°C. However, enzyme assay procedures were described here in brief.

The reaction mixture for PK contained 1 mM ADP, 0.5 mM PEP, 10 mM $MgCl_2$, 40 mM KCI, 0.1 mM NADH, 4 units of lactate dehydrogenase, 50 mM TES-NaOH (pH 7.9) and 200 µL tissue extract in a total volume of 1 ml. PEPtase activity was measured by omitting ADP from the assay mixture.

The assay mixture for PEPC contained, in a total volume of 1.0 ml, 0.1 M HEPES-NaOH (pH 7.4), 1 mM $KHCO_3$, 10 mM $MgS0_4$, 2 mM phospho*enol*pyruvate, 0.2 mM NADH, 1.5 units malate dehydrogenase and 200 µL tissue extract.

Decarboxylase activity of PEPCK was measured in a continuous assay at 25°C including 65 mM Tris-acetate (pH 7.4), 100 mM KCl, 0.3 mM OAA, 1 mM ATP, 10 µM $MnCl_2$,4 mM $MgCl_2$, 71.5 mM mecaptoethanol, 0.1 mM NADH, 2 units of pyruvate kinase, 5 units of lactate dehydrogenase and 200 µL tissue extract. After adding pyruvate kinase and lactate dehydrogenase, the change in absorbance was measured for 10 min before the addition of tissue extract to correct for the nonenzymatic decarboxylation of OAA to pyruvate. One unit

of enzyme (PK, PEPtase, PEPC and PEPCK) activity corresponds to the production of 1μmol product per one minute at 25°C.

Statistical Analysis

All data were compared using one-way factorial ANOVA (SPSS, IBM, Armonk, NY, USA). Tukey's honest significance posthoc tests were used to identify significantly different means. Significant differences between means were calculated at *P* = 0.05.

Determination of protein concentrations

Protein concentrations of isolated mitochondria and crude extracts were determined by the BCA (bicinchoninic acid) method using a Pierce^{TM} Protein Assay Kit (Thermo SCIENTIFIC) as described previously (Kakizaki et al., 2012)

Results

Enzymes Assay

As the expression level of *SrPEPC* transcripts showed higher than other identified genes (chapter 2), we determined the enzyme activity of PK, PEPtase, PEPC and PEPCK. For the enzymes assay, I used the crude extracts of cytosolic fraction of thermogenic florets (details in material and methods) for three different preparations to know the enzymes activity in three

different samples. In this assay system, I used the PEP as a substrate to start the reaction to know the activity of these enzymes. It was shown that PEPC activity is higher than PK, PEPtase, PEPCK (decarboxylation) in three crude enzyme preparation (Table 1-3).

Discussion

It has been reported that overexpression of C4-type PEPC in C3 plant redirected the carbon metabolism and fixed the CO_2 that can be produced organic acids of TCA cycle to play the anaplerotic role (Ku et al., 1999; Rademacher et al., 2002; Paulus et al., 2013). Generally, PEPC plays a very important role to fix the atmospheric CO_2 in CAM and C4 plants for photosynthesis (Rajagopalan et al., 1994; Chollet et al., 1996), but PEPC in thermogenic plants fixed the CO_2 produced in TCA cycle by the reaction with H_2O as HCO_3^- which is the unique feature for thermogenic tissues (ap Rees et al., 1981b). The enzyme activity data suggested that *SrPEPC* might play predominant role to recycle the internal CO_2 of TCA cycle, because PEPC showed higher enzyme activity than PK, PEPtase, and PEPCK which shows similarity with previous findings that PEPC activity was found high in *Arum maculatum* (ap Rees et al., 1981). The function of non-photosynthetic PEPC from C3, C4 and CAM plants are associated with the anaplerosis of TCA cycle (O'Leary et al., 2011). Notably, enzymatic characteristics revealed that post-translation modification of PEPC achieved this phenomena (Chollet et al.,

1996). Because it has been reported that substitution of single amino acid dramatically changed its enzymatic properties from C3 to C4 type –PEPC and *vice-versa* (Paulus et al., 2013). Besides that, C3-type PEPC has high affinity to PEP as substrate (Masumoto et al., 2010) and high sensitive to malate as inhibitor than C4-type (Paulus et al., 2013) which also suggested that PEPC has high affinity to the PEP that will be interesting findings to explain the regulatory activity. Expression level and enzyme activities data suggested that *SrPEPC* is the major enzyme which may convert PEP to OAA and then to malate.

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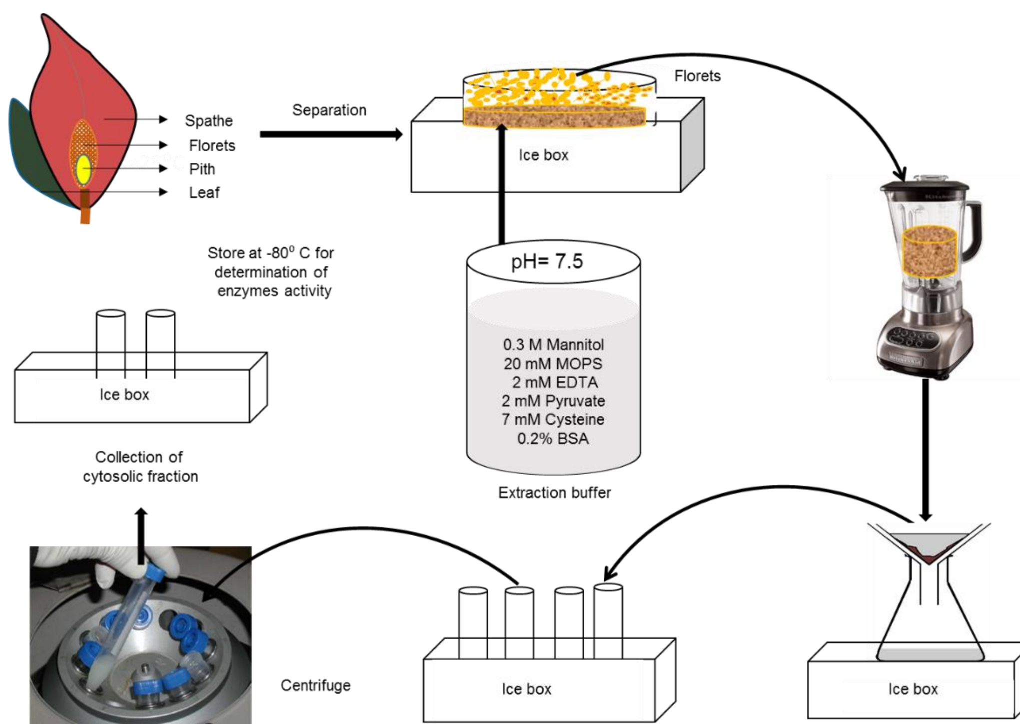

Figure 1. Schematic presentation of crude enzyme purification steps in the thermogenic florets of *S. renifolius.*

Table 1. Enzyme activities of PK, PEPtase, PEPC, and PEPCK in cytosolic fraction of thermogenic florets of *S. renifolius.*

Enzyme	Specific activity (nmol min^{-1} mg^{-1} protein)
PK	9.5 ± 2.1 [a]
PEPtase	10.2 ± 4.3 [a]
PEPC	107.5 ± 13.7 [b]
PEPCK	14.8 ± 5.7 [a]

Value for PEPCK is depicted as decarboxylation activity. Assays were performed in triplicate for each sample. Data are expressed as mean ± standard deviations. Values with different letters indicate that they are statistically significantly different (n = 3; P<0.05). PK, pyruvate kinase; PEP, phosphoenolpyruvate; PEPtase, PEP phosphatase; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.

Table 2. Enzyme activities of PK, PEPtase, PEPC, and PEPCK in cytosolic fraction of thermogenic florets of *S. renifolius.*

Enzyme	Specific activity (nmol min^{-1} mg^{-1} protein)
PK	30.4 ± 12.7 [a]
PEPtase	18.9 ± 10.1 [a]
PEPC	101.6 ± 10.8 [b]
PEPCK	8.5 ± 3.3 [a]

Value for PEPCK is depicted as decarboxylation activity. Assays were performed in triplicate for each sample. Data are expressed as mean ± standard deviations. Values with different letters indicate that they are statistically significantly different (n = 3; P<0.05). PK, pyruvate kinase; PEP, phosphoenolpyruvate; PEPtase, PEP phosphatase; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.

Table 3. Enzyme activities of PK, PEPtase, PEPC, and PEPCK in cytosolic fraction of thermogenic florets of *S. renifolius.*

Enzyme	Specific activity ($nmol\ min^{-1}\ mg^{-1}$ protein)
PK	12.48 ± 7.84 [a]
PEPtase	15.68 ± 7.00 [a]
PEPC	136.56 ± 17.30 [b]
PEPCK	7.87 ± 3.39 [a]

Value for PEPCK is depicted as decarboxylation activity. Assays were performed in triplicate for each sample. Data are expressed as mean ± standard deviations. Values with different letters indicate that they are statistically significantly different (n = 3; P<0.05). PK, pyruvate kinase; PEP, phosphoenolpyruvate; PEPtase, PEP phosphatase; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.

Chapter 4

Substrate dependent oxygen consumption of intact mitochondria purified from thermogenic florets of skunk cabbage, *Symplocarpus renifolius*

Chapter 4

Summary

Mitochondria purified from the florets exhibited an extremely low respiration activity upon pyruvate was added as a substrate, whereas a significant malate-mediated respiration was found in the same mitochondria. I changed the respiration buffer into different pHs and added co-factors that are essential for pyruvate metabolism to examine the effects of pyruvate on respiration. These data also showed that pyruvate did not increase the respiration. I hypothesize that post translational activation of AOX protein by pyruvate is facilitated by intra-mitochondrial produced pyruvate in this plant.

Introduction

In most of the plant mitochondria contains cyanide and antimycin-resistant alternative oxidase (AOX) and their functional characteristics have been known as alternative respiratory pathway in plants, fungi and algae (Moore and Siedow, 1991). Alternative respiratory pathway was the best reported in thermogenic inflorescences of Araceae such as *Sauromatum guttatum* (Meeuse, 1975). Alternative pathway respiration branches from the cytochrome pathway at ubiquinone and donates electrons directly to oxygen to form water instead of produced energy by cytochrome oxidase pathway (COX) by-passing the electron (Siedow and Moore, 1993). This type of bypass reaction has been regulated by some organic acids like pyruvate in plant

mitochondria of soybean (Day et al., 1994). Stimulation of cyanide resistant AOX respiration after adding the NADH has been occurred by the succinate, malate and pyruvate in the potato (*Solanum tuberosum*) tuber and pyruvate showed the higher activation than malate and succinate (Wagner et al., 1995). It is revealed that pyruvate is a good stimulatory effector in the respiration of potato and soybean mitochondria (Millar et al., 1993). In addition to that, some α-keto acid like pyruvate has also been shown to be able to stimulate the AOX activity whereby a half-maximal AOX activity stimulated by pyruvate reached at approximately 50 μM in thermogenic plant *S. renifolius* (Onda et al., 2007). In contrast, pyruvate as a substrate for AOX mediated respiration in thermogenic tissue of *A. maculatum* (Proudlove et al. 1987). It has been found that different thermoregulatory activities existed in between *A. maculatum* and *S. renifolius,* which is characterized by gene expression pattern of their mitochondrial complexes varies from species to species (Kakizaki et al., 2012). Furthermore, thermogenic plants of *Philodendron selloum,* and *Dracunculus vulgaris,* showed the different expression pattern of uncoupling protein (UCP) and AOX genes depending on the lipids and carbohydrates as substrate revealed that thermoregulatory activities are bit different for these two species (Ito and Seymour, 2005). Thermoregulatory activity has also been found in *N. nucifera* that is not similar with other thermogenic plants (Grant et al., 2008). All of these data suggested the first hand idea to identify the possible metabolic pathway of PEP metabolism which will pave the way for understanding the thermoregulatory phenomena in *S. renifolius.*

Materials and Methods

Plant materials

Mitochondria were purified from spadices of *S. renifolius* sampled at Omori on March 27, 2015. The spadices were collected from the field and brought into the laboratory in ice box prior to start mitochondria purification.

Isolation of intact mitochondria and respiration analyses

Mitochondria were isolated from *S. renifolius* spadices as described previously (Umekawa et al., 2016). Oxygen uptake by mitochondria was then measured according to our previous reports (Ito et al., 2011; Umekawa et al., 2016) at 25°C. The details procedures of mitochondria purification were described here. About 10-15 g fresh florets were diced and suspended in 20 ml ice cold grinding buffer containing 0.3 M mannitol, 20 mM MOPS (pH 7.5), 2 mM EDTA, 2 mM pyruvate, 7 mM cysteine and 0.2 % (w/v) bovine serum albumin. These thermogenic florets extract were then homogenized by blender for 5 seconds repeated with 3 times.

Extracts were filtered through 8 layers of Miracloth (EMD Millipore, Billerica, MA, USA). Filtrates were collected in 50 ml tubes and centrifuged at 120 x g for 10 min at 4°C. Supernatants were collected and centrifuged again at 12,000 x g for 10 min at 4°C, discarded the supernatant and pellets were re-suspended with minimal amount of washing buffer containing 0.3 M mannitol, 20 mM MOPS (pH 7.5), 2 mM EDTA, 2 mM pyruvate, and 0.2 %

(w/v) bovine serum albumin. The homogenized mixtures were then transferred into 50 ml tube and centrifuged at 12,000 x g for 10 minutes. Again, supernatant were discarded and pellets were re-suspended with 35 ml Percoll gradients into 50 ml tubes and centrifuged at 24,000 x g for 30 minutes. Mitochondria were transferred (near bottom of the tube) into 50 ml tube and washing buffer were added. These were centrifuged at 12,000 x g for 10 minutes and supernatant were gently discarded from the tube. Mitochondrial pellets were re-suspended in washing buffer and washing were repeated until pellets were solid. Mitochondrial suspension were kept in ice for determination of protein and respiration analysis.

Oxygen consumption rates of isolated mitochondrial protein (90 µg) were assayed with a Clark type oxygen electrode (Hansatech) in 1.5 ml of buffer containing 3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS and 10 mM KH_2PO_4 at pH 6.8, 7.2 and 7.6 at 25°C. Substrates and inhibitors concentration were 10 mM pyruvate, 1 mM pyruvate, 10 mM malate, 0.3 mM NAD^+, 0.1 mM TPP, 0.5 mM ADP, 0.5 mM KCN, 20 µM UK5099 and 0.1 mM *n*-PG.

Determination of protein concentrations

Protein concentrations of isolated mitochondria and crude extracts were determined as described previously (Kakizaki et al., 2012).

Results

Both of the gene expression and enzyme assays showed that *SrPEPC* is expressed not only mRNA transcript level, but also in protein level (Chapter 2 & 3). These data suggest that PEP metabolism may follow the PEPC mediated pathway. To proof this hypothesis, I purified and isolated intact mitochondria from the thermogenic floret of *S. renifolius* to measure the different substrate dependent oxygen consumption. Previously published data showed that pyruvate is the substrate for the mitochondrial respiration in thermogenic florets of *A. maculatum.* To check the mitochondrial respiration activity, I used the known substrate NADH for the AOX-mediated respiration as a control as it showed a higher oxygen uptake rate (data not shown). I determined the oxygen consumption rate by adding pyruvate without pyruvate transporter inhibitor (UK5099) to check the exogenous effects of pyruvate. My data showed that pyruvate did not increase the oxygen consumption rate (Fig. 1 a). Because, in this experimental condition, at first I used mitochondria only for starting material and observed initial oxygen consumption rate. Obtained data showed that initial oxygen consumption rate did not change after adding pyruvate. I conducted another set of oxygen uptake experiment with sequentially added pyruvate and pyruvate transporter inhibitor (UK5099), I could not get any change of oxygen consumption rate and indicated that pyruvate is not good substrate for mitochondrial respiration (Fig. 1 b). Interestingly, rate of oxygen uptake significantly increased immediate after

adding of NADH, which is known substrate for alternative respiratory pathway in both cases (Fig. 1 a-b). Oxygen consumption rate was decreased while used KCN is an inhibitor of cytochrome oxidase (COX) pathway. Finally respiratory reaction was stopped by adding *n*-propyl gallate is an inhibitor of AOX pathway.

To confirm whether pyruvate is a substrate or not for AOX mediated respiration, we changed the pH of the assay buffer at 6.8, 7.2 and 7.6 for the respiration assay as well as added co-factors NAD^+ and TPP (thiamine pyro-phosphate) to assist the reaction capacity of pyruvate dehydrogenase (Fig. 2 a-c). Considering the pH and co-factors, pyruvate did not increase the respiration as a substrate.

Discussion

Expression level and enzyme activity data suggested that SrPEPC is the major enzyme which may convert PEP to OAA and then to malate. To confirm these data, I determined the substrate dependent oxygen consumption using intact mitochondria. I first testified the measurement of respiration rate using previously described reaction buffer and pH in the thermogenic tissue mitochondria of *A. maculatuam* and they reported that pyruvate was the substrate for mitochondrial respiration (Proudlove et al.,

1987). Obtained data showed that pyruvate did not increased the respiration irrespective of different pH 6.8, 7.2 and 7.6 (Day and Hanson, 1977). I did not get any significant changed of respiration rate for pyruvate as substrate, even after adding the co-factors which are involved in the reaction of pyruvate to acetyl-CoA (Fig. 2 a-c). In contrast, pyruvate is the substrate of the thermogenic tissue-mitochondria of *A. maculatum*, which is the difference from the *S. renifolius*. This study further revealed that pyruvate has stimulatory functions to activate the non-covalently associated dimer form of AOX in the thermogenic spadix of *S. renifolius* to produce metabolic heat (Onda et al., 2007). It has been reported that rapidly increased the oxygen consumption rate after addition of pyruvate which revealed that pyruvate is the stimulator of AOX capacity (Moore and Siedow 1991). The present study revealed that PEP is converted into OAA by the SrPEPC and thereafter, OAA is converted into malate by the malate dehydrogenase and malate replenish the TCA cycle intermediates to continue the metabolic heat production of *S. renifoilius*. Obtained data further suggested that pyruvate is produced inside the mitochondria through the PEPC mediated pathway from PEP.

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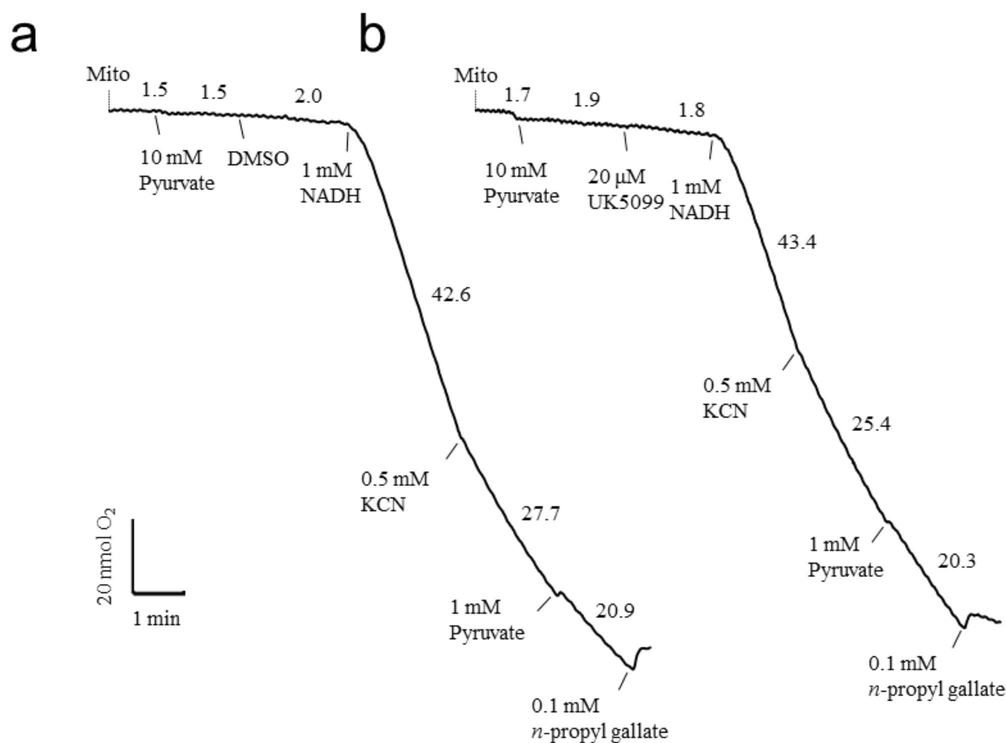

Figure 1. Oxygen consumption of intact mitochondria purified from thermogenic florets of *S. renifolius*. Substrates and compounds were added using freshly prepared mitochondria. Values over the traces indicate oxygen consumption in nmol of O_2 per min. Mitochondrial respiration rates with pyruvate as a substrate in a buffer without cofactors at pH 7.2 (a, b). All assays were performed at 25°C. Typical results are shown, representing three independent respiration assays.

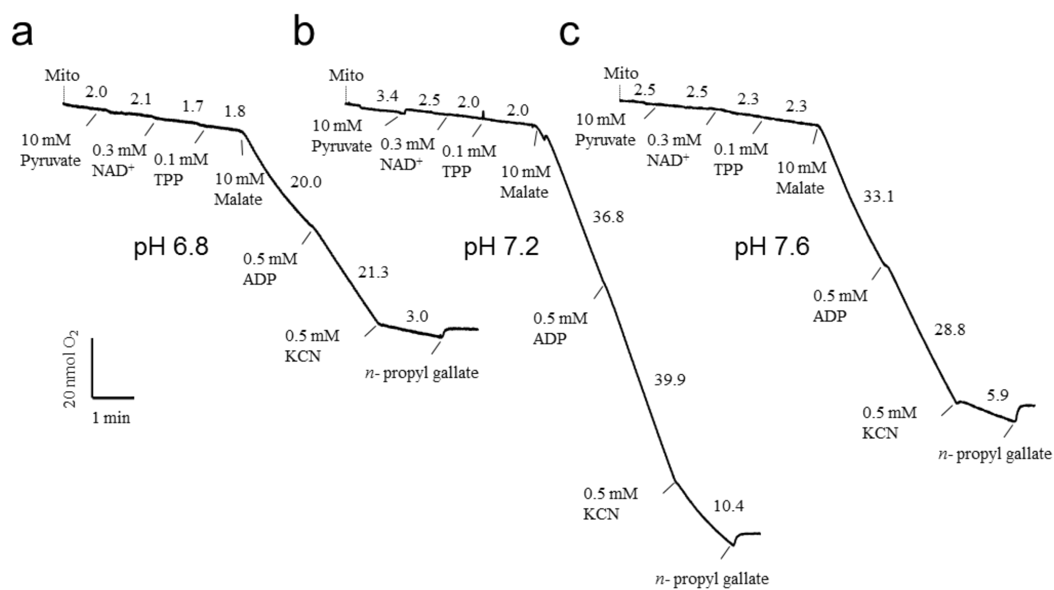

Figure 2. Oxygen consumption of intact mitochondria purified from thermogenic florets of *S. renifolius*. Substrates and compounds were added using freshly prepared mitochondria. Values over the traces indicate oxygen consumption in nmol of O_2 per min. Mitochondrial respiration rates with pyruvate as a substrate in the presence of cofactors NAD^+ and TPP at pH 6.8 (a), 7.2 (b), and 7.6 (c). All assays were performed at 25°C. Typical results are shown, representing three independent respiration assays. NAD, nicotinamide adenine; TPP, thiamine pyrophosphate.

Chapter 5

General discussion

Chapter 5

Summary of the thesis

In this thesis, I focused on PEP metabolism to unlock the major pathway in the thermogenic spadices of *S. renifolius*. To uncover predominant metabolic pathway, I cloned, sequenced and deposited full length cDNAs of PK, PEPtase, PEPC and PEPCK which are associated with PEP metabolism in glycolysis. Tissue specific level of mRNA expression analysis revealed that *SrPEPC* co-expressed abundantly with *SrAOX* in the thermogenic florets; more specifically in petal and pistil (Chapter 2). Intriguingly, enzyme assay of crude extracts also showed that SrPEPC activity is higher than other enzymes (Chapter 3). More interestingly, substrate dependent oxygen consumptions of intact mitochondria showed that cyanide-insensitive AOX respiration increased by malate rather than pyruvate (Chapter 4) which indicated that SrPEPC mediated pathway plays predominant role for replenishing the TCA cycle intermediates to continuous supply of reducing equivalent to the AOX respiration for producing sufficient heat in thermogenic tissues of *S. renifolius*.

General discussions

Before this study there was no information about the PEP metabolism pathway in the spadices of *S. renifolius.* Moreover, there was no data base sequence of full length cDNAs and information of enzyme activities of PK, PEPtase, PEPC and PEPCK in this plant. I reported first time that pyruvate is

not a respiratory substrate in thermogenic inflorescence of *S. renifolius.* Hence, collectively, these data revealed that PEP metabolism follows PEPC mediated pathway rather than PK, PEPtase and PEPCK to heat production through AOX-mediated respiration. Plant mitochondria comprises several complexes in the electron transport chain (ETC). In plant, ETC comprises classical oxidoreductase complexes (I-IV) and AOX located in matrix and intermembrane space, provides electron to the ETC for oxidative phosphorylation which are linked to the mobile electron transporter cytochrome *c* and ubiquinone (Schertl and Braun, 2014). For oxidative phosphorylation, complex-I has 50 subunits plays major role to supply electron into the ETC (Braun et al., 2014). Some functional domain have been characterized in complex I likely as γ-type carbonic anhydrase in *Arabidopsis* and their functions are associated with plant reproductive development through recycling CO_2 (Fromm et al., 2016a; Fromm et al., 2016b). All of these published data suggested that reproductive organ like florets, petal, and pistil may have major role of carbonic anhydrase in complex I in *S. renifolius* to receive continuous supply of respiratory substrate through PEP metabolism.

Herein, we showed that transcripts encoding the C3-type of PEPC protein SrPEPC were specifically and highly co-expressed with those encoding SrAOX in the petals and pistils of thermogenic florets, whereas the expression levels of *SrPK*, *SrPEPtase*, and *SrPEPCK* transcripts were extremely low in all tissues examined (Fig. 17 & 18, Chapter 2). Moreover,

enzymatic activities of PEPC in thermogenic florets were higher than those of PK, PEPtase, and PEPCK (Table 1-3, Chapter 3), indicating that PEP is predominantly catabolized by PEPC in thermogenic tissues of *S. renifolius*. Although extremely high enzymatic activities of PEPC have been reported in other thermogenic plants including *A. maculatum* (ap Rees et al., 1981), this is the first study to show tissue-specific co-expression of *PEPC* and *AOX* in quantitative gene expression analyses of thermogenic plants.

Because the AOX-mediated energy-dissipative respiration pathway contributes significantly to cellular thermogenesis in plants, co-expression of *SrAOX* and *SrPEPC* detected in the present study may be critical for metabolic cross-talk between the cytosol and AOX-expressing mitochondria in thermogenic cells (Fig. 1, In this chapter). In thermogenic cells, PEP was primarily catabolized by PEPC to produce OAA, which is used directly as a mitochondrial respiration substrate or is converted to malate by malate dehydrogenase(Fernie et al., 2004; Sweetlove et al., 2010) for use as a respiratory substrate (Fig. 1 & 2, Chapter 4). In either case, such PEPC-mediated metabolism may contribute significantly to continuous carbon flow in furnishing C4-dicarboxylic acids that maintains increased respiration for thermogenesis in *S. renifolius*. These data are consistent with those of previous report of constitutive PEPC-overexpressing transgenic plants, in which carbon flow was redirected from soluble sugars to organic acids (Rademacher et al., 2002). More importantly, because PEPC catalyzes the

addition of HCO_3^- to PEP (Davies, 1979), excess CO_2 that is liberated with increased mitochondrial respiration in thermogenic cells may be catabolized by complex I-integrated mitochondrial γ-carbonic anhydrases to form HCO_3^- (Sunderhaus et al., 2006; Fromm et al., 2016b), which is subsequently converted to oxaloacetic acid by PEPC. Previously, it was shown that PEPC is highly expressed and participates in the recycling of respired CO_2 in the spikelets of C3-type plants (Imaizumi et al., 1997). Similarly, fruiting plants possess a system known as fruit photosynthesis(Blanke and Lenz, 1989), in which CO_2 from mitochondrial respiration is refixed by PEPC. These data indicate that thermogenic plants express higher levels of PEPC and AOX enzymes in their non-photosynthetic organs, such as in the spadices of *S. renifolius* which developed specialized metabolisms not only for recycling of excess CO_2 similar to that seen in other C3 plants but also for energy-dissipating AOX-mediated mitochondrial respiration during evolution. Accordingly, integration of PEPC-mediated CO_2 assimilation and AOX-mediated mitochondrial respiration probably act as substantial carbon resources in thermogenic plants.

In the present study, mitochondria purified from thermogenic florets of *S. renifolius* did not oxidize exogenous pyruvate as a respiratory substrate in the presence or absence of cofactors (Fig. 1 & 2, Chapter 4). In contrast, mitochondria from thermogenic spadices of *A. maculatum* were previously shown to oxidize exogenous pyruvate in the absence of exogenous cofactors

(Proudlove and Moore, 1984). In addition, rapid oxidation of pyruvate by isolated *A. maculatum* mitochondria was sensitive to UK5099, suggesting the presence of mitochondrial pyruvate carriers, as shown recently in *Arabidopsis* (Li et al., 2014). Hence, expression of mitochondrial pyruvate carriers in thermogenic florets of *S. renifolius* may be lower than in those of *A. maculatum*. Because pyruvate has been identified as an allosteric activator of AOX in *S. renifolius* (Onda et al., 2007). AOX activities in this plant may be post-translationally regulated by intra-mitochondrially produced pyruvate via malic enzyme (Wedding and Whatley, 1984; Edwards et al., 1998) that uses malate from the PEPC-mediated metabolic pathway. It should be noted here that NAD-dependent malic enzyme α-and/or β-subunits have also been identified in mitochondria from thermogenic florets of *S. renifolius* (Kakizaki et al., 2012).

In conclusion, I found that *SrPEPC* is abundantly co-expressed with *SrAOX* in thermogenic florets of *S. renifolius*. These results further suggest that PEPC plays a role in metabolic heat-production in furnishing C4-dicarboxylic acids to AOX-expressing mitochondria in other thermogenic plants.

Conclusion remarks

In this thesis, I have summarized the PEP metabolism pathway based on the gene expression, enzyme activity and mitochondrial respiration analysis. PEPC is the major enzyme which can able to refix mitochondrial CO_2

via γ-carbonic anhydrase of complex I, as already discussed above. Several further studies need to elucidate the actual mechanism of PEPC mediated pathway. Molecular study of complex I can be the best target for elucidation this mechanism. It will be interesting topics for the study to know the role of γ-carbonic anhydrase as well as mitochondrial pyruvate carrier proteins for the find out heat regulatory metabolism in thermogenic plants.

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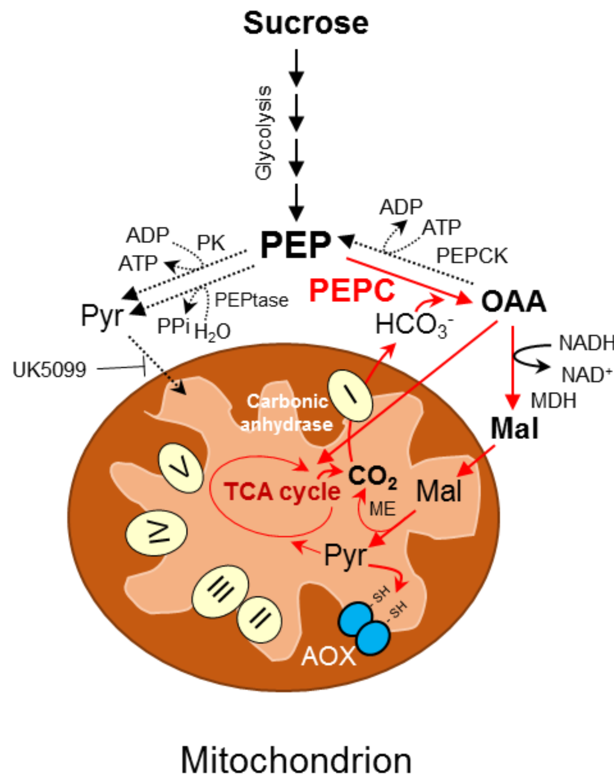

Figure 1. PEP-mediated metabolism in thermogenic cells of *S.renifolius.* PEP yielded by glycolytic pathway is predominantly catabolized by PEPC to yield oxaloacetic acid (OAA). OAA is either directly oxidized in the mitochondria or converted to malate (Mal) by cytosolic malate dehydrogenase (MDH). Malate is catabolized by the malic enzyme (ME) in mitochondria to produce pyruvate (Pyr) and carbon dioxide (CO_2). Intra-mitochondrially produced pyruvate can either enter the TCA cycle or allosterically activate the alternative oxidase (AOX). The CO_2 produced in the TCA cycle and by the malic enzyme is catabolized by complex I-integrated mitochondrial carbonic anhydrase to form bicarbonate (HCO_3^-) and is catabolized by PEPC to yield OAA. Expression of PEPC and AOX can enhance the recycling of respired CO_2 and energy dissipative mitochondrial respiration. Major metabolic pathways predicted in the present study are depicted by red arrows. Complexes I–V of the electron transport chain are shown. UK5099, an inhibitor of the pyruvate transporter, is also shown. PK, pyruvate kinase; PEP, phospho*enol*pyruvate; PEPCK, phospho*enol*pyruvate carboxykinase (PEPCK); PEP phosphatase (PEPtase); PPi, pyrophosphate; Suc, succinate; Cit, citrate.

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