Functional Analyses of Phosphoenolpyruvate Carboxykinase (PEPCK) in Tomato (*Solanum lycopersicum* L.) Plant

September 2015

Yongxing HUANG

Functional Analyses of Phosphoenolpyruvate Carboxykinase (PEPCK) in Tomato (*Solanum lycopersicum* L.) Plant

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Agricultural Science
(Doctoral Program in Biosphere Resource Science and Technology)

Yongxing HUANG

Content

Abbreviations

CaMV: cauliflower mosaic virus

CAM: Crassulacean Acid Metabolism

DAF: days after flowering

DAS: days after sowing

EC: electrical conductivity

HPLC: high performance liquid chromatography

ME: malic enzyme

mMDH: mitochondrial malate dehydrogenase

OAA: oxaloacetate

PEP: phosphoenolpyruvate

PEPCK: phosphoenolpyruvate carboxykinase

PK: pyruvate kinase

RI: refractive index

RNAi: RNA interference

RT-PCR: reverse transcription polymerase chain reaction

SGN: Solanaceae genome network

WT: wild type

List of table and figures

Chapter1 General introduction

Phosphoenolpyruvate carboxykinase (PEPCK) is the ATP dependent (EC 4.1.1.49) or GTP dependent (EC4.1.1.32) enzyme of lyase family (Krupa and Srinivasan, 2006), which converts the decarboxylation reaction from oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and carbon dioxide. As the key rate-limiting enzyme, PEPCK is regulating gluconeogenesis metabolic pathway in many organisms including animals, microorganisms and plants (Chakravarty et al., 2005; Aich et al., 2003; Matín et al., 2011).

In animals, two GTP-dependent isoforms are separately localized in cytosol and mitochondria separately and those function to keep the glucose homeostasis in blood as a part of glucose synthesis modulators in the liver, kidney and adipose (Hanson and Patel, 1994; Hanson and Reshef, 1997). Over-expression of PEPCK in a mouse with type II diabetes mellitus (Valera et al., 1994; Sun et al., 2002), which is the most common form of human diabetes, highlighted the key regulatory role of PEPCK in the blood glucose level. On the other hand, overexpression of cytosolic PEPCK in skeletal muscle in a mouse brought itself more active and longer life. For example, the transgenic mice can run totally more than 5 km for 4 h without feeding (Parvin et al., 2007), suggesting the importance of PEPCK in carbonaceous energy supply. In bacteria （*Mycobacterium tuberculosis*), researchers discovered the function of PEPCK participating in DNA recombination and the immune system by enhancing cytokine

activity (Liu et al., 2006; Aich et al., 2003).

In plants *PEPCK* gene has been known to express in a tissue specific manner (Furumoto et al., 1999; Finnegan and Burnell 1995; Finnegan et al., 1999; Martín et al., 2011) and under specific conditions such as asparagine, 5% CO_2, higher pH, drying and salty stress condition can promote the PEPCK abundance (Sáez-Vásquez et al., 1995; Walker and Chen, 2002; Walker et al., 1999; Martín et al., 2007; Wringler et al., 1999; Saito et al., 2008). Recent studies on PEPCK mostly focused on role(s) in the regulation of gluconeogenesis in *Arabidopsis thaliana*, cucumber and maize (Fig. 1. 1). Additional functions of PEPCK were also studied in plants. It is known that PEPCK plays a key role in production of carbohydrate from storage lipid and protein during the germination of fat-storing seeds (Leegood and Rees, 1978; Rylott et al., 2003; Penfield et al., 2004). PEPCK also plays an important role in photosynthetic carbon assimilation in C_4 and Crassulacean Acid Metabolism (CAM) plants providing partial decarboxylation of C_4 acids (Reiskind and Bowes, 1991; Furumoto et al., 1999). Besides, PEPCK also functions in adjusting pH balance, anaplerotic reactions in some C_3 plants (Lea et al., 2001; Walker et al., 2001).

In fresh berry-type fruits, it has been reported that PEPCK dominantly expresses during fruit ripening (Walker et al., 2011a; Famiani et al., 2005, 2009). The researchers had mainly worked on isolation, characterization of genes, and analyzing biochemical properties in fruits of cherry, strawberry, blackberry, red currant and Japanese plum

(Walker et al., 2012; Famiani et al., 2009, 2012). Regarding the physiological function, PEPCK was predicted to be involved in the organic acid metabolism in cherry fruit because the change of malate dissimilation ratio was well consistent with the PEPCK protein abundance during fruit development (Famiani et al., 2005).

In tomato, PEPCK is a 74kDa polypeptide and abundantly expressed in pericarp of ripening fruits, and germinating seeds (Walker et al., 1999; Bahrami et al., 2001; Saito et al., 2008; Yin et al., 2010a). In physiological study of PEPCK in tomato fruits, Osorio (2013) confirmed increase of malate and of soluble sugar in *E8* promoter-driven RNAi transgenic lines in which *SlPEPCK* expression was suppressed. PEPCK depression additionally affected starch conservation, which caused starch degradation in mature-red fruits.

Despite PEPCK locates at the crossover point on the primary metabolism pathway including glycolysis / gluconeogenesis in various organisms, the physiological functions of PEPCK in a whole plant still remains uncertain in fresh berry type crops because of lack of a functional analysis with those crop species.

In order to clarify the physiological function of PEPCK, in chapter II, our research team generated the RNA interference (RNAi) transgenic tomato lines in which *SlPEPCK* expression was suppressed by either CaMV *35S* constitutive promoter or *E8* fruit-specific promoter (Sanuki, 2010). In this research, as subsequent analyses, the author performed detailed characterization of those RNAi transgenic lines in Chapter II.

The systemic suppression by the *35S* promoter firstly revealed PEPCK contributes to seedling establishment and the vegetative growth during juvenile stage in tomato. Furthermore, it was demonstrated that PEPCK also affects sugar / amino acid accumulations in ripening fruit of tomato probably by suppression of the gluconeogenesis pathway.

Taking the results of Chapter II, it is likely that the excessive expression of PEPCK during fruit ripening would create high quality fruit with higher sugar / acid ratio. In order to examine this hypothesis, characterization of a tomato plant overexpressing PEPCK will be indispensable. Although the PEPCK overexpression super mouse was already created for studying physiological function of PEPCK in animals (Hanson and Hakimi, 2008), actually a PEPCK-overexpressing plant has not been produced up to date. In this context, in Chapter III, I generated the transgenic tomato lines over-expressing *SlPEPCK* by either CaMV*35S* or *E8* promoter. Detailed characterization of the phenotypic and metabolic properties of the *35S* promoter-driven lines demonstrated that the transgenic seedlings exhibited earlier germination and better seedling growth compared with the wild type. Furthermore, post-germination growth of the transgenic lines was enhanced by an exogenous sucrose supply. In the fruits, increased soluble sugars and decreased malate contents were observed in red-ripe stage in both the *35S* and *E8* promoter-driven lines, indicating the participation of gluconeogenesis in sugar / acid metabolism during fruit ripening.

The author will discuss about the function of PEPCK not only in tomato but also other fresh berry fruit in Chapter IV. A burst of PEPCK expression during fruit ripening is widely observed in horticultural crops having berry type fruits. Therefore, the PEPCK-mediated regulation mechanism through the gluconeogenesis pathway in sugar accumulation would be common in the berry type fruits. This study provides important new and notable information, which will contribute to understand the regulation mechanisms for sugar and organic acid metabolism and its balance in developing fruits not only in tomato but also in crops having berry-type fruits. Furthermore, this study demonstrates the physiological function of PEPCK in sugar / acid content in tomato fruits and seedling growth during post-germination and juvenile stage.

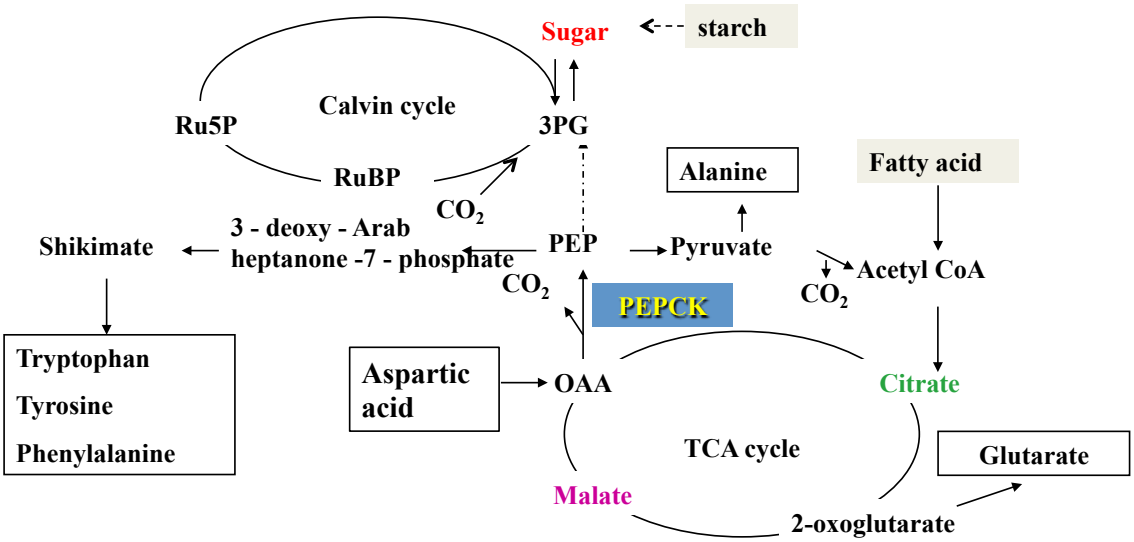

Figure 1.1 Sugar biosynthesis and catabolic pathways in tomato schematic illustration of the metabolism pathway of PEPCK.

PEPCK reacts as the rate-limiting enzyme in gluconeogenesis and also indirectly influences the glycolysis and TCA cycle, which works as a key role for sugar, organic- and amino acid accumulation.

Table 1-1 The *SlPEPCK* gene expression in different plant species.

English name	Scientific name	Gene	Expression	Reference
Cucumber	*Cucumis sativus*	1	Root, Stem, Leaf, Cotyledon	Kim and Smith, 1994
Tomato	*Lycopersicon esculentum*	1	Fruit, Root, Stem	Bahrami et al., 2001
	Panicum maximum	1	Root	Bailey et al., 2007
Corn	*Zea mays*	2	Root, Stem, Flower, Cotyledon	Furomoto et al.,1999 Dong et al., 2001
Pineapple	*Ananas comosus*	2	Leaf	Martin et al., 2011
	Urochloa panicoides	4	Leaf	Finnegan and Burnell, 1995
Rice	*Oryza sativa*	2	Leaf	Nomura et al., 2005
	Arabidopsis thaliana	3	Root, Stem, Flower, Cotyledon	Malone et al., 2007 Brown et al., 2010 Penfield et al., 2012

Chapter 2 Phosphoenolpyruvate carboxykinase (PEPCK) deficiency affects the germination, growth and sugar / acid ratio in tomato fruit (*Solanum lycopersicum* L.)

2.1 Introduction

In plants, phosphoenolpyruvate carboxykinase (PEPCK) [EC 4.1.1.49] is localized in the cytosol and catalyzes the ATP-dependent decarboxylation of oxaloacetate (OAA) into phosphoenolpyruvate (PEP) (OAA + ATP → PEP +CO_2 + ADP) (Leegood and Walker, 2003; Watanabe et al., 1984). PEPCK is considered a key rate-limiting enzyme that regulates an early step in the gluconeogenesis pathway in many organisms, including higher plants (Leegood and ap Rees, 1978). Furthermore, PEPCK is utilized as a decarboxylase in the photosynthetic CO_2-concentrating mechanisms of some CAM plants and C_4 plants (Dittrich et al., 1973; Furumoto et al., 1999; Hansen and Juni, 1974; Martín et al., 2011; Reiskind and Bowes, 1991; Walker and Leegood, 1996; Wingler et al., 1999). PEPCK is present in a range of plant tissues (Mazelis and Vennesland, 1957; Walker and Chen, 2002), including many fruits, such as grapes, tomatoes, oranges, soft fruits, apricots, cherries and plums (Baldicchi et al., 2015; Bahrami et al., 2001; Echeverria and Valich, 1989; Famiani et al., 2005, 2009, 2012; Famiani and Walker, 2009; Martín et al., 2007; Ruffner and Kliewer, 1975; Walker et al., 2011a; Walker and Leegood, 1995). On the other hand, PEPCK

expression is regulated in tissue specific and developmental stage-specific manners in flowering plants. For example, PEPCK is highly expressed in germinating seedlings of fat-storing seed plants (Leegood and ap Rees, 1978; Martín et al., 2007) and has been proposed to be involved in carbohydrate supply from lipids and proteins through a regulatory role in the gluconeogenesis pathway (Leegood and ap Rees, 1978; Penfield et al., 2004; Rylott et al., 2003). However, in spite of the intense research activity on PEPCK, no reports are available on possible roles of this enzyme and gluconeogenesis on total plant growth / development.

In the flesh of many berry-type (including tomatoes, gooseberries, blueberries and red currants) the abundance of PEPCK increases greatly during ripening (Bahrami et al., 2001; Famiani et al., 2005, 2009). In tomato fruits, suppression of PEPCK expression by RNA interference driven by fruit ripening-specific promoter resulted in an accumulation of malate with a decrease in the sugar and starch content (Osorio et al., 2013). Those studies suggest a regulatory role of PEPCK in the metabolism of carbohydrates and organic acids during ripening fruit. On the other hand, because there were only reports focusing on the fruit metabolism in berry fruits, effects of gluconeogenesis and a role of PEPCK have been still unclear in those plants including tomato.

In this chapter, in order to obtain more detailed information on the physiological function of PEPCK and the role of gluconeogenesis in the tomato plant, I

generated RNAi transgenic plants with suppressed PEPCK expression, driven by the CaMV *35S* constitutive promoter and the fruit-ripening-specific *E8* promoter. I performed a detailed characterization of these RNAi plants by evaluating seedling and plant growth and measuring fruit primary metabolites such as sugars and organic acids. In this chapter, I determined that down-regulation of PEPCK altered the accumulation profiles of soluble sugars and organic acids in red-ripe fruits and negatively affected early stage of plant growth, especially seedling growth. These results indicate multiple roles of PEPCK in the development of the tomato plant.

2.2 Materials and methods

2.2.1 Plant materials

The tomato (*Solanum lycopersicum* L.) cultivar 'Micro-Tom' (Scott and Harbaugh, 1989) was used in this study. For germination, surface-sterilized seeds were sown on moist paper in a culture room at 25ºC under a light intensity of 130 μmol m^{-2} s^{-1} with a 16/8 h light/dark cycle with a relative humidity of 55% in the daytime and 65% at night. One week after germination, the seedlings were transplanted into rock wool pots (5×5×5 cm). The plants were hydroponically cultivated in plastic trays (534×348×600 mm) and were supplied with a commercial nutrient solution (Otsuka A; Otsuka Chemical Co. Ltd., Osaka, Japan) adjusted to an electrical conductivity (EC) of 1.5 dS m^{-1}, which was maintained at a constant volume of 2 l. For phenotype analysis, the height, and fresh and dry weight of the tomato plants were recorded every 10 days from 40 to 100 days (plant height) and from 50 to 100 days (fresh and dry weight) after sowing. For each measurement, at least 7 plants were individually sampled for fresh weight and then dried at 80ºC for 48 h to measure the dry weight. Plant heights were recorded for more than 7 plants during each evaluation. For primary metabolite measurements, fruits were sampled at 38, 42 and 46 days after flowering (DAF) and stored at -80ºC until use.

2.2.2 Vector construction and transformation

The RNAi constructs used to suppress the mRNA expression of *SlPEPCK* gene (accession no. AY007226) were created under the control of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter or the fruit-ripening-specific *E8* promoter (Deikman and Fischer 1988) (Fig 2.1). To create RNAi constructs targeted toward *PEPCK* suppression, the RNAi-targeted region of *PEPCK* (1575 bp – 1872 bp) was amplified with gene-specific primers as follows, FW 5'-TATCATGCTTCATCCAACCAA-3' and RW 5'-ATCCTTGTGTGCCTTCTTGTCG-3'. The PCR fragment was directly cloned into the entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA) and transferred into the Gateway vector pBI sense-antisense GW (Inplanta Innovations Inc., Yokohama, Japan) using the Gateway LR Clonase enzyme (Invitrogen, Carlsbad, CA, USA). This construct was designated as *35Spro::SlPEPCK*^{RNAi}. To create RNAi constructs under the control of the *E8* promoter, the region (accession number AF515784) was amplified using specific primers containing *Bln*I and *Xho*I sites. The fragment was cloned in place of the CaMV *35S* promoter in the pBI sense-antisense GW vector. Subsequent procedures were performed using this same strategy, and the vector was designed as *E8pro::SlPEPCK*^{RNAi}. These constructs were then transformed into *Agrobacterium Rhizobium radiobacter* GV2260 using the electroporation method. The transformation with these constructs was performed according to a procedure described by Sun et al.

(2006). The transgenic plants were selected on Murashige and Skoog (MS) agar plates containing kanamycin (100 mg l^{-1}). Transgenic plants regenerated from calli were defined as the T_0 generation. Plants at the T_3 and the T_4 generations were submitted to the analyses in the present work.

2.2.3 RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from frozen samples using the RNeasy plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in RNase free-water and stored at -80ºC until use. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using the First Strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instruction. The quantitative RT-PCR (qRT-PCR) reactions were carried out using fruits from wild type (WT), *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} plants at T_0 and/or T_1 generations according to Yin et al. (2010a), using an Mx 3000P qRT-PCR system (Stratagene, San Diego, CA, USA). The gene-specific primers were designed based on the *SlPEPCK* cDNA sequence (Bahrami et al., 2001) and the putative mRNA sequence obtained from the tomato genome sequence database (http://solgenomics.net/organism/Solanum_lycopersicum/genome) (Tomato Genome Consortium, 2012). For the normalization of the qRT-PCR reactions, the actin gene (*Tom52*, accession number U60482; Petreikov et al., 2006) was used as an internal standard. The following sequence of primer sets were utilized for the RT-PCR reactions;

FW 5’-GAATACAAGAAGACCGAGGTA-3’, Rev 5’-CTCAAAATTCTTCCTAAATAGG-3’ for *SlPEPCK*, and FW 5’-CACCATTGGGTCTGAGCGAT-3’, Rev 5’-GGGCGACAACCTTGATCTTC-3’ for *Tom52*. For all of the genes, the PCR reaction was performed with following cycles: 95ºC for 10 min for the initial denaturation; 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec; and 1 cycle of 95ºC for 1 min, 55 ºC for 30 sec, and 95ºC for 30 sec. The specific amplification of a single transcript was confirmed by single dissociation peaks and calibration curves as well as agarose gel electrophoresis. The gene expression was calculated relative to the transcript levels of the actin gene, according to the instructions provided by Stratagene and the method reported by Pfaffl (2001).

2.2.4 Measurement of PEPCK activity

For enzyme extraction, 0.2 g of tomato fruit tissue was ground in a chilled mortar with 1 ml of ice-cold extraction medium containing 200 mM Bicine-KOH (pH 9.8) and 50 mM dithiothreitol (DTT). The PEPCK activity in the Amicon Ultra-4-treated fresh fruit extracts was measured in the carboxylating direction following the oxidation of NADH at 340 nm and 25ºC (Walker et al., 1999). The reaction buffer contained 100 mM HEPES (pH7.0), 100 mM KCl, 90 mM $KHCO_3$, 0.5 mM PEP, 1.0 mM ADP, 5 μM $MnCl_2$, 0.14 mM NADH, and 6 unit ml^{-1} malate dehydrogenase for the optimum reaction (Bailey et al., 2007).

2.2.5 Measurement of Brix (%), soluble sugars, malate and citrate contents

The Brix (%) of red-ripe fruit (42 DAF) was measured using a Portable Brix Meter (RA-250HE, KEM, Japan). For sugar and organic acid measurements, 500 mg of frozen fruit was ground in liquid nitrogen and 500 µl of MilliQ water. The extract was incubated at 99ºC for 5 min to inactivate the sugar degradative enzymes and then centrifuged for 10 min at 13, 200 rpm at 4ºC. The supernatant was transferred to a new plastic tube and maintained at -30ºC until use.

The malate and citrate content was measured using a P/ACE MDQ capillary electrophoresis (CE) system (Beckman Coulter, Brea, CA, USA). The supernatant collected from the upper section was diluted 50-fold using MilliQ water and was filtered through a 45-µm filter paper. Organic acids were detected at 25ºC with a capillary electrophoresis system (75 µm×50 cm, Beckman Coulter, Brea, CA, USA), and a refractive index (RI) detector was used to obtain the signal. The reaction buffer was supplied by a commercial Anion analysis kit (Beckman Coulter, Brea, CA, USA).

The glucose, fructose and sucrose content were determined using high-performance liquid chromatography (HPLC) as described by Zushi and Matsuzoe (2006). The supernatant was filtered through 0.45-µm filter paper. Soluble sugars were separated at 80°C using HPLC in a Shim-Pack SCR-101C column (7.9×300 mm, Shimadzu, Tokyo, Japan), and the signal was detected using an RI detector. The mobile phase was 100% MilliQ water at a flow rate of 0.8 ml min^{-1}.

2.3 Results

2.3.1 The expression of the *SlPEPCK* genes and enzymatic activity in the transgenic lines

For this research, transgenic RNAi lines with suppressed *PEPCK* expression (Fig. 2.1) were generated utilizing miniature tomato, 'Micro-Tom' under the control of the constitutive CaMV *35S* promoter or *E8* promoters (designed as *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi}) (Fig. 2.2). T_0 generation plants were subjected to a polyploidy check and Southern blot analyses, and the following diploid individuals harbouring a single copy of the transgene were selected: no. 22, 30 and 42 in *35Spro::SlPEPCK*^{RNAi} and no. 13 and 19 in *E8* promoter-driven *E8pro::SlPEPCK*^{RNAi}. To evaluate the suppression of *SlPEPCK* gene expression through RNAi, qRT-PCR was performed using ripening fruits (42 DAF) from wild type (WT), *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} plants (Fig. 2.3). The expression levels in each line were calculated relative to those of the *SlPEPCK* gene in the WT, which was established as 100%. The endogenous *PEPCK* transcriptional levels in RNAi transgenic individuals were suppressed to less than 10% and 20% of the wild-type level in *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} plants, respectively (Fig. 2.3A and B). The PEPCK enzymatic activity in the red-ripe fruit was also significantly suppressed in both the *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} plants compared with the wild type, i.e., 17% (no. 22), 34% (no. 30) and 19% (no.42) in *35Spro::SlPEPCK*^{RNAi} and

50% (no. 13) and 36% (no. 19) in *E8pro::SlPEPCK*^{RNAi} plants (Fig. 2.3C and D).

2.3.2 Seedling and vegetative growth in the transgenic lines

To evaluate the effect of PEPCK suppression on seedling growth and subsequent vegetative growth and fruit development, the seedling length, plant height, fresh/dry weight and fruit weight of the T_3 plants were evaluated in the transgenic lines every 10 days from 40 to 100 days after sowing (DAS) (Figs. 2.4, 2.5 and 2.6).

The seedling growth of the *35Spro::SlPEPCK*^{RNAi} lines was significantly suppressed compared with the wild-type seedlings (Fig. 2.4A). The suppression level was much prominent in root, but there were not significant differences in shoot length (Fig. 2.4C and E). The average root lengths of the transgenic lines at 10 DAS were 2.7 cm, 2.6 cm and 2.7 cm in nos. 22, 30 and 42, respectively, whereas that of the wild type was 6.3 cm (Fig. 2.4C). On the other hand, the *E8pro::SlPEPCK*^{RNAi} lines did not exhibited remarkable suppression as observed in the*35Spro::SlPEPCK*^{RNAi} lines in both of the root and shoot lengths (Fig. 2.4B), although slight suppression occurred in root of no. 13 and root and shoot of no. 19 (Figs. 2.4D and F).

The subsequent vegetative growth of the transgenic lines is shown in Fig. 3 and 4. The growth suppression appeared at 10 DAS in the *35Spro::SlPEPCK*^{RNAi} lines was maintained until 50 DAS (Fig. 2.5A). The plant height of the *35Spro::SlPEPCK*^{RNAi} lines, measured from the base to the growing point, was suppressed by 23-30% at 40

DAS compared with the wild-type plants (Fig. 2.5A), although no morphological changes were observed except for the height in the transgenic plants. The growth suppression in the *35Spro::SlPEPCK*^{RNAi} lines tended to recover; these plants grew normally, similarly to the wild-type plants, after 70 DAS. The average plant height at 70 DAS was 21.5 cm in the wild type, whereas 21.0 cm, 27.0 cm and 26.1 cm in *35Spro::SlPEPCK*^{RNAi} nos. 22, 30 and 42, respectively. On the other hand, during fruit ripening, the height tended to be suppressed slightly in no. 22 and 42, however the effect was different depending on the line (Fig. 2.5A). In contrast to the *35Spro::SlPEPCK*^{RNAi} lines, plant height of *E8pro::SlPEPCK*^{RNAi} lines was not suppressed in early growth stages. However, it was significantly suppressed (by 13% to 17%) in the *E8pro::SlPEPCK*^{RNAi} lines compared with the wild type, from 80 DAS to 100 DAS, which corresponded with fruit ripening (Fig. 2.5B).

Plant biomass tended to decrease in the *35Spro::SlPEPCK*^{RNAi} lines after 70 DAS in no. 22 and 42 for both fresh and dry weight compared with the wild-type plants although this effect was not significant and was unclear in no. 30 (Fig. 2.5C and E). By contrast, the fresh and dry weights of the *E8pro::SlPEPCK*^{RNAi} plants were not suppressed compared with the wild type in most of the stages (Fig. 2.5D and F). Especially, no. 13 showed an increase in both the fresh and dry biomass compared with the wild type plants after 80 DAS. To evaluate the effect of *SlPEPCK* suppression on fruit size, the fruit weight in the *RNAi* transgenic lines was also measured. Although

several lines, such as the *35Spro::SlPEPCK*^{RNAi} line no. 30 at 42 day after flowering (DAF) and no. 22 at 46 DAF and the *E8pro::SlPEPCK*^{RNAi} line no. 13 at 38 and 42 DAF, showed an increase in the average fruit weight, a similar tendency was not observed in the transgenic lines (Fig. 2.6A and B).

2.3.3 Fruit-soluble sugar contents

To clarify the physiological function of PEPCK in fruit sugar accumulation, the fruit brix (%) and soluble sugar content, glucose, fructose and sucrose, were measured in the RNAi transgenic lines at 42 DAF (Figs. 2.7 and 2.8) when endogenous *SlPEPCK* was highly expressed (Fig. 2.11A). As reported in previous works, the major fruit-soluble sugars were glucose and fructose; the amount of sucrose was relatively low. Among the *35Spro::SlPEPCK*^{RNAi} lines, the fruit brix (%) ranged from 3.5 to 3.7, but no remarkable differences were observed (Fig. 2.7A). By contrast, the major soluble sugars fructose and glucose decreased by 65% and 54%, respectively, in no. 30, by 43% and 48%, respectively, in no. 42, and by 38% and 75%, respectively, in no. 22 compared with the wild type fruit at 42 DAF (Fig. 2.7B and C). The total soluble sugar content decreased by 59%, 46% and 55% in no. 30, 42 and 22, respectively, compared with the wild-type fruit. The fruit brix (%) of the *E8pro::SlPEPCK*^{RNAi} lines ranged from 3.8 to 4.9, and suppression of the sugar content was observed in no. 13 at 42 DAF (Fig. 2.8A). In the *E8pro::SlPEPCK*^{RNAi} fruit, the fructose and glucose content tended to decrease at

42 DAF by 18% and 12%, respectively, in no. 13 and by 18% and 7%, respectively, in no. 19 compared with the wild type, although those differences were not significant (Fig. 2.8B). The total soluble sugar content decreased by 16% and 8% in no. 19 and 13, respectively, at 42 DAF.

2.3.4 Organic acid contents

The amount of malate and citrate, i.e., the major organic acids in tomato fruit, were also evaluated in the transgenic lines (Fig. 2.9). As the fruit ripened, the malate content decreased from 38 DAF to 46 DAF in all the RNAi lines as well as in the wild type (Fig. 2.9A). In the *35Spro::SlPEPCK*^{RNAi} lines, the malate content was 24% to 58% higher in the RNAi transgenic lines at 38 DAF than in the wild type. However, there was almost no difference at 42 DAF and 46 DAF, although there was a slight decrease in no. 30 at 46 DAF and no. 30 and 42 at 46 DAF compared with the wild-type fruits (Fig. 2.9A). By contrast, the citrate content tended to increase during ripening in most of the tested lines. Although these contents were not clearly different between the wild type and the *35Spro::SlPEPCK*^{RNAi} lines at 38 DAF, they tended to be lower in the RNAi lines compared with the wild type at 46 DAF (Fig. 2.9C). In the *E8pro::SlPEPCK*^{RNAi} lines, the malate content decreased during fruit ripening in all the lines (Fig. 2.9B). However, the contents were higher in both no. 13 and 19 at 42 and 46 DAF compared to those of the wild type. It was 129% and 33% higher at 42 DAF and

51% and 32% higher at 46 DAF in nos. 13 and 19, respectively, than the wild type (Fig. 2.9B). On the other hand, the citrate content in the *E8pro::SlPEPCK*^{RNAi} lines increased by 14–25% at 42 DAF and 40–62% at 46 DAF in no. 13 and 19, respectively, although there was not significant difference among the wild type and the RNAi lines at 38 DAF (Fig. 2.9D).

2.4 Discussion

Although the high expression level of PEPCK during tomato fruit ripening has been reported in previous work (Bahrami et al., 2001; Saito et al., 2008; Yin et al., 2010a), its physiological role in plant and fruit development remained to be elucidated in tomato. In this research, I generated transgenic lines in which PEPCK expression was strongly suppressed by RNAi gene silencing, and I revealed the pleiotropic effect of PEPCK on tomato plant development and fruit metabolism. Prior to conducting the present work, I surveyed the *SlPEPCK* gene family in the Sol genome network (SGN; http://solgenomics.net/) database and found two homologues; one was previously reported by Bahrami et al. (2001) (*SlPEPCK*; accession no. AY007226 / Solyc04g076880.2.1) and the other (Solyc12g088160.1.1) was unknown. Solyc12g088160.1.1 was 86% identical to *SlPEPCK* at the amino acid level (Fig. 2.11). However, because its transcription level was extremely low compared with *SlPEPCK* (Fig. 2.12), and the enzymatic analysis showed that PEPCK activity was significantly reduced by *SlPEPCK* suppression (Fig. 2.3C and D), I concluded that Solyc12g088160.1.1 is not functional in tomato.

The phenotypic analyses of the *35Spro::SlPEPCK*^{RNAi} lines showed that systemic suppression of PEPCK reduced the growth of the seedlings (Fig. 2.4). This result indicates that PEPCK plays a role in the early development of tomato seedlings. In *Arabidopsis*, a PEPCK mutant (*pck1*) showed a reduction in the length of the

hypocotyl during germination, which recovered when exogenous sucrose was supplied (Rylott et al., 2003, Penfield et al., 2004). These results indicate that PEPCK functions in that catabolization of both storage lipid and protein through gluconeogenesis to produce soluble sugars as a carbon source. In tomato, insufficient information about the metabolism of germinating seedlings has prevented an in-depth understanding of the mechanism of carbohydrate supply during germination. Our results revealed that PEPCK and gluconeogenesis are involved in the germination process in tomato, probably through a mechanism similar to that in *Arabidopsis*. Interestingly, in the early germination stage (10 DAS), the effect of PEPCK suppression was much more prominent in root elongation compared with the aerial parts (Fig. 2.4A and C). It was reported that transgenic tomato plants in which mitochondrial malate dehydrogenase (*mMDH*) expression was systemically suppressed exhibited reduced length and dry mass in a root-specific manner (van der Merwe et al., 2009). In this transgenic line, the respiration ratio was dramatically reduced in the root, suggesting that the energy metabolism in the root slows in transgenic plants (van der Merwe et al., 2009). Although it is unclear how the modification of malate metabolism specifically affected root development, it is likely that a similar mechanism underlies both phenomena. Although the systemic PEPCK suppression in the *35Spro::SlPEPCK*^{RNAi} lines also caused growth suppression in the height of aerial part of the juvenile plants by 50 DAS, obvious growth suppression was not observed after 60 DAS (Fig. 2.5A). These results

suggest that the energy source for plant growth switches from the PEPCK-mediated gluconeogenesis pathway to another pathway around 40–50 DAS. Since PEPCK deficiency affected the plant height but not clearly influenced both the fresh and dry weight (Fig. 2.5), I can not exclude a possibility that PEPCK affects tissue elongation rather than plant biomass.

Considering the results of seedling growth (Fig. 2.4), it is very interesting that the PEPCK deficiency specifically affected seed and seedling root development, which have strong sink strength in the sink organs such seedling and fruit. Those results suggest that PEPCK would function in carbohydrate partitioning in a sink tissue, which originated from fatty acids and/or organic acids.

Radiolabelling studies provided evidence that gluconeogenesis from malate occurs in the flesh of ripening tomatoes (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991). However, the function of PEPCK in ripening fruit is not fully understood, although gene expression and the enzymatic activity pattern suggest that PEPCK is involved in gluconeogenesis in tomato (Bahrami et al., 2001). In the present study, the total soluble sugar content in the fruit of the *SlPEPCK*^{RNAi} lines decreased during the ripening stages by an average of 53% and 12% in the *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} lines, respectively, compared with the wild type (calculated using Figs. 2.7 and 2.8). Additionally, the suppression of PEPCK affected the malate and soluble sugar content in the fruit. The malate content in the fruit tended to increase

during the ripening stages, although this varied somewhat between the *35S* and *E8* promoter-driven lines (Fig. 2.9A and B). PEPCK is thought to function in the net dissimilation of malate (Famiani et al., 2005). The present results provide direct evidence of the involvement of PEPCK in the sugar accumulation process as well as in malate metabolism during fruit ripening. PEP is the key intermediate in the conversion of malate to sugars by gluconeogenesis, and PEP is produced either by the combined action of the malate dehydrogenase and PEPCK, or the malic enzyme and pyruvate orthophosphate dikinase (Kliewer, 1965; Ruffner, 1982; Walker et al., 2011b; Famiani et al., 2014). In tomato, PEPCK is highly expressed in ripening fruit, whereas the transcriptional and protein levels of the malic enzyme decrease during fruit ripening as the malate content decreases (Bahrami et al., 2001; Saito et al., 2008; Yin et al., 2010b; Osorio et al., 2013). Osorio et al. (2013) also reported a significant reduction of fructose and glucose levels accompanied by malate accumulation in the red-ripe fruit of transgenic tomato in which PEPCK expression was suppressed. Interestingly, RNAi transgenic lines in which plastidic ME expression was suppressed did not exhibit any significant changes in the soluble sugars or malate content (Osorio et al., 2013). These results suggest that the major metabolic pathway of malate during fruit ripening in tomato is the malate dehydrogenase/PEPCK-mediated pathway and, as a result of this, the reduction in the sugar content and the accumulation of malate observed in our RNAi lines could be due, at least in part, to the suppression of the gluconeogenesis pathway

caused by *SlPEPCK* deficiency.

However, in contrast to the malate content, there was no clear tendency in the citrate content among the three genotypes, the wild type, and the *35S* and *E8* promoter-driven RNAi lines (Fig. 2.9 C and D). Compared with malate, which decreased during ripening, the citrate content either did not change or increased slightly during ripening. If the gluconeogenesis pathway is suppressed in fruit, a decrease in the citrate content can be expected in the RNAi lines compared with WT plants. Although such a tendency was observed in *35Spro::SlPEPCK*^{RNAi} no. 42 and *E8pro::SlPEPCK*^{RNAi} no. 13 (Fig. 2.9C and D), I could not confirm whether this was a common tendency due to PEPCK suppression. The reason that the visible influence of the *SlPEPCK* suppression was weaker with respect to citrate compared with malate could be explained by the difference in the absolute amounts of both metabolites. According to our previous work, the citrate content was 5–10 times higher than the malate content (Akihiro et al., 2008; Saito et al., 2008). It is likely that a large pool of citrate absorbed the impact of gluconeogenesis suppression. Regardless, the role of gluconeogenesis and PEPCK in the regulation of organic acid accumulation during fruit ripening cannot be ignored.

The present research demonstrated the massive effect that reducing the amount of PEPCK in tomato fruits has on their sugar content. It is widely known among tomato farmers that the fruit sugar / acid ratio increases around harvesting time and is affected

by environmental conditions. Although the stress response of PEPCK has been reported in other plants (Penfield et al., 2012; Sáez-Vásquez et al., 1995), the abiotic and/or biotic stress response of PEPCK in tomato is not fully understood. In our previous work, PEPCK showed a response to salinity stress (Saito et al., 2008). It is likely that PEPCK affects the sugar / acid ratio of ripening fruit through the regulation of gluconeogenesis in response to various environmental conditions. This also suggests that the excessive expression of PEPCK during fruit ripening results in high-quality fruit with a high sugar/acid ratio. To confirm this, characterization of a tomato plant over-expressing PEPCK is required.

2.5 Figure legends

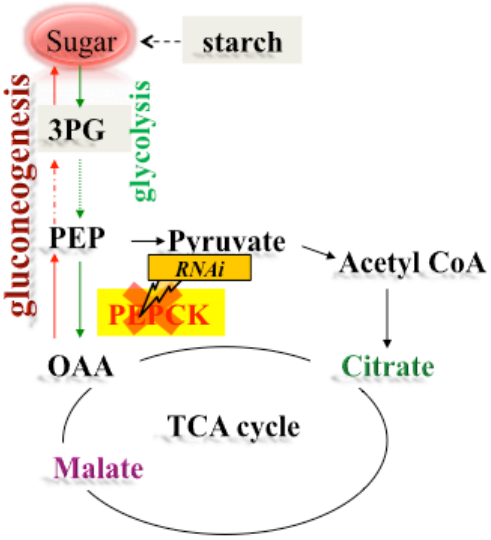

Fig. 2.1 Design of *35Spro::SlPEPCK*^{RNAi} and *E8pro::SlPEPCK*^{RNAi} based on the metabolic pathway.

PEPCK is the irreversible enzyme in gluconeogenesis pathway to synthesis sugars. Besides gluconeogenesis pathway, sugar accumulation comes also from starch assimilation, photosynthesis, et al. Besides, glycolysis pathway utilize the sugars during fruit development. By knocking down PEPCK, this work aims to evaluate the contribution of gluconeogenesis on sugar accumulation during plant development.

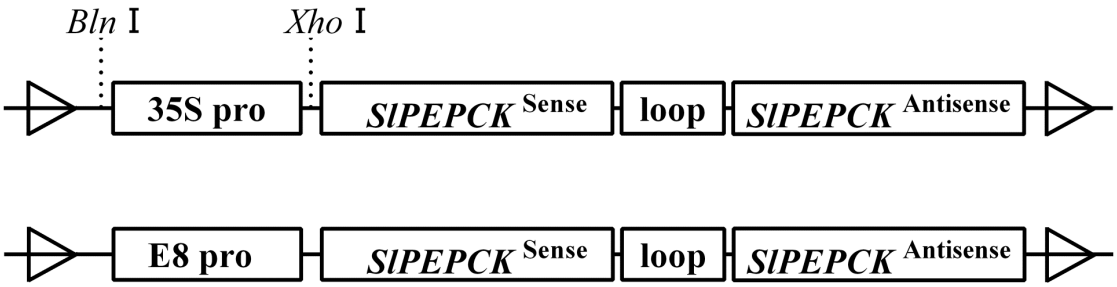

Fig. 2.2 The structure of the RNAi transformation vectors.

SlPEPCK was introduced into tomato using *Agrobacterium*-mediated transformation. *SlPEPCK* was driven by the constitutive expression promoter CaMV *35S* promoter and the fruit-specific *E8* promoter.

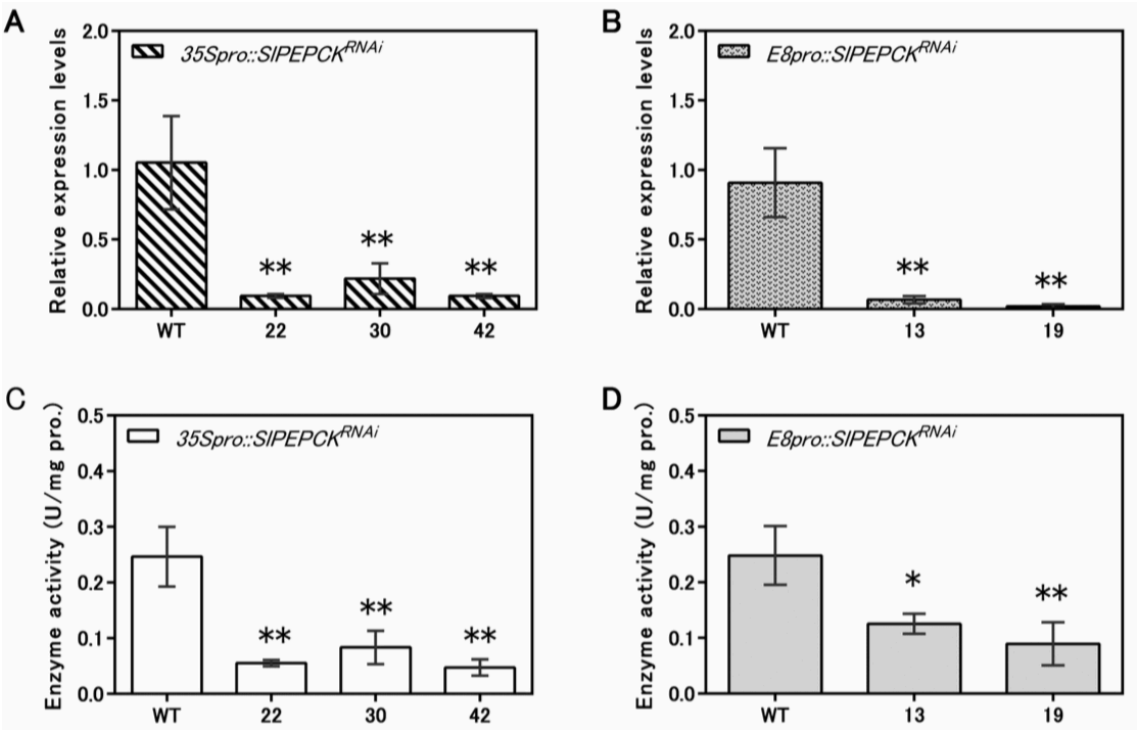

Fig. 2.3 Relative expression levels of the *SlPEPCK* gene and PEPCK enzyme activity in RNAi transgenic lines.

(A) and (B), Relative expression levels of the *SlPEPCK* gene. Values are means ±SD (n=6). (C) and (D), PEPCK enzyme activity. Values are means ±SE (n=3). The labels below the horizontal axis indicate genotypes of the transgenic lines. WT, wild type. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

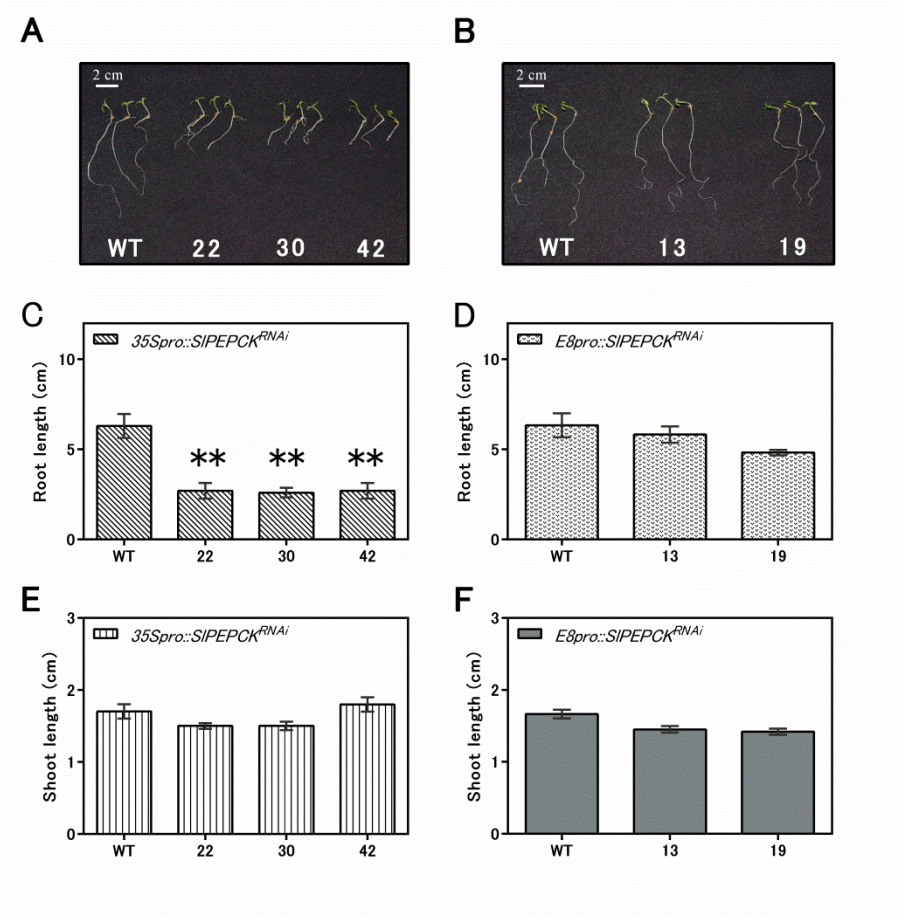

Fig. 2.4 Seedling growth of the *SlPEPCK*^{RNAi} transgenic lines.

(A) and (B), Seedlings at 10 DAS. Bar = 2 cm. (C) and (D), Root length of seedlings at 10 DAS. (E) and (F), Shoot length of seedlings at 10 DAS. The labels below the horizontal axis in (C) to (F) indicate genotypes of the transgenic lines. Values are means ±SE (n=10). (A), (C) and (E), *35Spro::SlPEPCK*^{RNAi}. (B), (D) and (F), *E8pro::SlPEPCK*^{RNAi} lines. WT, wild type. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$,**$P < 0.01$).

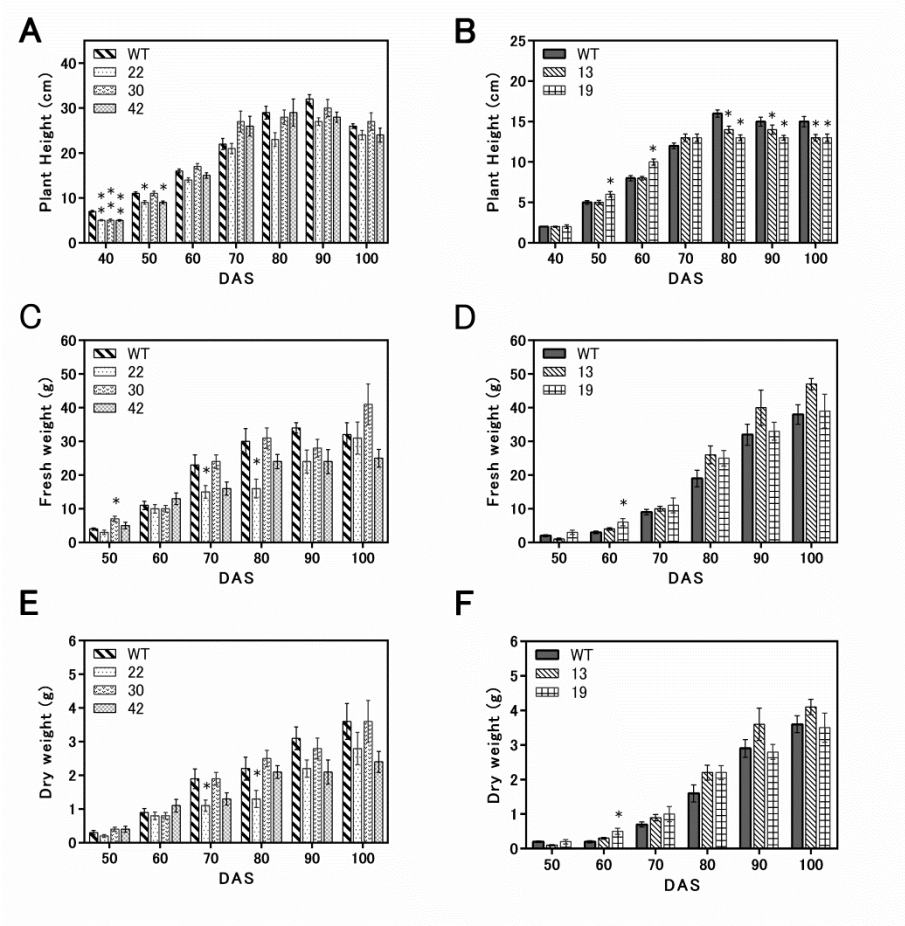

Fig. 2.5 Vegetative growth of the *SlPEPCK*^{RNAi} transgenic lines.

(A) and (B), Plant height from 40 DAS to 100 DAS. (C) and (D), Plant fresh weight, and (E) and (F), Plant dry weight from 50 DAS to 100 DAS. The labels below the horizontal axis indicate DAS. Values are means ±SD (n=7). (A), (C) and (E) from *35Spro::SlPEPCK*^{RNAi} and (B), (D) and (F) from *E8pro::SlPEPCK*^{RNAi} lines, respectively. WT, wild type. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

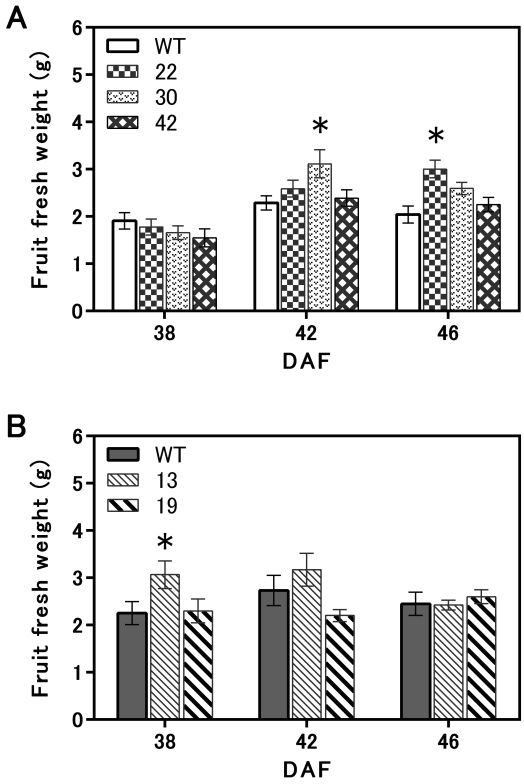

Fig. 2.6 Fruit weight of the *SlPEPCK*^{RNAi} transgenic lines.

(A) and (B), Fruit weight from 38 DAF to 46 DAF. The labels below the horizontal axis indicate DAF. The labels below the horizontal axis indicate genotypes of the transgenic lines. Seeds were sampled from fruit at 42 DAF. Values are means ±SE (n=9). The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

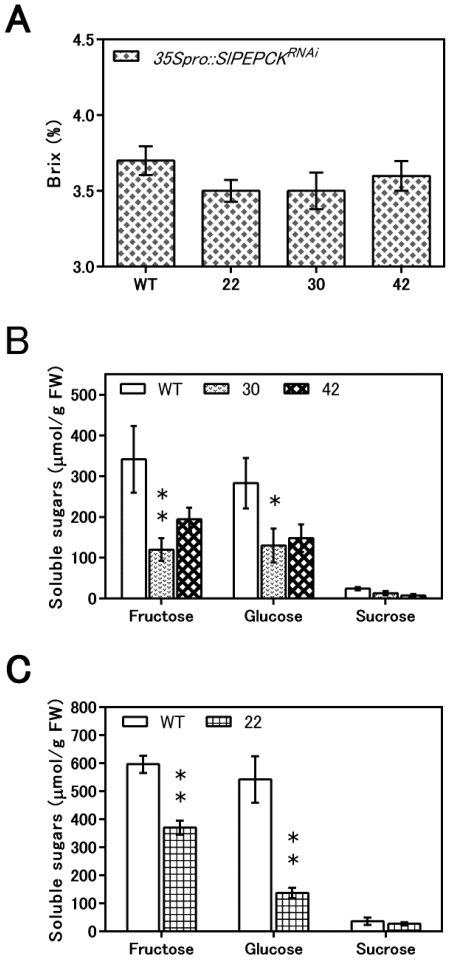

Fig. 2.7 Brix (%) and soluble sugar contents in *35Spro::SlPEPCK*^{RNAi} fruits.

(A) Brix (%), (B) soluble sugar contents in no. 30 and no. 42, and (C) in no. 22 in fruit at 42 DAF. Because the fruits for soluble sugar content measurements were sampled from different cultivations, results from no. 30 and no.42, and no. 22 lines are separately displayed. The labels below the horizontal axis indicate genotypes of the transgenic lines. WT, wild type. Values are means ±SE (n=7). The asterisks indicate significant differences between the means of the transgenic lines and the wild-type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

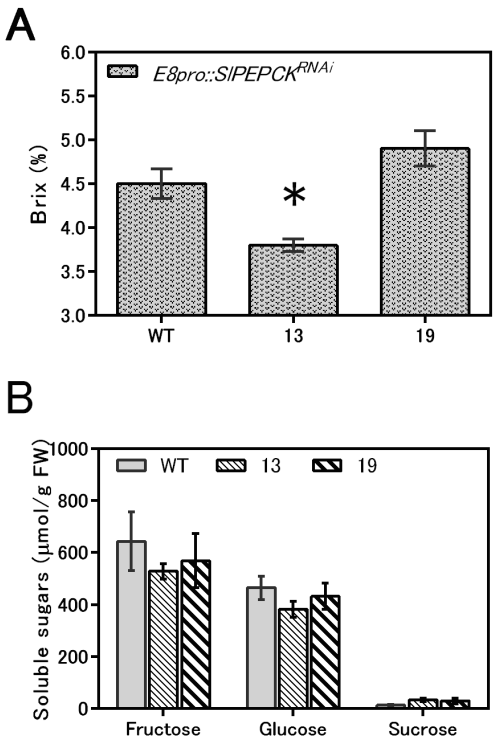

Fig. 2.8 Brix (%) and soluble sugar contents in *E8pro::SlPEPCK*^{RNAi} fruits.

(A) Brix (%), (B) soluble sugar contents in fruit at 42 DAF. The labels below the horizontal axis indicate genotypes of the transgenic lines. WT, wild type. Values are means ±SE (n=7). The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$).

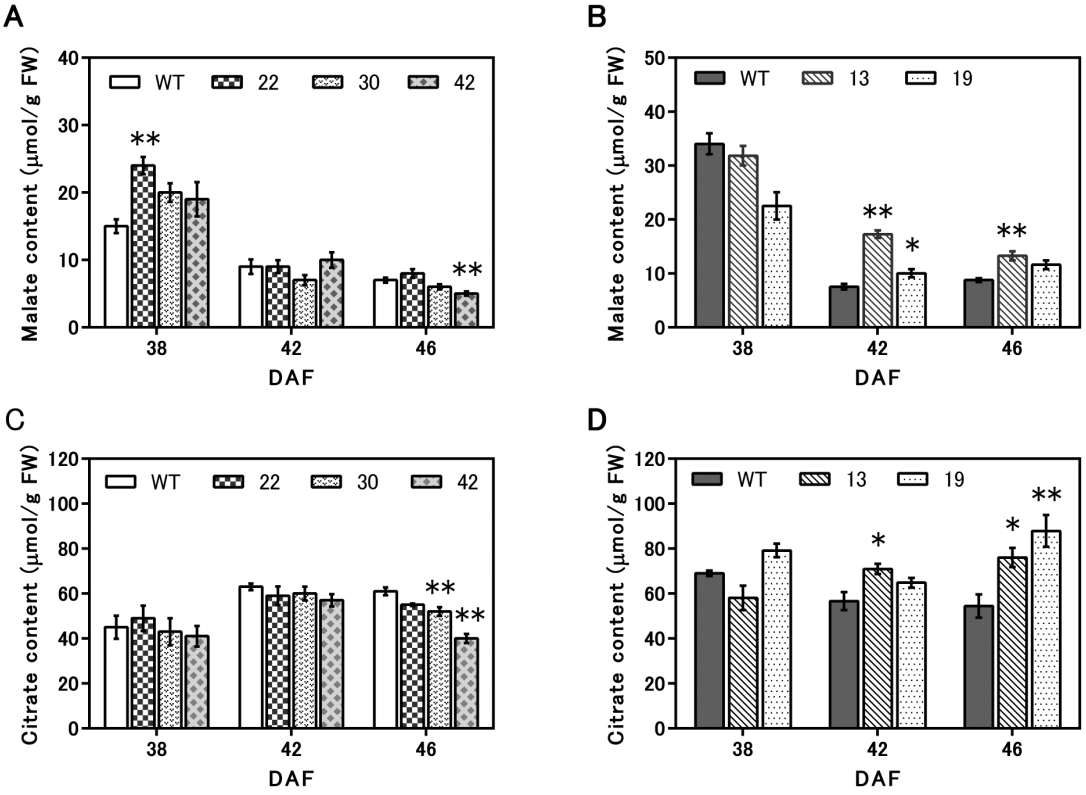

Fig. 2.9 Malate and citrate contents in fruit of the transgenic lines.

(A) and (B), Malate, (C) and (D), Citrate. The labels below the horizontal axis indicate DAF. (A) and (C), *35Spro::SlPEPCK*^{RNAi}. (B) and (D), *E8pro::SlPEPCK*^{RNAi} lines. WT, wild type. Values are means ±SE (n=7). The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

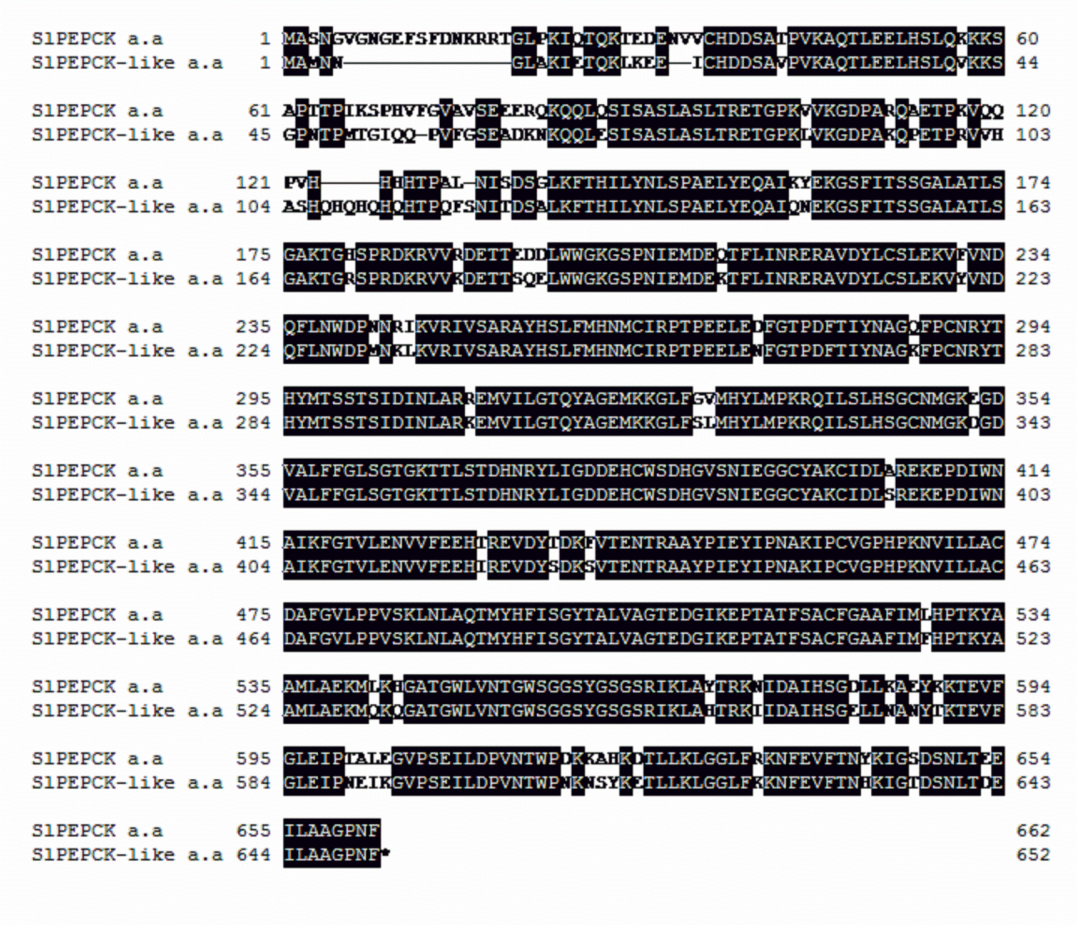

Fig. 2.10 Comparison of deduced peptide sequences of tomato *SlPEPCK* and *SlPEPCK*-like genes.

A gene previously reported by Bahrami et al. (2001, accession no. AY007226) and newly identified in this research is designed as *SlPEPCK* and *SlPEPCK*-like (Solyc12g088160.1.1), respectively. Identical amino acid residues are shaded.

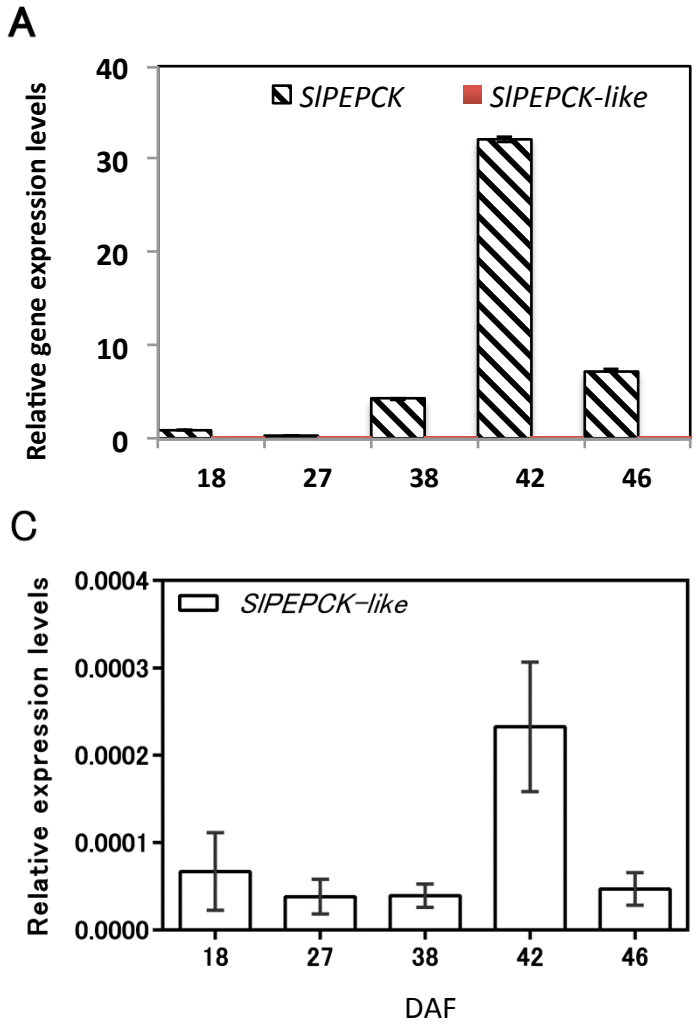

Fig. 2.11 Endogenous expression level of *SlPEPCK* and *SlPEPCK*-like gene in developing tomato fruits.

The horizontal axis indicates fruit developing stages (DAF). For the normalization of the qRT-PCR reactions, the actin gene (Tom52, accession number U60482; Petreikov et al., 2006) was used as an internal standard. The labels below the horizontal axis indicate DAF. Values are means ±SE (n=3).

Chapter 3 Overexpression of phosphoenolpyruvate carboxykinase gene (*SlPEPCK*) promotes soluble sugar accumulation in fruit and post-germination growth in tomato (*Solanum lycopersicum* L.)

3.1 Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) [EC 4.1.1.49] is an enzyme that belongs to the protein lyase family (Krupa and Srinivasan, 2006) and catalyses the ATP-dependent decarboxylation of oxaloacetate (OAA) into phosphoenolpyruvate (PEP) (OAA + ATP → PEP +CO_2 + ADP). It works as a key rate-limiting enzyme that regulates the gluconeogenesis pathway in wide range of organisms (Martín et al., 2011). In plants, PEPCK is localized in the cytosol (Leegood and Walker, 2003) and expressed in specific tissues in different species (Bahrami et al., 2001; Famiani et al., 2012). It is known that PEPCK functions in decarboxylation through CO_2-concentrating mechanisms to enhance photosynthesis in C_4 and CAM (Crassulacean Acid Metabolism) plants (Hansen and Juni, 1974; Reiskind and Bowes, 1991; Walker and Leegood, 1996; Furumoto et al., 1999). In fat-storing seed plants, PEPCK is highly expressed in germinating seedlings (Martín et al., 2007) and has been thought to be involved in carbohydrate supply from lipids and proteins through a regulatory role in the gluconeogenesis pathway because the reaction speed of gluconeogenesis was in good agreement with its expression level (Leegood and ap Rees, 1978; Rylott et al., 2003; Penfield et al., 2004; Martín et al., 2007).

In fresh berry-type fruit, PEPCK is dominantly expressed in ripening fruit and is

proposed to function in the dissimilation of malate/citrate during ripening (Ruffner and Kliewer, 1975; Famiani et al., 2005, 2009, 2012; Walker et al., 2011; Baldicchi et al., 2015). In tomato, high level of expression level of *SlPEPCK* was reported in ripening tomato fruit (Bahrami et al., 2001; Saito et al., 2008; Yin et al., 2010a). Recent studies on tomato lines in which endogenous *SlPEPCK* was suppressed by RNAi proved the involvement of PEPCK in soluble sugar accumulation and dissimilation of organic acids during fruit ripening (Osorio et al., 2013; Huang et al., 2015). Furthermore, systemic suppression of *SlPEPCK* by the constitutive *35S* promoter tomato resulted in suppression of post-germination growth of transgenic seedlings (Huang et al., 2015), suggesting that PEPCK and gluconeogenesis also participate in carbohydrate supply during tomato germination in tomato through a similar mechanism to that which occurs in fat-storing seed plants. On the other hand, there was quite poor information about the effect(s) of excessive PEPCK on fruit metabolism and post-germination growth of plants is limited.

To investigate the effect(s) of excessive PEPCK, in the present study, transgenic tomato plants overexpressing *SlPEPCK* by either CaMV *35S* or fruit-ripening specific *E8* promoters were generated, and the influences on soluble sugars and organic acids contents, germination/post-germination growths and fruit weight were investigated. Excessive PEPCK promoted the accumulation of soluble sugars accompanied by organic acids in red-ripe fruits, and also enhanced germination and post-germination growth. In particular, the seedling growth was enhanced by exogenous sugar supply compared with the wild type plants. The effects of excessive PEPCK were completely

opposite to those observed in the PEPCK-suppressed RNAi lines and the present results prove evidence of the regulatory role of PEPCK in carbohydrate synthesis in seedlings and balance keeping between sugar and acid accumulation in ripening tomato fruits.

3.2 Materials and methods

3.2.1 Plant materials

Dwarf tomato (*Solanum lycopersicum* L.) 'Micro-Tom' was used as the wild type plant in the current study. After sterilization, seeds were sown on moist filter paper and germinated at 25ºC under a light intensity of 130 μmol m^{-2} s^{-1} and a 16/8 h light/dark photoperiod with a humidity of 55% in the daytime and 65% at night. One week after cultivation, the seedlings were transplanted to plastic pots (120 mm in diameter × 90mm deep), filled with commercial culture soil. The cultivation pots were placed inside of plastic trays (534×348×600 mm) and were irrigated using a commercial nutrient solution (Otsuka A; Otsuka Chemical Co. Ltd., Osaka, Japan) adjusted to an EC of 1.5 dS m^{-1} and maintained at a 2 l volume in each tray. For phenotypic analyses, the fruits were harvest at 42 DAF to check fruit number and weight. Plant biomass was measured on the same day as the fruit harvest.

3.2.2. Generation and screening of transgenic tomato lines

The transformation vectors for *SlPEPCK* overexpression were created under the control of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter or the fruit-ripening-specific *E8* promoter (accession number AF515784) (Fig. 3.1). Prior to the constructions, a whole cDNA sequence of *SlPEPCK* (accession no. AY007226, Bahrami et al., 2001) was cloned by RT-PCR using total RNA extracted from tomato seeds or fruits with gene-specific primers as follows: FW 5'-

CACCATGGCGTCGAACGGAGTC -3' and RW 5'-TTAGAAGTTTGGACCAGCTGCC -3' and transferred into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) by TOPO reaction. After inspecting the accuracy of the DNA sequence, the targeted sequence was finally transferred into the destination vector pBI-OX-GW (Inplanta Innovations Inc. Yokohama, Japan) by LR Clonase enzyme (Invitrogen, Carlsbad, CA, USA) and was designated as *35Spro::SlPEPCK*^{OX}. To construct the *E8pro::SlPEPCK*^{OX} vector, the CaMV *35S* promoter sequence was replaced by the *E8* promoter sequence with *Bln*I and *Xho*I. Transformation into tomato cv. 'Micro-Tom' was conducted with seedling cotyledons using *Agrobacterium (Rhizobium) radiobacter* GV2260 according to a procedure outlined by Sun et al. (2006). Screening for homozygous lines harbouring a single transgene was conducted using the T_0 and T_1 generations and Southern blot analyses and quantitative RT-PCR (qRT-PCR).

3.2.3. Measurement of *PEPCK* expression and enzyme activity

To determine the PEPCK gene expression in the transgenic lines, qRT-PCR was performed. Total RNA was extracted from frozen samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The cDNA was synthesized from 1 μg of total RNA by the First Strand cDNA Synthesis kit (Takara Bio Inc. Otsu, Japan) according to the manufacturer's instructions. The cDNA was diluted by 10 times with MilliQ water and

was used as the template for qRT-PCR. The PCR reactions were performed using seeds or fruits from wild type (WT), *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} plants from the T_0 and/or T_1 generations, using a Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) with SYBR Premix Ex Taq II (Takara-Bio Inc.). The PCR reaction was subjected to following cycling conditions: 95ºC for 10 min for the initial denaturation followed by 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, and then 1 cycle of 95ºC for 1 min, 55ºC for 30 sec, and 95ºC for 30 sec. The endogenous actin gene (*Tom52*, accession number U60482; Petreikov et al., 2006) was used as an internal standard, with the following primers: FW 5'-CACCATTGGGTCTGAGCGAT-3' and Rev 5'-GGGCGACAACCTTGATCTTC-3'. The *SlPEPCK* cDNA sequence (Bahrami et al., 2001) was amplified using the following sequence of the primer set: FW 5'-GAATACAAGAAGACCGAGGTA-3' and Rev 5'-CTCAAAATTCTTCCTAAATAGG-3'. The specific amplification of a single transcript was confirmed by single dissociation peaks and calculated based on calibration curves.

A PEPCK enzyme assay was performed following a procedure described by Huang et al. (2015). Tomato seed or fruit tissue (200 mg) was homogenized in a chilled mortar containing 1 ml of ice-cold 200 mM Bicine-KOH (pH 9.8) and 50 mM dithiothreitol (DTT). The carboxylation PEPCK activity was measured in the Amicon Ultra-4-treated fresh extracts at 340 nm and 25ºC. The assay mixture contained 100 mM HEPES (pH7.0), 100 mM KCl, 90 mM $KHCO_3$, 0.5 mM PEP, 1.0 mM ADP, 5 μM

$MnCl_2$, 0.14 mM NADH, and 6 unit ml^{-1} malate dehydrogenase for the optimum reaction (Bailey et al., 2007).

3.2.4. Seed germination and seedling growth investigation

Tomato seeds were surface-sterilized by sink in a 0.5% sodium hypochlorite solution on a rotary for 30 min. Subsequently, the seeds were washed 3 times using distilled sterile water. After sterilization, the seeds were soaked into distilled water for 4 h. After sufficient imbibition, seeds of each transgenic line and the WT were transferred onto 3 different media, i.e., MS with 3% sucrose, MS with 1.5% sucrose and MS without sucrose (0%). The seed germination ratio was evaluated by root appearance at 2 DAS, and post-germination growth was evaluate by root and shoot length at 10DAS in the T_3 generation of the *35Spro::SlPEPCK*^{OX} lines.

3.2.5. Measurement of Brix (%), soluble sugars, malate and citrate contents

In red-ripe fruit (42 DAF), the soluble solid content (Brix%), was measured using a Portable Brix Meter (RA-250HE, KEM, Japan). Soluble sugars, malate and citrate contents were measured following a procedure described by Huang et al. (2015). For sugar and organic acid measurements, 500 mg of frozen fruit powder was suspended in 500 µl of MilliQ water and incubated at 99 ºC for 5 min to inactivate the sugar degradative enzymes and then centrifuged at 13,200 rpm for 10 min at 4ºC. The supernatant was stored at -30ºC until use.

The malate and citrate content was measured using a P/ACE MDQ capillary electrophoresis (CE) system (Beckman Coulter, Brea, CA, USA). The supernatant collected from the upper section was 50-fold diluted using MilliQ water and was purified through a 0.45-μm filter. Organic acids were measured at 25ºC using a CE system (75 μm×50 cm capillary, Beckman Coulter, Brea, CA, USA) and anion analysis kit (Beckman Coulter, Brea, CA, USA). Organic acids peaks were separate using an applied voltage of 30.0 K. Glucose, fructose and sucrose content were separated after passed the supernatant through 0.45-μm membrane filter. While the condition for soluble sugars separation was at 80°C using a Shim-Pack SCR-101C column (7.9×300 mm, Shimadzu, Tokyo, Japan), installed in a high-performance liquid chromatography (HPLC, LC-2010A, Shimadzu, Kyoto, Japan). The signal was detected using an RI detector. The mobile phase was MilliQ water at a flow rate of 0.8 ml min^{-1}.

3.3 Results

3.3.1 Expression levels of *SlPEPCK* genes and enzymatic activity in germinating seeds of the *SlPEPCK*^{OX}-overexpressing lines

In this research, the *SlPEPCK* overexpressing Micro-Tom lines *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} were generated using either the CaMV *35S* promoter or the fruit ripening-specific *E8* promoter, respectively. A polyploidy check and genomic Southern blot analyses were conducted using the T_0 generation, and individuals harboring a single copy of the diploid transgene were selected as experimental lines as follows; no. 1, 3, 10 and 24 in the *35Spro::SlPEPCK*^{OX} lines and no. 1, 4, 5, 6 and 10 in the *E8pro::SlPEPCK*^{OX} line. The *SlPEPCK* expression level in germinating seeds was determined using qRT-PCR at 2 DAS with the gene specific primers in the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines (Fig. 3.2.A and B). The *SlPEPCK* expression levels of each line were represented as relative level to that of the WT plants. The endogenous *SlPEPCK* transcriptional levels in *35Spro::SlPEPCK*^{OX} lines were 3.3- to 9.2-fold higher than in the wild-type (Fig. 3.2.A), while no significant difference was observed in *E8pro::SlPEPCK*^{OX} lines (Fig. 3.2.B). At the same germination stage, PEPCK enzymatic activity also increased by 3.5-, 3.4-, 4.1- and 2.9-fold in the no. 1, 3, 10, and 24 in *35Spro::SlPEPCK*^{OX} lines, whereas that of the *E8pro::SlPEPCK*^{OX} lines did not show significant changes compared with the WT (Fig. 3.2.C and D).

3.3.2 Germination and post-germination growth in the *35Spro::SlPEPCK^{OX}* lines

To evaluate the effect of overexpression PEPCK on germination and subsequent seedling growth, the germination ratio at 2 DAS and seedling height and root length at 10 DAS were measured in T_3 generation plants of the *35Spro::SlPEPCK^{OX}* lines (Figs. 3.3 and 3.4). At 2DAS, the primary root had already extended in the *35Spro::SlPEPCK^{OX}* lines while the WT root had only emerged under the 0% and 1.5% Sucrose conditions whereas germination was obviously suppressed in both the wild-type and *35Spro::SlPEPCK^{OX}* lines under the 3% sucrose condition (Fig. 2A). The germination ratio was also significantly higher in *35Spro::SlPEPCK^{OX}* lines compared with the wild type under all three conditions, in which the ratio in the *35Spro::SlPEPCK^{OX}* lines, the ratio ranged from 73% to 80% for the 0% sucrose condition, 64% to 83% for the 1.5% sucrose condition and 1.9% to 16% for the 3% sucrose condition, whereas it was 59% (0% suc), 54% (1.5% suc) and 1.7 % (3% suc) in the wild-type, respectively (Fig. 3.3.B). On the other hand, no significant change was observed between the 0% and 1.5% sucrose conditions in each transgenic line and the wild type, while the germination ratio under the 3% sucrose condition was much lower than that measured in the other treatments in all tested line and the wild type (Fig. 3.3B).

The post-germination growth of the *35Spro::SlPEPCK^{OX}* lines were also investigated at 10 DAS (Fig. 3.4). The seedling growth at this time was very similar to that measured under the 0% sucrose condition. However, although both the 1.5% and

3% sucrose conditions enhanced seedling growth in all of the tested lines, the effect of enhancement by sugar was obviously greater in the *35Spro::SlPEPCK*^{OX} lines than in the wild type (Fig. 3.4.A). The root length measured in the *35Spro::SlPEPCK*^{OX} lines under the 0% sucrose condition was 50 mm, 48 mm, 68 mm and 55 mm in lines 1, 3, 10 and 24, respectively, compared with 27 mm in the wild type. However, under the 1.5% sucrose condition, the root length of the transgenic lines was 52 mm, 53 mm, 53 mm and 56 mm in no 1, 3, 10 and 24, respectively, compared with 36 mm in the wild type. Under the 3% sucrose condition, the average root length of the transgenic lines was 64 mm, 58 mm, 72 mm and 65 mm in lines 1, 3, 10 and 24, respectively, whereas that of the wild type was 39 mm (Fig. 3.4.B). All *35Spro::SlPEPCK*^{OX} lines exhibited significantly longer roots compared with the WT (Fig. 3.4.B). Shoot length was ranged from 15.7 mm to 17.4 mm, 22.8 mm to 26.2 mm and 30.9 mm to 33.7 mm in the 0%, 1.5% and 3% sucrose conditions, respectively (Fig. 3.4.C). As for the shoot length, although the sucrose supply obviously enhanced shoot growth, there were not remarkable differences between the WT and the *35Spro::SlPEPCK*^{OX} lines for any of the sucrose conditions (Fig. 3.4.C). On the other hand, seedling vigor was considerably better in the *35Spro::SlPEPCK*^{OX} lines compared with the WT in the presence of sucrose (Fig. 3.4.A).

3.3.5 The expression of *SlPEPCK* genes and the enzymatic activity in *SlPEPCK*-overexpressing lines in fruits

SlPEPCK gene expression in ripening fruits at 42 DAF was investigated in both

of the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines and the WT using qRT-PCR (Fig. 3.5.A and B). The endogenous *SlPEPCK* transcriptional level in the transgenic fruits were promoted to more than 3.2- to 9.9-fold higher in both of the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines compared with the wild type (Fig. 3.5.A and B). PEPCK enzymatic activity in the red-ripe fruit was also significantly increased in both of the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines compared with that of the wild type, i.e., 12-, 12-, 6- and 7-fold higher in lines 1, 3, 10 and 24 of *35Spro::SlPEPCK*^{OX} and 6-, 8-, 6-, 4- and 4-fold higher in lines 1, 4, 5, 6 and 10 of *E8pro::SlPEPCK*^{OX}, respectively (Fig. 3.5.C and D). Fruit number and weight were also evaluated in the *SlPEPCK*^{OX} lines at the same fruit stage (42 DAF) (Fig. 3.6). Although plant biomass and fruit number tended to increase slightly in the transgenic lines, it was not significant except for no. 24 in *35Spro*::*SlPEPCK*^{OX} (Fig. 3.6.A and C) and line 4 in *E8pro*::*SlPEPCK*^{OX} (Fig. 3.6.B and D). No common tendency was not observed among the transgenic lines with respect to fruit weight (Fig. 3.6.E and F).

3.3.6 Fruit soluble sugar contents

To evaluate the effect of PEPCK-overexpression on the fruit sugar levels, fruit brix (%) and the soluble sugar contents, i.e., glucose, fructose and sucrose, was measured at 42 DAF were measured (Fig. 3.7). Similar to the results reported in earlier work, the major soluble sugars in tomato fruit were glucose and fructose, and the sucrose content was much lower than the glucose and fructose content. In the *35Spro::SlPEPCK*^{OX} lines, the fruit brix (%) was ranged from 4.0 to 4.5, and a

remarkable increase was observed in lines 1 and 3 (Fig. 3.7.A). In *E8pro::SlPEPCK*^{OX} fruits, the brix (%) value ranged from 4.0 to 4.4 (Fig. 3.7.B). Although the value measured in line 4 increased 1.14-fold, the other lines had levels that were similar to the WT. However, in the *35Spro::SlPEPCK*^{OX} lines, the major soluble sugar contents, fructose, glucose and sucrose contents measured 42 DAF increased by 33%, 42% and 52% in no.1, 20%, 36% and 37% in no. 3, 10%, 21% and 98% in no. 10, and 9%, 10% and 50% in no. 24, respectively (Fig. 3.7.C). The total soluble sugar content was 37%, 27%, 18% and 16% higher compared to the wild type fruit in no. 1, 3, 10 and 24, respectively, compared with the wild-type. The content of all three sugars increased in the *E8pro::SlPEPCK*^{OX} lines (Fig. 3.7.D). The total soluble sugar content increased by 59%, 27%, 9%, 35% and 9% compared to the wild type fruit in no. 1, 4, 5, 6 and 10, respectively, in comparison with the wild-type fruits.

3.3.7 Organic acid contents

The contents of malate and citrate, i.e., major organic acids in tomato fruit, were also evaluated in the transgenic lines (Fig. 3.8). The fruit malate contents measured at 42 DAF decreased in all of the transgenic lines compared with the WT, by 35%, 44%, 40% and 14% in no. 1, 3, 10 and 24 in *35Spro::SlPEPCK*^{OX} lines, and by 16%, 26%, 11%, 41% and 32 % in no. 1, 4, 5, 6 and 10 in *E8pro::SlPEPCK*^{OX} lines, respectively (Fig. 3.8.A and B). Fruit citrate content also decreased in most of the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines except for *35Spro::SlPEPCK*^{OX} no. 24. It decreased by 6%, 23% and 33% in no. 1, 3 and 10 of *35Spro::SlPEPCK*^{OX} lines,

and by 26%, 37%, 17%, 50% and 31% in no. 1, 4, 5, 6 and 10 of *E8pro::SlPEPCK*^{OX} lines, respectively (Fig. 3.8.C and D). However, significant differences were only observed in *35Spro::SlPEPCK*^{OX} no. 10 and *E8pro::SlPEPCK*^{OX} no. 6 compared to the WT (Fig. 3.8.C and D).

3.4 Discussion

The high expression level of PEPCK during tomato fruit ripening has been reported in previous studies (Bahrami et al., 2001; Saito et al., 2008; Yin et al., 2010a). However, the physiological role of PEPCK in plant and fruit development remained to be elucidated in tomato. Recent studies on RNAi transgenic tomato plants revealed PEPCK suppression leads reduced sugar accompanied by increase of malate in ripening fruit (Osorio et al., 2013; Huang et al., 2015). These results indicate the involvement of PEPCK and gluconeogenesis in sugar accumulation and dissimilation of organic acid metabolism during fruit ripening. However, few studies have focused on the effect of excessive PEPCK on fruit metabolism and plant development. Therefore, in this work, I generated transgenic tomato lines overexpressing *SlPEPCK* gene driven by constitutive *35S* promoter and fruit-specific *E8* promoter, and investigated the effect(s) through the characterization of the transgenic plants.

Our recent study reported that *SlPEPCK* is only functional PEPCK gene in tomato plant (Huang et al. 2015). In accordance with the result, PEPCK activity was increased by the overexpression of *SlPEPCK* gene (Fig. 3.2 and 3.5). In the seedlings, both *SlPEPCK* expression and PEPCK activity increased markedly in the all *35Spro::SlPEPCK*^{OX} lines (Fig. 3.2.A and C). These lines exhibited earlier seed germination and faster seedling growth compared with the WT (Fig. 3.3 and 3.4), which is the opposite results to our previous work in which the reduction of PEPCK expression suppressed seedling growth (Huang et al., 2015). The close relationship between the PEPCK expression suppressed level and seedling germination and growth

indicates a regulatory role of PEPCK in the early development of tomato seedlings. This perspective is also supported by the *E8pro::SlPEPCK*^{OX} results, in which the PEPCK activity did not change significantly (Fig. 3.2.D) and the seedlings exhibited almost similar growth to the WT with and without exogenous sugar supply (data not shown).

The post-seedling growth (10DAS) of both the WT and the *35Spro::SlPEPCK*^{OX} lines was strongly enhanced by the exogenous sugar supply (Fig. 3.4.A). Interestingly, the effects were more prominent in root rather than aerial part (Fig. 3.4 A and B). Although the seedling height was not remarkably different between the WT and the *35Spro::SlPEPCK*^{OX} lines even in the presence of sucrose (Fig. 3.4.C), seedling vigour was considerably better in the transgenic lines compared with the WT in the presence of sucrose, and the effects of the sucrose treatment were more prominent in the root than in the shoot (Fig. 3.4.A). Similar differences between organs were observed in seedlings of the *35Spro::SlPEPCK*^{RNAi} lines (Huang et al., 2015). The *Arabidopsis* PEPCK mutant (*pck1*) showed a reduction in the length of the hypocotyl but not the root during germination; this reduction was reversed by the supply of exogenous sucrose (Rylott et al., 2003; Penfield et al., 2004). These differences in organ specificity can be explained by the expression pattern of the *PEPCK* gene, i.e., endogenous *SlPEPCK* is highly expressed in the seedling root rather than other tissues, including the shoot (Fig. 3.9), which suggests that a lower endogenous level would lead to excessive PEPCK in the shoot during post-germination growth.

The underlying mechanism(s) pertaining to how exogenous sucrose enhances

post-germination growth under excessive PEPCK levels remains unclear. In fat-storing seed plants, PEPCK has been proposed to be involved in carbohydrate supply from lipids and proteins through a regulatory role in the gluconeogenesis pathway (Leegood and ap Rees, 1978; Penfield et al., 2004; Rylott et al., 2003). In our previous work in which PEPCK suppression resulted in growth suppression of seedlings, we concluded a similar metabolic pathway functions in tomato seedling establishment (Huang et al,. 2015). However, the present results suggest that PEPCK did not enhance post-seedling growth by gluconeogenesis because if the gluconeogenesis pathway was involved in this process, seedling growth should have been promoted, even under the 0% sucrose condition in the transgenic lines. However, seedling growth was similar between the wild-type and the transgenic lines (Fig. 3.4.A). It is more likely that PEPCK promotes the provision of pyruvate for the TCA cycle through the PEPCK/ pyruvate kinase (PK) pathway rather than directly regulating the carbohydrate supply through gluconeogenesis during early seedling growth (Leegood and Walker, 2003; Yin et al., 2010a). On the other hand, in contrast to the post-seedling growth, the exogenous sucrose supply did not clearly affect the germination ratio in the WT and the *35Spro::SlPEPCK*^{OX} lines (Fig. 3.3.B). These results indicate that PEPCK functions differently during germination (2 DAS) and post-germination growth (10 DAS). A metabolic shift in the seedlings, for example from an autotrophic to a heterotrophic phase, could be involved in this change.

SlPEPCK expression and PEPCK activity in the fruit were significantly elevated in all *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines (Fig. 3.5) at 42DAF when

endogenous *SlPEPCK* exhibits the highest expression during fruit development (Huang et al., 2015). Although a phenotypic change was observed in the *35Spro::SlPEPCK*^{OX} seedlings, common responses in fruit number per plant and average fruit weight were not observed among the transgenic lines (Fig. 3.6.C to E). Because plant weight did not change markedly between the WT and the transgenic lines, these results indicate that an excess of PEPCK does not affect plant biomass, including fruit yield. However, the fruit soluble sugar and organic acid contents were altered in the *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines (Fig. 3.7 and 3.8). In our previous study on RNAi transgenic tomato, *SlPEPCK* suppression resulted in a reduced sugar and an increased malate level in red-ripe fruits (Osorio et al., 2013; Huang et al., 2015). In contrast to the results from the RNAi plants, the transgenic lines overexpressing *SlPEPCK* tended to exhibit increased sugar and reduced malate contents during fruit ripening (Fig. 3.7 and 3.8). While the average brix (%) in the WT was 3.9, it reached 4.4-4.5 in the fruits of the transgenic *35Spro::SlPEPCK*^{OX} lines 1 and 3 and the *E8pro::SlPEPCK*^{OX} line no. 4 (Fig. 3.7.A and B). Additionally, in most of the tested *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines, the soluble sugar contents were higher than those measured in the WT fruit at 42 DAF (Fig. 3.7.C and D).

In addition to the increase in soluble sugars, the malate and citrate contents decreased in the fruit of most of the PEPCK-overexpressing lines (Fig. 3.8). These results are opposite to those obtained with PEPCK-suppressed lines (Huang et al., 2015). This type of inverse correlation between soluble sugars and malate was also reported in fumarase and malate dehydrogenase-antisense transgenic tomato fruit (Centeno et al.,

2011). These results indicate that the modified PEPCK level directly affects the sugar and organic acid contents, likely through gluconeogenesis. While it has been reported that the predominant process in the flesh of tomato and grape is glycolysis (Famiani et al., 2014; Carrari et al., 2006), early labelling studies demonstrated that gluconeogenesis occurs during tomato fruit ripening (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991). Our present results indicate that gluconeogenesis is involved in sugar accumulation in fruit, and PEPCK plays a regulatory role in this process. This study demonstrated the considerable effect of excessive PEPCK on the sugar content of fruit, as well as the post-germination growth of seedlings.

3.5 Figure legends

Fig. 3.1 The structures of the transformation vectors overexpressing *SlPEPCK*.

SlPEPCK was introduced into tomato by Agrobacterium-mediated transformation. *SlPEPCK* was driven by CaMV*35S* promoter (A) and fruit-specific *E8* promoter (B). NTPII, neomycin phosphotransferase II, NOST, nopaline synthase terminator

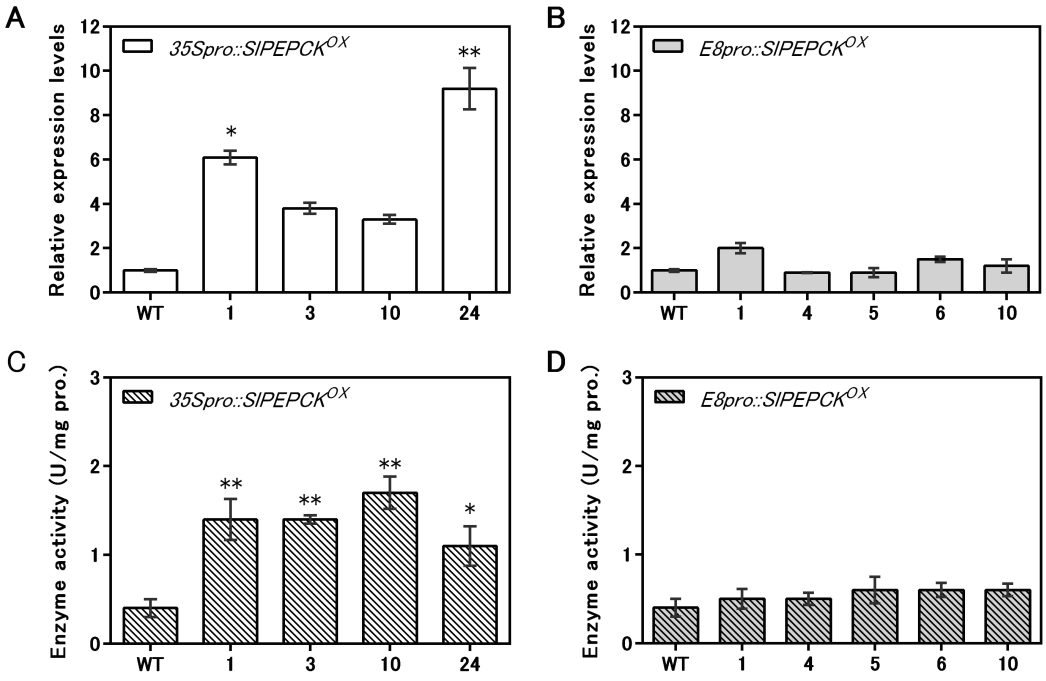

Fig. 3.2 Relative expression levels of the *SlPEPCK* gene and PEPCK enzyme activity in germinating seeds of *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines.

(A) and (B), Relative expression levels of the *SlPEPCK* gene, (C) and (D) PEPCK enzyme activity. Seeds at 2DAS were used for measurements. Genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate no. of the transgenic lines. WT, wild type. Values are means ±SE (n=3).The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$,**$P < 0.01$).

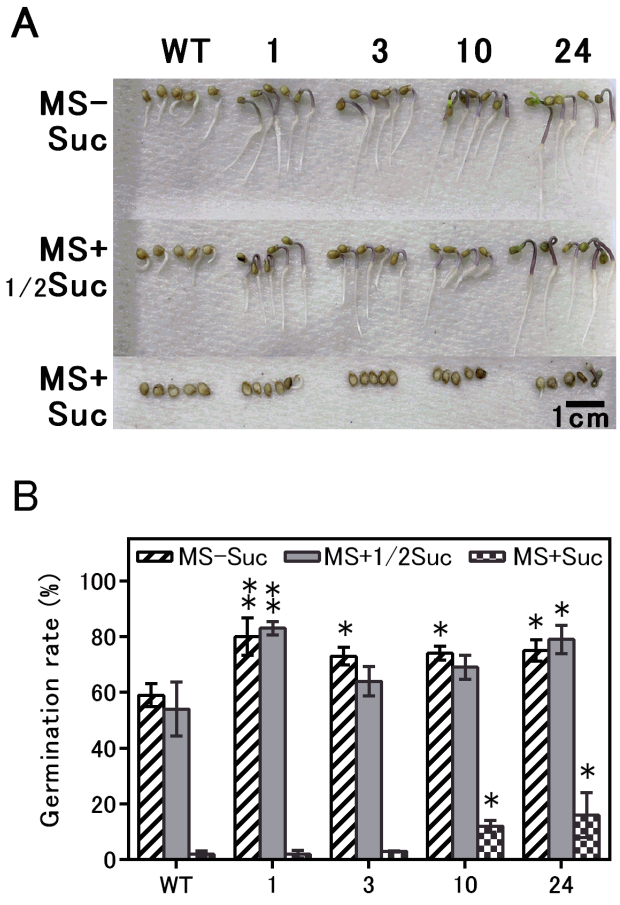

Fig. 3.3 Seed germination of *35Spro::SlPEPCK*^{OX} lines under different sucrose conditions.

(A) Germinating seed at 2 DAS on the medium containing 0%, 1.5% and 3%. The labels at top of the photo indicate no. of the transgenic lines. Bar=1cm. (B) Germination ratio on MS medium with various sucrose conditions. The labels below the horizontal axis indicate no. of the transgenic lines. WT, wild type. Values are means ±SE (n=4). The asterisks indicate significant differences between the means of the transgenic lines and the wild type in the each sucrose condition, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

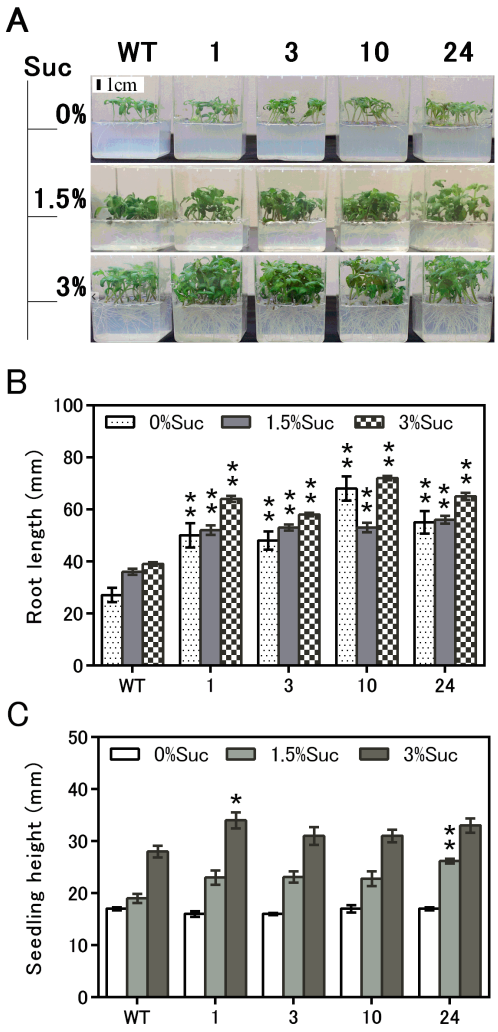

Fig. 3.4 Post-germination growth of the *35Spro::SlPEPCK*^{OX} transgenic lines under the different sucrose conditions.

(A) Seedling growth on MS medium containing 0%, 1.5% and 3% at 10 DAS. The labels at top of the photo indicate no. of the transgenic lines. Bar=1 cm. (B) Root length and (C) Seedling height (mm) at 10 DAS. The labels below the horizontal axis indicate no. of the transgenic lines. WT, wild type. The asterisks indicate significant differences between the means of the transgenic lines and the wild type in the each sucrose condition, estimated by the Dunnett (2-sided) test (*P < 0.05,**P < 0.01).

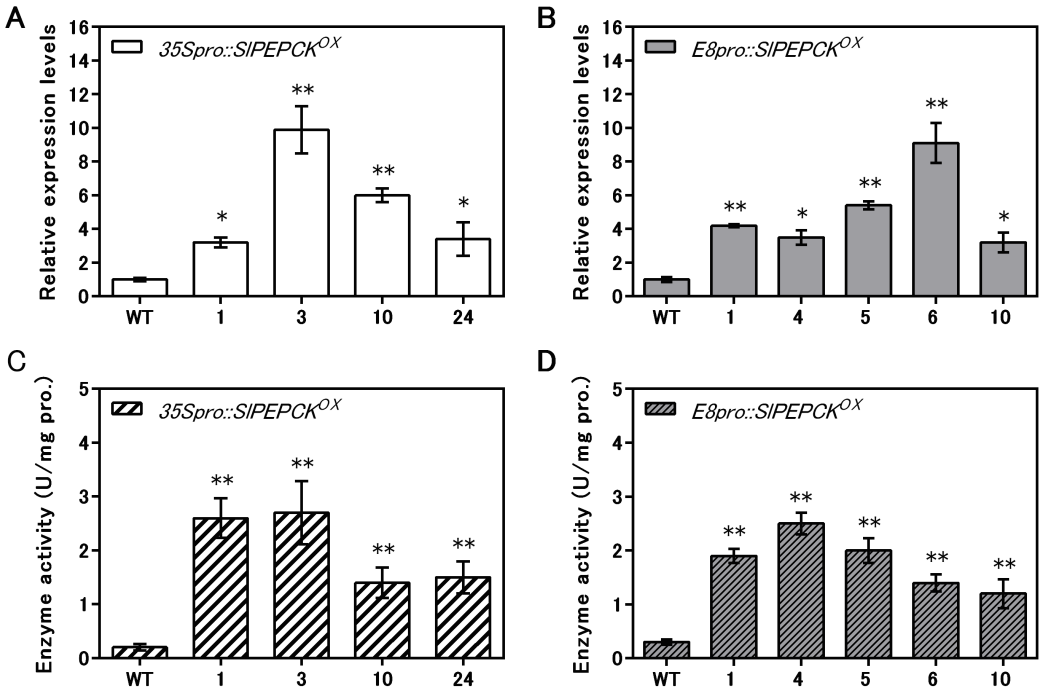

Fig. 3.5 Relative expression levels of the *SlPEPCK* gene and PEPCK activity in fruit of *35Spro::SlPEPCK*^{OX} lines in 42DAF.

(A) and (B), Relative expression levels of the *SlPEPCK* gene, (C) and (D) PEPCK enzyme activity. Values are means ±SE (n=3). Genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate genotypes of the transgenic lines. WT, wild type. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$,**$P < 0.01$).

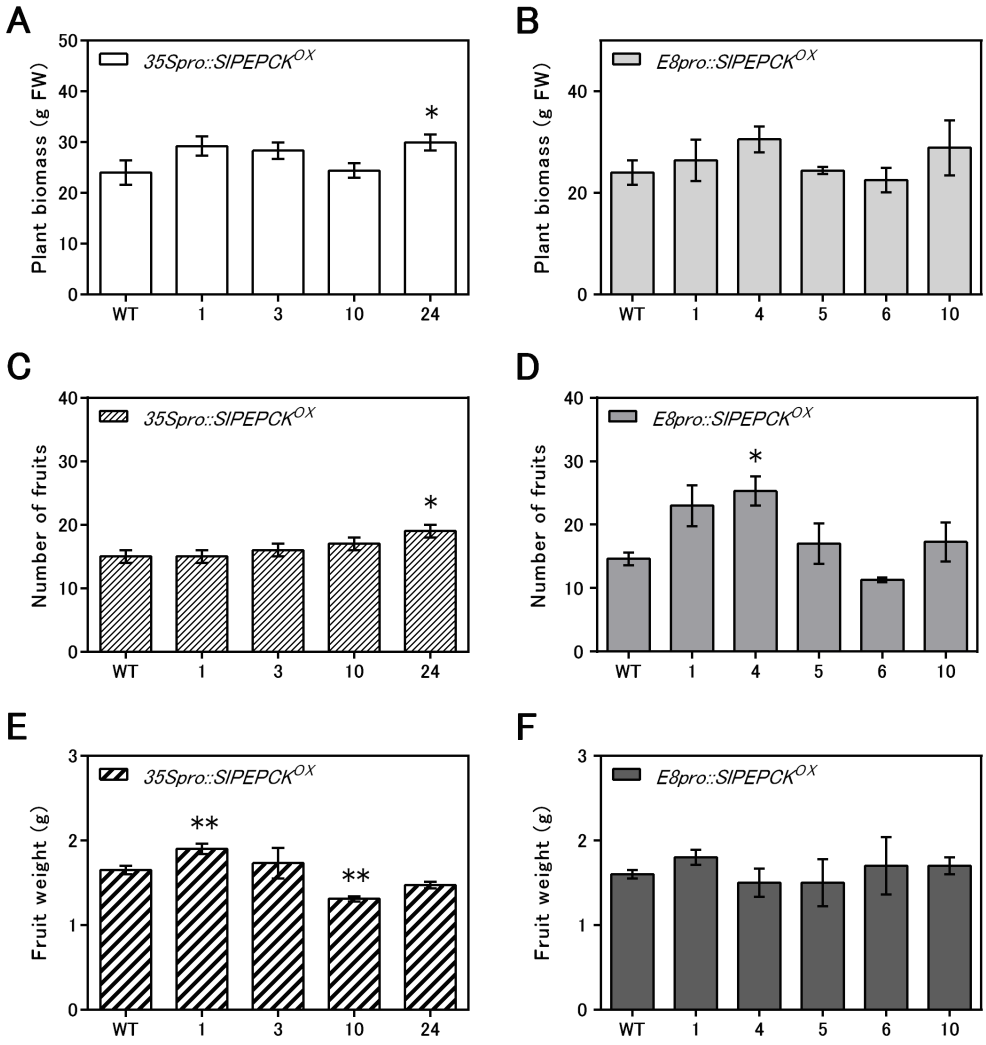

Fig. 3.6. Plant biomass, fruit number and fruit weight of the *SlPEPCK*^{OX} transgenic lines in 42DAF.

(A) and (B) Plant biomass/plant, (C) and (D) Fruit number/plant, (E) and (F) Fruit weight. Fruit samples were harvest from fruit at 42 DAF. (A), (C) and (E), *35Spro*::*SlPEPCK*^{OX}. (B), (D) and (F), *E8pro*::*SlPEPCK*^{OX} lines. Values are means ±SE (n=16). Genotype of the transgenic lines is represented in each graph. The labels below the horizontal axis indicate no. of the transgenic lines. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$,**$P < 0.01$).

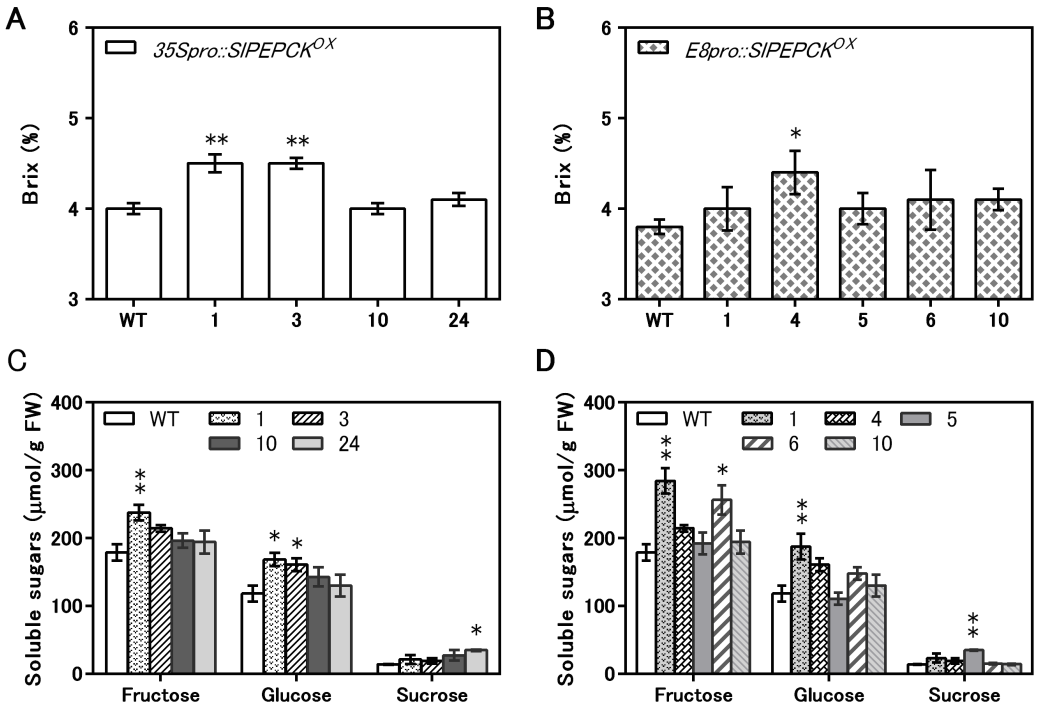

Fig. 3.7. Fruit brix (%) and soluble sugar contents in *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines in 42DAF.

(A) and (B) brix (%), (C) and (D) soluble sugar contents in fruit at 42 DAF, respectively. Genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate the no. of the transgenic lines. WT, wild type. Values are means ±SE (n=7). The asterisks indicate significant differences between the means of the transgenic lines and the wild-type, estimated using the Dunnett (2-sided) test (*$P < 0.05$,**$P < 0.01$).

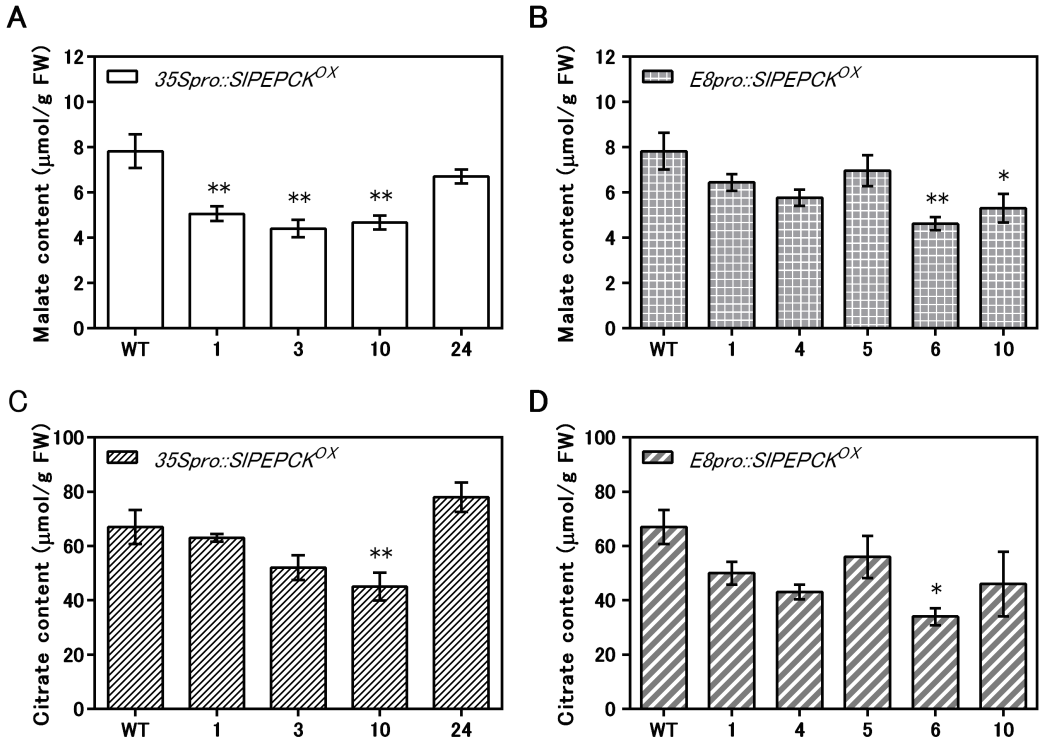

Fig. 3.8. Malate and citrate contents in fruit at 42DAF in *35Spro::SlPEPCK*^{OX} and *E8pro::SlPEPCK*^{OX} lines in 42DAF.

(A) and (B) Malate, (C) and (D) citrate contents in fruit at 42 DAF, respectively. Genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate genotypes of the transgenic lines. WT, wild type. Values are means ±SE (n=7). The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).

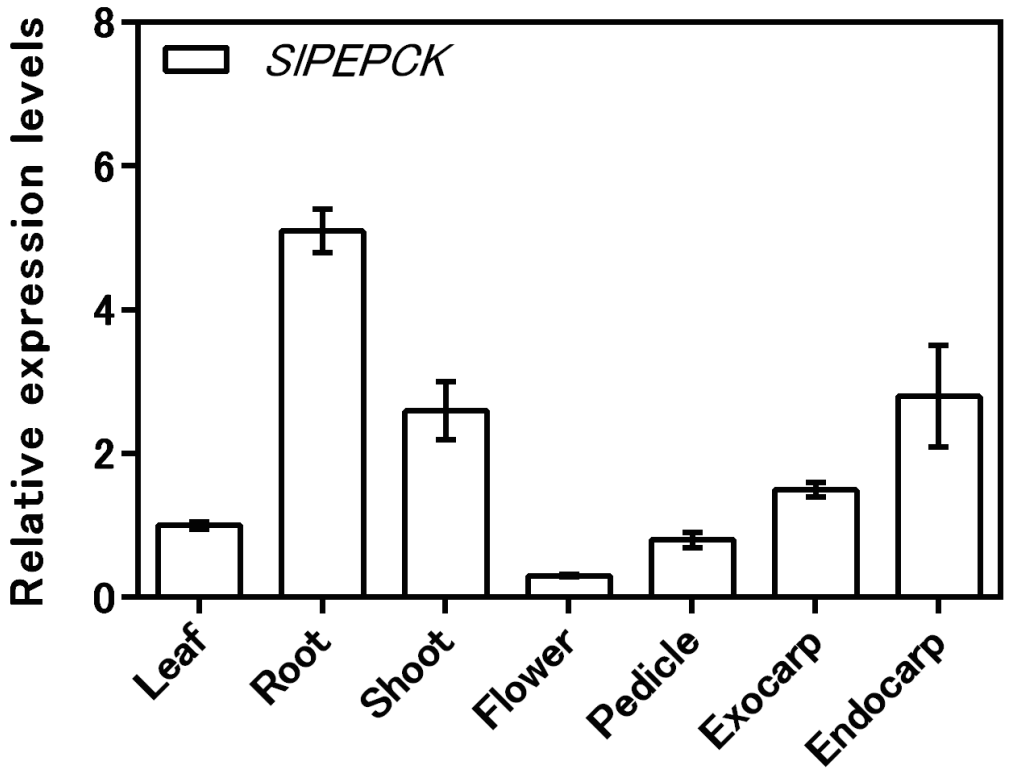

Fig. 3.9. Endogenous expression levels of the *SlPEPCK* gene in tomato plants.

The horizontal axis indicates leaf, root, shoot, flower, pedicle, exocarp and endocarp of green tomato fruits (27DAF). For the normalization of the qRT-PCR reactions, the actin gene (*Tom52*, accession number U60482; Petreikov et al., 2006) was used as an internal standard. WT tomato plant growing at 80DAS was used in this experiment. Values are means ±SE (n=3).

Chapter 4. General discussion

As a key enzyme in gluconeogenesis pathway, PEPCK has been detected with the high level expression during tomato fruit ripening (Bahrami et al., 2001, Saito et al., 2008, Yin et al., 2010a). Although it has been wildly studied in fresh-berry type fruits, the physiological roles of PEPCK in the whole plant and fruit development are still elucidated in tomato. In this research, I generated transgenic lines in which the *SlPEPCK* expression was suppressed by RNAi or over-expressed by either *35S* constitutive- or *E8* fruit-specific promoters. The results revealed that PEPCK effects on seed germination, growth of seedling and juvenile plant and accumulation of sugars and organic acids in fruit.

During plant development, the suppression of PEPCK resulted in the depressed growth in seedlings of the *35Spro::SlPEPCK*^{RNAi} lines (Fig. 2.4), by contrast, the *35Spro::SlPEPCK*^{OX} lines exhibited faster seed germination and better seedling growth comparing with the WT (Fig. 3.3 and 3.4). On the other hand, the expression of *SlPEPCK* and enzymatic activity in germinating seeds did not show significant differences between the WT and *E8* promoter-driven transgenic lines (Fig. 3.2.B and D), resulting in the similar growth vigor at 10DAS comparing with the WT and the transgenic lines (Fig. 2.4 B). These corresponding results indicate PEPCK plays a critical role in early seedling development in tomato.

In the early germination stage (10 DAS), the effect of the PEPCK was more prominent in root elongation rather than that in the aerial part (Figs. 2.4.C and 3.4.B). These differences in organ specificity can be explained by the endogenous *SlPEPCK* expression, which shows higher expression level of *SlPEPCK* in root than shoot (Fig. 3.9). It was also reported that the suppressed expression of mitochondrial malate dehydrogenase (*mMDH*) in tomato plant led reduced root length and dry mass, which was followed by the dramatically reduced respiration ratio in root, suggesting that the arrest of energy metabolism of root slows the root growth of the transgenic plants (van der Merwe et al., 2009). Although there is a limited evidence that *SlPEPCK* regulates root growth, it is likely that similar mechanism is underlying the varied root elongation in the *SlPEPCK* transgenic tomato plants as reported in the *mMDH* suppressed tomato plants.

The seedling growth in the *SlPEPCK*-excessive lines was strongly promoted by the exogenous sugar supply (Fig. 3.4). However, the underlying mechanism has been still unclear, although early studies in fat storing seedlings indicated PEPCK functions in assimilation of storage lipid and protein through gluconeogenesis to supply soluble sugars (Penfield et al., 2004; Rylott et al., 2003). However, in this study, the 35S promoter-driven lines exhibited enhanced seedling growth compared to the WT even under the existence of exogenous sucrose (Fig. 3.4), suggesting gluconeogenesis pathway is not directly involved in the promotion of seedling growth. The previous

work by my research group studying on GABA metabolism in tomato fruit reported that the bypass from malate to pyruvate via PEP mediated by PEPCK/PK, which flows back to TCA cycle, was strongly activated during ripening (Yin et al., 2010a). It would be a possible explanation for the results of Fig. 3.4 that the same pathway participates in tomato seedling growth and it is activated by the overexpression of *PEPCK,* which causes active pyruvate provision to TCA cycle, resulting in the promotion of seedling growth.

Although the suppression- or overexpression of *SlPEPCK* affected plant growth in seedlings and the plant height by 50 DAS (Fig. 2.5.A), clear differences in fruit number per plant and fruit weight was not observed between the WT and the transgenic lines even in *E8* promoter-driven lines (Fig. 2.6; Fig. 3. 6C to E). Most of the transgenic lines showed no significant difference in fruit biomass, fruit number and fresh/dry weight per plant comparing with WT (Fig. 2.5.C to F; Fig. 3.6.A and B). Therefore, I conclude that PEPCK mainly affects the growth at juvenile stages but not in mature plant growth including fruit setting and subsequent fruit development.

Early studies reported that gluconeogenesis occurs during fruit ripening in tomato (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991). However, the contribution of gluconeogenesis and a regulatory role of PEPCK during plant development and fruit metabolism have not been fully understood to date. In tomato fruit, *SlPEPCK* was highly expressed during ripening stage in 42 DAF (Fig. 2.11.A). In

this stage, soluble sugar and organic acid contents in fruit were largely different in transgenic lines compared with that of WT. Total soluble sugar contents in fruit of the *SlPEPCK*-suppressed RNAi lines decreased during ripening stages by 53% and 12% in *35S* and *E8* promoter driven lines compared to the WT (Fig. 2.7 and 2.8). By contrast, the *SlPEPCK*-excessive lines showed higher sugar accumulation than the WT fruits (Fig. 3.7). In addition, PEPCK expression level affected malate contents in fruit. The malate contents tended to be higher during ripening in *SlPEPCK*-suppression lines, while the *SlPEPCK*- overexpressing fruit exhibited lower malate accumulation comparing with the WT (Fig. 2.9.A and B; Fig.3.8.A and B). In contrast to *SlPEPCK*, which highly expresses in ripening fruit, the transcriptional and protein levels of malic enzyme decreased during ripening accompanied by reduced malate content in tomato fruit (Bahrami et al., 2001; Osorio et al., 2013; Saito et al., 2008; Yin et al., 2010a). On the other hand, Osorio et al. (2013) also reported a significant reduction of fructose and glucose levels accompanied by malate accumulation in red-ripe fruit of transgenic tomato in which PEPCK expression was suppressed. Meanwhile, RNAi transgenic lines for malic enzyme did not show any significant difference in stock of sugars and malate (Osorio et al., 2013). Those results indicate that the major metabolic pathway of malate is the malate dehydrogenase / PEPCK-mediated pathway but not the malic enzyme-mediated pathway during fruit ripening in tomato. The contrasting tendency of sugar and malate accumulations observed in the transgenic lines which I tested in this

study strongly suggests the suppression of gluconeogenesis pathway occurs by the *SlPEPCK* down regulation.

Despite the malate content clearly altered in the transgenic fruits, citrate content did not show significant changes among the all tested lines including wild type, *35S* promoter-driven and *E8* promoter-driven transgenic lines (Fig. 2.9.C and D; Fig. 3. 8.C and D). The reason why the apparent influence of the PEPCK was weaker in citrate than in malate can be explained by the difference of absolute amount of the each metabolite. According to the previous studies by my research group, the citrate content was 5 to 10-times higher than the malate content (Akihiro et al., 2008; Saito et al., 2008). It is likely that a large pool of citrate absorbed an impact of the suppression of gluconeogenesis. In conclusion, the contribution of gluconeogenesis and PEPCK on regulation of organic acid accumulation cannot be ignored in ripening of fruit.

Throughout the whole research, I focused on the physiological function of PEPCK in the whole tomato plant. My results revealed the regulatory roles of PEPCK in ripening fruit metabolism and seed establishment in tomato. Firstly, my present results demonstrated the gluconeogenesis is involved in sugar accumulation in tomato fruit and this process is, at least partially, regulated by PEPCK. PEPCK also affects the sugar / acid ratio in ripening fruit through the regulation of gluconeogenesis in tomato fruits. In addition, PEPCK influences seed germination and post-seedling growth in plant development in tomato.

A burst of PEPCK expression during fruit ripening is widely observed in crops having berry type fruit. Therefore, the mechanism that the gluconeogenesis participates in sugar accumulation would be common in the berry type fruit. Meanwhile, in the *SlPEPCK*-RNAi/OX tomato lines, seedling growth was altered according to *SlPEPCK* expression levels. Consequently, the results indicate the regulatory role of PEPCK in post-germination growth and sugars / organic acids accumulation in ripening tomato fruit. In a word, the present research will contribute to understand the regulation mechanisms for sugar / organic acid metabolism in developing fruit and seedlings not only in tomato but also in crops having berry-type fruit.

Summary

Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme regulating the metabolic pathway of gluconeogenesis, which converts the decarboxylation reaction from oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and carbon dioxide. In fresh berry-type fruit, it has been reported that PEPCK dominantly expresses during fruit ripening. The researchers had mainly worked on isolation, characterization of *SlPEPCK* genes, and analyzing of biochemical properties. However, its physiological role has not been fully understood. The purpose of the research for my doctor thesis is to clarify the physiological function of PEPCK in fruit through detail characterization on the transgenic tomato plants.

In Chapter II, the RNAi transgenic tomato lines down-regulating *PEPCK* gene driven either by CaMV *35S* constitutive promoter or fruit-specific *E8* promoter was generated. I evaluated phenotypic properties of the RNAi transgenic plants from the aspects of seedling establishment, plant height, fresh- and dry-weight and fruit weight. The *35S* promoter-driven RNAi transgenic plant exhibited growth suppression during germination period and the effect was prominent in root but not in shoot growth. Subsequent growth suppression was observed in both *35S* and *E8* promoter driven lines in ripening period. This result indicated that gluconeogenesis pathway is a main sugar resource for seedling establishment and PEPCK play a key role in these processes in tomato. The major fruit contents, soluble sugars such as glucose, fructose and sucrose, and organic acids such as malate and citrate were measured. The results showed that the

soluble sugar contents 12-53% decreased in ripening fruit in the RNAi transgenic lines compared to the wild-type fruit, which was accompanied by increased malate content. Those results indicate the gluconeogenesis is important for soluble sugar accumulation in ripening fruit and PEPCK plays a regulatory role in the gluconeogenesis in tomato. To further identify the physiological function of PEPCK in the seedling establishment and sugar/acid balance in tomato fruits, the performance of PEPCK in overexpression lines is demanded.

In order to further investigate the physiological function of PEPCK, in chapter III, I newly generated transgenic lines overexpressing PEPCK gene utilizing CaMV *35S* and fruit-specific *E8* promoter. More than 3 transgenic lines harboring single transgene were acquired in each promoter construct after genotyping by southern blot analyses and submitted to the further detail characterization in order to check whether any phenotypic and metabolic changes occur in the transgenic lines. In the results, the *35S* promoter-driven overexpression lines exhibited the earlier germination and longer root elongation compared to those of wild type. Interestingly, the post-germination growth was promoted by sucrose existence. Those results suggest the PEPCK not only functions in direct supply of carbon source through gluconeogenesis but also extend a carbohydrate availability during germination. While the fruit weight and size were not affected by PEPCK overexpression. Regarding fruit metabolism, the opposite results were acquired, that is, the increased soluble sugars and decreased malate contents were

observed in the transgenic lines. Those results prove the regulatory role of PEPCK in controlling the sugar / acid balance and seedling growth in ripening fruit in tomato.

Acknowledgements

At the ending of my student time, I wish to express my special and heartfelt thanks to my academic supervisor Dr. C, Matsukura, Professor, Graduate School of Life and Environmental Sciences, University of Tsukuba. Thank you very much for your sincere guidance and open-armed encouragement during the past five years for my master and doctor research. Thanks for your always understanding and tolerant in my living days in Japan. I am really so proud and lucky to be your student. Really thank you for your continuous support to my experiment, the spiritual support, and also the daily life. Otherwise, I would like to bring my great thanks to Dr. H. Ezura, Professor, Graduate School of Life and Environmental Sciences, University of Tsukuba. Thank you very much for your magnanimous understanding, guidance and support. Moreover, I intended to show my reverence to broad-minded Dr. N. Fukuda, Dr. T. Ariizumi, Associate Professor, Graduate School of Life and Environmental Science, University of Tsukuba; Dr. S. Sugaya, Dr. H. Miyazaki, Professor, Graduate School of Life and Environmental Science, University of Tsukuba. I appreciate your sincere supervision and guidance on my research and thanks for your hard work very much on the revise of my doctor thesis. Otherwise, I am particularly grateful to Dr. S. Nonaka; Dr. N. Wang; Dr. K. Izawa; Dr. Y. Okabe, Assistant Professors, Graduate School of Life and Environmental Science, University of Tsukuba; Assistant Professor, Graduate School of Life and Environmental Science, University of Tsukuba; Dr. K. Tanase, Dr. K.

Hosikawa, Graduate School of Life and Environmental Science, University of Tsukuba. Thanks for your genuine recommendations and sincere assistance during my doctor research. By this special moment, I truly thanks for all the teachers who gave me cheerful lessons in my doctor course. I am grateful to your beneficial instruction.

Besides, I would like to communicate my great thanks to my seniors and coworkers in our harmonious research team. Thank you to Ms. A. Sanuki, Ms. M. Takayama, Ms. M. Sato, Mr. Y. Goto, Graduate School of Life and Environmental Science, University of Tsukuba. Thanks a lot for your help in my experiment and your great contribution for the cordial and friendly working atmosphere. Best wishes for your own research and truly expect our joyful cooperation in the future.

Truly thanks to the MEXT scholarship and the strong backing of my family, love you all, my dear husband Eikyo and cute daughter Chiki.

Special thanks to my senior Dr YG Yin, by providing lots of help to my study and papers' preparation.

References

Aich S, Imabayashi F and Delbaere LT (2003) Expression, purification, and characterization of a bacterial GTP-dependent PEP carboxykinase. *Protein Expres Purify.* **31:** 298–304

Akihiro T, Koike S, Tani R, Tominaga T, Watanabe S, Iijima Y, Aoki K, Shibata D, Ashihara H, Matsukura C, Akama K, Fujimura T and Ezura H (2008) Biochemical mechanism on GABA accumulation during fruit development in tomato. *Plant and Cell Physiol.* **49:** 1378–1389

Bahrami AR, Chen Z-H, Walker RP, Leegood RC and Gray JE (2001) Ripening-related occurrence of phosphoenolpyruvate carboxykinase in tomato fruit. *Plant Mol Biol.* **47:** 499–506

Bailey KJ, Gray JE, Walker RP and Leegood RC (2007) Coordinate regulation of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase by light and CO_2 during C_4 photosynthesis. *Plant Physiol.* **144:** 479–486

Baldicchi A, Farinelli D, Micheli M, Di Vaio C, Moscatello S, Battistelli A, Walker RP and Famiani F (2015) Analysis of seed growth, fruit growth and composition and phosphoenolpyruvate carboxykinase (PEPCK) occurrence in apricot (*Prunus armeniaca* L.). *Sci. Hortic*. **186:** 38-46.

Brown NJ, Palmer BG, Stanley S, Hajaji H, Janacek SH and Astley HM (2010) C_4 acid decarboxylases required for C_4 photosynthesis are active in the mid-vein of the C_3 species *Arabidopsis thaliana*, and are important in sugar and amino acid metabolism. *Plant J.* **61**(1)**:** 122–133

Carrari F, Baxter C, Usadel B, Urbanczyk-Wochniak E, Zanor MI, Nunes-Nesi A, Nikiforova V, Centero D, Ratzka A, Pauly M, Sweetlove LJ and Fernie AR (2006) Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. *Plant Physiol.* **142**(4**):** 1380-1396

Centeno DC, Osorio S, Nunes-Nesi A, Bertolo ALF, Carneiro RT, Arau´jo WL, Steinhauser MC, Michalska J, Rohrmann J, Geigenberger P, Oliver SN, Stitt M, Carrari F, Rose JKC and Fernie AR (2011) Malate plays a crucial role in starch metabolism, ripening and soluble solid content of tomato fruit and affects postharvest softening. *Plant Cell.* **23:** 162-184

Chakravarty K, Cassuto H, Reshef L and Hanson RW (2005) Factors that control the tissue-specific transcription of the gene for phosphoenolpyruvate carboxykinase-C. *Crit Rev Biochem Mo. Biol.* **40** (3)**:** 129–54.

Deikman J and Fischer RL (1988) Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. *EMBO J.* **7:** 3315–3320

Dittrich P, Campbell WH and Black CC (1973) Phosphoenolpyruvate Carboxykinase in plants exhibiting crassulacean acid Metabolism. *Plant Physiol.* **52:** 357–361

Dong L, Ermolova NV and Chollet R (2001) Partial purification and bio- chemical characterization of a heteromeric protein phosphatase 2A holoenzyme from maize (*Zea mays* L.) leaves that dephosphorylates C4 phosphoenolpyruvate carboxylase. *Planta*. **213:** 379–389.

Echeverria E and Valich J (1989) Enzymes of sugar and acid metabolism in stored "Valencia" oranges. *J. Amer. Soc*. *Hort. Sci*. **114:** 445-449

Famiani F, Cultrera NGM, Battistelli A, Casulli V, Proietti P, Standardi A, Chen Z-H, Leegood RC and Walker RP (2005) Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. *J. Exp. Bot.* **56:** 2959–2969

Famiani F and Walker RP (2009) Changes in abundance of enzymes involved in organic acid, amino acid and sugar metabolism, and photosynthesis during the ripening of blackberry fruit. *J Am Soc Hortic Sci.* **134:**167–175

Famiani F, Baldicchi A, Battistelli A, Moscatello S and Walker RP (2009) Soluble sugar and organic acid contents and the occurrence and potential role of

phosphoenolpyruvate carboxykinase (PEPCK) in gooseberry (*Ribes grossularia* L.). *J Hortic Sci Biotechnol*. **84:** 249-254

Famiani F, Casulli V, Baldicchi A, Battistelli A, Moscatello S and Walker RP (2012) Development and metabolism of the fruit and seed of the Japanese plum Ozark premier (Rosaceae). *J Plant Physiol.* **169:** 551-560

Famiani F, Moscatello S, Ferradini N, Gardi T, Battistelli A and Walker RP (2014) Occurrence of a number of enzymes involved in either gluconeogenesis or other processes in the pericarp of three cultivars of grape (*Vitis vinifera* L.) during development. *Plant Physiol Biochem.* **84:** 261–270

Farineau J and Laval-Martin D (1977) Light versus dark carbon metabolism in cherry tomato fruits II. Relationship between malate metabolism and photosynthetic activity. *Plant Physiol.* **60:** 877–880

Finnegan PM and Burnell JN (1995) Isolation and sequence analysis of cDNAs encoding phospho*enol*pyruvate carboxykinase from the PCK-type C_4 grass *Urochloa panicoides*. *Plant Mol Biol.* **27:** 365–376

Finnegan PM, Suzuki S, Ludwig M and Burnell JN (1999) Phospho*enol*pyruvate carboxykinase in the C_4 monocot *Urochloa panicoides* is encoded by four differentially expressed genes. *Plant Physiol.* **120**(4)**:** 1033–1042

Furumoto T, Hata S and Izui K (1999) cDNA cloning and characterization of maize phosphoenolpyruvate carboxykinase, a bundle sheath cell-specific enzyme. *Plant Mol Biol*. **41:** 301–311

Halinska A and Frenkel C (1991) Acetaldehyde stimulation of net gluconeogenic carbon movement from applied malic acid in tomato fruit pericarp. *Plant Physiol.* **95:** 954–960

Hansen EJ and Juni E (1974) Two routes for synthesis of phospoenolpate from C4-dicarboxylic acids in *Eschkrichia coli*. *Biochem. Biophs. Res. Commun.* **59(4):** 1204–1210

Hanson RW and Reshef L (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem.* **66:** 581-611

Hanson RW and Patel YM (1994) Phosphoenolpyruvate carboxykinase (GTP): the gene and the enzyme. *Adv Enzymol Relat Areas Mol Biol.* **69:** 203-281

Hanson RW and Hakimi P (2008) Born to run: the story of the PEPCK-Cmus mouse. *Biochimie.* **90:** 838-842

Huang YX, Yin YG, Sanuki A, Fukuda N, Ezura H and Matsukura C (2015) Phosphoenolpyruvate carboxykinase (PEPCK) deficiency affects the germination,

growth and fruit sugar content in tomato (*Solanum lycopersicum* L.). *Plant Physiol Bioch.* **96:** 417-425

Krupa A and Srinivasan N (2006) Genome-wide comparative analyses of domain organization of repertoires of protein kinases of *Arabidopsis thaliana* and *Oryza sativa.* *Gene.* **380:** 1-13

Kim DJ and Smith SM (1994) Molecular cloning of cucumber phosphoenolpyruvate carboxykinase and developmental regulation of gene expression. *Plant Mol Biol.* **26:** 423–434.

Kliewer WM (1965) Changes in the concentration of malates, tartrates, and total free acids in flowers and berries of *Vitis vinifera*. *Am J Enol Vitic.* **16:** 92–100.

Lea PJ, Chen ZH, Leegood RC and Walker RP (2001) Does phosphoenolpyruvate carboxykinase have a role in both amino acid and carbohydrate metabolism? *Amino Acid.* **20:** 225-241

Leegood RC and ap Rees TA (1978) Phosphoenolpyruvate carboxykinase and gluconeogenesis in cotyledons of *Cucurbita pepo*. *Biochim Biophys Acta*. **524:** 207–218

Leegood RC and Walker RP (2003) Regulation and roles of phosphoenolpyruvate carboxykinase in plants. *Arch Biochem Biophys.* **414:** 204–210

Liu K, Ba X, Yu J, Li J, Wei Q, Han G, Li G and Cui Y (2006). The phosphoenolpyruvate carboxykinase of *Mycobacterium tuberculosis* induces strong cell-mediated immune responses in mice. *Mol Cell Biochem.* **288 (1-2):** 65–71

Malone S, Chen ZH, Bahrami AR, Walker RP, Gray JE and Leegood RC (2007) phoenolpyruvate carboxykinase in Arabidopsis: changes in gene expression, protein and activity during vegetative and reproductive development. *Plant Cell Physiol.* **48 (3):** 441–450

Mati E, Gisbert C, Bishop GJ, Dixon MS and García-Marínez JL (2006) Genetic and physiological characterization of tomato cv. Micro-Tom. *J Exp Bot.* **57:** 2037-2047

Martín M, Plaxton WC and Podesta FE (2007) Activity and concentration of non-proteolyzed phosphoenolpyruvate carboxykinase in the endosperm of germinating castor oil seeds: effects of anoxia on its activity. *Plant Physiol.* **130:** 484–494

Martín M, Riua PS and Podesta E (2011) Two phosphoenolpyruvate carboxykinases coexist in the Crassulacean acid metabolism plant *Ananas comosus*. Isolation and characterization of the smaller 65kDa form. *Plant Physiol Biochem.* **49:** 646–653

Mazelis M and Vennesland B (1957) Carbon dioxide fixation into oxalacetate in higher plants. *Plant Physiol*. **32:** 591-600

Nomura M, Higuchi T, Katayama K, Taniguchi M, Miyao-Tokutomi M, Matsuoka M and Tajima S (2005) The promoter for C4-type mitochondrial aspartate aminotransferase does not direct bundle sheath-specific expression in transgenic rice plants. *Plant Cell Physiol.* **46:** 743–753

Osorio S, Vallarino JG, Szecowka M, Ufaz S, Tzin V, Angelovici R, Galili G and Fernie AR (2013) Alteration of the interconversion of pyruvate and malate in the plastid or cytosol of ripening tomato fruit invokes diverse consequences on sugar but similar effects on cellular organic acid, metabolism, and transitory starch accumulation. *Plant Physiol.* **161:** 628–643

Parvin H, Yang J, Hakimi P, Casadesus G, Massillon D, Tolentino-Silva F, Nye CK, Cabrera ME, Hagen DR, Utter CB, Baghdy Y, Johnson DH, Wilson DL, Kirwan JP, Kalhan SC and Hanson RW (2007) Overexpression of the Cytosolic of Phosphoenolpyruvate Carboxykinase(GTP) in Skeletal Muscle Repatterns Energy Metabolism in the Mouse. *J Bio Che.* **282:** 32844–32855

Penfield S, Rylott EL, Gilday AD, Graham S, Larson TR and Graham IA (2004) Reserve mobilization in the Arabidopsis endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires Phosphoenolpyruvate carboxykinase. *Plant Cell.* **16:** 2705–2718

Penfield S, Clements S, Bailey KJ, Gilday AD, Leegood RC, Gray JE and Graham IA (2012) Expression and manipulation of *PHOSPHOENOLPYRUVATE CARBOXYKINASE 1* identifies a role for malate metabolism in stomatal closure *Plant J.* **69:** 679–688

Petreikov M, Shen S, Yeselson Y, Levin I, Bar M and Schaffer AA (2006) Temporally extended gene expression of the ADP-Glc pyrophosphorylase large subunit *(AgpL1)* leads to increased enzyme activity in developing tomato fruit. *Planta.* **224:** 1465–1479

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29(9):** e45

Reiskind JB and Bowes G (1991) The role of phosphoenolpyruvate carboxykinase in a marine macroalga with C_4-like photosynthetic characteristics. *Proc Natl Acad Sci USA.* **88:** 2883–2887

Ruffner HP and Kliewer WM (1975) Phosphoenolpyruvate carboxykinase activity in grape berries. *Plant Physiol.* **56:** 67–71

Ruffner HP (1982) Metabolism of tartaric and malic acids in Vitis: a review, Part A. *Vitis.* **21:** 247–259

Rylott EL, Gilday AD and Graham LA (2003) The gluconeogenic enzyme phosphoenolpyruvate carboxykinase in Arabidopsis is essential for seedling establishment. *Plant Physiol.* **131:** 1834–1842

Sáez-Vásquez J, Raynal M and Delseny M (1995) A rapeseed cold-inducible transcript encodes a phospohoenolpyruvate carboxykinase. *Plant Physiol.* **109:** 611–618

Saito T, Matsukura C, Ban Y, Shoji K, Sugiyama M, Fukuda N and Nishimura S (2008) Salinity stress assimilate metabolism at the gene-expression level during fruit development and improves fruit quality in tomato (*Solanum licopersicum L.*). *J J Soc Hortc Sci.* **77:** 61–68

Sanuki A (2010) Elucidation of the regulation mode of gene expression of ADP-Glucose pyrophosphorylase and phosphoenolpyruvate carboxykinase in tomato fruit. Master thesis of the Graduate School of Life and Environmental Sciences, the University of Tsukuba

Scott JW and Harbaugh BK (1989) Micro-Tom. A miniature dwarf tomato. *Florida Agricultural Experimental Station*, *Institute of Food and Agricultural Sciences*, *University of Florida Circular*. **S-370**, 1–6

Sun HJ, Uchii S, Watanabe S and Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell*

Physiol. **47(3):** 426–31

Sun Y, Liu S, Ferguson S, Wang LQ, Klepcyk P, Yun JS and Friedman JE (2002) Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice, *J Bio Chem.* **277(26):** 23301–23307

Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution, *Nature.* **485:** 635–641

van der Merwe MJ, Osorio S, Moritz T, Nunes-Nesi A and Fernie AR (2009) Decreased mitochondrial activities of malate dehydrogenase and fumarase in tomato lead to altered root growth and architecture via diverse mechanisms. *Plant Physiol.* **149:** 653–669

Walker RP and Leegood RC (1996) Phosphorylation of phosphoenolpyruvate carboxykinase in plants Studies in plants with C_4 photosynthesis and Crassulacean acid metabolism and in germinating seeds. *Biochem J.* **317:** 653–658.

Walker RP, Chen ZH, Técsi LI, Famiani F, Lea PJ and Leegood RC (1999) Phosphoenolpyruvate carboxykinase plays a role in interactions of carbon and nitrogen metabolism during grape seed development. *Planta*. **210:** 9-18.

Walker RP, Chen ZH, Johnson KE, Famiani F, Tecsi L and Leegood RC (2001) Using immunohistochemistry to study plant metabolism: the examples of its use in the localization of amino acids in plant tissues, and of phosphoenolpyruvate carboxykinase and its possible role in pH regulation. *J Exp Bot.* **52:** 565-576

Walker RP and Chen Z-H (2002) Phosphoenolpyruvate carboxykinase: Structure, function and regulation. *Adv Bot Res.* **38:** 93–189

Walker RP, Battistelli A, Moscatello S, Chen Z-H, Leegood RC and Famiani F (2011a) Phosphoenolpyruvate carboxykinase in cherry *(Prunus avium L.*) fruit during development. *J Exp Bot.* **62:** 5357–5365

Walker RP, Famiani F, Baldicchi A, Cruz-Castillo J-G and Inglese P (2011b) Changes in enzymes involved in photosynthesis and other metabolic processes in the fruit of *Opuntia ficus-indica* during growth and ripening. *Sci Hortc.* **128:** 213–219

Watanabe M, Ohnishi J and Kanai R (1984) Intracellular localization of phosphoenolpyruvate carboxykinase in bundle sheath cells of C_4 plants. *Plant Cell Physiol.* **25:** 69–76

Wringler A, Walker RP, Chen Z-H and Leegood RC (1999) Phosphoenolpyruvate carboxykinase is involved in the decarboxylation of aspartate in the bundle sheath of maize. *Plant Physiol.* **120:** 539–545

Yin Y-G, Tominaga T, Iijima Y, Aoki K, Shibata D, Ashihara H, Nishimura S, Ezura H and Matsukura C (2010a) Metabolic alterations in organic acids and γ-Aminobutyric acid in developing tomato (*Solanum lycopersicum L.*) fruits. *Plant Cell Physiol.* **51:** 1300–1314

Yin Y-G, Kobayashi Y, Sanuki A, Kondo S, Fukuda N, Ezura H and Matsukura C (2010b) Salinity induces carbohydrate accumulation and sugar-regulated starch biosynthetic genes in tomato (*Salanum lycopersicum* L. cv. 'Micro-Tom') fruits in an ABA- and osmotic stress-independent manner. *J Exp Bot.* **61:** 563–574

Zushi K and Matsuzoe N (2006) Postharvest changes in sugar, organic acid, glutamic acid and antioxidant contents in tomato fruit grown under salinity stress. *Environ Control Biol.* **44:** 111–117