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***Original Paper***

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

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**Abstract** Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that regulates the gluconeogenesis pathway in plants. While the biochemical properties of PEPCK have been reported for many species, its physiological function is not fully understood in plants with fresh berry-type fruit. To clarify its physiological role(s) in the tomato plant, the effect of excessive PEPCK was investigated using transgenic lines overexpressing *SlPEPCK* by either the CaMV *35S* constitutive promoter or the fruit-specific *E8* promoter. Detailed characterization of the phenotypic and metabolic properties of the *35S* promoter-driven lines revealed that the transgenic seedlings exhibited earlier germination and better seedling growth compared with the wild type. Interestingly, seedling growth at 10 days after sowing of the transgenic lines was enhanced by an exogenous sucrose supply. These results suggest that PEPCK enhances seedling growth through PEPCK/pyruvate kinase-mediated pathway rather than gluconeogenesis during germination. In addition, increased soluble sugars and decreased malate contents were observed in red-ripe fruit in both the *35S* and *E8* promoter-driven lines, indicating the participation of gluconeogenesis in sugar/acid metabolism during fruit ripening. The present results are totally opposite to those observed in PEPCK-suppressed RNAi lines, which were investigated in our previous work. The results indicate the regulatory role of PEPCK in post-germination growth and sugar/organic acid accumulation in ripening tomato fruit.

**Key words:** Fruit, gluconeogenesis, PEPCK, seedling growth, tomato.

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 a 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 (Furumoto et al. 1999; Hansen and Juni 1974; Reiskind and Bowes 1991; Walker and Leegood 1996). 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; Martín et al. 2007; Penfield et al. 2004; Rylott et al. 2003).

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 (Baldicchi et al. 2015; Famiani et al. 2005, 2009, 2012; Ruffner and Kliewer 1975; Walker et al. 2011). In tomato, a high expression level of *SlPEPCK* was reported in ripening tomato fruit (Bahrami et al. 2001; Saito et al. 2008; Yin et al. 2010). Recent studies on tomato lines in which endogenous *SlPEPCK* was suppressed by RNA interference (RNAi) proved the involvement of PEPCK in soluble sugar accumulation and dissimilation of organic acids during fruit ripening (Huang et al. 2015;

Abbreviations: CaMV, cauliflower mosaic virus; CAM, Crassulacean Acid Metabolism; CE, capillary electrophoresis; DAF, days after flowering; DAS, days after sowing; EC, electrical conductivity; HPLC, high performance liquid chromatography; RI, refractive index; OAA, oxaloacetate; OX, overexpression; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; WT, wild type.

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Osorio et al. 2013). Furthermore, systemic suppression of *SlPEPCK* by the constitutive *35S* promoter resulted in suppression of post-germination growth of transgenic tomato seedlings (Huang et al. 2015), suggesting that PEPCK and gluconeogenesis also participate in carbohydrate supply during tomato germination through a similar mechanism to that which occurs in fat-storing seed plants. On the other hand, 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 influence on germination/post-germination growth, soluble sugars and organic acid contents, and fruit number and weight were investigated. Excessive PEPCK promoted the accumulation of soluble sugars, accompanied by reduced 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. The present results provide evidence of the regulatory role of PEPCK in seedling growth and sugar and acid accumulation in ripening tomato fruit.

## Materials and methods

### *Plant materials*

Dwarf tomato (*Solanum lycopersicum* L.) cv. '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 25°C under a light intensity of 130 $\mu$mol $m^{-2}s^{-1}$ 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×90 mm deep) filled with commercial culture soil. The cultivation pots were placed inside 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 electrical conductivity (EC) of 1.5 dS $m^{-1}$ and maintained at a 2 l volume in each tray. For metabolic analyses, the fruits were harvested at 42 days after flowering (DAF) and stored at −80°C until use. For phenotypic analysis, 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.

### *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) (Figure S1). 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 fruit with gene-specific primers as follows: FW 5′-CAC CAT GGC GTC GAA CGG AGT C-3′ and RW 5′-TTA GAA GTT TGG ACC AGC TGC C-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 transferred into the destination vector pBI-OX-GW (Inplanta Innovations Inc. Yokohama, Japan) using the 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).

### *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 $\mu$g of total RNA using the First Strand cDNA Synthesis Kit (Takara Bio Inc. Otsu, Japan) according to the manufacturer's instructions. The cDNA was diluted 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 the 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: 95°C for 10 min for the initial denaturation followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and then 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The endogenous actin gene (*Tom52*, accession number U60482; Petreikov et al. 2006) was used as an internal standard with the following primers: FW 5′-CAC CAT TGG GTC TGA GCG AT-3′ and Rev 5′-GGG CGA CAA CCT TGA TCT TC-3′. The *SlPEPCK* cDNA sequence (Bahrami et al. 2001) was amplified using the following sequence of the primer set FW 5′-GAA TAC AAG AAG ACC GAG GTA-3′ and Rev 5′-CTC AAA ATT CTT CCT AAA TAG G-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 Amicon Ultra-4-treated fresh extracts at 340 nm and 25°C. The assay mixture contained 100 mM HEPES (pH 7.0), 100 mM KCl, 90 mM $KHCO_3$, 0.5 mM PEP, 1.0 mM ADP, 5 $\mu$M $MnCl_2$, 0.14 mM NADH, and 6 unit $ml^{-1}$ malate dehydrogenase for the optimum reaction (Bailey et al. 2007).

### *Seed germination and seedling growth investigation*

Tomato seeds were surface-sterilized 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 in distilled water for 4 h. After sufficient imbibition, seeds of each transgenic line and the WT were separately 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 evaluated by root and shoot length at 10 DAS in the $T_3$ generation of the *35Spro::SlPEPCK$^{OX}$* lines.

### *Measurement of Brix (%), soluble sugars, malate and citrate content*

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 $\mu$l of MilliQ water and incubated at 99°C for 5 min to inactivate the sugar degradative enzymes and then centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant was stored at −30°C 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 filtrated through a 0.45-$\mu$m membrane filter. Organic acids were measured at 25°C using a CE system (75 $\mu$m×50 cm capillary, Beckman Coulter, Brea, CA, USA) and an anion analysis kit (Beckman Coulter, Brea, CA, USA). Organic acid peaks were separated using an applied voltage of 30.0 K. Glucose, fructose and sucrose contents were separated at 80°C using a Shim-Pack SCR-101C column (7.9×300 mm, Shimadzu, Tokyo, Japan) installed in a high-performance liquid chromatograph (HPLC, LC-2010 A, 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 $min^{-1}$.

## Results

### *Expression levels of SlPEPCK genes and enzymatic activity in germinating seeds of the SlPEPCK-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 harbouring a single copy of the diploid transgene were selected as experimental lines as follows: no. 1, 3, 10 and 24 in the *35Spro::SlPEPCK$^{OX}$* line 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 days after sowing (DAS) with the gene specific

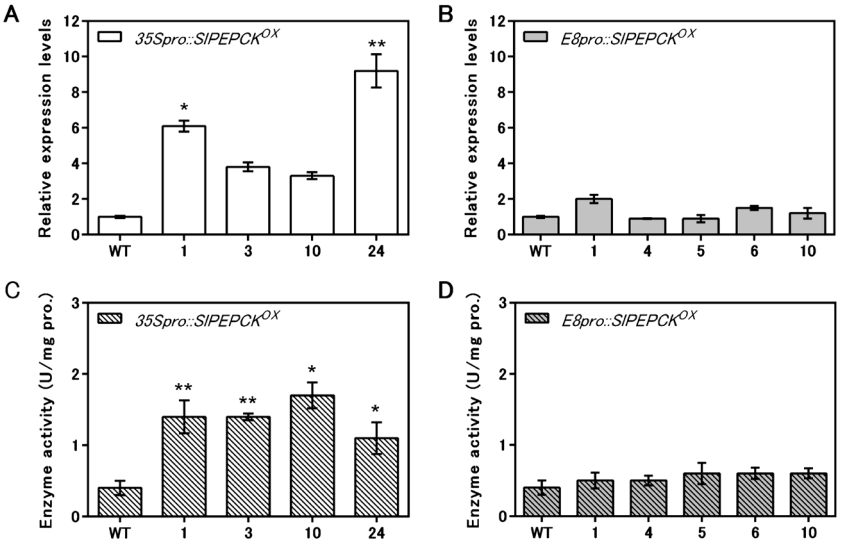


Figure 1. Relative expression levels of the *SlPEPCK* gene and PEPCK enzyme activity in germinating seeds of the *35Spro::SlPEPCK$^{OX}$* and *E8pro::SlPEPCK$^{OX}$* lines. (A) and (B) Relative expression levels of the *SlPEPCK* gene, (C) and (D) PEPCK enzyme activity. Seeds at 2 DAS were used for measurements. The genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate the no. of the transgenic line. Pro, protein. 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 (* $p<0.05$, ** $p<0.01$).

primers in the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines (Figure 1A and B). The *SlPEPCK* expression levels of each line were represented relative to that of the WT plants. The endogenous *SlPEPCK* transcriptional levels in the *35Spro::SlPEPCK*$^{OX}$ lines were 3.3- to 9.2-fold higher than in the wild type (Figure 1A), while no significant difference was observed for the *E8pro::SlPEPCK*$^{OX}$ lines (Figure 1B). 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 *35Spro::SlPEPCK*$^{OX}$ lines, whereas that of the *E8pro::SlPEPCK*$^{OX}$ lines did not show significant changes compared with the WT (Figure 1C and D).

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

To evaluate the effect of PEPCK overexpression on germination and subsequent seedling growth, the germination ratio at 2 DAS and the seedling height and root length at 10 DAS were measured in $T_3$ generation plants of the *35Spro::SlPEPCK*$^{OX}$ lines (Figures 2 and 3). At 2 DAS, the primary root had already extended in the *35Spro::SlPEPCK*$^{OX}$ lines while the WT root had only just 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 (Figure 2A). The germination ratio was also significantly higher in most of the

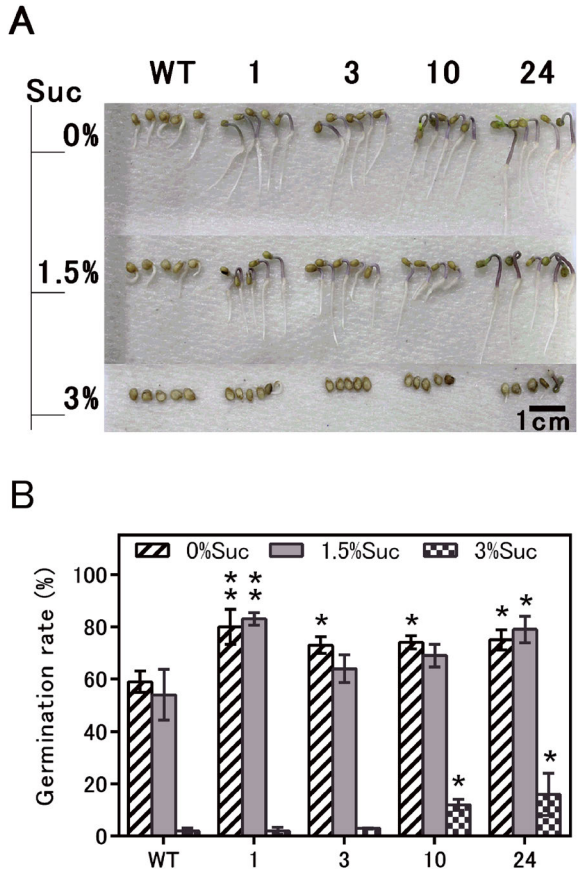


Figure 2. Seed germination of the *35Spro::SlPEPCK*$^{OX}$ lines under different sucrose conditions. (A) Germinating seed at 2 DAS on the medium containing 0%, 1.5% and 3%. The labels at the top of the photograph indicate the no. of the transgenic line. Bar=1 cm. (B) Germination ratio with respect to the MS medium under various sucrose conditions. The labels below the horizontal axis indicate no. of the transgenic line. 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 for each sucrose condition, estimated using the Dunnett (2-sided) test (* $p$<0.05, ** $p$<0.01).

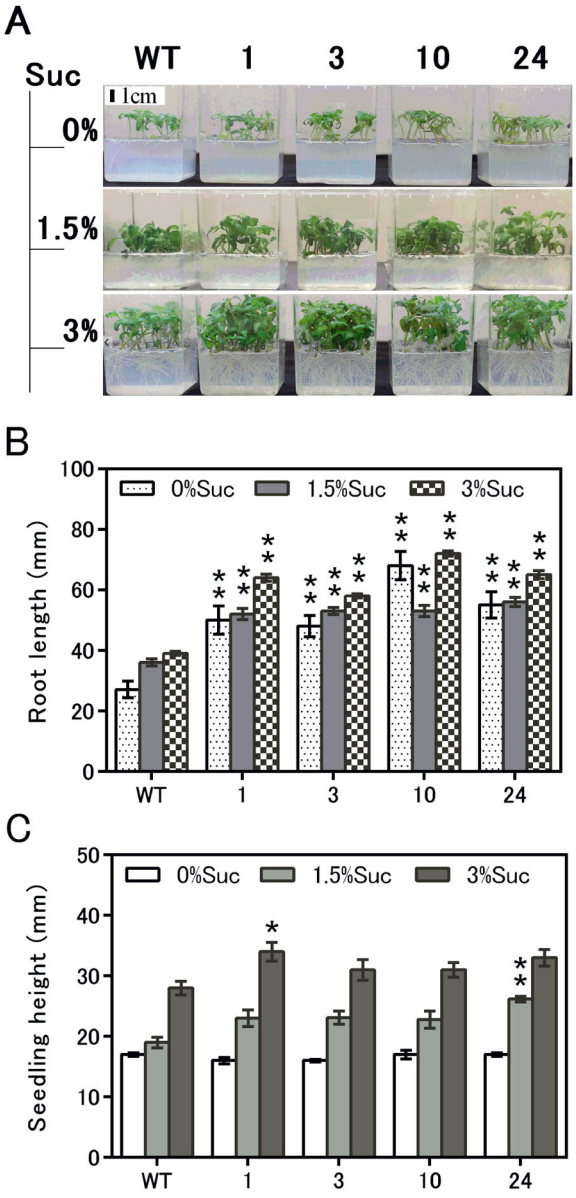


Figure 3. Post-germination growth of the *35Spro::SlPEPCK*$^{OX}$ lines under the different sucrose conditions. (A) Seedling growth on MS medium containing 0%, 1.5% and 3% at 10 DAS. The labels at the top of the photograph indicate the no. of the transgenic line. Bar=1 cm. (B) Root length and (C) seedling height (mm) at 10 DAS. The labels below the horizontal axis indicate the no. of the transgenic line. WT, wild type. Values are means ±SE ($n$=10). The asterisks indicate significant differences between the means of the transgenic lines and the wild type for each sucrose condition, estimated using the Dunnett (2-sided) test (* $p$<0.05, ** $p$<0.01).

*35Spro::SlPEPCK*$^{OX}$ lines compared with the wild type under all three conditions. In the *35Spro::SlPEPCK*$^{OX}$ lines, this 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 (Figure 2B). 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 lines and the wild type (Figure 2B). However, after 3 DAS, seeds began to germinate and grew faster than 0% and 1.5% Suc conditions.

The post-germination growth of the *35Spro::SlPEPCK*$^{OX}$ lines was also investigated at 10 DAS (Figure 3). 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, this effect was obviously greater in the *35Spro::SlPEPCK*$^{OX}$ lines than in the wild type (Figure 3A). 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 lines 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 (Figure 3B). All *35Spro::SlPEPCK*$^{OX}$ lines exhibited significantly longer roots compared with the WT (Figure 3B). Seedling height ranged from 15.7 to 17.4 mm, 22.8 to 26.2 mm and 30.9 to 33.7 mm in the 0%, 1.5% and 3% sucrose conditions, respectively (Figure 3C). Although the sucrose supply obviously enhanced seedling height, there were no remarkable differences between the WT and the *35Spro::SlPEPCK*$^{OX}$ lines for any of the sucrose conditions compared to the root (Figure 3C). On the other hand, seedling vigour was considerably better in the *35Spro::SlPEPCK*$^{OX}$ lines compared with the WT in the presence of sucrose, whereas it was similar in the absence of sucrose (Figure 3A).

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

*SlPEPCK* gene expression in ripening fruits at 42 days after flowering (DAF) was investigated in the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines and the WT using qRT-PCR (Figure 4A and B). The endogenous *SlPEPCK* transcriptional levels in the transgenic fruits were 3.2- to 9.9-fold higher in both the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines compared with the wild type (Figure 4A and B). PEPCK enzymatic activity in the red-ripe fruit was also significantly increased in both the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines compared with 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*$^{OX}$

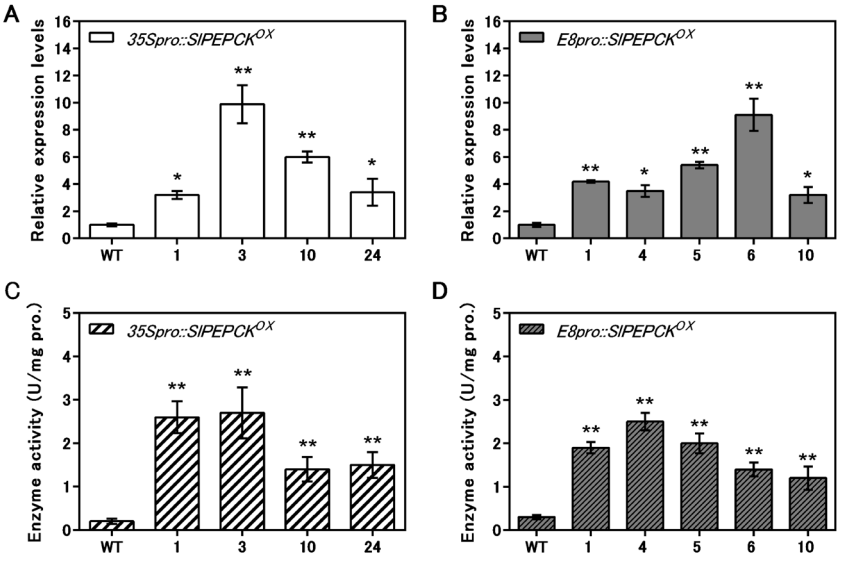


Figure 4. Relative expression levels of the *SlPEPCK* gene and PEPCK enzyme activity in the fruit of the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines. (A) and (B), relative expression levels of the *SlPEPCK* gene, (C) and (D), PEPCK enzyme activity at 42DAF fruits. Values are means ±SE ($n$=3). The genotype of the transgenic lines is represented in each graph. The labels below the horizontal axis indicate the no. of the transgenic line. Pro, protein. 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 (* $p<0.05$, ** $p<0.01$).

(Figure 4C and D). Fruit number and weight and plant biomass were also evaluated in the *SlPEPCK*$^{OX}$ lines at the same fruit stage (42 DAF) (Figure S3). Although fruit number and plant biomass tended to increase slightly in the transgenic lines, it was not significant except for line no. 24 in *35Spro*::*SlPEPCK*$^{OX}$ and line no. 4 in *E8pro*::*SlPEPCK*$^{OX}$ (Figure S3A–D). No common tendency was observed among the transgenic lines with respect to fruit weight (Figure S3E and F).

### *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, were measured at 42 DAF (Figure 5). 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,

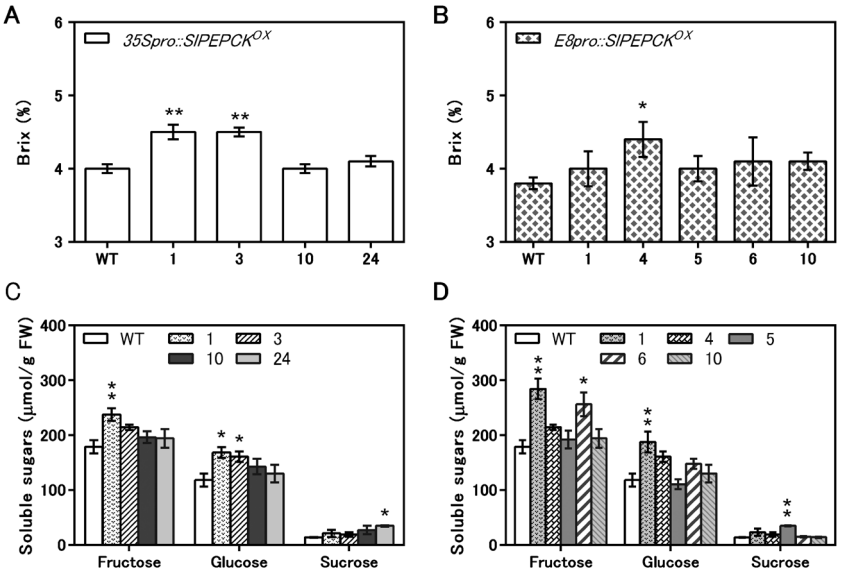


Figure 5. Fruit brix (%) and soluble sugar contents in the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines. (A) and (B) brix (%), (C) and (D) soluble sugar contents in the fruit at 42 DAF. The genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate the no. of the transgenic line. FW, fresh weight. 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).

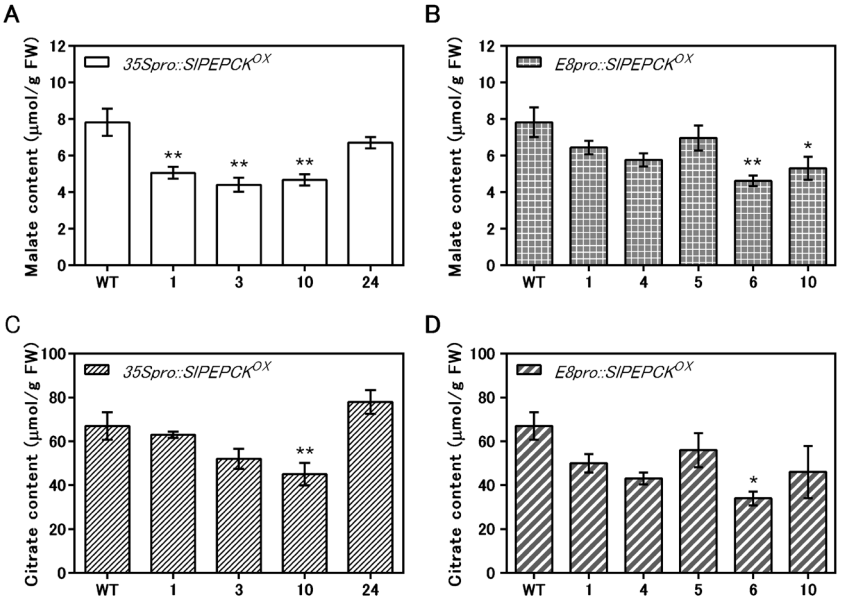


Figure 6. Malate and citrate contents measured in the fruit of the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines at 42 DAF. (A) and (B) Malate contents, (C) and (D) citrate contents in the fruit at 42 DAF. The genotype of the transgenic lines is shown in each graph. The labels below the horizontal axis indicate the 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).

fruit brix (%) ranged from 4.0 to 4.5, and a remarkable increase was observed in lines 1 and 3 (Figure 5A). In *E8pro::SlPEPCK*$^{OX}$ fruits, the brix (%) value ranged from 4.0 to 4.4 (Figure 5B). Although the value measured in line no. 4 increased 1.14-fold, the other lines had levels that were similar to the WT. However, in the *35Spro::SlPEPCK*$^{OX}$ lines, the fructose, glucose and sucrose contents measured 42 DAF increased by 33%, 42% and 52% in line no. 1; 20%, 36% and 37% in line no. 3; 10%, 21% and 98% in line no. 10; and 9%, 10% and 50% in line no. 24, respectively (Figure 5C). The total soluble sugar content was 37%, 27%, 18% and 16% higher in lines 1, 3, 10 and 24, respectively, compared with the wild type. The content of all three sugars increased in the *E8pro::SlPEPCK*$^{OX}$ lines (Figure 5D). The total soluble sugar content increased by 59%, 27%, 9%, 35% and 9% in lines 1, 4, 5, 6 and 10, respectively, compared with the wild-type fruits.

### *Organic acid contents*

The contents of malate and citrate, i.e., the major organic acids in tomato fruit, were also evaluated in the transgenic lines (Figure 6). The fruit malate contents measured at 42 DAF decreased in all transgenic lines compared with the WT, i.e., by 35%, 44%, 40% and 14% in *35Spro::SlPEPCK*$^{OX}$ lines 1, 3, 10 and 24, and by 16%, 26%, 11%, 41% and 32% in *E8pro::SlPEPCK*$^{OX}$ lines 1, 4, 5, 6 and 10, respectively (Figure 6A and B). The fruit citrate content also decreased in most of the *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines except for *35Spro::SlPEPCK*$^{OX}$ no. 24. The citrate content decreased by 6%, 23% and 33% in *35Spro::SlPEPCK*$^{OX}$ lines 1, 3 and 10, and by 26%, 37%, 17%, 50% and 31% in *E8pro::SlPEPCK*$^{OX}$ lines 1, 4, 5, 6 and 10, respectively (Figure 6C and D). However, significant differences were only observed between the WT and *35Spro::SlPEPCK*$^{OX}$ line no. 10 and *E8pro::SlPEPCK*$^{OX}$ line no. 6 (Figure 6C and D).

## 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. 2010). 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 that PEPCK suppression leads to reduced sugar contents accompanied by an increase in malate in ripening fruit (Huang et al. 2015; Osorio et al. 2013). 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, we generated transgenic tomato lines overexpressing the *SlPEPCK* gene driven by the constitutive *35S* promoter and fruit-specific *E8* promoter, and investigated the effect(s) through 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 (Figures 1 and 4). In the seedlings, both *SlPEPCK* expression and PEPCK activity increased markedly in the all *35Spro::SlPEPCK*$^{OX}$ lines (Figure 1A and C). These lines exhibited earlier seed germination and faster seedling growth compared with the WT (Figures 2 and 3). Those results were consistent with the previous study in which the reduction in PEPCK expression suppressed seedling growth (Huang et al. 2015). The close relationship between the PEPCK expression 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 (Figure 1D) and the seedlings exhibited growth similar to the WT, with and without exogenous sugar supply (data not shown).

The post-seedling growth (10 DAS) of both the WT and the *35Spro::SlPEPCK*$^{OX}$ lines was strongly enhanced by the exogenous sugar supply (Figure 3). Interestingly, the effects of the sucrose treatment were more prominent in the *35Spro::SlPEPCK*$^{OX}$ lines (Figure 3A 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 (Figure 3C), seedling vigour was considerably better in the transgenic lines compared with the WT in the presence of sucrose (Figure 3A). 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 (Penfield et al. 2004; Rylott et al. 2003). 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 (Figure S2), which suggests that a lower endogenous level would lead the remarkable effect of the excessive PEPCK in 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 activation of 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 (Figure 3A). 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. 2010). 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 (Figure 2B). These results indicate that PEPCK functions differently during germination and post-germination growth. A metabolic shift in the seedlings, for example from an autotrophic to a heterotrophic phase, could be involved in this difference.

*SlPEPCK* expression and PEPCK activity in the fruit were significantly elevated in all *35Spro::SlPEPCK*$^{OX}$ and *E8pro::SlPEPCK*$^{OX}$ lines (Figure 4) at 42 DAF when endogenous *SlPEPCK* exhibits the highest expression during fruit development (Huang et al. 2015). Although a vegetative growth 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 (Figure S3C to E). Because plant weight also 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 (Figures 5 and 6). In our previous study on RNAi transgenic tomato, *SlPEPCK* suppression resulted in a reduced sugar and an increased malate level in red-ripe fruits (Huang et al. 2015; Osorio et al. 2013). 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 (Figures 5 and 6). 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 no. 1 and 3 and the *E8pro::SlPEPCK*$^{OX}$ line no. 4 (Figure 5A 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 (Figure 5C 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 (Figure 6). 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 metabolic flow in the flesh of tomato and grape is glycolysis (Carrari et al. 2006; Famiani et al. 2014), 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.

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***Supplementary Materials***

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

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Fig.S1

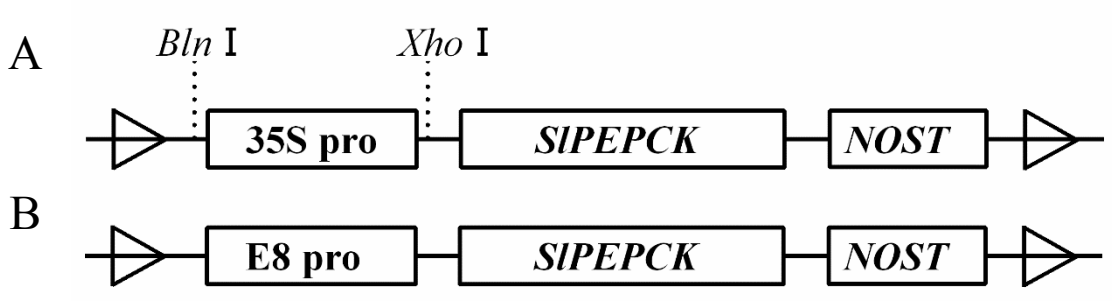


Figure S1. The structures of the transformation vectors overexpressing *SlPEPCK*

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

Fig.S2

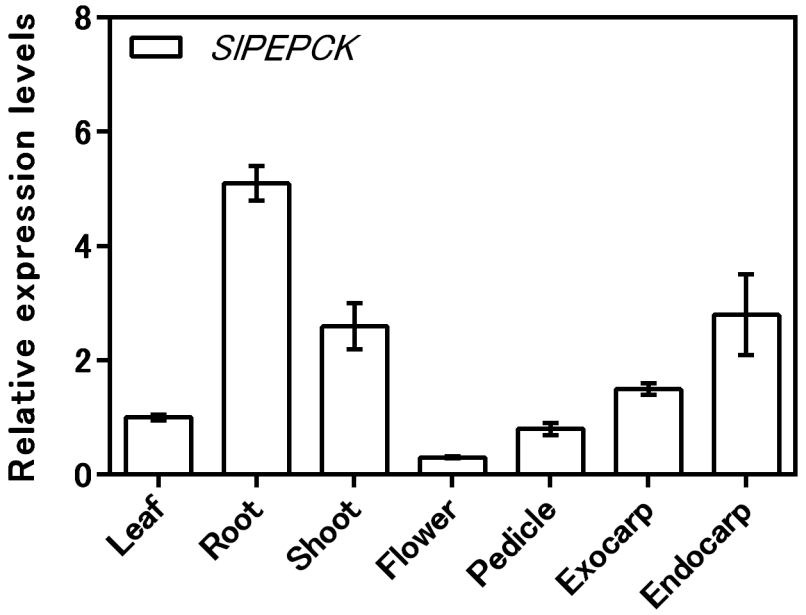


Figure S2. Endogenous expression levels of the *SlPEPCK* gene in tomato plants

The horizontal axis indicates the tested organs, i.e., leaf, root, stem, flower, pedicle, exocarp and endocarp of mature green fruits (27 DAF). The actin gene (*Tom52*, accession number U60482) was used as an internal standard. Values are means ±SE (n=3).

Fig.S3

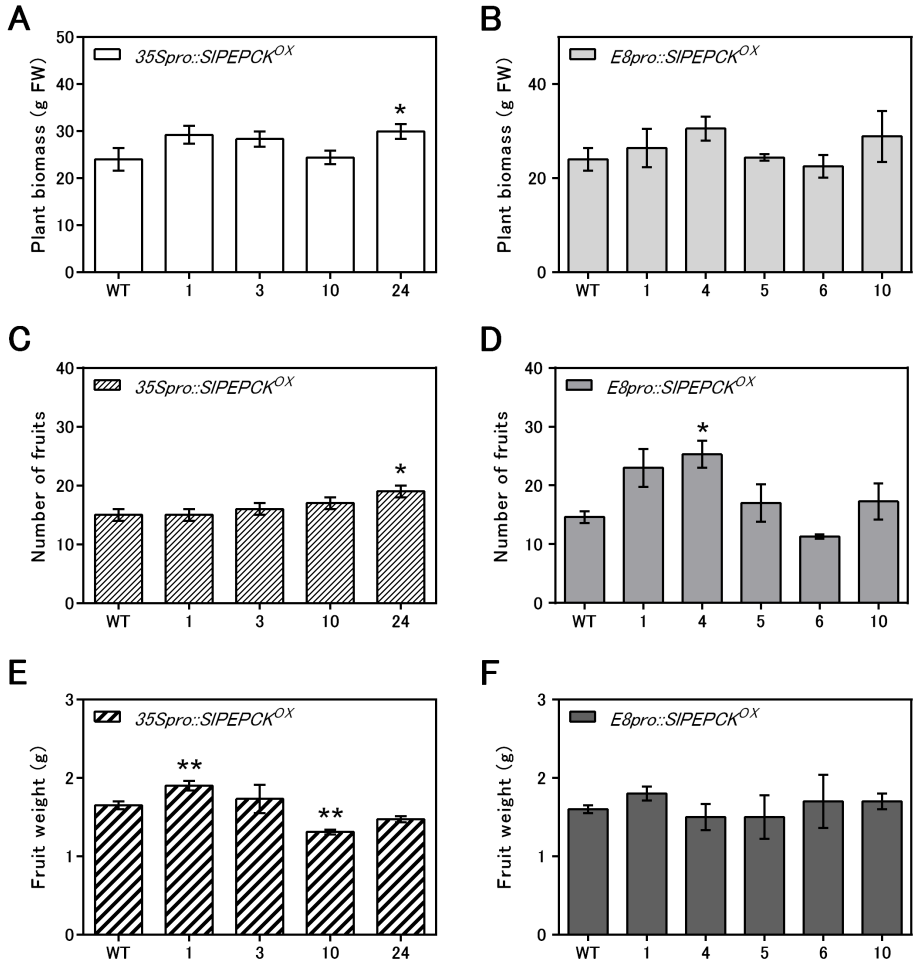


Figure S3. Plant biomass, fruit number and fruit weight of the transgenic lines

(A) and (B), Plant biomass/plant; (C) and (D), fruit number/plant; (E) and (F), fruit weight/fruit. Fruit samples were collected from fruit at 42 DAF. Plant biomass was measured on the same day as the fruit harvest. Values are means ±SE (n=16). The genotype of the transgenic lines is represented in each graph. The labels below the horizontal axis indicate the no. of the transgenic line. The asterisks indicate significant differences between the means of the transgenic lines and the wild type, estimated using the Dunnett (2-sided) test (*$P < 0.05$, **$P < 0.01$).