

RESEARCH PAPER

# Phosphoenolpyruvate carboxykinase in cherry (*Prunus avium* L.) fruit during development

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## Abstract

In this study the abundance and location of phosphoenolpyruvate carboxykinase (PEPCK) was determined in the flesh and skin of the sweet cherry (*Prunus avium* L.) cultivar Durone Nero II during development. PEPCK was not present in young fruit but appeared in both tissues as the fruit increased in size. In these there was no net dissimilation of malic acid, which accounts for the bulk of their organic acid contents when PEPCK was present. To assist in understanding the function of PEPCK, the abundance of a number of other enzymes was determined. These enzymes were aspartate aminotransferase (AspAT), glutamine synthetase (GS), phosphoenolpyruvate carboxylase (PEPC), pyruvate, orthophosphate dikinase (PPDK), and ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). A potential role for PEPCK in the regulation of pH and the utilization of malate in gluconeogenesis in the flesh and skin of cherries is presented.

**Key words:** Cherry, gas exchange, fruit, malate, pH, phosphoenolpyruvate carboxykinase.

## Introduction

Cherries, *Prunus avium* L. (sweet) and *Prunus cerasus* L. (sour), are an important fruit crop. The fruit wall (pericarp) comprises the skin (epicarp), the flesh (mesocarp), and an inner endocarp which becomes lignified to form the stone. The stone encloses a single seed (Marshall, 1954). The growth of whole cherries can usually be described as consisting of three stages (I–III) that are depicted as a double-sigmoidal curve. This arises from a slowing of the growth of the flesh and endocarp during stage II (Marshall, 1954). During each growth stage, distinct changes occur. During stage I, there is a large increase in size of each part of the pericarp; and cell division in this is largely restricted to this period. In the seed, the integuments and nucellus reach their final size. During stage II, the endocarp hardens

and the endosperm and embryo grow. During stage III, there is a large increase in fruit volume brought about by cell expansion in the flesh, and it ripens (Marshall, 1954). In the edible part softening, coloration, and the accumulation of glucose, fructose, and sorbitol occur during ripening (Marshall, 1954; Gao *et al.*, 2003). During ripening there is a large up-regulation of genes encoding expansins and enzymes involved in cell wall modification and these are thought to function in softening (Yoo *et al.*, 2003). Malic acid accounts for the bulk of the organic acid content of cherries, and whether it decreases during ripening depends on the variety (Marshall, 1954; Girard and Kopp, 1998).

In plants, phosphoenolpyruvate carboxykinase (PEPCK) is a cytosolic enzyme that catalyses the ATP-dependent

Abbreviations: AspAT, aspartate aminotransferase; DAB, days after full bloom; GS, glutamine synthetase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PPDK, pyruvate, orthophosphate dikinase; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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decarboxylation of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) (Leegood and Walker, 2003). Phosphoenolpyruvate carboxylase (PEPC) is a cytosolic enzyme that catalyses the carboxylation of PEP to form OAA (Chollet *et al.*, 1996). Thus PEPCK and PEPC both catalyse the interconversion of OAA and PEP in the cytosol but in opposite directions. PEPC is present in the flesh of all fruit examined (Chollet *et al.*, 1996; Famiani *et al.*, 2000, 2005, 2009; Famiani and Walker, 2009). By contrast, PEPCK is present in the flesh of some but not all fruit, and in a number of these its abundance increases at the start of ripening during which there is a decrease in their malate and/or citrate content (Famiani *et al.*, 2005, 2009; Famiani and Walker, 2009). This has led to the suggestion that PEPCK functions in this dissimilation, and that a proportion of these dissimilated organic acids may be used in gluconeogenesis (Leegood and Walker, 2003; Famiani *et al.*, 2005, 2009; Famiani and Walker, 2009). In plants, gluconeogenesis from malate/citrate requires the presence of either PEPCK or PPDCK (pyruvate, orthophosphate dikinase) and PPDCK was not detected in the flesh of these fruit (Leegood and Walker, 2003; Famiani *et al.*, 2005, 2009; Famiani and Walker, 2009). In this study, the function of PEPCK in fruit was investigated further using cherry.

## Materials and methods

### Plant material

Fruit and leaves of cherries (*Prunus avium* L., cv. Durone Nero II) were collected from plants growing in an experimental orchard of the Faculty of Agriculture of the University of Perugia, in Deruta (PG)–central Italy, in 2005. The development stage of the fruit was based on days after full bloom (DAB), and this is when 50% of flowers are open. Only healthy fruit were used and these were taken from several positions on the plant. *Panicum maximum* was grown in a greenhouse in Perugia, Italy.

### Measurement of fresh and dry weights

At each stage of development, the weights of 10 whole fruit and their flesh plus skin were determined. This was done for both freshly harvested material and for the same samples after being dried to constant weight in a forced-air oven at 90 °C.

### Preparation of a nitrogen powder

For measurements of sugar and organic acid contents, PEPCK activity, and electrophoresis, a nitrogen powder was prepared. This was done to ensure that the samples analysed were representative of a number of fruit. The flesh plus skin, was quickly dissected from the fruit and frozen in liquid nitrogen. Then, the flesh plus skin was removed from liquid nitrogen and skin and flesh were separated as soon as their consistency allowed this, but before they melted and then they were refrozen. Samples of each tissue were then ground in a mortar containing liquid nitrogen and the resulting powder was used either immediately or after storage at –80 °C. For each stage of development three samples of 10–20 fruit were used.

### Measurement of soluble sugars and malate

Frozen powder (50 mg) was added to 1.5 ml of 80% ethanol, 20% 100 mM HEPES-KOH (pH 7.1), and 20 mM MgCl<sub>2</sub> in an Eppendorf tube. It was then incubated at 80 °C for 1 h and then centrifuged at 12 000 g for 5 min. One-hundred-fifty µl of charcoal

suspension (100 mg ml<sup>-1</sup>) was added to the supernatant, vortexed, and then centrifuged at 12 000 g for 5 min. The supernatant was stored at –20 °C until required for metabolite determinations. Glucose, fructose, and sucrose were measured in the supernatant using an enzyme-coupled spectrophotometric method (Jones *et al.*, 1977; Antognozzi *et al.*, 1996). The assay mixture contained 100 mM HEPES-KOH (pH 7.0), 5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.02% (w/v) BSA, 1 mM ATP, 0.5 mM NAD<sup>+</sup>, and 3 U of hexokinase. The reaction was initiated by adding 1 U of glucose-6-phosphate dehydrogenase to measure glucose. Fructose and sucrose were then measured in sequence, after the addition of 1 U of phosphoglucose isomerase and 100 U of invertase, respectively. Malate was measured using an enzyme-coupled method (Lowry and Passonneau, 1972). The assay mixture contained 50 mM 2-amino-2-methylpropanol (pH 9.9), 40 mM glutamate, and 1 mM NAD<sup>+</sup>. The reaction was initiated by adding 10 U of glutamate oxalacetate transaminase and 1 U of malate dehydrogenase.

### Enzyme assay

200 mg of frozen powder was ground in a mortar containing 800 µl of ice-cold 200 mM Bicine-KOH (pH 9.0) and 50 mM DTT and then clarified by centrifugation at 12 000 g for 5 min. Enzyme activity in the supernatants was then measured. The activity of PEPCK was measured in the carboxylation direction as described by Walker *et al.* (1999). One unit of activity is that which produces 1 µmol product min<sup>-1</sup> at 25 °C.

### SDS-PAGE and immunoblotting

For flesh 500 mg, skin 250 mg, and leaf 60 mg, of frozen powder was added to 500 µl electrophoresis buffer [62.5 mM TRIS-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 50 mM ascorbate, 5% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue] contained in a mortar, and ground with a pestle. If the extract became yellow, several microlitres of 20% (w/v) NaOH were added until it just became blue. The suspension was immediately poured into an Eppendorf tube, which was then incubated at 100 °C for 5 min and then centrifuged at 12 000 g for 5 min. The supernatant was separated from the pellet and stored at –20 °C until required. After centrifugation at 10 000 g for 5 min, 1–5 µl of extract were loaded onto each track of SDS-PAGE gels. SDS-PAGE and immunoblotting were done as described in Walker and Leegood (1996). Briefly SDS-PAGE was performed in a Hoefer mini-gel apparatus (SE 250 mighty Small II; Hoefer Scientific Instruments, San Francisco, USA) and western transfer done using a Pharmacia Multiphor device (Multiphor II Electrophoresis System; Pharmacia Biotech, Uppsala, Sweden) in conjunction with Millipore Immobilon-P membrane (Millipore, Billerica, Massachusetts, USA). Anti-rabbit peroxidase (diluted 1/1000) was used in conjunction with an ECL kit (GE Healthcare, Little Chalfont, UK) to visualize immunoreactive polypeptides.

### Source and specificity of antibodies

All antisera were polyclonal and raised in rabbits. PEPCK antiserum was raised in a rabbit against the enzyme from *P. maximum* leaves (Walker *et al.*, 2002). PEPC, aspartate aminotransferase (AspAT), and ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) antisera were raised in rabbits against the enzymes from *P. maximum* leaves (RP Walker, unpublished work). PPDCK antiserum was raised against the enzyme from maize and had been affinity purified (Chastain *et al.*, 2002). Glutamine synthase (GS) antiserum was raised against plastidic GS from *Sinapis alba* (Höpfner *et al.*, 1990). To assess the specificity of antisera for enzymes, tissues were used in which the enzymes are abundant and their mass known. For PEPCK, PEPC, PPDCK, and AspAT, which function in C<sub>4</sub> photosynthesis, leaves of the C<sub>4</sub> plant *P. maximum* were used and their abundance was compared with that in cherry (C<sub>3</sub>) leaves. For rubisco and GS, cherry leaves at different stage of development were used.

## Results

### Changes in fresh and dry weights during development

The increase in FW of both whole fruit and flesh plus skin during growth was double sigmoidal. Stage I was from approximately 0–20 DAB, stage II 20–36 DAB, and stage III after 36 DAB (Fig. 1). The rate of increase in the FW of the flesh plus skin slowed during stage II and then increased after 36 DAB (Fig. 1).

### Changes in the content of sugars and malate during development

For measurement of sugars and malate, recovery experiments were done. A known amount of metabolite was co-extracted with either the flesh or skin of cherries harvested at 27 DAB. For both tissues, recovery of metabolites was between 85% and 95% (data not shown). In both flesh and skin glucose and fructose were more abundant than sucrose, and on a whole fruit basis the greatest increase in these occurred during stage III (Fig. 2). On a  $\text{g}^{-1}$  FW basis these increases were not as pronounced, and this was a result of the large increase in the FW of these tissues at this time (Figs 1, 2). The abundance of malate was highest in the flesh, and on a whole fruit basis most of it accumulated during stage III (Fig. 3). In the skin, malate accumulated earlier in development (Fig. 3). There was a decline in malate  $\text{g}^{-1}$  FW in both tissues during stage III, however, this was a result of the large increase in FW of these tissues (Figs 1, 3, 7).

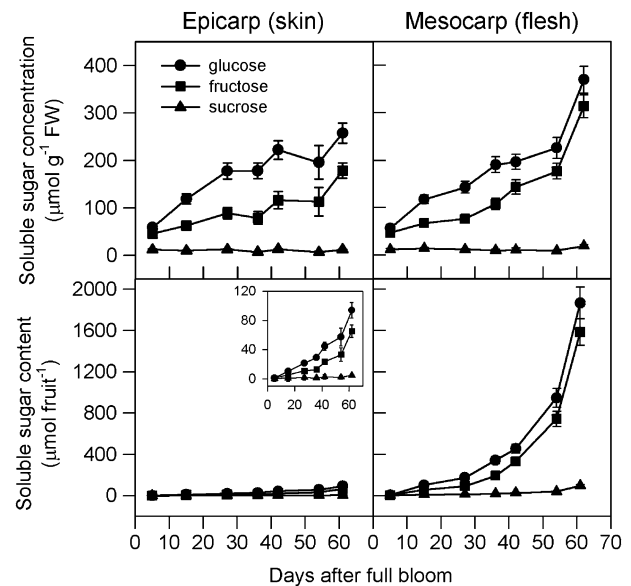
### Assessment of extracts for SDS-PAGE

Homogenization of extracts in the buffer used for denaturing samples for SDS-PAGE gels was satisfactory because the polypeptide pattern on gels was sharp and well

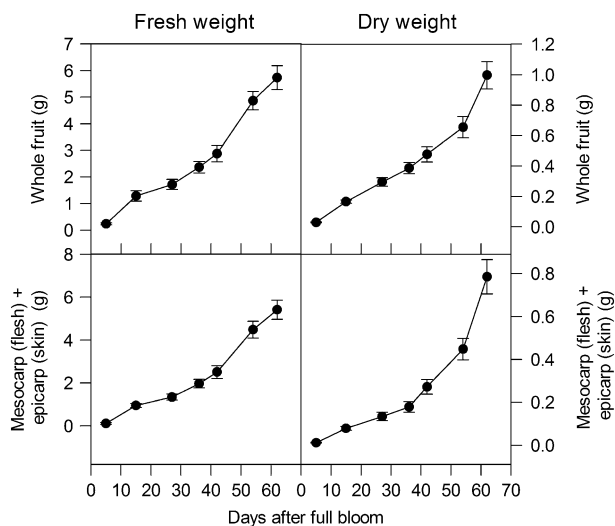
separated (Fig. 5). Further, co-extraction of fruit tissues with *P. maximum* leaves whose polypeptide pattern is known (Walker *et al.*, 2002), did not lead to the loss of polypeptides present in these leaves (data not presented).

### Assessment of antibody specificity

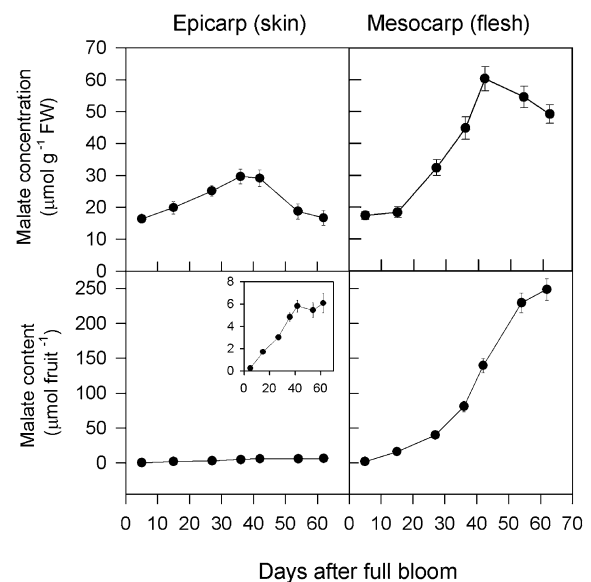
The PEPCK antiserum recognized a 74 kDa polypeptide in extracts of leaves of *P. maximum* but not in those of cherry (Fig. 4). The PEPC antiserum recognized a 110 kDa



**Fig. 2.** Abundance of soluble sugars in the flesh and skin of cherries through development. Sucrose is expressed as  $\mu\text{mol}$  of glucose. Each point on the graph shows the mean and standard error of three separate extractions.



**Fig. 1.** Fresh and dry weights of whole cherries and their flesh and skin through development. Each point on the graph shows the mean and standard error of 10 fruit or their component parts.



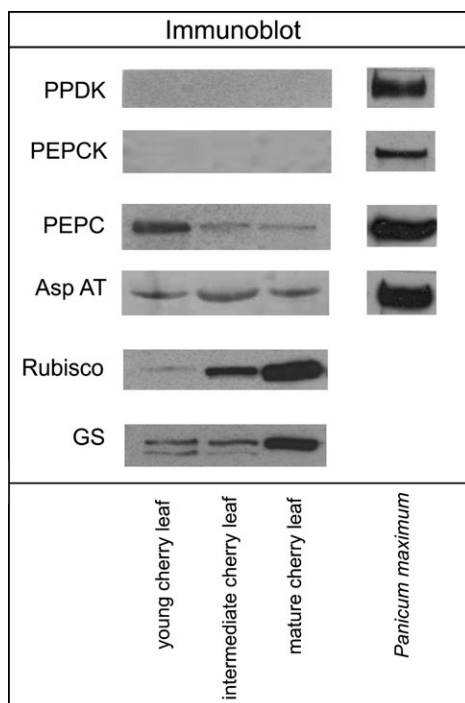
**Fig. 3.** Abundance of malate in the flesh and skin of cherries through development. Each point on the graph shows the mean and standard error of three separate extractions.

polypeptide in extracts of leaves of both *P. maximum* and cherry, however, it was less abundant in cherry (Fig. 4). The PPKK antiserum recognized a 95 kDa polypeptide in extracts of *P. maximum* leaves but not in those of cherry leaves (Fig. 4). The antiserum to AspAT recognized a 42 kDa polypeptide in extracts of all tissues but was much more abundant in leaves of *P. maximum* (Fig. 4). The antiserum against the large subunit of rubisco recognized a 55 kDa polypeptide in cherry leaves at all stages of development (Fig. 4). The antiserum against GS recognized both a 39 kDa and a 43 kDa polypeptide in immature leaves (Fig. 4). In mature leaves, the 39 kDa polypeptide was not detected and the 43 kDa polypeptide had greatly increased in abundance. The 39 kDa polypeptide is GS1 the cytosolic isoform whereas the 43 kDa polypeptide is plastidic GS2. The mass and occurrence of the polypeptides recognized by these antisera are consistent with previous studies using these antibodies (Famiani and Walker, 2009).

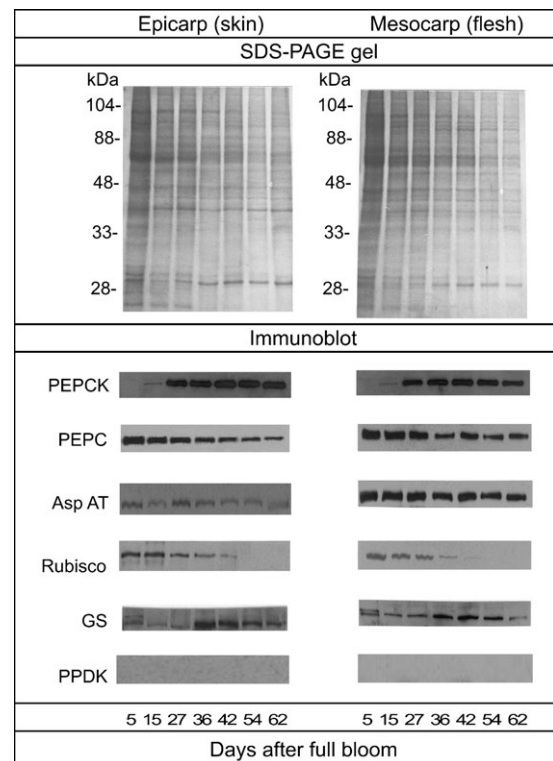
#### Enzyme abundance in the skin and flesh during development

Polypeptides were well resolved after SDS-PAGE analysis of either skin or flesh (Fig. 5). Staining of tracks on gels was greatest for both tissues from fruit 5 DAB, this declined by 27 DAB and then remained similar, apart for a decline in the intensity of staining of tracks loaded with flesh 54–62 DAB. In extracts of either flesh or skin there were few changes in polypeptide composition, the most notable being

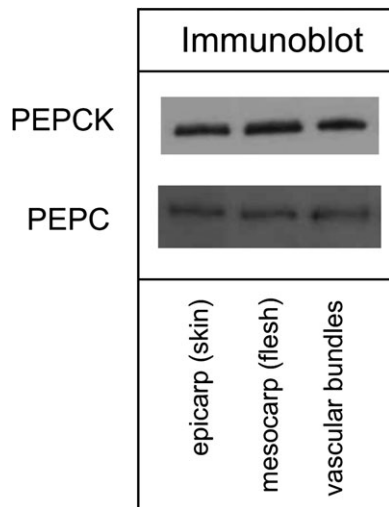
an increase of a polypeptide with a molecular mass slightly higher than 28 kDa and a decrease of a polypeptide with a molecular mass between 48 and 88 kDa and a polypeptide with a molecular mass slightly lower than 104 kDa in both tissues (Fig. 5). Changes in enzyme abundance were investigated using immunoblots of replicas of these gels. PEPCK was not detected in either tissue at 5 DAB, small amounts were present in both by 15 DAB and by 27 DAB it was approaching maximum abundance in both tissues (Fig. 5). After this, PEPCK abundance in skin changed little, whereas it had declined slightly by 54 DAB in flesh (Fig. 5). In ripe cherries, dissection showed that similar amounts of PEPCK were present in the skin, flesh, and vascular bundles (Fig. 6). PEPC was detected in both the skin and the flesh in which it declined during development, however, this decrease was less in flesh (Fig. 5). In ripe cherries, similar amounts of PEPC were present in the skin, flesh, and vascular bundles (Fig. 6). In both skin and flesh, rubisco was most abundant at 5 DAB and then declined until it was not detected after 42 DAB (Fig. 5). The 39 kDa cytosolic GS was detected in both skin and flesh and was present throughout development (Fig. 5). The 43 kDa plastidic GS was only detected in flesh and skin at 5 DAB. AspAT was present in both the flesh and skin throughout development (Fig. 5). PPKK was not detected in either tissue (Fig. 5).



**Fig. 4.** Evaluation of antibody specificity. Extracts corresponding to 1 mg FW tissue were subjected to SDS-PAGE and then transferred to Immobilon-P membrane and enzymes detected using specific antisera.



**Fig. 5.** Polypeptide and enzyme abundance in cherry flesh and skin through development. Extracts corresponding to 4 mg FW tissue were subjected to SDS-PAGE. Polypeptides were then either stained using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes detected using specific antisera.



**Fig. 6.** Abundance of PEPCK and PEPC in different tissues of ripe cherries. Skin and central vascular bundles were separated from the flesh of red fruit at 62 DAB by dissection. Extracts of these were subjected to SDS-PAGE. Then polypeptides were transferred to Immobilon-P membrane and enzymes detected using specific antisera. Equal amounts of total protein were loaded on each track of the gel.

#### *Changes in PEPCK activity in the flesh and the accumulation/dissimilation of malate during development*

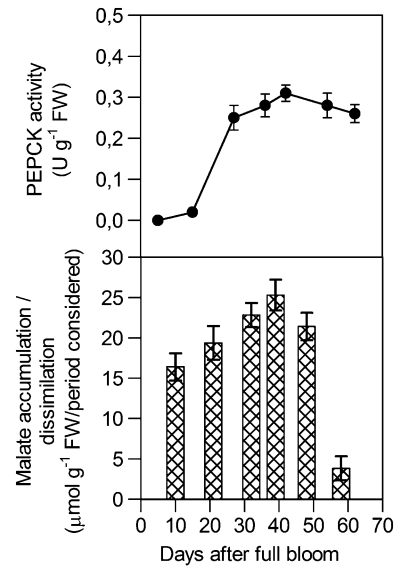
Recovery of enzyme activity from flesh was more than 80% as determined by co-extraction of the tissue and *P. maximum* leaves, a tissue for which the extraction of the enzymes studied has been carefully optimized (Walker *et al.*, 1997). In the flesh, PEPCK activity was not detected at 5 DAB, small amounts were present at 15 DAB, and by 27 DAB its abundance had increased greatly (Fig. 7). The maximum amount,  $0.31 \text{ U g}^{-1} \text{ FW}$ , was present at 42 DAB and, after this, its abundance decreased slightly. The intensity of staining of PEPCK on immunoblots mirrored the abundance of PEPCK as determined by enzyme assay (Figs 5, 7). In ripe fruit, the specific activity of PEPCK was  $0.33 \text{ U mg}^{-1}$  total protein in the flesh and  $0.27 \text{ U mg}^{-1}$  total protein in the skin. In the flesh there was a net synthesis of malate throughout development (Figs 3, 7).

## Discussion

The aim of this study was to investigate whether PEPCK is involved in organic acid dissimilation in cherry flesh and skin. To assist in this, some related aspects of the metabolism of these tissues were investigated.

#### *Changes in fresh and dry weights and metabolite contents*

The increase in FW of both whole fruit and flesh was double sigmoidal (Fig. 1). These changes in FW and DW are consistent with previous studies (Marshall, 1954). In both the flesh and skin glucose and fructose were more



**Fig. 7.** Abundance of PEPCK and the accumulation/dissimilation of malate in cherry flesh during development. For PEPCK activity, each point on the graph shows the mean and standard of measurements made on three separate extractions. When considering the decrease in malate content of the flesh on a  $\text{g}^{-1} \text{ FW}$  basis, it is important to allow for the decrease brought about by dilution arising from growth. To allow for this dilution, the results were adjusted using the equation: (amount  $\text{g}^{-1} \text{ FW}$  at the present time point) - [(weight of flesh at the previous time point/weight of flesh at the present time point)  $\times$  amount  $\text{g}^{-1} \text{ FW}$  at the previous time point]. This difference gives the approximate amount of synthesis of malate on a per fruit basis, if the difference is positive then it means there has been synthesis and if it is negative there has been dissimilation. This assumes that there is no import or export of malate and, in the flesh of fruit in which it has been studied such as grape, it is thought that the bulk of organic acids is synthesized within the fruit from imported sugars, and that these are not then exported (Famiani *et al.*, 2005).

abundant than sucrose (Fig. 2). The greatest increase in the abundance of these, in both the skin and flesh, occurred during stage III. By contrast, peach flesh accumulates sucrose, glucose, and fructose; the lower accumulation of sucrose in cherry may be because its soluble acid invertase activity is at least 50-times higher (Krishnan and Pueppke, 1990; Moriguchi *et al.*, 1990). In cherry flesh, malate/malic acid accounts for most of its organic acid content (Girard and Kopp, 1998). The abundance of malate was highest in the flesh and, on a whole fruit basis, most of it accumulated during stage III (Fig. 3). In the skin, malate accumulated earlier in development (Fig. 3). Where comparable data exist the changes in sugar and malate contents were consistent with previous studies (Marshall, 1954; Girard and Kopp, 1998; Gao *et al.*, 2003). The intensity of staining of total polypeptides on gels, loaded with extracts of either flesh or skin, decreased during development (Fig. 5), and this decrease mirrored that of changes in total protein content measured by protein assay (Krishnan and Pueppke, 1990).

*Amino acid metabolism*

Amino acids can be divided into families on the basis of their biosynthetic pathway, with each family having a different precursor at its start. Glutamate, aspartate, and pyruvate are the precursors for the synthesis of many amino acids (Lea, 1993). The carbon skeletons of glutamate and aspartate are the organic acids 2-oxoglutarate and OAA respectively, hence the metabolism of many organic and amino acids are closely linked. GS catalyses the incorporation of  $\text{NH}_4^+$  into glutamate to form glutamine. There are two forms of GS, a plastidic form that functions in the assimilation of photorespiratory  $\text{NH}_4^+$ , and a cytosolic form that assimilates  $\text{NH}_4^+$  arising in other ways. Glutamate synthase then catalyses the transfer of the amide group of glutamine to 2-oxoglutarate to yield two molecules of glutamate. AspAT converts a proportion of this glutamate to aspartate (Lea, 1993). PEPC catalyses the carboxylation of PEP to form OAA, and is necessary for the synthesis of Krebs cycle acids and the carbon skeletons of many amino acids (Chollet *et al.*, 1996). In cherry, asparagine and glutamine are major forms of transported nitrogen (Millard *et al.*, 2006). However, since fruit is growing, a wide range of amino acids is required for protein synthesis. The presence of the above enzymes throughout the development of cherry flesh and skin is in keeping with a potential function in the conversion of imported amino acids to those required for protein synthesis. Further, the increase in the abundance of cytosolic GS at 36 DAB in both the flesh and skin corresponded with an increase in the rate of growth of these tissues (Figs 1, 5). This may be because there is an increased demand to assimilate  $\text{NH}_4^+$  arising from amino acid metabolism.

*Potential functions of PEPCK in cherry flesh and skin*

There was no net dissimilation of malate, when measured over a period of days, in either the flesh or skin of the sweet cherry cultivar Durone Nero II when PEPCK was present (Figs 3, 7). Therefore, it is unlikely that PEPCK functions to catalyse a net dissimilation of malate in these tissues. This raises the question of its function. Further, it raises the possibility that, in the flesh of fruit in which the appearance of PEPCK coincides with a net dissimilation of malate/citrate, PEPCK functions in more than this. In cherry flesh and skin, as in other fruit studied (Famiani *et al.*, 2005), PEPCK was either not present early in development or at much lower abundance (Fig. 5). In both cherry flesh and skin, the appearance of PEPCK coincided with disappearance of plastidic GS (Fig. 5). In photosynthetic tissues of other fruit and seeds, the absence of plastidic GS is attributed to a high concentration of  $\text{CO}_2$  suppressing photorespiration, and hence the need for plastidic GS to reassimilate  $\text{NH}_4^+$  released by it (King *et al.*, 1998; Famiani and Walker, 2009). This raises the possibility that the internal concentration of  $\text{CO}_2$  in cherries increases at this stage of development.

A feature of many fruit is that their internal concentration of  $\text{CO}_2$  is high (Burton, 1982), and in both peach and

tomato flesh PEPC rapidly fixes radiolabelled  $\text{CO}_2$  to form malate (Farineau and Laval-Martin, 1977; Moing *et al.*, 1999). Sycamore cell suspension cultures fed with 5 mM  $\text{CO}_2/\text{HCO}_3^-$  rapidly synthesize malate utilizing PEPC over a range of cytoplasmic pH values. It appears that under these particular conditions a high cytosolic  $\text{HCO}_3^-$  concentration is the dominant factor in determining flux through PEPC (Gout *et al.*, 1993). In some tissues, PEPC, malate dehydrogenase, malic enzyme (ME) operate to bypass cytosolic pyruvate kinase (Plaxton, 1996). In tomato flesh, malate is used by the Krebs cycle, both directly and after its conversion to pyruvate by NADP-ME, and this provides evidence for the operation of this bypass (Farineau and Laval-Martin, 1977), which might also operate in peach flesh (Borsani *et al.*, 2009). Potentially, this bypass can regulate the tissues' malate content, however, it also converts excess  $\text{H}^+/\text{HCO}_3^-$  resulting from the reaction  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$  back to  $\text{CO}_2$ . High concentrations of  $\text{CO}_2$  lower cytosolic pH because of this reaction (Gout *et al.*, 1993), and the bypass may, therefore, be important in pH regulation. When malate synthesized in response to a high  $\text{HCO}_3^-$  concentration exceeds a certain concentration in the cytoplasm it accumulates in the vacuole (Gout *et al.*, 1993). The increase in cytoplasmic malate may be determined, in part, by the tissues ability to utilize it in metabolism. In tomato fruit, this malate is metabolized by NADP-malic enzyme, stored in the vacuole or utilized in gluconeogenesis (Farineau and Laval-Martin, 1977). Similarly, gluconeogenesis from malate occurs in both cherry and peach flesh (Leegood and Walker, 1999; Moing *et al.*, 1999). If  $\text{C}_3$  plants are placed in an atmosphere containing 5%  $\text{CO}_2$  PEPCK appears in their leaves (Chen *et al.*, 2004). In cherry flesh and skin, as in the other tissues discussed above, the presence of PEPCK allows malate to be used in gluconeogenesis and other pathways requiring PEP.

The presence of PEPCK is induced by asparagine or  $\text{NH}_4^+$  but not glutamine or aspartate in developing seeds of both grape and pea (Walker *et al.*, 1999; Delgado-Alvarado *et al.*, 2007). In developing soybean seeds, asparaginase converts asparagine to  $\text{NH}_4^+$  and aspartate, and this contrasts with glutamine which is metabolized by glutamate synthase and does not produce  $\text{NH}_4^+$  (Skokut *et al.*, 1982). PEPCK is induced in the vasculature of roots and stems fed with  $\text{NH}_4^+$  at low pH, which lowers intracellular pH, but not in those fed with  $\text{NH}_4^+$  at high pH or with  $\text{NO}_3^-$ , which increase intracellular pH. Other treatments that lower cytosolic pH, such as feeding butyric acid, also result in the appearance of PEPCK. This suggests that this induction of PEPCK by  $\text{NH}_4^+$  is related to a lowering of pH. In cherry, asparagine is a major form of transported nitrogen and it is abundant in the flesh (Drawert *et al.*, 1970; Millard *et al.*, 2006). In cherry fruit and seeds, as in developing leaves (Sieciechowicz *et al.*, 1988), asparagine catabolism is likely to be co-ordinated with their ability to utilize it. The  $\text{NH}_4^+$  and aspartate produced by asparaginase may not be utilized at the same time, or even in the same tissue. For instance, in some developing seeds, a proportion of  $\text{NH}_4^+/\text{NH}_3$  produced

by asparaginase exits the seed coat and outer tissues of the cotyledons and accumulates in the apoplast before being utilized later (Skokut *et al.*, 1982; Delgado-Alvarado *et al.*, 2007). Similarly, large amounts of  $\text{NH}_4^+$  can accumulate in the flesh of fruit and this is likely to be located largely in the apoplast and vacuole (Roubelakis-Angelakis and Kliever, 1992; Loqué *et al.*, 2005). In both the fruit and seed it is likely that  $\text{NH}_3$  is the form that leaves the cytoplasm and a proton therefore remains (Schjoerring *et al.*, 2002; Loqué *et al.*, 2005). AspAT is present in cherry flesh (Fig. 5) and the utilization of aspartate or 2-oxoglutarate, arising from asparaginase and glutamate dehydrogenase, by AspAT will generate OAA. Radiolabelling studies in peach flesh (Moing *et al.*, 1999), suggest that OAA rapidly equilibrates with both aspartate and the cytoplasmic malate pool because of the action of malate dehydrogenase and AspAT. Further, protons and malate may also arise as a result of the high concentration of  $\text{CO}_2$  in the tissue (see above). To regulate the malate content of the tissue and the pH of the vacuole and cytosol in this complex situation may require a combination of proton pumps at the plasma membrane/tonoplast and the biochemical pH stat.

A widespread mechanism utilized in pH regulation in plants is the biochemical pH stat. In this, malic acid is synthesized in the cytosol utilizing PEPC when cytosolic pH is high, malate is then transported into the vacuole leaving the protons produced by its synthesis in the cytosol. Malate is metabolized utilizing malic enzyme when cytosolic pH is low and this consumes protons. It has been suggested that this mechanism is utilized in the pH regulation in fruit (Law and Plaxton, 1995; Sweetman *et al.*, 2009). In addition to malic enzyme, PEPCK might also act as a decarboxylase in this mechanism under certain conditions (Walker and Chen, 2002). In *Arabidopsis* cell suspension cultures, Chen *et al.* (2008) provided evidence of the co-ordinate regulation of PEPCK and PEPC in response to pH changes. Further, in these there is a significant reverse flux through PEPC that can be attributed to PEPCK and/or malic enzyme (Williams *et al.*, 2008). In cherry flesh, the abundance of NADP-malic enzyme increases as the fruit becomes larger (Hartmann, 1975). The utilization of both PEPCK and malic enzyme as decarboxylases could be advantageous in certain situations. This is because malic enzyme produces pyruvate whereas PEPCK produces PEP. Gluconeogenesis from pyruvate requires PPDK. Gluconeogenesis from malate occurs in cherry flesh (Leegood and Walker, 1999), and this would appear to require PEPCK because PPDK was either not present or at low abundance (Fig. 5).

Gluconeogenesis, or potentially other processes that utilize PEP, may be important for two reasons. The first arises when the requirement of the tissue to utilize malate exceeds its need to use it in other ways. In support of this the fate of malate depends on temperature in ripening grapes. At lower temperatures, there is a decreased demand for pyruvate, and an increased proportion of malate is used in gluconeogenesis (Ruffner, 1982). Similarly, in developing oilseed rape seeds gluconeogenesis from lipid occurs in seeds with elevated malate synthase and isocitrate lyase activities

but not in those with lower activities. An increase in the activity of these glyoxylate cycle enzymes may result in more OAA being produced from lipid breakdown (Chia *et al.*, 2005). A second reason why gluconeogenesis might be important is that it largely avoids the production of NADH and the consequent  $\text{O}_2$  utilization that occurs if malate is utilized by the Krebs cycle (Delgado-Alvarado *et al.*, 2007). In hypoxic tissues, the production of pyruvate is carefully controlled in order to regulate  $\text{O}_2$  concentration (Zabalza *et al.*, 2009). The assimilation of  $\text{NH}_4^+$  consumes large amounts of  $\text{O}_2$  (Matsumoto and Tamura, 1981), and the induction of PEPCK in some hypoxic tissues fed with  $\text{NH}_4^+$  at low pH may serve to counteract low intracellular pH without driving the tissue into anoxia. By contrast, PEPCK is not induced during the senescence of leaves in which  $\text{O}_2$  concentrations are unlikely to be low. In these, a substantial amount of  $\text{NH}_4^+$  is produced by amino acid catabolism, and a proportion of this is lost to the atmosphere as  $\text{NH}_3$  leaving a proton in the tissue. It appears that protons are consumed by the oxidation of amino acid skeletons rather than by gluconeogenesis in this situation (Chen *et al.*, 2000). In tissues under anoxia, malic enzyme has been shown to act as a decarboxylase in the malate pH stat (Edwards *et al.*, 1998). PEPCK is not induced by anaerobic conditions in roots (Chen *et al.*, 2004). However, under anoxia, an important use of pyruvate derived from glycolysis is in fermentation reactions, and glycolysis is an important source of ATP when oxidative phosphorylation is inhibited under anoxia.

In summary, in this study, evidence is provided that PEPCK does not simply function in the net dissimilation of malate in cherry flesh and skin. A potential role for PEPCK in the regulation of pH and the utilization of malate in gluconeogenesis in cherry flesh and skin has been outlined. Further, it is suggested that PEPCK may also function in these processes in fruit in which there is a net dissimilation of malate/citrate during ripening, and other plant tissues in which gas exchange is restricted such as developing seeds, vascular tissues, and roots (Leegood and Walker, 2003).

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