Plant Molecular Biology **47:** 499–506, 2001.
© 2001 Kluwer Academic Publishers. Printed in the Netherlands.

Ripening-related occurrence of phospho*enol*pyruvate carboxykinase in tomato fruit

Ahmad R. Bahrami[+], Zhu-Hui Chen[+], Robert P. Walker, Richard C. Leegood* and Julie E. Gray

*Robert Hill Institute, University of Sheffield, Sheffield, S10 2TN, UK (*author for correspondence; e-mail r.leegood@shef.ac.uk); [+]joint first authors*

Received 28 December 2000; accepted in revised form 5 June 2001

Key words: fruit ripening, phospho*enol*pyruvate carboxykinase, tomato (*Lycopersicon*)

Abstract

Phospho*enol*pyruvate carboxykinase (PEPCK) is present in ripening tomato fruits. A cDNA encoding PEPCK was identified from a PCR-based screen of a cDNA library from ripe tomato fruit. The sequence of the tomato PEPCK cDNA and a cloned portion of the genomic DNA shows that the complete cDNA sequence contains an open reading frame encoding a peptide of 662 amino acid residues in length and predicts a polypeptide with a molecular mass of 73.5 kDa, which corresponds to that detected by western blotting. Only one PEPCK gene was identified in the tomato genome. PEPCK is shown to be present in the pericarp of ripening tomato fruits by activity measurements, western blotting and mRNA analysis. PEPCK abundance and activity both increased during fruit ripening, from an undetectable amount in immature green fruit to a high amount in ripening fruit. PEPCK mRNA, protein and activity were also detected in germinating seeds and, in lower amounts, in roots and stems of tomato. The possible role of PEPCK in the pericarp of tomato fruit during ripening is discussed.

Introduction

The development of fruits such as tomato is accompanied by large changes in the content of sugars and organic acids. Organic acids, but little non-structural carbohydrate, accumulate before ripening, making the fruit unpalatable. In tomato fruit, the major organic acids are malate and citrate (Hobson and Davies, 1971). During ripening, several processes occur: the fruit softens, it begins to accumulate soluble carbohydrate, the organic acid content declines, and respiration increases dramatically in climacteric fruit, such as tomato (Seymour *et al.*, 1993). In many varieties of tomato, the major forms of carbohydrate that accumulate are glucose and fructose, which are mainly from imported sucrose. The dissimilation of organic acids such as malate may be mediated by either NADP-malic enzyme or NAD-malic enzyme, followed by metabolism in the Krebs cycle (Ruffner *et al.*, 1984). Alternatively, it has been proposed that phospho*enol*pyruvate carboxykinase (PEPCK) may be involved in the metabolism of malate, followed by gluconeogenesis from the phospho*enol*pyruvate that is formed (Ruffner and Kliewer, 1975; Ruffner, 1982b; Knee and Finger, 1992). The synthesis of organic acids is also likely to decline as a result of a decrease in phospho*enol*pyruvate carboxylase activity (Hawker 1969) and an inhibition of glycolysis (Ruffner and Hawker, 1977). However, the relative importance of each of these pathways in the metabolism of organic acids in fruits is poorly understood. PEPCK is present in a wide range of fruits, including grape berries (Ruffner and Kliewer 1975), apples, kiwi fruit and aubergine (Blanke *et al.*, 1988) and blueberry, orange, pineapple, apricot, peach and cherry (Leegood and Walker, 1999; data not shown), but its function and relationship to fruit ripening remain unclear.

The nucleotide sequence data reported will appear in the EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AY007226 and AF327432.

In this study we have identified a tomato cDNA encoding a polypeptide with extensive homology to other plant PEPCKs. We have investigated the occurrence of PEPCK mRNA and protein, and its activity, in the pericarp of ripening tomato fruits.

Materials and methods

Plant material

Tomato seeds (*Lycopersicon esculentum* cv. Ailsa Craig and cv. Gold Star) were obtained from Steve Howe Seeds, Boston, UK. Plants were maintained in a growth room at 25 °C with a 16 h day light and 8 h night cycle. Tissues were sampled by cutting representative segments from the plant and were frozen in liquid N_2, and stored at −70 °C for RNA and protein extraction.

Measurement of PEPCK activity

About 10 g of the fruit pericarp was frozen and ground in a mortar containing liquid N_2 and the powder stored at −70 °C. At least three fruit from different plants were sampled for each stage of ripening. 0.5 g of frozen powder was mixed with 800 μl of ice-cold 0.5 M Bicine-KOH pH 9.0, 1 mM EDTA, 1% w/v PEG-4000, 50 mM DTT. The extract was centrifuged at 12 000 × *g* for 15 min and 500 μl of the supernatant mixed with 850 μl of ice-cold 0.5 M Bicine KOH pH 9.0, 1 mM EDTA, 55% w/v PEG-4000, 5 mM DTT, to give a final concentration of 35% PEG. The mixture was incubated on ice for 10 min and then centrifuged for 25 min at 12 000 × *g*. The pellet was suspended in 100 μl of 10 mM Bicine-KOH pH 9.0, 5 mM DTT and stored at −70 °C. The homogenate was clarified by centrifugation at 12 000 × *g* for 5 min and the supernatant stored at −70 °C. The carboxylation reaction of PEPCK was measured as described by Walker *et al.* (1995). (One unit of PEPCK activity corresponds to the production of 1 μmol product per minute at 25 °C).

Western blotting

For SDS-PAGE, fruit pericarp was homogenised as described above. A 300 μl portion of clarified homogenate was added to 1.2 ml of 80% v/v acetone and placed in liquid N_2 for 10 min. After thawing, the mixture was centrifuged at 12 000 × *g* for 10 min and the pellet resuspended in 100 μl 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue (solubilisation buffer). Samples were incubated at 100 °C for 3 min and then stored at −20 °C until required. Before electrophoresis insoluble material was removed by centrifugation at 12 000 × *g* for 3 min. Protein was measured as described by Walker *et al.* (1995). SDS-PAGE and immunoblotting were performed as described by Walker and Leegood (1996). The antiserum specific for PEPCK was raised against the enzyme purified from cotyledons of germinating cucumber seedlings (Walker *et al.*, 1995).

Identification of a tomato PEPCK cDNA

Tomato genomic DNA was used as template in degenerate PCR with forward, CAA(G)TAC(TGCA(C,T)GGA(C,G,T)GAA(G)ATG and reverse, ATG(A)AAG(A)TGG(A)TACATA(C,G,T)GTT(C)TG primers designed to match regions highly conserved between the cucumber, *Arabidopsis*, and *Urochloa* PEPCK genes. A band of 805 bp was amplified and sequenced to verify that it was a fragment of a putative PEPCK gene. To obtain the full-length cDNA, a tomato fruit cDNA library, kindly provided by Dr S. Chengappa, Unilever, Bedford, UK, was screened by PCR with an internal reverse primer (CAGCAGTGTTCATCATC) and a T3 vector primer, and an internal forward (TAATGCACTATCTAATGCC) primer with a T7 vector primer to amplify the 5′ and 3′ ends of the cDNA respectively. These fragments were sequenced and primers designed to amplify the full-length cDNA. In addition, ca. 60 000 cDNA clones were screened with a random-primed DNA probe labelled from the PCR product.

Six PEPCK-like cDNAs were identified by this screen, all of which had identical nucleotide sequences to the cDNA sequence identified by PCR, over ca. 300 nucleotides examined at the 5′ end. The complete DNA sequence was obtained and compared to EMBL and GenBank databases with BLASTx and BLASTn algorithms (Altschul *et al.*, 1997) and sequence alignment performed using the Multialin program (Human Genome Sequencing Center, Houston, TX).

Genomic DNA analysis

Tomato genomic DNA, incubated with appropriate restriction enzymes and used for Southern blot analysis as described (Bahrami and Gray 1999) with an antisense RNA digoxygenin-labelled tomato PEPCK probe.

A fragment of the 3′ end of the tomato PEPCK genomic DNA was amplified with internal primers designed from the cDNA sequence DNA sequencing of this genomic fragment showed the presence of several introns, which were used to design primers that would allow identification of false PCR products from genomic DNA contamination in RT-PCR experiments.

Analysis of mRNA abundance

Both northern blotting and RT-PCR were used to estimate the levels of PEPCK mRNA in tomato. Total RNA was extracted from tomato roots, germinating seeds, stems, leaves, and pericarp at different ripening stages using an acid guanidinum thiocyanate phenol-chloroform extraction method (Chomczynski *et al.*, 1987) and precipitated with 4 M lithium chloride and resuspended in sterile H_2O.

Northern blotting and RT-PCR

Total RNA (10 μg) was electrophoresed on a 1.2% denaturing agarose gel transferred onto Hybond-N membrane as described by Bahrami and Gray (1999). A probe was generated from the full-length cDNA by random priming using a New England Biolabs kit and dCT[^{32}P]. Filters were prehybridised and hybridised at 55 °C, as described by Bahrami and Gray (1999), and washed to a final stringency of 0.1× SSC, 0.1% SDS at 55 °C before exposure to X-ray film.

RT-PCR was performed on total RNA by means of reverse transcriptase (Promega) and forward TCG-TAACAGAGAACACC and reverse GGACCAGCT-GCCAGGATCTC primers, designed to flank introns on the tomato genomic DNA. At the same time a PCR was performed on the genomic DNA and total RNA, without the reverse transcription reaction, as size marker and negative control respectively. To confirm that equal amounts of RNA were present in reactions separate RT-PCR reactions were carried out on the same RNA samples using primers complementary to the tomato ubiquitin gene sequence.

Source of antibodies

The antiserum specific for NAD-malic enzyme was raised against the enzyme purified from *Eleusine coracana* leaves (Murata *et al.*, 1989). The antiserum specific for NADP-malic enzyme was raised against the enzyme purified from maize (Langdale *et al.*, 1988). The antiserum specific for PEPCK was raised against the enzyme purified from cotyledons of germinating cucumber seedlings (Walker *et al.*, 1995).

Results

The tomato PEPCK cDNA and peptide sequence

A putative PEPCK-encoding cDNA was identified from a PCR-based screen of a ripe tomato cDNA library. The complete sequence of this cDNA was determined. The sequence of the PEPCK cDNA and a cloned portion of the genomic DNA shows that the complete cDNA sequence contains an 1988 bp open reading frame and 5′ and 3′-untranslated regions of 93 and 73 bp including a 19 bp poly(A) tail (data not shown). Comparison of the cDNA sequence with that of the partial genomic clone revealed the presence of at least 4 introns in this gene. The derived amino acid sequence of the tomato PEPCK was 662 amino acid residues in length (Figure 1) and predicts a polypeptide with a molecular mass of 73.5 kDa, which corresponds to that detected by western blotting (Figure 2B).

PEPCK is encoded by a single gene in tomato

Screening of the tomato fruit cDNA library by both PCR and homology-based approaches identified only one PEPCK cDNA sequence. In addition, PCR with tomato genomic DNA as template and degenerate primers amplified only a fragment of the identical sequence to this cDNA (data not shown). Genomic Southern blotting analysis with the PEPCK cDNA revealed a single homologous tomato genomic DNA band after separate digestion with three different restriction enzymes (none of which had restriction sites within the probe) (data not shown).

PEPCK is present in tomato fruit and its abundance increases during ripening

Changes in the activity and amount of PEPCK at different stages of fruit ripening were studied. PEPCK activity was not detectable in either immature or mature green fruits pericarp (Figure 2A). Activity was first detected at the onset of fruit colour change at the breaker stage and reached a maximum in orange ripening fruit. Activity then declined in red fruit as they over-ripened. The variety Gold Star had appreciably higher activities of PEPCK (about 4-fold) at ripening than did fruits of cv. Ailsa Craig. We showed

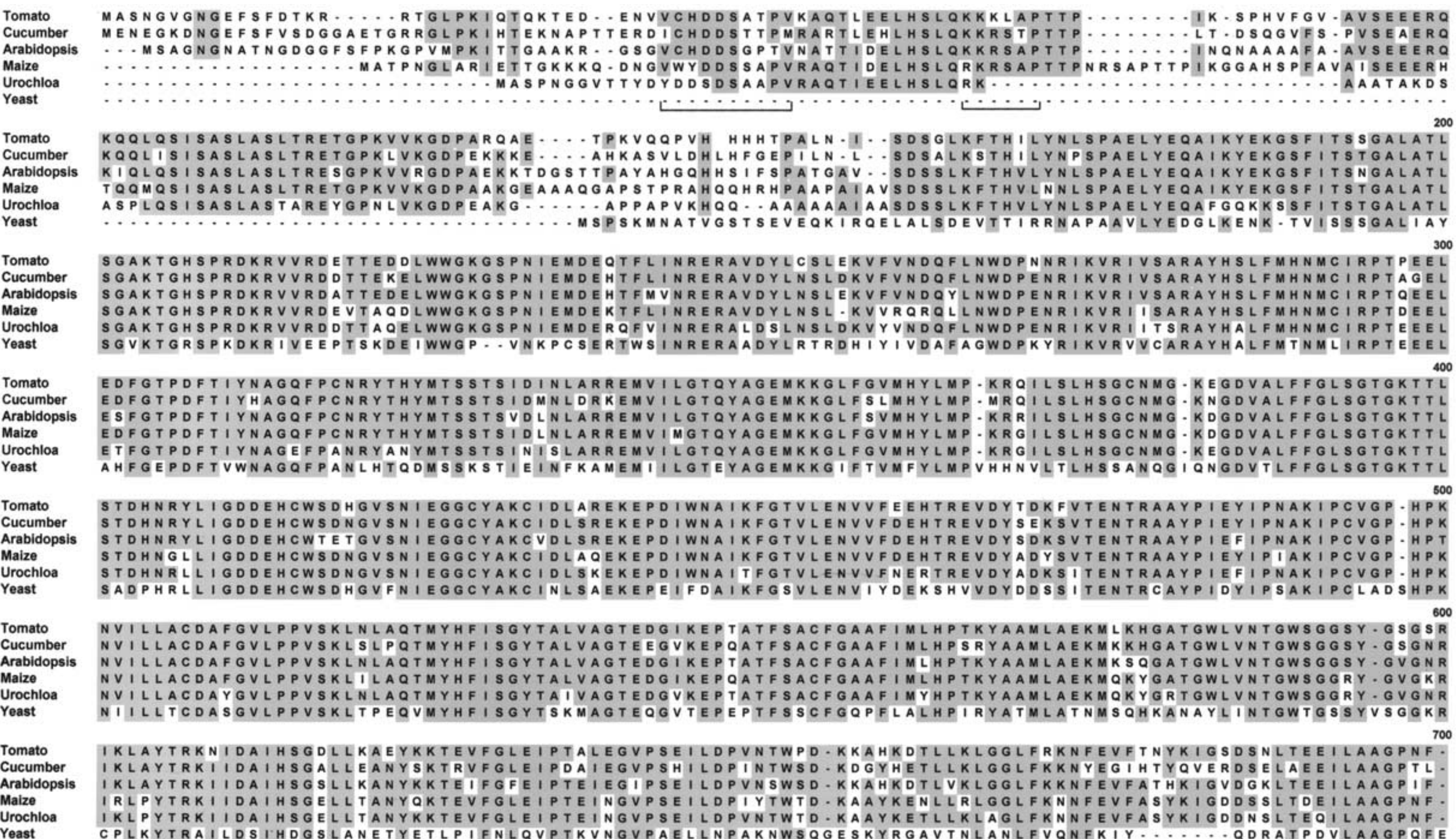

Figure 1. Alignment of derived peptide sequences of plant and yeast PEPCKs. Shaded amino acid residues indicate positions where four out of five sequences show similarity. Potential phosphorylation sites are underlined.

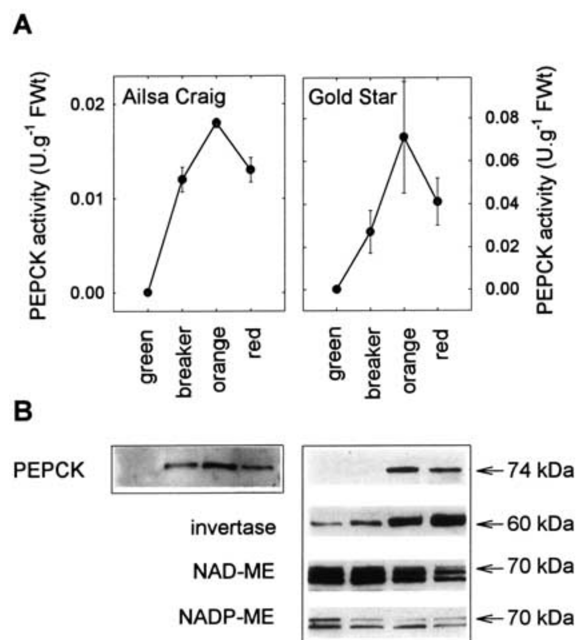

Figure 2. The abundance of PEPCK and other enzymes changes during tomato fruit ripening. A. Fruits of Ailsa Craig and Gold Star were sampled at different stages of ripening (orange = ripe, red = over-ripe) and the activity of PEPCK determined. Values represent means ± SE of three different extractions. B. Extracts containing 20 μg of protein were subjected to SDS-PAGE, and after transfer of the fractionated polypeptides onto Immobilon-P membrane, enzyme protein was detected using antiserum raised to cucumber PEPCK. For both NAD-ME and NADP-ME two bands were observed with molecular masses of 70 and 68 kDa.

that our measurements of PEPCK activity in fruit and seeds were reliable by experiments in which fruits were co-extracted with a small amount of germinated cucumber cotyledon, a tissue for which we have carefully optimised the extraction and assay of PEPCK. Recoveries were greater than 90%. While the activity of PEPCK expressed on a fresh weight basis in fruit was substantially less than in leaves of a C_4 plant or a germinating cucumber cotyledon, it was similar to the amount in cucumber leaves, which contains PEPCK in both the trichomes and phloem (Leegood and Walker, 1999).

A western blot (Figure 2B) confirmed that PEPCK protein (74 kDa) increased during tomato fruit ripening in both varieties, Ailsa Craig and Gold Star. The intensity of the signal on immunoblots corresponded to the activity of PEPCK. We also determined how the abundance of soluble acid invertase, NAD-malic enzyme and NADP-malic enzyme changed during the development of fruits of Gold Star by using immunoblotting (Figure 2B). The amount of soluble acid

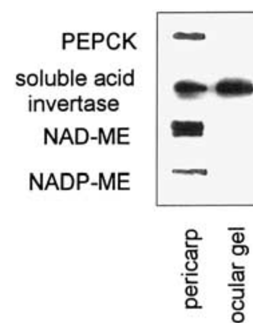

Figure 3. Several enzymes have an unequal distribution between the pericarp and locular gel of ripe tomatoes. Extracts of either pericarp or locular gel (from tomato cv. Gold Star, from which seeds had been removed) each containing 20 μg of protein were subjected to SDS-PAGE. After transfer of the fractionated polypeptides onto Immobilon-P membrane, enzyme protein was detected using specific antisera.

invertase increased, whereas in all the fruit the abundance of the malic enzymes remained constant or declined during ripening. NAD-ME is seen as a doublet on immunoblots because it is composed of two subunits of different size and the antibody used was raised against both subunits (Murata *et al.*, 1989). The cytosolic and plastidic forms of NADP-ME were also detected.

The location of PEPCK was investigated in ripening tomatoes. Figure 3 shows a western blot that compares the abundance of PEPCK, soluble acid invertase and the malic enzymes in the pericarp and locular gel. PEPCK, NADP-malic enzyme and NAD-malic enzyme were only present in the pericarp, whereas soluble acid invertase was present in both compartments.

PEPCK mRNA increases in ripening tomato fruit

The tomato PEPCK cDNA probe hybridised to a single mRNA species of 2.2 kb which corresponds closely to the size of the cloned cDNA. Northern blotting (Figure 4A) and PCR products extended from oligo-dT reverse transcription of total RNA (RT-PCR) (Figure 4B) showed that this tomato PEPCK mRNA was present in relatively high amounts in seeds 4 days after germination and ripe fruit pericarp, lower amounts in roots and stems, but it was undetectable in mature tomato leaves.

Amounts of PEPCK mRNA increased during tomato fruit ripening (Figure 5), in agreement with the activity measurements presented in Figure 2A. No PEPCK mRNA signal could be detected either by northern blotting (Figure 5A) or by RT-PCR (Figure 5B) in either immature or mature green fruit

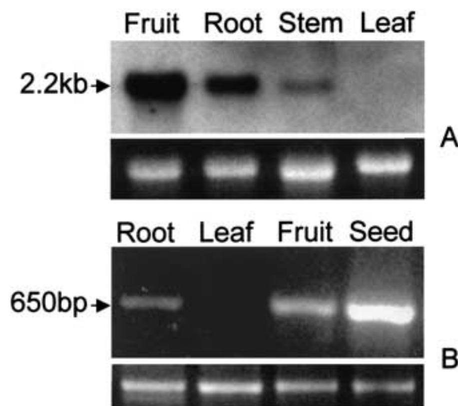

Figure 4. PEPCK mRNA is present at high levels in fruit and seed. A. Northern blot analysis of total RNA in (upper panel) probed with PEPCK cDNA. Lower panel: ethidium bromide staining of 28S ribosomal band. B. PCR products extended from oligo-dT reverse transcription of total RNA. Specific gene primers for PEPCK (upper panel) and ubiquitin, as a control (lower panel), were used.

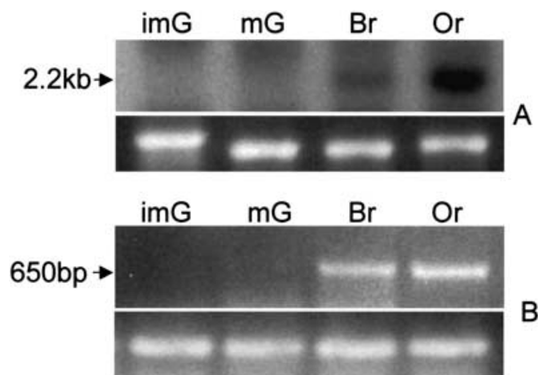

Figure 5. PEPCK mRNA amounts increase during fruit ripening. A. Northern blot analysis of total pericarp RNA (upper panel) at different ripening stages probed with tomato PEPCK cDNA. Lower panel: ethidium bromide staining of 28S ribosomal band. B. RT-PCR products extended from oligo-dT reverse transcription of total RNA. Specific primers for PEPCK (upper panel) and ubiquitin, as a control (lower panel) were used. imG, immature green; mG, mature green; Br, breaker; Or, orange (ripe) fruit.

pericarp samples. PEPCK mRNA became detectable at the onset of ripening, at the breaker stage, and was present throughout fruit ripening.

Discussion

Our data strongly suggest that there is a single gene encoding PEPCK in tomato that is expressed in different organs such as fruit, seeds and roots. Only one PEPCK gene has been detected in cucumber (Kim and Smith 1994). On the other hand, analysis of several other plants indicates the presence of multigene families encoding PEPCK. Genome sequencing of *Arabidopsis* has revealed at least three PEPCK-like sequences, although only one has a corresponding EST sequence and is, therefore, known to be expressed. Genomic Southern hybridisation analysis also indicates that *Brassica napus*, and its progenitor species, *B. campestris* and *B. oleracea*, contain PEPCK multigene families (Sáez-Vásquez *et al.*, 1995). The C_4 plant, *Urochloa panicoides*, has four PEPCK genes: two transcripts are expressed predominantly in leaves and two transcripts are expressed predominantly in roots (Finnegan *et al.*, 1999).

The derived amino acid sequence of the tomato PEPCK is 662 amino acid residues long and has 74%, 76%, 75%, 73%, and 50% identity to PEPCK sequences from cucumber (Kim and Smith, 1994), *Arabidopsis* (T06034), maize (BAA36483), *Urochloa panicoides* (Finnegan and Burnell, 1995) and yeast (CAA82177), respectively (Figure 1). Alignment of these derived peptide sequences revealed both conserved and variable regions between the sequences, with the N-terminus of plant PEPCKs being considerably more variable than the C-terminus. PEPCK has been purified from plants and shown to be considerably larger in size than that of yeast and *Escherichia coli* (Walker and Leegood 1996). Deduced protein sequences of cDNAs cloned from different plant species also show the presence of a stretch of amino acids in the N-terminal region that is missing in the yeast and *Escherichia coli* sequences (Kim and Smith, 1994). PEPCKs from many plants are known to be phosphorylated (Walker *et al.*, 1995, 1997; Walker and Leegood, 1996). Inspection of the sequence of tomato PEPCK reveals one potential phosphorylation site in the N-terminal region, a motif (residues 35–44; VCHDDSATPV) recognised by SNF-1-related protein kinases in higher plants (Halford and Hardy, 1998). These kinases are thought to be global regulators of carbon metabolism in plants, being implicated in the regulation of sucrose-phosphate synthase, nitrate reductase and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses a key step in the synthesis of isoprenoids (Halford and Hardy, 1998). Another potential cAMP-dependent protein kinase phosphorylation site is present in cucumber PEPCK (residues 64–69, KKRSTP; Kim and Smith 1994), but lacks the crucial serine and threonine in the tomato sequence (residues 57–62, KKKLAP). The tomato PEPCK sequence contains no obvious potential membrane-spanning regions, consistent with PEPCK being a cytoplasmic enzyme.

We have shown that PEPCK is present in the pericarp of ripening tomato fruits by activity measurements, western blotting and mRNA analysis. The occurrence and location of PEPCK is consistent with the view that it may be involved in malate dissimilation during ripening. During the ripening of tomatoes there is a large decrease in the organic acid content of the pericarp (Knee and Finger, 1992). This decrease in organic acids could be brought about by either NAD-malic enzyme, NADP-malic enzyme or PEPCK. However, the amounts of the malic enzymes decrease during ripening (Figure 2; Goodenough *et al.*, 1985; Knee and Finger, 1992), in contrast to the large increase in PEPCK that occurs during ripening. The amount of PEPCK in ripe tomatoes (ca. 4 μmol/h per gram fresh weight) greatly exceeded that needed to support the reported rate of dissimilation of organic acids (0.05 μmol h^{-1} g^{-1}; Knee and Finger, 1992). PEP that is formed by PEPCK could be utilised either in gluconeogenesis or respiration (Ruffner and Kliewer, 1975; Ruffner, 1982b; Knee and Finger, 1992). However, there is evidence from labelling studies that gluconeogenesis occurs in ripening tomato fruit (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991). The availability of PEP for gluconeogenesis must involve either PEPCK or the sequence of NAD(P) malic enzymes and pyruvate, Pi dikinase. There is no evidence for the last being present in tomato fruit (data not shown). The identification and characterisation of the tomato PEPCK gene will allow further studies of its role in transgenic tomato plants.

Acknowledgements

This research was supported by the Biotechnology and Biological Sciences Research Council, UK (Research Grant PO9495, and by a David Phillips Fellowship to R.P.W.) and by the British Council (Research Attachment for Z.-H.C.).

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389–3402.

Bahrami, A.R. and Gray, J.E. 1999. Expression of a proteasome α-type subunit gene during tobacco development and senescence. Plant Mol. Biol. 39: 325–333.

Blanke, M.M., Hucklesby, D.P. and Notton, B.A. 1988 Phospho*enol*pyruvate carboxykinase in aubergine, kiwi and apple fruit. Gartenbauwissenschaft 53: 65–70.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–159.

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

Finnegan, P.M. and Burnell, J.N. 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, P.M., Suzuki, S., Ludwig, M. and Burnell, J.N. 1999. Phospho*enol*pyruvate carboxykinase in the C_4 monocot *Urochloa panicoides* is encoded by four differentially expressed genes. Plant Physiol. 120: 1033–1041.

Goodenough, P.W., Prosser, I.M. and Young, K. 1985. NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. Phytochemistry 24: 1157–1162.

Halford, N.G. and Hardie, D.G. 1998, SNF1-related protein kinases: global regulators of carbon metabolism in plants? Plant Mol. Biol. 37: 735–748.

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

Hawker, J.S. 1969. Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry. 8: 19–23.

Hobson, G.E. and Davies, J.N. 1971. The tomato. In: A.C. Hulme (Ed.) The Biochemistry of Fruits and their Products. Academic Press, London, pp. 437–482.

Knee, M. and Finger, F.L. 1992. $NADP^+$-malic enzyme and organic acid levels in developing tomato fruits. J. Am. Soc. Hort. Sci. 117: 799–801.

Langdale, J.A., Rothermel, B.A. and Nelson, T. 1988. Cellular patterns of photosynthetic gene expression in developing maize leaves. Genes Dev. 2: 106–115.

Leegood, R.C. and Walker, R.P. 1999. Phospho*enol*pyruvate carboxykinase: iIts role and regulation. In: J.A. Bryant, M.M. Burrell and N.J. Kruger (Eds.) Plant Carbohydrate Biochemistry Bios Scientific Publishers, Oxford, pp. 201–213.

Murata, T., Ohsugi, R., Matsuoka, M. and Nakamoto, H. 1989. Purification and characterization of NAD malic enzyme from leaves of *Eleusine coracana* and *Panicum dichotomiflorum*. Plant Physiol. 89: 316–324.

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

Ruffner, H.P. 1982b. Metabolism of tartaric and malic acids in *Vitis*: a review – Part B. Vitis 21: 346–358.

Ruffner, H.P. and Kliewer, W.M. 1975. Phospho*enol*pyruvate carboxykinase activity in grape berries. Plant Physiol. 56: 67–71.

Ruffner, H.P. and Hawker, J.S. 1977. Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochemistry 16: 1171–1175.

Ruffner, H.P., Possner, D., Brem, S. and Rast, D.M. 1984. The physiological role of malic enzyme in grape ripening. Planta 160: 444–448.

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

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Ccloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Seymour, G.B., Taylor, J.E. and Tucker, G.A. 1993. Biochemistry of Fruit Ripening. Chapman and Hall, London.

Walker, R.P. and Leegood, R.C. 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, R.P., Trevanion, S.J. and Leegood, R.C. 1995. Phosphoenolpyruvate carboxykinase from higher plants: purification from cucumber and evidence of rapid proteolytic cleavage in extracts from a range of plant tissues. Planta 196: 58–63.

Walker, R.P., Acheson, R.M., Técsi, L.I. and Leegood, R.C. 1997. Phospho*enol*pyruvate carboxykinase in C_4 plants: its role and regulation. Aust. J. Plant Physiol. 24: 459–468.