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Phosphoenolpyruvate carboxykinase, pyruvate orthophosphate dikinase and isocitrate lyase in both tomato fruits and leaves, and in the flesh of peach and some other fruits

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ABSTRACT

In this study the occurrence of a number of enzymes involved in gluconeogenesis was investigated in both tomato fruits and leaves during their development and senescence and in some other fruits. The enzymes studied were phosphoenolpyruvate carboxykinase (PEPCK), pyruvate orthophosphate dikinase (PPDK) and glyoxysomal isocitrate lyase (ICL). PPDK was detected in the ripe flesh of tomato, and much smaller amounts were detected in the flesh of both peach and pepper, whereas it was not detected (not present or at very low abundance) in the other fruits which were investigated (apricot, aubergine, blackberry, blueberry, cherry, grape, plum, raspberry and red current). By contrast PEPCK was present in the flesh of all the fruits investigated. Very small amounts of ICL were detected in ripe tomato flesh. PEPCK was present in the skin, flesh, locular gel and columella of tomato fruit, and in these its abundance increased greatly during ripening. PPDK showed a similar distribution, however, its abundance did not increase during ripening. PEPCK was not detected in tomato leaves at any stage of their development or senescence. The content of PPDK g^{-1} fresh weight (FW) increased in tomato leaves as they matured, however, it declined during their senescence. In tomato leaves the content of ICL g^{-1} FW increased until the mid-stage of development, then decreased as the leaf matured, and then increased during the latter stages of senescence. In the flesh of tomato fruits the contents of PPDK and PEPCK g^{-1} FW decreased during senescence.

The results suggest that in fruits other than tomato the bulk of any gluconeogenic flux proceeds via PEPCK, whereas in tomato both PEPCK and PPDK could potentially be utilised. Further, the results indicate that the conversion of pyruvate/acetyl-CoA to malate by the glyoxylate cycle, for which ICL is necessary, is not a major pathway utilised by gluconeogenesis in fruits under normal conditions of growth. Finally, the results contribute to our understanding of the role of several enzymes in the senescence of both leaves and fruits.

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1. Introduction

Gluconeogenesis from malate occurs in the flesh of ripening grape, tomato and cherry fruits (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991; Huang et al., 2015a, 2015b; Leegood and Walker, 1999; Osorio et al., 2013; Ruffner, 1982). In plants gluconeogenesis from malate can occur by two alternate pathways. One pathway utilises malate dehydrogenase (MDH) in conjunction with phosphoenolpyruvate carboxykinase (PEPCK). The other pathway utilises malic enzyme (ME) in conjunction with pyruvate orthophosphate dikinase (PPDK) (Famiani et al., 2015, 2014b; Leegood and Walker, 2003). In the fruits of both cherry and grape it appears that the PEPCK pathway is used in gluconeogenesis, and this is because PPDK is not present (or is at very low abundance) (Famiani et al., 2014b; Walker et al., 2011a). PEPCK is also present in ripening tomato flesh in which it catalyses a gluconeogenic flux

Abbreviations: ICL, isocitrate lyase; MS, malate synthase; MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase.

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from malate/citrate (Bahrami et al., 2001; Huang et al., 2015a, 2015b; Osorio et al., 2013). In tomato, radiolabelling experiments have shown that gluconeogenesis from pyruvate can also occur, and this requires either PPDK or PEPCK (Farineau and Laval-Martin, 1977). If PEPCK is utilised, pyruvate can be converted to malate by either the Krebs cycle or the glyoxylate cycle. Isocitrate lyase (ICL) is an essential component of the glyoxylate cycle (Eastmond and Graham, 2001). In both cucumber flesh and ripening banana flesh very low amounts of ICL are present (Liu et al., 2004; Yang et al., 1998). However, glyoxysomal ICL was not detected in either grape flesh or that of some soft fruits (Famiani et al., 2014b, 2005). In tomato fruits the occurrence of both PPDK and the glyoxylate cycle is uncertain. The first aim of the present study was to investigate the occurrence of PEPCK, PPDK and glyoxysomal ICL in tomato and other fruits. There is contradictory information regarding the occurrence of PPDK in senescing leaves (Chen et al., 2000; Taylor et al., 2010), and very little is known about the occurrence of either PEPCK or PPDK in most fruits during their senescence. The second aim of this study was to determine the abundance of PEPCK and PPDK in tomato fruits and leaves during their senescence.

2. Materials and methods

2.1. Plant material

Tomato plants (*Solanum lycopersicum*, cultivar Moneymaker) were grown in a greenhouse in Perugia and fruits were collected at four stages of development. These were: 1, small-green (20% final FW); 2, medium-green (50% final FW); 3, breaker (turning colour); 4, full colouration (red). Plants of sweet pepper (*Capsicum annuum*, cv. World Beater), aubergine (*Solanum melongena*, cv. Black Enorma), *Hoya carnosa* and maize were grown in pots of garden soil in a greenhouse in Perugia. In 2004, fruits of both peach (*Prunus persica* L. Batsch) cultivar Adriatica and other fruits were collected from mature trees growing in the experimental orchard of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia, in Deruta (PG), central Italy. The fruits of all species were harvested from several positions on the plant. Maize seedlings (*Zea mays*) were germinated and grown in trays of perlite in a greenhouse in Perugia, Italy, and they were then fed NH_4Cl as previously described (Walker et al., 2001). Cucumber cotyledons (*Cucumis sativus*) were obtained from seeds germinated in perlite at 25 °C under darkness for 4 d. For both tomato leaf and fruit senescence experiments mature leaves or ripe fruit (development stage 4) were detached and placed on moist filter paper in petri dishes. These were then placed in an incubator under darkness at 25 °C.

2.2. Preparation of a nitrogen powder

To ensure that the sample was representative of the tissue a nitrogen powder was prepared. Tissues were frozen in liquid nitrogen. When required for analysis, the tissues were removed from the liquid nitrogen. For fruits three samples of the components of the pericarp (skin, flesh, locular gel and columella), each composed of subsamples of 5–10 fruits, were used. For each sample, tissues were then ground together in a mortar containing liquid nitrogen and the resulting powder was used either immediately or after storage at −80 °C. This powder was used for electrophoretic analysis. Each set of samples was analysed separately by SDS-PAGE and immunoblotting. For all figures similar results were obtained with each set of samples and representative gels and western blots are shown.

2.3. Enzyme assays

Two hundred mg of frozen powder of fruit flesh was ground in a mortar containing 800 μl of ice cold 200 mM Bicine-KOH (pH 9.0), 50 mM DTT and clarified by centrifugation at 12,000*g* for 5 min. PEPCK activity in the supernatant was measured, using an enzyme coupled method, in the carboxylation direction as described by Walker et al. (1999) and Famiani et al. (2005). For the measurement of PPDK activity plant material was extracted and enzyme activity determined in the forward direction (PEP formation) using an enzyme-coupled spectrophotometric method as described previously (Ashton et al., 1990). For both PEPCK and PPDK one unit of activity is that which produces 1 μmol product min^{-1} at 25 °C.

2.4. SDS-PAGE and immunoblotting

For pericarp 500 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. Other tissues were extracted in the same way except that for leaves 100 mg, for roots 250 mg and for either developing or germinating seeds 50 mg of frozen powder was used. 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, 4 μl of extract was loaded onto each track of SDS-PAGE gels. SDS-PAGE, staining of gels with Coomassie Brilliant Blue dye and immunoblotting were done as described in Walker and Leegood (1996). Protein measurement was done using the Lowry method as previously described (Walker et al., 1995). 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, Massachutes, USA). Anti-rabbit peroxidase (diluted 1/1,000) was used in conjunction with an ECL kit (GE Healthcare, Little Chalfont, UK) to visualize immunoreactive polypeptides. The antisera to aldolase, PEPCK, phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) were produced using as antigens the enzymes that had been purified from *Panicum maximum* leaves (Walker et al., 2002; R.P. Walker, unpublished work). The antisera to PPDK was that used in a previous study of grape (Chastain et al., 2002; Famiani et al., 2014b). ICL antiserum was raised against the enzyme from castor bean endosperm (Maeshima et al., 1988). The glutamine synthetase (GS) antiserum was raised against the plastidic enzyme from *Sinapsis alba* (Höpfner et al., 1990).

3. Results and discussion

3.1. The occurrence of PPDK and PEPCK in the ripe flesh of both tomato and some other fruits

Many studies have established the specificity of both the PEPCK and PPDK antibodies used in the present study for the enzymes from a wide range of plant species (Chastain et al., 2006, 2002; Famiani et al., 2014b, 2005; Taylor et al., 2010; Walker et al., 2011a, 2011b, 2011c). PPDK was detected in both tomato flesh and leaves, and was of the same molecular mass (95-kDa) as PPDK from both maize and *Hoya carnosa* leaves (Famiani and Walker,

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