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Changes in Abundance of Enzymes Involved in Organic Acid, Amino Acid and Sugar Metabolism, and Photosynthesis during the Ripening of Blackberry Fruit

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Abstract. **Although information is available regarding the content of various metabolites such as sugars and organic/amino acids in blackberry (*Rubus* L.), little is known about its enzyme composition. The aim of this study was to investigate changes in the abundance of various enzymes during the ripening of blackberry. Blackberry is an aggregate fruit, composed of a receptacle and several drupelets attached to it, which in turn, are composed of the flesh (mesocarp plus epicarp) and seed enclosed in the endocarp; therefore, these parts were analyzed separately along with the pedicel. The enzymes studied participate in organic/amino acid and sugar metabolism and photosynthesis, processes known to be important in fruit development. These enzymes were phosphoenolpyruvate carboxykinase [PEPCK (EC:4.1.1.49)], phosphoenolpyruvate carboxylase [PEPC (EC:4.1.1.31)], pyruvate, orthophosphate dikinase [PPDK (EC:2.7.9.1)], cytosolic aspartate aminotransferase [cyt AspAT (EC:2.6.1.1)], aldolase (EC:4.1.2.13), glutamine synthetase [GS (EC:6.3.1.2)], and ribulose-1,5-bisphosphate carboxylase/oxygenase [RUBISCO (EC:4.1.1.39)]. To avoid problems in measuring enzyme activity, the approach taken was to use antibodies specific for each enzyme in conjunction with immunoblotting of sodium dodecyl sulfate polyacrylamide gel electrophoresis. During ripening, there were marked changes in abundance of several of these enzymes and these changes were dependent on the tissue investigated. PEPCK appeared when organic acids decreased in the flesh and was only detected in this tissue, whereas PPDK was not detected in any tissue. In the flesh, there was a large decrease in abundance of RUBISCO, plastidic GS, and plastidic aldolase, but little change in cytosolic GS, cytosolic aldolase, and PEPC. In seeds, there was a decrease in the abundance of all enzymes. In the receptacle and pedicel, apart from a large decrease in RUBISCO in the receptacle, there was little change in enzyme abundance.**

The ripening of blackberry, like many other fruits, is accompanied by color, respiratory, and compositional changes, together with softening (Perkins-Veazie et al., 2000; Tosun et al., 2008; Wrolstad et al., 1980). There is a large decrease in titratable acidity concomitant with a substantial increase in the content of soluble solids and anthocyanins (Perkins-Veazie et al., 2000; Tosun et al., 2008; Walsh et al., 1983). The large increase in sugar content that occurs during ripening is mostly a result of the accumulation of fructose and glucose (Kafkas et al., 2006; Perkins-Veazie et al., 2000; Wrolstad et al., 1980). Isocitric, lactoisocitric, and malic acids make up the bulk of organic acids in blackberry; however, the abundance of each of these is dependent on the cultivar or species (Kafkas et al., 2006; Whiting, 1958; Wrolstad et al., 1980). Blackberry contains a range of amino acids (Green, 1971).

The metabolism of organic acids/sugars and photosynthesis are important processes in fruit development and ripening (Blanke and Lenz, 1989; Famiani et al., 2000, 2005, 2009). However, very little is known about the occurrence of enzymes involved in these processes in blackberry. The aim of this study was to determine how the abundance of enzymes involved in these processes changed during the ripening of blackberry. The enzymes studied were phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase, cytosolic aspartate aminotransferase, aldolase, glutamine synthetase, and ribulose-1,5-bisphosphate carboxylase/oxygenase. It is difficult to measure the activity of enzymes in extracts of many soft fruit (Manning, 1993). Therefore, in this study, the approach taken was to use western blotting of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with specific antisera, an approach that has proved successful for a number of soft fruit (Famiani et al., 2005, 2009). To use this approach, it is essential that yields of polypeptides on SDS-PAGE gels are good and that the antibodies are specific for their antigen. Experiments were done to ensure that these conditions were met. To assess the specificity of antisera, tissues in which the considered enzymes are abundant were used [i.e., maize (*Zea mays* L.) leaves, *Panicum maximum* L. leaves, blackberry leaves, and maize roots].

The blackberry is an aggregate fruit that is composed of a central receptacle to which drupelets are attached. Vascular tissue passes from the receptacle to each drupelet. Each drupelet contains a seed that is surrounded by a stony endocarp that in turn is enclosed by the flesh (mesocarp plus epicarp) of the drupelet (Perkins-Veazie et al., 2000). The enzyme content of these structures is likely to differ; therefore, the receptacle, flesh, and seed plus stony endocarp were dissected and analyzed separately, along with the pedicel. In addition, the amount of PEPC and PEPCK in blackberry fruit was compared with that in other soft fruit, such as ripe blueberries (*Vaccinium corymbosum* L.) and red currants (*Ribes rubrum* L.), in which the abundance of these enzymes was established in a previous study (Famiani et al., 2005).

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Materials and Methods

Plant material. Fruit and leaves of blackberry (cv. Black Satin) were collected from plants growing in central Italy in 2005. The fruit were collected at four stages of development, based on their color, and they were: 1 = green, 2 = onset of coloration, 3 = half coloration (all drupelets were red), 4 = maximum coloration (all drupelets were black). This approach to distinguish the different stages of development/ripening of blackberry was also used in previous studies (Perkins-Veazie et al., 2000; Tosun et al., 2008; Wrolstad et al., 1980) and in a study of other soft fruit (Famiani et al., 2005). Only healthy fruit were used, and these were taken from several positions on the plant. Ripe blueberries (cv. Blueray) and red currants (cv. Junifer) were collected from plants growing in central Italy in 2005. Leaves and roots of maize (cv. Golden Giant) and leaves of *P. maximum* were collected from plants growing in a greenhouse in Perugia, Italy.

Measurement of fresh and dry weights. At each stage of development, the weights of 30 intact blackberries were each measured separately. This was done for freshly harvested fruit and for the same fruit after being dried to constant weight in a forced-air oven at 90 °C.

Measurement of soluble solids content. Soluble solids content of the drupelets was measured using a refractometer. For each stage of development, this was done on fresh-squeezed juice sample of drupelets of each of 30 fruit.

Measurement of titratable acidity. Three 10-g samples of drupelets collected from several fruit were each homogenized in a mortar containing 50 mL of distilled water, passed through Miracloth (Calbiochem–EMS Biosciences, San Diego), and the free total acidity in the filtrate was neutralized with 100 mM NaOH using phenolphthalein as acid-base indicator. Results are expressed as a percentage or milligrams of malic acid.

Measurement of anthocyanin content. Three 10-g samples of drupelets, collected from several fruit, were each homogenized in a mortar containing 40 mL of distilled water and were then centrifuged at 10,000 g_n for 5 min. The supernatant was passed through Miracloth and 5 mL of the filtrate was then added to 10 mL of distilled water. The absorbance of this solution at 520 nm was determined using a spectrophotometer.

Preparation of a nitrogen powder. For electrophoresis and measurement of sugar content, a nitrogen powder was prepared to ensure that the sample was representative of the tissue. For each stage of development, three samples of 10 blackberries were used. Drupelets and pedicels were removed from receptacles and the seed plus endocarp were then removed from the flesh of the drupelets. The removal of the seed plus endocarp was done on drupelets semifrozen in liquid nitrogen; that is, the seed plus endocarp were dissected as soon as drupelets taken out from liquid nitrogen had a consistency that allowed this operation, but before they melted. Samples (pedicel, receptacle, seed plus endocarp and flesh) were each ground in a mortar containing liquid nitrogen and the resulting powder was used immediately or after storage at –80 °C. Leaf discs of maize, *P. maximum* and blackberry, roots of maize, and flesh of ripe blueberries and red currants were also ground in a mortar containing liquid nitrogen and powder stored at –80 °C.

Measurement of soluble sugars. Frozen powder (50 mg) was added to an Eppendorf tube containing 1.5 mL of 80% ethanol/20% water (v/v), 100 mM HEPES-KOH (pH 7.1), and 20 mM $MgCl_2$, incubated at 80 °C for 1 h and then centrifuged at 12,000 g_n for 5 min. One-hundred fifty microliters of charcoal suspension (100 mg·mL^{-1}) was added to the supernatant, vortexed, and then centrifuged at 12,000 g_n for 5 min. The supernatant was stored at –20 °C until used. The total soluble sugars were measured in the supernatant colorimetrically with a spectrophotometer using the anthrone reagent (Morris, 1948). The calibration curve used as reference to determine the amount of sugars was prepared using glucose.

SDS-PAGE and immunoblotting. Frozen powder of flesh (500 mg), receptacle (250 mg), pedicel (250 mg), roots (125 mg), and other tissues (60 mg) was added to 500 µL of 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 microliters of 20% (w/v) NaOH was 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_n for 5 min. The supernatant was separated from the pellet and stored at –20 °C until used. For SDS-PAGE, 1 to 5 µL of extract was loaded onto each track of the gel after centrifugation at 10,000 g_n for 5 min. SDS-PAGE and immunoblotting were done as described in Walker and Leegood (1996). Briefly, SDS-PAGE was performed in a mini-gel apparatus (SE 250 Mighty Small II; Hoefer Scientific Instruments, San Francisco) and western transfer was done using a Pharmacia Multiphor device (Multiphor II Electrophoresis System; Pharmacia Biotech, Uppsala, Sweden) in conjunction with Millipore Immobilon-P membrane (Millipore, Billerica, MA). Antirabbit 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, cyt AspAT, aldolase, and RUBISCO antisera were raised in rabbits against the enzymes from *P. maximum* leaves (R.P. Walker, unpublished data). PPDK antiserum was raised against the enzyme from maize and was affinity purified (Chastain et al., 2002). GS antiserum was raised against plastidic GS from *Sinapsis alba* L. (Höpfner et al., 1990).

To assess the specificity of antisera for enzymes, tissues were used in which the enzymes considered are abundant and their mass is known. For PEPCK, PEPC, PPDK, and cyt AspAT, which function in C_4 photosynthesis, leaves of the C_4 plants maize and *P. maximum* were used. For enzymes that are associated with the C_3 photosynthetic pathway, such as RUBISCO, GS, and aldolase, blackberry leaves were used. To have a more complete picture about antibody specificity, maize roots were also used.

Results

Changes in fresh and dry weights, soluble solids content, titratable acidity, and anthocyanin content during fruit development. The fresh and dry weights and anthocyanin content of blackberries increased during the period of development considered (Fig. 1). Soluble solids content increased markedly between stage 3 and 4 of development (Fig. 1). Titratable acidity, per fruit and per gram fresh weight,

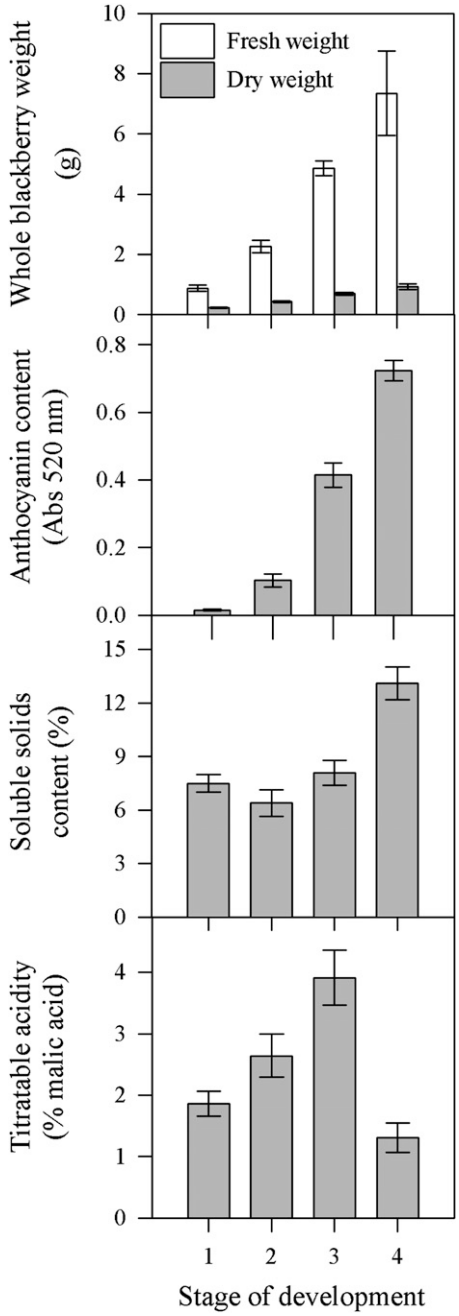

Fig. 1. Fresh and dry weights of whole blackberry and anthocyanin content, soluble solids content, and titratable acidity of blackberry drupelets at different stages of development. Blackberries were collected from plants of cultivar Black Satin. For weights, each bar on the histogram shows the mean and SE of 30 whole fruit. For the soluble solids content, each bar on the histogram shows the mean and SE of two readings done on the juice of drupelets of each of 30 fruit. For anthocyanin content, expressed as absorbance (Abs) at 520 nm of the extracts, and titratable acidity each bar on the histogram shows the mean and SE of two measurements done on each of three 10-g samples of drupelets. The development stage of the fruit was based on their color: 1 = green, 2 = onset of coloration, 3 = half-maximum coloration (all drupelets were red), 4 = maximum coloration (all drupelets were black).

increased until stage 3 of development and then declined (Figs. 1 and 2).

Changes in the content of total sugars in the flesh during development. The content of total sugars, per fruit and per gram fresh weight, increased between stages 2 and 3 of development and showed a large increase between stages 3 and 4 (Fig. 2).

Antibody specificity. The PEPCK antiserum recognized a 74-kDa polypeptide in extracts of leaves of maize and *P. maximum*, but not in that of maize root that had not been fed with ammonium (Fig. 3). The PEPC antiserum recognized a 110-kDa polypeptide in extracts of leaves of maize and *P. maximum* and maize roots, however, it was most abundant in mature leaves of maize and *P. maximum* (Fig. 3). The PPDK antiserum recognized a 95-kDa polypeptide in extracts of maize and *P. maximum* leaves (Fig. 3). However, it was less abundant in immature maize leaves and was not detected in maize roots or leaves of the C_3 plant blackberry (Figs. 3 and 4). The antiserum to cyt AspAT recognized a 42-kDa polypeptide in extracts of all tissues, but was much more abundant in leaves of *P. maximum* (Fig. 3). The antiserum against the large subunit of RUBISCO recognized a 55-kDa polypeptide in blackberry leaves, but it was more abundant in mature leaves (Fig. 4). The antiserum against GS recognized a 39- and 43-kDa polypeptide in immature blackberry leaves (Fig. 4). In mature blackberry leaves, the 39-kDa polypeptide was not detected and the 43-kDa polypeptide had greatly increased in abundance (Fig. 4). The aldolase antiserum recognized a 38- and 40-kDa polypeptide in immature blackberry leaves (Fig. 4). In mature blackberry leaves, the 40-kDa polypeptide was not detected, probably because it was present in small amounts, and the abundance of the 38-kDa polypeptide had greatly increased (Fig. 4).

Enzyme abundance in different tissues of blackberry. SDS-PAGE gels were loaded with extracts of pedicel, flesh, receptacle, and seed plus endocarp of ripe blackberry (stage 4 of development), immature and mature blackberry leaves, and mature maize leaves (Fig. 4). Staining of polypeptides in the gel, using Coomassie Brilliant Blue dye, showed that the polypeptides were well resolved and that the polypeptide composition of the tissues was different (Fig. 4). In extracts of seed plus endocarp, several abundant polypeptides were present, and in blackberry leaves, there was a large increase in abundance of a 13-, 30-, and 55-kDa polypeptide as they developed (Fig. 4). On immunoblots, PEPCK was only detected in extracts of ripe blackberry flesh and maize leaves, while PPDK was only detected in maize leaves (Fig. 4). Coextraction of maize leaves and each tissue of blackberry did not lead to a loss of PEPCK or PPDK on immunoblots (data not presented). RUBISCO was only detected in leaves and pedicel, but not in other tissues of the ripe fruit (Fig. 4). The 43-kDa plastidic GS was most abundant in mature leaves of blackberry, but was also present in immature leaves and maize leaves (Fig. 4). In the flesh, pedicel, and receptacle of ripe berries, only the 39-kDa cytosolic GS was detected (Fig. 4). PEPC was most abundant in mature maize leaves; in contrast, its abundance decreased during the development of blackberry leaves (Figs. 3 and 4). In blackberry, its abundance was similar in immature leaves, and the pedicel, flesh, and receptacle of ripe fruit (Fig. 4). Only the 40-kDa cytosolic aldolase was detected in the flesh and receptacle of ripe blackberries (Fig. 4). However, in the pedicel, a longer exposure of the blot showed that small amounts of the 38-kDa plastic aldolase was present (Fig. 5). In immature blackberry leaves the 40-kDa cytosolic aldolase together with the 38-kDa plastidic aldolase was present, and in mature blackberry and maize leaves, only the 38-kDa plastidic enzyme was detected (Fig. 4).

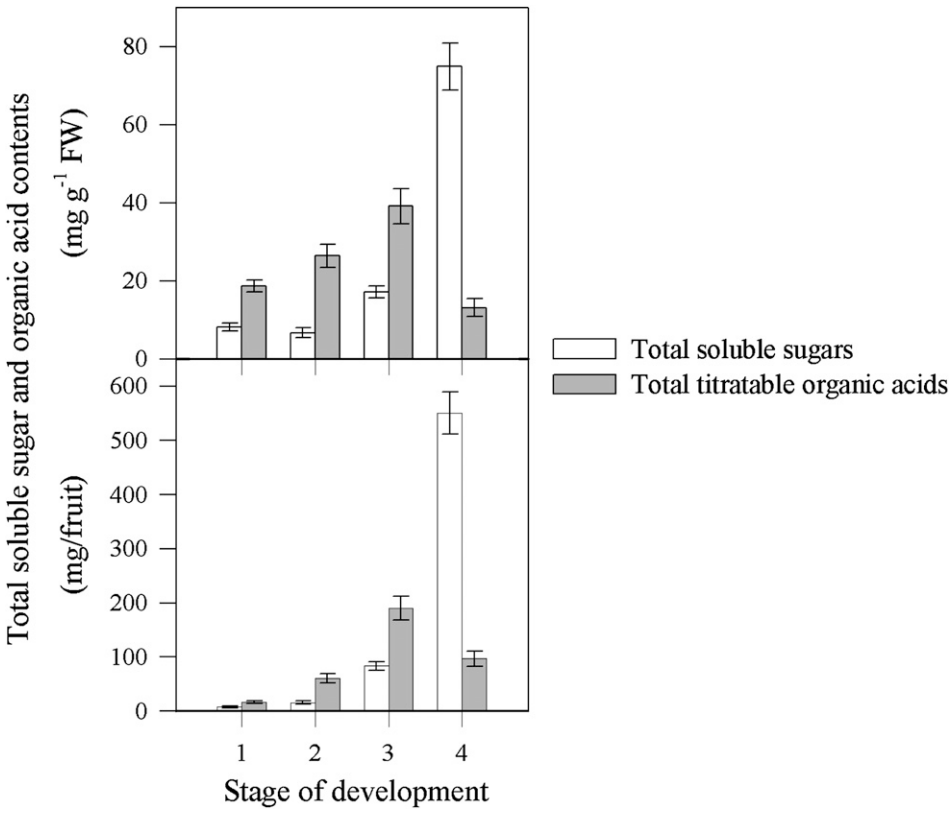

Fig. 2. Changes in the abundance of total sugars and total titratable organic acids during the development of blackberry, both on per gram and per whole blackberry basis. Blackberries were collected from plants of cultivar Black Satin. Each point on the graph shows the mean and SE of two measurements done on each of three separate extractions of flesh (total sugars) or drupelets (titratable organic acids). The development stage of the fruit was based on their color: 1 = green, 2 = onset of coloration, 3 = half-maximum coloration (all drupelets were red), 4 = maximum coloration (all drupelets were black).

Changes in enzyme abundance during fruit development. SDS-PAGE gels were loaded with extracts of the flesh, receptacle, pedicel, and seed plus endocarp of blackberries at different stages of development, and after electrophoresis, polypeptides were visualized using Coomassie Brilliant Blue dye. In all cases, bands on gels were sharp and well separated (Fig. 5) and coextraction of fruit tissues and maize leaves, whose polypeptide pattern after SDS-PAGE is known, did not result in a loss of polypeptides present in maize leaves (data not presented). The intensity of staining of the tracks for all tissues at different stages of development was similar, and the polypeptide composition of the flesh, receptacle, pedicel, and seed plus endocarp was different (Fig. 5). There were no marked changes in polypeptide composition of any tissue except for the decrease in abundance of three low mass (18–21 kDa) polypeptides in the receptacle and an increase in abundance of 15-, 25-, 39-, and 45-kDa polypeptides in the seed plus endocarp (Fig. 5). Changes in enzyme abundance were investigated using immunoblots of the previous gels. PEPCK was only present in the flesh of blackberries at stage 4 of development (Fig. 5). The failure to detect PEPCK in flesh earlier in development was not a result of loss of enzyme after extraction because coextraction of ripe flesh with flesh earlier in development did not lead to a loss of PEPCK (data not presented). PEPC was detected in all tissues at all stages of development. In flesh and receptacle, its abundance changed little during development, whereas it increased in the pedicel and decreased in the seed plus endocarp (Fig. 5). RUBISCO was detected in all tissues, however, except for pedicel in which its abundance declined slightly during development, it was not detected at later stages of development (Fig. 5). The 39-kDa cytosolic GS was detected in all tissues in which abundance changed little during development, except for seed plus endocarp in which it was undetectable after stage 2. The 43-kDa plastidic GS was only detected in flesh at stage 1 of development (Fig. 5). The 40-kDa cytosolic aldolase was detected in all tissues in which abundance changed little during development, except for seed plus endocarp in which it was undetectable after stage 1. The 38-kDa plastidic aldolase was present in flesh at stages 1 and 2 and in the pedicel throughout development (Fig. 5). Cytosolic AspAT was detected in all tissues in which abundance changed little during development, except for seed plus endocarp in which it was undetectable after stage 1. PPDK was not detected in any tissue (data not presented).

Comparison of the abundance of PEPCK and PEPC in the ripe flesh of blackberry, blueberry, and red current. Immunoblots of the SDS-PAGE gels loaded with extracts of the ripe flesh of blackberry, blueberry, and redcurrant gave signals of similar strength for PEPCK and PEPC (Fig. 6).

Discussion

The aim of this study was to investigate the occurrence of enzymes involved in photosynthesis and the metabolism of organic/amino acids and sugars in different tissues of blackberries, and how their abundance in these changed during ripening. This was addressed by using antisera specific for key enzymes used in these processes to determine their abundance on blots of SDS-PAGE gels.

The results regarding changes in fresh and dry weights, titratable acidity, and contents in anthocyanins, soluble solids and soluble sugars are, in general, comparable to those of previous studies (Perkins-Veazie et al., 2000; Tosun et al., 2008; Walsh et al., 1983). In ripe blackberries, Kafkas et al. (2006) found lower amounts of total sugars than those observed in this study; however, different genotypes of blackberry were used.

For many fruit, problems are encountered in the preparation of extracts for SDS-PAGE (Famiani et al., 2000; Manning, 1993). As in the case of several other soft fruit (Famiani et al., 2005, 2009), homogenization in a modified SDS-PAGE extraction buffer was satisfactory. The suitability of this method was shown by two observations. First, the polypeptide pattern on gels was sharp and well separated (Figs. 4 and 5). Second,

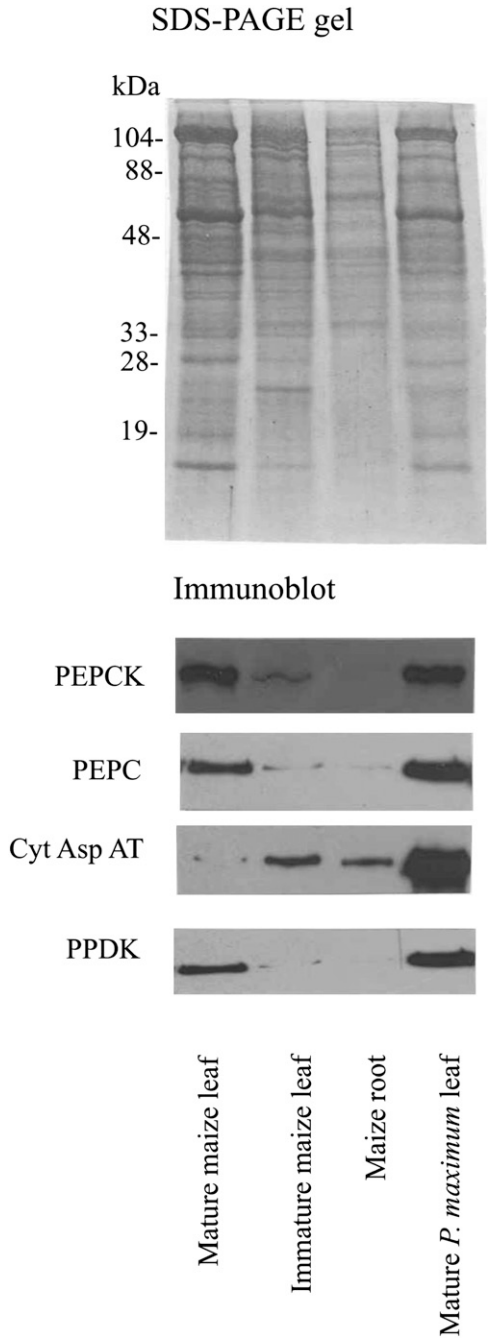

Fig. 3. Evaluation of antibody specificity by immunoblots that used tissues in which the enzymes considered are abundant and their mass is known. Extracts of mature and immature maize leaves, mature *Panicum maximum* leaves, and maize roots were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then stained using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes were detected using specific antisera. PEPCK = phosphoenolpyruvate carboxykinase, PEPC = phosphoenolpyruvate carboxylase, cyt AspAT = cytosolic aspartate aminotransferase, PPDK = pyruvate, orthophosphate dikinase.

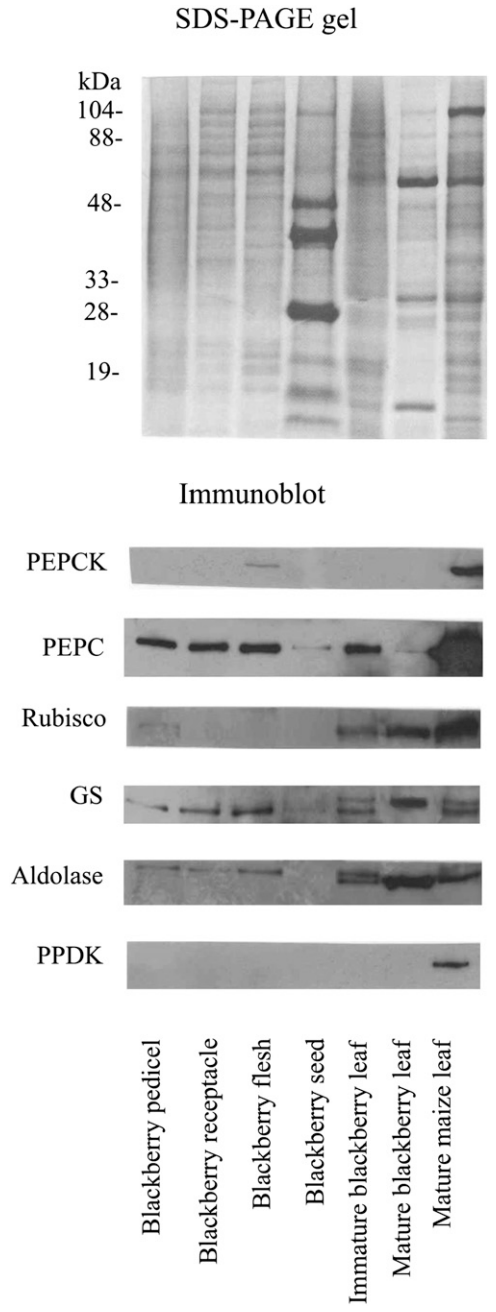

Fig. 4. Comparison of enzyme abundance in different parts of ripe blackberry, immature (5% full size) and mature blackberry leaves, and mature maize leaves to assess antibody specificity. Blackberry and blackberry leaves were collected from plants of cultivar Black Satin. Extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The amount of extract loaded on gels corresponded to 4.6 mg fresh weight for flesh, 2.4 mg for receptacle and pedicel, and 0.3 mg for seed and leaf. Polypeptides were then stained using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes were detected using specific antisera. PEPCK = phosphoenolpyruvate carboxykinase, PEPC = phosphoenolpyruvate carboxylase, RUBISCO = ribulose-1,5-bisphosphate carboxylase/oxygenase, GS = glutamine synthetase, PPDK = pyruvate orthophosphate dikinase.

coextraction of fruit tissues and maize leaves, whose polypeptide pattern after SDS-PAGE is known (Wingler et al., 1999), did not result in a loss of polypeptides present in maize leaves (data not presented).

The antisera were specific for their target enzymes (Figs. 3 and 4). The mass and occurrence of the polypeptides recognized by the PEPCK, PEPC, PPDK, Cyt AspAT, and RUBISCO antisera are consistent with previous studies of PEPCK (Walker and Chen, 2002), PEPC (McNaughton et al., 1989), PPDK (Chastain et al., 2002; Chen et al., 2000), Cyt

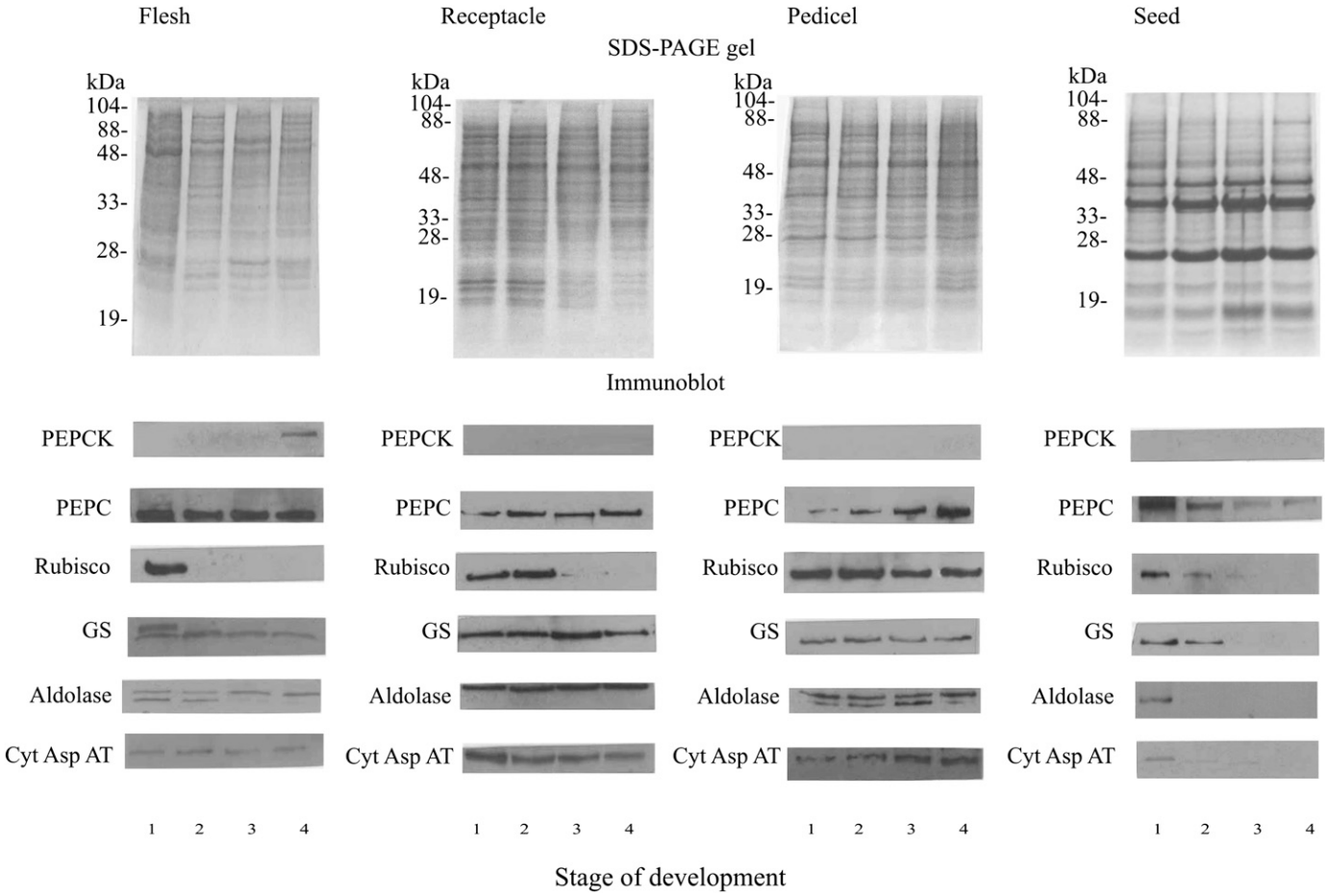

Fig. 5. Polypeptide and enzyme abundance in the flesh (mesocarp plus epicarp), receptacle, pedicel and seed plus endocarp of 'Black Satin' blackberry at different stages of development. Extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then stained using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes were detected using specific antisera. The amount of extract loaded on gels corresponded to 4.6 mg fresh weight for flesh, 2.4 mg for receptacle and pedicel, and 0.3 mg for seed. The development stage of the fruit was based on their color: 1 = green, 2 = onset of coloration, 3 = half-maximum coloration (all drupelets were red), 4 = maximum coloration (all drupelets were black). PEPCK = phosphoenolpyruvate carboxykinase, PEPC = phosphoenolpyruvate carboxylase, RUBISCO = ribulose-1,5-bisphosphate carboxylase/oxygenase, GS = glutamine synthetase, cyt AspAT = cytosolic aspartate aminotransferase.

AspAT (Miesak and Coruzzi, 2002; Numazawa et al., 1989), and RUBISCO (Spreitzer, 2003). The mass and distribution of polypeptides recognized by the GS antiserum are consistent with previous studies of GS in which a 38- to 40-kDa polypeptide represents GS1, a cytosolic isoform not associated with photosynthesis, while a 43-kDa polypeptide represents plastidic GS2 associated with photosynthesis (Ochs et al., 1999). The mass and distribution of polypeptide recognized by aldolase are consistent with previous studies of this enzyme in which the 40-kDa polypeptide is the cytosolic enzyme, not associated with photosynthesis, while the 38-kDa polypeptide is plastidic aldolase that functions in the Calvin cycle (Lebherz et al., 1984).

PEPC was detected in all parts of blackberry fruit and at all stages of development (Fig. 5), and this is consistent with the view that it functions in the synthesis of stored organic acids and in the anaplerotic replenishment of the Krebs cycle necessary for biosynthesis (Guillet et al., 2002; Law and Plaxton, 1995). PEPC was also found in the flesh of other fruit species throughout ripening (Famiani et al., 2000, 2005, 2009; Law and Plaxton, 1995; Moing et al., 2000). In maize leaves, PEPC increased during development, while in blackberry leaves, it decreased (Figs. 3 and 4). This is consistent with maize being a C_4 photosynthesis plant in which PEPC functions as a carboxylase in the C_4 cycle (Walker et al., 1997). In contrast, blackberry leaf does not possess C_4 photosynthesis and an important function of PEPC is anaplerotic replenishment of the Krebs cycle necessary to produce precursors for growth (Law and Plaxton, 1995). Visualization of total polypeptides revealed that the seed plus endocarp contained a number of very abundant polypeptides (Fig. 5), which, as the endocarp was stony, are likely to be seed storage proteins; storage proteins are also abundant in the seeds of other fruit (Famiani et al., 2000, 2005; Walker et al., 1999). These proteins were present at all stages of ripening and only showed a small increase in abundance (Fig. 5). This suggests that the seeds were approaching maturity before ripening, as is the case in grape (*Vitis vinifera* L.) (Famiani et al., 2000; Walker et al., 1999), and this is consistent with a previous study of blackberry in which seed dry weight increased little after the green-red stage of development (Perkins-Veazie et al., 2000). For the seed plus endocarp, the detected enzymes are likely to be in the seed, as the endocarp was stony. In seeds, PEPC and other enzymes showed a large decrease in abundance during ripening, and

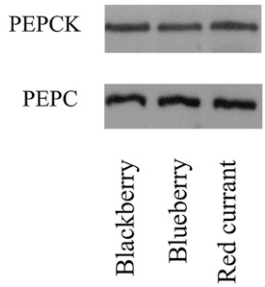

Fig. 6. Comparison of the abundance of PEPCK and PEPC in the ripe flesh of 'Black Satin' blackberry, 'Blueray' blueberry, and 'Junifer' red currant. Extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membrane, and enzymes detected using specific antisera. PEPCK = phosphoenolpyruvate carboxykinase, PEPC = phosphoenolpyruvate carboxylase.

often to undetectable amounts (Fig. 5). Similarly, in grape, the abundance of many enzymes decreases greatly after storage protein deposition is complete (Famiani et al., 2000; Walker et al., 1999). In seeds of grape and pea (*Pisum sativum* L.), PEPCK is thought to function in the metabolism of imported assimilates, and its abundance decreases greatly after the deposition of storage proteins is complete (Delgado-Alvarado et al., 2007; Walker et al., 1999). Therefore, it is possible that PEPCK might occur in blackberry seeds before ripening. In the other tissues of blackberry that were examined, PEPCK was only detected in ripe flesh (Figs. 4 and 5). In raspberry (*Rubus idaeus* L.), blueberry, and tomato (*Solanum lycopersicum* L.), PEPCK is absent in green fruit but appears during ripening; however, it is present in green red currant (Bahrami et al., 2001; Famiani et al., 2005). PEPCK was absent in blackberry leaves (Fig. 4). In the leaves of other plants that use C_3 photosynthesis, PEPCK is present in some species, but not others, and when present, it appears to be localized in certain tissues such as trichomes or vasculature (Chen et al., 2004). PEPCK was abundant in the leaves of maize and *P. maximum* in which it functions as a decarboxylase in C_4 photosynthesis (Walker et al., 1997). In the ripe flesh, the abundance of PEPC and PEPCK was similar in blackberry, blueberry, and red currant (Fig. 6). A previous study that measured enzyme activity showed that the amount of PEPC and PEPCK in the ripe flesh of blueberry and red currant was in the range 0.07 to 0.15 U g^{-1} fresh weight (Famiani et al., 2005). Evidence has been provided that PEPCK may function in the dissimilation of organic acids during the ripening of some fruit (Bahrami et al., 2001; Famiani et al., 2005, 2009; Ruffner and Kliewer, 1975). In blackberry, the appearance of PEPCK when the organic acid content of the flesh decreased is consistent with this (Figs. 2 and 5). A number of fates for dissimilated organic acids are possible in fruit such as utilization by the Krebs cycle and gluconeogenesis (Famiani et al., 2005; Halinska and Frenkel, 1991; Leegood and Walker, 1999; Ruffner et al., 1975; Ruffner, 1982; Tucker, 1993). If gluconeogenesis occurs in blackberry flesh, then it would use PEPCK. This is because in plants, PEPCK or PPDK are required for gluconeogenesis from malate, citrate, and isocitrate (Walker and Chen, 2002), and PPDK was not detected in blackberry. In addition to being used in the Krebs cycle and for gluconeogenesis, organic acids could be used for fermentation to ethanol, anthocyanin biosynthesis, and/or amino acid metabolism (Famiani et al., 2000; Farineau and Laval-Martin, 1977; Ruffner, 1982).

The metabolism of nitrogenous compounds such as amino acids and ammonium is an important process in fruit (Loulakakis and Roubelakis-Angelakis, 2001; Van Heeswijck et al., 2001), and central to this is the glutamine synthase/glutamate synthase cycle. In this cycle, GS catalyzes the incorporation of ammonium into glutamate to form glutamine. Glutamine is then converted to two glutamates by glutamate synthase. The plastidic form of GS is important in reassimilating ammonium released as a consequence of photorespiration in green tissues, whereas cytosolic GS is important in many plant tissues in producing glutamine to fuel biosynthetic pathways (Loulakakis and Roubelakis-Angelakis, 2001). Cytosolic AspAT functions in the conversion of glutamate to aspartate, which is required for the synthesis of many compounds such as the aspartate family of amino acids (Miesak and Coruzzi, 2002). Cytosolic GS, the smaller GS polypeptide, and cytosolic AspAT were present in the flesh, receptacle, pedicel, and seed of blackberries, and apart from in the seed, showed little difference in abundance during development (Fig. 5); similar results were obtained in the flesh and seed of grape (Famiani et al., 2000). The presence of these enzymes is in keeping with the pivotal position they occupy in nitrogen metabolism. Cytosolic aldolase can function in gluconeogenesis and in catalyzing a glycolytic flux; a significant proportion of the latter can be used in providing precursors for biosynthesis (Law and Plaxton, 1995; Plaxton, 1996). Cytosolic aldolase, the larger aldolase polypeptide, was present in the flesh, receptacle, and pedicel throughout ripening and showed little change in abundance (Fig. 5). Similarly, in some other fruit, a number of enzymes that function in glycolysis show little change in abundance during ripening (Law and Plaxton, 1995; Ruffner and Hawker, 1977). In blackberry leaves, cytosolic aldolase was detected in immature but not mature leaves (Fig. 4). In the seed, it was present early in ripening and then it was not detected (Fig. 5); a similar pattern was observed in grape seed (Famiani et al., 2000). In blackberry seeds, the decline in cytosolic aldolase and other enzymes is likely because they had reached maturity as shown by changes in their weight (Perkins-Veazie et al., 2000) and content of seed storage proteins (Fig. 5). During ripening, there is a large increase in weight of blackberry fruit (Fig. 1; Perkins-Veazie et al., 2000). However, there was little change in abundance of total protein per gram of fresh weight as shown by the intensity of staining of total polypeptides on gels (Fig. 5). This suggests that proteins were synthesized throughout ripening. It is likely that one function of PEPC, cytosolic GS, cytosolic AspAT, and cytosolic aldolase is providing substrates for growth.

Three enzymes involved in photosynthesis were studied, and these were plastidic GS, and the Calvin cycle enzymes RUBISCO and plastidic aldolase. RUBISCO was present in the flesh receptacle, pedicel, and seed of blackberries at early stages of ripening and, apart from in the pedicel in which it persisted, declined to undetectable amounts as ripening progressed (Fig. 5). A similar pattern was observed in grape flesh (Famiani et al., 2000). In contrast, in blackberry leaves, the abundance of RUBISCO greatly increased as the leaf matured (Fig. 4). In the flesh of blackberries, plastidic GS was only detected at stage 1 of development. Similarly, in tomato, plastidic GS disappears during ripening, while the cytosolic form persists (Gallardo et al., 1988). Plastidic GS was not detected in receptacle, pedicel, or seed of blackberries (Figs. 4 and 5). This was not a result of loss of plastidic GS after

extraction because when these tissues were coextracted with flesh at stage 1 of development, there was no loss of plastidic GS (data not presented). In pea, plastidic GS is absent or at very low abundance in the pod wall and seedcoat despite RUBISCO being present (Delgado-Alvarado et al., 2007). In blackberry leaves, the abundance of plastidic GS greatly increased as the leaf matured (Fig. 4). The abundance of plastidic GS in mature maize leaves was less than in that of blackberry and this is consistent with the observation that the abundance of plastidic GS is lower in the leaves of some C_4 plants than in C_3 plants (McNally et al., 1983). This may be because in leaves of C_4 plants, elevated CO_2 concentrations inhibit photorespiration, and plastid GS functions in reassimilating ammonium produced by this process (McNally et al., 1983). In bulky plant tissues such as fruit, concentrations of CO_2 can be high and those of O_2 low (Blanke and Lenz, 1989), and this raises the possibility that in the receptacle, pedicel, and seed, plastid GS is not present because photorespiration is low. Plastidic aldolase was detected in the flesh and pedicel but not in the receptacle or seed (Figs. 4 and 5). In the flesh, it declined during development, however, like RUBISCO it persisted in the pedicel. In blackberry leaves, the abundance of plastidic aldolase greatly increased as the leaf matured (Fig. 4). The absence of plastidic aldolase in the receptacle was not the result of loss after extraction because when these tissues were coextracted with flesh at stage 1 of development, there was no loss of plastidic aldolase (data not presented). The failure to detect plastidic aldolase in seeds could be because they were approaching maturity; however, the reason for its absence in the receptacle is unknown. It should be noted, however, that recent studies have shown that RUBISCO functions in a metabolic pathway without plastidic aldolase and the Calvin cycle in developing oilseed rape seeds (Schwender et al., 2004).

Conclusions

During the ripening of blackberries, a first group of enzymes show little change in abundance in the flesh and receptacle. These enzymes were PEPC, cytosolic GS, cytosolic aldolase, and cytosolic AspAT, and one function of these may be the provision of substrates necessary for growth and maintenance. A second group of enzymes—RUBISCO, plastidic GS, and plastidic aldolase—decreased to undetectable amounts in the flesh as ripening progressed. This, apart from a dilution effect caused by growth, likely reflects the decrease in photosynthetic capacity of the flesh that occurs during ripening of other fruit such as tomato (Piechulla et al., 1987). In contrast, these photosynthetic enzymes showed a very large increase in abundance during leaf development. The third group was enzymes that appeared during ripening, and in this study, only PEPCK belonged to this group. Previous work has provided evidence that this enzyme functions in the catabolism of organic acids in the flesh of some ripening fruit, and the appearance of PEPCK at the onset of the decrease in organic acid content suggests that it might function similarly in blackberry.

Literature Cited

Bahrami, A.R., Z.-H. Chen, R.P. Walker, R.C. Leegood, and J.E. Gray. 2001. Ripening-related occurrence of phosphoenolpyruvate carboxykinase in tomato fruit. Plant Mol. Biol. 47:499–506.

Blanke, M.M. and F. Lenz. 1989. Fruit photosynthesis. Plant Cell Environ. 12:31–46.

Chastain, C.J., J.P. Fries, J.A. Vogel, C.L. Randklev, A.P. Vossen, S.K. Dittmer, E.E. Watkins, L.J. Fiedler, S.A. Wacker, K.C. Meinhover, G. Sarath, and R. Chollet. 2002. Pyruvate, orthophosphate dikinase in leaves and chloroplasts of C_3 plants undergoes light-/dark-induced reversible phosphorylation. Plant Physiol. 128:1368–1378.

Chen, Z.-H., R.P. Walker, L.I. Técsi, P.J. Lea, and R.C. Leegood. 2004. Phosphoenolpyruvate carboxykinase in cucumber plants is increased both by ammonium and by acidification, and is present in the phloem. Planta 219:48–58.

Chen, Z.-H., R.P. Walker, R.M. Acheson, L.I. Técsi, A. Wingler, P.J. Lea, and R.C. Leegood. 2000. Are isocitrate lyase and phosphoenolpyruvate carboxykinase involved in gluconeogenesis during senescence of barley leaves and cucumber cotyledons? Plant Cell Physiol. 41:960–967.

Delgado-Alvarado, A., R.P. Walker, and R.C. Leegood. 2007. Phosphoenolpyruvate carboxykinase in developing pea seeds is associated with tissues involved in solute transport and is nitrogen responsive. Plant Cell Environ. 30:225–235.

Famiani, F., A. Baldicchi, A. Battistelli, S. Moscatello, and R.P. Walker. 2009. Soluble sugar and organic acid contents and the occurrence and potential role of phosphoenolpyruvate carboxykinase (PEPCK) in gooseberry (*Ribes grossularia* L.). J. Hort. Sci. Biotechnol. 3:249–254.

Famiani, F., N. Cultrera, A. Battistelli, V. Casulli, P. Proietti, A. Standardi, Z.-H. Chen, R.C. Leegood, and R.P. Walker. 2005. Phosphoenolpyruvate carboxykinase and its potential role in the catabolism of organic acids in the flesh of soft fruit during ripening. J. Expt. Bot. 421:2959–2969.

Famiani, F., R.P. Walker, L.I. Técsi, Z.-H. Chen, P. Proietti, and R.C. Leegood. 2000. An immunohistochemical study of the compartment of metabolism during the development of grape (*Vitis vinifera* L.) berries. J. Expt. Bot. 345:675–683.

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

Gallardo, F., S. Gálvez, M.A. Quesada, F.M. Cánovas, and I. Núñez de Castro. 1988. Glutamine synthetase activity during the ripening of tomato. Plant Physiol. Biochem. 26:747–752.

Green, A. 1971. Soft fruits, p. 375–410. In: A.C. Hulme (ed.). The biochemistry of fruits and their products. Vol. 2. Academic Press, London/New York.

Guillet, C., D. Just, N. Bénard, A. Destrac-Irvine, P. Baldet, M. Hernould, M. Causse, P. Raymond, and C. Rothan. 2002. A fruit-specific phosphoenolpyruvate carboxylase is related to rapid growth of tomato fruit. Planta 214:717–726.

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

Höpfner, M., G. Ochs, and A. Wild. 1990. Glutamine synthetase of green and etiolated leaves of *Sinapsis alba*. Evidence of the identity of the respective enzyme proteins. Planta 181:155–161.

Kafkas, E., M. Koşar, N. Türemiş, and K.H.C. Başer. 2006. Analysis of sugars, organic acids and vitamin C contents of blackberry genotypes from Turkey. Food Chem. 97:732–736.

Law, R.D. and W.C. Plaxton. 1995. Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit. Biochem. J. 307:807–816.

Lebherz, H.G., M.M. Leadbetter, and R.A. Bradshaw. 1984. Isolation and characterization of the cytosolic and chloroplast forms of spinach leaf fructose diphosphate aldolase. J. Biol. Chem. 259:1011–1017.

Leegood, R.C. and R.P. Walker. 1999. Phosphoenolpyruvate carboxykinase in plants: Its role and regulation, p. 201–211. In: J.A. Bryant, M.M. Burrel, and N.J. Kruger (eds.). Plant carbohydrate biochemistry. Bios Scientific, Oxford, UK.

Loulakakis, K.A. and K.A. Roubelakis-Angelakis. 2001. Nitrogen assimilation in grapevine, p. 59–85. In: K.A. Roubelakis-Angelakis

(ed.). Molecular biology and biotechnology of the grapevine.. Kluwer, London.
Manning, K. 1993. Soft fruit, p. 347–348. In: G.B. Seymour, J.E. Taylor, and G.A. Tucker (eds.). Biochemistry of fruit ripening.. Chapman and Hall, London.
McNally, S.F., B. Hirel, P. Gadal, A.F. Mann, and G.R. Stewart. 1983. Glutamine synthetases of higher plants. Plant Physiol. 72:22–25.
McNaughton, G.A.L., C.A. Fewson, M.B. Wilkins, and H.G. Nimmo. 1989. Purification, oligomerization state and malate sensitivity of maize leaf phosphoenolpyruvate carboxylase. Biochem. J. 261:349–355.
Miesak, B.H. and G.M. Coruzzi. 2002. Molecular and physiological analysis of *Arabidopsis* mutants defective in cytosolic or chloroplastic aspartate aminotransferase. Plant Physiol. 129:650–660.
Moing, A., C. Rothan, L. Svanella, D. Just, P. Diakou, P. Raymond, J.-P. Gaudillère, and R. Monet. 2000. Role of phosphoenolpyruvate carboxylase in organic acid accumulation during peach fruit development. Physiol. Plant. 108:1–10.
Morris, D.L. 1948. Quantitative determination of carbohydrates with Dreywoods anthrone reagent. Science 107:254–255.
Numazawa, T., S. Yamada, T. Hase, and T. Sugiyama. 1989. Aspartate aminotransferase from *Panicum maximum* Jacq. var. *trichoglume* Eyles, a C_4 plant: Purification, molecular properties, and preparation of antibody. Arch. Biochem. Biophys. 270:313–319.
Ochs, G., G. Schock, M. Tirscher, K. Kosemund, and A. Wild. 1999. Complexity and expression of the glutamine synthetase multigene family in the amphidiploid crop *Brassica napus*. Plant Mol. Biol. 39:395–405.
Perkins-Veazie, P., J.R. Clark, D.J. Huber, and E.A. Baldwin. 2000. Ripening physiology in 'Navaho' thornless blackberries: Color, respiration, ethylene production, softening, and compositional changes. J. Amer. Soc. Hort. Sci. 125:357–363.
Piechulla, B., R.E. Glick, H. Bahl, M. Anastasios, and W. Gruissem. 1987. Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. Plant Physiol. 84:911–917.
Plaxton, W.C. 1996. The organization of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47:185–214.
Ruffner, H.P. 1982. Metabolism of tartaric and malic acid in *Vitis*: A review. Part A. Vitis 21:346–358.
Ruffner, H.P. and J.S. Hawker. 1977. Control of glycolysis in ripening berries of *Vitis vinifera*. Phytochemistry 16:1171–1175.
Ruffner, H.P. and M.W. Kliewer. 1975. Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiol. 56:67–71.
Ruffner, H.P., W. Koblet, and D. Rast. 1975. Gluconeogenese in reifenden Beeren von *Vitis vinifera*. Vitis 13:319–328.
Schwender, J., F. Goffman, J.B. Ohlrogge, and Y. Schachar-Hill. 2004. Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature 432:779–782.
Spreitzer, R.J. 2003. Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch. Biochem. Biophys. 414:141–149.
Tosun, I., N. Sule Ustun, and B. Tekguler. 2008. Physical and chemical changes during ripening of blackberry fruits. Scientia Agricola (Piracicaba, Brazil) 65:87–90.
Tucker, G.A. 1993. Introduction, p. 1–51. In: J.B. Seymour, J. Taylor, and G.A. Tucker (eds.). Biochemistry of fruit ripening. Chapman and Hall, London.
Van Heesswijck, R., A.P. Stines, J. Grubb, I. Skrumsager Møller, and P.B. Høj. 2001. Molecular biology and biochemistry of proline accumulation in developing grape berries, p. 87–108. In: K.A. Roubelakis-Angelakis (ed.). Molecular biology and biotechnology of the grapevine. Kluwer, London.
Walker, R.P. and R.C. Leegood. 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. and Z.H. Chen. 2002. Phosphoenolpyruvate carboxykinase: Structure, function and regulation. Adv. Bot. Res. 38:93–189.
Walker, R.P., R.M. Acheson, L.I. Técsi, and R.C. Leegood. 1997. Phosphoenolpyruvate carboxykinase in C_4 plants: Its role and regulation. Aust. J. Plant Physiol. 24:459–468.
Walker, R.P., Z.-H. Chen, L.I. Técsi, F. Famiani, P.J. Lea, and R.C. Leegood. 1999. Phosphoenolpyruvate carboxykinase plays a role in interactions of carbon and nitrogen metabolism during grape seed development. Planta 210:9–18.
Walker, R.P., Z.-H. Chen, R.M. Acheson, and R.C. Leegood. 2002. Effects of phosphorylation on phosphoenolpyruvate carboxykinase from the C_4 plant, guinea grass. Plant Physiol. 128:165–172.
Walsh, C.S., J. Popenoe, and T. Solomos. 1983. Thornless blackberry is a climacteric fruit. HortScience 18:482–483.
Whiting, G.C. 1958. The non-volatile organic acids of same berry fruits. J. Sci. Food Agr. 9:244–248.
Wingler, A., R.P. Walker, Z.-H. Chen, and R.C. Leegood. 1999. Phosphoenolpyruvate carboxykinase is involved in the decarboxylation of aspartate in the bundle sheath of maize. Plant Physiol. 120:539–545.
Wrolstad, R.E., J.D. Culbertson, D.A. Nagaki, and C.F. Madero. 1980. Sugars and non-volatile acids of blackberries. J. Agr. Food Chem. 28:553–558.