

An immunohistochemical study of the compartmentation of metabolism during the development of grape (*Vitis vinifera* L.) berries

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Abstract

The compartmentation of key processes in sugar, organic acid and amino acid metabolism was studied during the development of the flesh and seeds of grape (*Vitis vinifera* L.) berries. Antibodies specific for enzymes involved in sugar (cell wall and vacuolar invertases, pyrophosphate:fructose 6-phosphate phosphotransferase, aldolase, NADP-glyceraldehyde-P dehydrogenase, cytosolic fructose 1,6-bisphosphatase), photosynthesis (Rubisco, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase), amino acid metabolism (cytosolic and mitochondrial aspartate aminotransferases, alanine aminotransferase, glutamate dehydrogenase, glutamine synthetase), organic acid metabolism (phosphoenolpyruvate carboxylase, NAD- and NADP-dependent malic enzyme, ascorbate peroxidase), and lipid metabolism (acetyl CoA carboxylase, isocitrate lyase) were used to determine how their abundance changed during development. There were marked changes in the abundance of many of these enzymes in both the flesh and seeds. The inter-cellular location of some enzymes was investigated using immunohistochemistry. Several enzymes (e.g. phosphoenolpyruvate carboxylase and those involved in amino acid metabolism) were associated with tissues likely to function in the transport of imported assimilates, such as the vasculature. Although other enzymes (e.g. NADP-malic enzyme and soluble acid invertase, involved in the metabolism of sugars and

organic acids) were largely present in the parenchyma cells of the flesh, their distribution was extremely heterogeneous. This study shows that when considering the metabolism of complex structures such as fruit, it is essential to consider how metabolism is compartmentalized between and within different tissues, even when they are apparently structurally homogeneous.

Key words: *Vitis vinifera*, fruit, invertase, NADP-malic enzyme, phosphoenolpyruvate carboxylase, glutamine synthetase.

Introduction

The development of grape berries, like many other fruit, is accompanied by large changes in the content of sugars and organic acids. Organic acids, but little non-structural carbohydrate, accumulate before ripening (Ruffner and Hawker, 1977; Davies and Robinson, 1996). Ripening, which starts 6–8 weeks after full bloom (50% of flowers open), lasts for 4–10 weeks, depending on the cultivar. Several processes occur during ripening. The berry softens and it begins to accumulate soluble carbohydrate (Davies and Robinson, 1996), the organic acid content decreases (Ruffner and Hawker, 1977), the content of free amino acids increases (Kliwer, 1968) and, in red grape varieties, the skin becomes coloured due to the accumulation of substances such as anthocyanins and flavonols (Kanellis and Roubelakis-Angelakis, 1993; Boss *et al.*, 1996).

In grapes the major forms of stored carbohydrate are

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Abbreviations: GS, glutamine synthetase; PEPC, PEP carboxylase; NADP-ME, NADP-malic enzyme.

glucose and fructose, which are derived mainly from imported sucrose (Kanellis and Roubelakis-Angelikis, 1993). Vacuolar acid invertase is the major sucrose-degrading enzyme involved in sucrose breakdown (Hawker, 1969a; Davies and Robinson, 1996). A substantial amount of acid invertase activity is present in grapes throughout development and it appears that, before véraison (the start of ripening), hexoses produced by the action of vacuolar acid invertase are metabolized, but that after véraison this utilization is greatly reduced and hexoses accumulate. The observation that glycolysis becomes inhibited after véraison supports this view (Ruffner and Hawker, 1977; Robinson *et al.*, 1997).

The organic acid content increases up to véraison and then declines (Kanellis and Roubelakis-Angelikis, 1993). The content of organic acids is determined by a balance between their synthesis and degradation. For example, there is a decrease in PEPC activity, an enzyme involved in malate synthesis, during ripening (Hawker 1969b). Malate, the major organic acid in many grape cultivars, has been proposed to be degraded by both cytosolic NADP-malic enzyme (Ruffner *et al.*, 1984) and PEP carboxykinase (PEPCK) (Ruffner and Kliewer, 1975). It is thought that malate metabolized by NADP-malic enzyme is utilised in biosynthesis and respiration (Ruffner *et al.*, 1984), whereas that metabolized by PEPCK is utilized in gluconeogenesis (Ruffner *et al.*, 1975).

Although these studies illustrate changes in the abundance of various enzymes during the development of grape berries, little attention has been paid to their location and, when such studies have been done in fruits, they have usually been limited to measurement of enzymes in dissected tissues (Seymour *et al.*, 1993). This approach inevitably suffers from a lack of resolution. For example, many studies on the metabolism of grape flesh have involved measuring the activity of various enzymes in extracts of whole berries and have ignored the contribution of the activity present in the seed, which can be substantial (Walker *et al.*, 1999).

The aims of this study of grape berries were 2-fold. Firstly, to use antisera, each specific for an enzyme which is indicative of the operation of an important metabolic processes, to probe changes in the enzymic capacity of grape berries harvested throughout development. Secondly, these antisera were used, in conjunction with immunohistochemistry, to determine the location of a selection of these enzymes in order to relate structure and function in the developing fruit.

Materials and methods

Plant material

Berries of grape (*Vitis vinifera* L.) of the cv. Pinot noir were collected throughout the 1996 season from vines growing in the vineyard of the University of Perugia, Italy. Berries were also

collected during 1995 from the cv. Black Hamburg growing in a greenhouse in Bolton, England. At each sampling, healthy berries from different bunches and from different parts of the bunches were collected. Seeds were separated from berries.

Measurement of fresh and dry weights

Fruit unit weight was determined by weighing three samples of 10 berries each. Then seeds were removed and seeds and deseeded berries were weighed either immediately or after drying them at 105 °C in a forced-air oven to constant weight.

SDS-PAGE, immunoblotting and immunohistochemistry

Deseeded berries: Approximately 10 g of deseeded berries were ground in a mortar containing liquid nitrogen and the powder stored at -80 °C. Then 0.5 g of nitrogen powder were mixed, with a mortar and pestle, with 800 µl of ice-cold 0.5 M AMPS (pH 10.8), 1% (w/v) SDS, 1% (w/v) PEG-6000, and 25 mM DTT. The extract was centrifuged at 12 000 g for 5 min, then 300 µl of clarified homogenate was added to 1.2 ml of 80% (v/v) acetone and placed in liquid nitrogen for 10 min. After thawing, the mixture was centrifuged at 12 000 g for 10 min and the pellet resuspended in 75 µl 62.5 mM TRIS-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue (solubilization buffer). For cell wall invertase, the pellet was washed with 1 ml of extraction buffer and centrifuged at 12 000 g for 5 min. This procedure was repeated and the pellet was resuspended in 400 µl of 1 M NaCl, 20 mM K phosphate, pH 7.0, placed on a shaker at 25 °C for 1 h and centrifuged at 12 000 g for 5 min. Assay of the pellet before washing and of the pellet after washing showed recovery of invertase activity within 10% of the expected values.

Seeds: Approximately 10 g of seeds were ground in a mortar containing liquid nitrogen and the powder stored at -80 °C. Then 0.05 g of nitrogen powder were mixed, with a mortar and pestle, with 400 µl of ice-cold 0.5 M AMPS (pH 10.8), 1% (w/v) SDS, 1% (w/v) PEG-6000, and 50 mM DTT. The extract was centrifuged at 12 000 g for 5 min. For soluble protein, 150 µl of clarified homogenate was added to 600 µl of 80% (v/v) acetone and placed in liquid nitrogen for 10 min. Protein in the supernatant was precipitated by acetone as described for soluble protein. For both samples, after thawing, the mixture was centrifuged at 12 000 g for 5 min and the pellet resuspended in 100 µl 62.5 mM TRIS-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue (solubilization buffer). Cell wall invertase was extracted as described above.

After resuspension of the pellet in the solubilization buffer both deseeded berry and seed 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 5 min.

SDS-PAGE and immunoblotting were done as described previously (Walker and Leegood, 1996). For SDS-PAGE, 20 µl of sample containing approximately 20 µg of protein was loaded onto each track of the gel. Anti-rabbit peroxidase (diluted 1/1000) was used in conjunction with an ECL kit (Amersham, UK) to visualize immunoreactive polypeptides. All antibodies were polyclonal, raised in rabbit and were used at a dilution of 1/1000, except for that against pyrophosphate:fructose 6-phosphate phosphotransferase, which was used at a dilution of 1/50. Immunohistochemistry was performed as described previously (Walker *et al.*, 1997), using antibodies at a dilution of 1/1000. In immunoblot studies, the specificity of the antibody for the target polypeptide was assessed by ensuring that it

cross-reacted specifically with a polypeptide of the correct molecular mass. In addition, samples of tissue known to contain the target antigen at high abundance were assessed alongside samples of grape tissue.

Carbohydrate and malate extraction and measurements

50 mg of frozen powder of deseeded berries were extracted in 1.5 ml of 80% ethanol and 20% water containing 100 mM HEPES-KOH (pH 7.1) and 20 mM $MgCl_2$, for 1 h at 80 °C. After cooling to room temperature, the extract was centrifuged at 12 000 g for 5 min. The supernatant was recovered and after adding 150 μ l of charcoal suspension (100 mg ml^{-1}) it was stirred and centrifuged at 12 000 g for 5 min. The supernatant was stored at -20 °C until required.

Glucose, fructose and sucrose were measured using an enzyme-coupled method described previously (Jones *et al.*, 1977), with minor modifications. The assay mixture was 100 mM HEPES-KOH (pH 7.0), 5 mM $MgCl_2$, 0.5 mM DTT, 0.02% (w/v) BSA, 100 mM ATP, and 40 mM NAD^+ . Glucose was measured initiating the reaction with 3 U of hexokinase (from yeast) and 1 U of glucose-6-phosphate dehydrogenase (from *Leconostoc mesenteroides*). Fructose and sucrose were analysed in sequence after glucose, following the addition to the assay mixture of 1 U of phosphoglucose isomerase (from yeast) and 100 U of invertase (from yeast), respectively.

Malate was measured using the enzyme-coupled method (Lowry and Passonneau, 1972). The assay mixture contained, in 1 ml: 50 mM 2-amino-2-methylpropanol and 40 mM glutamate (pH 9.9), and 1 mM NAD^+ . The reaction was initiated by adding 10 U of glutamate oxalacetate transaminase (from pig heart) and 0.7 U of malate dehydrogenase (from pig heart) to the assay mixture. All coupling enzymes were from Boehringer, Mannheim.

Protein determination

Protein was determined by the Lowry method (as described by Walker *et al.*, 1995).

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 (*Zea mays*) leaves (Langdale *et al.*, 1988). The antiserum specific for soluble acid invertase was a gift from A Sturm (Basel) and was raised against carrot (*Daucus carota*) isoform II which had been over-expressed in *E. coli*. The antiserum specific for alanine aminotransferase was raised against AlaAT purified from barley (*Hordeum vulgare*) roots (Good and Muench, 1992). The antiserum specific for cytosolic aspartate aminotransferase was raised against purified AspAT from leaves of *Panicum maximum* (Numazawa *et al.*, 1989). The antiserum specific for the α -CT subunit of acetyl CoA-carboxylase was raised against the enzyme from pea (Shorrosh *et al.*, 1996). The following antisera were also gifts: plastidic aldolase and NADP-glyceraldehyde 3-phosphate dehydrogenase (K-H Süß, Gatersleben), SBPase and plastidic FBPase (T Dyer, Cambridge), soybean ascorbate peroxidase (D Dalton, Portland), potato (*Solanum tuberosum*) tuber pyrophosphate:fructose 6-phosphate phosphotransferase (W Plaxton, Kingston), spinach leaf cytosolic FBPase (J Daie, Wisconsin) and mitochondrial aspartate aminotransferase (M Taniguchi, Nagoya), potato tuber pyrophosphate:fructose 6-phosphate phosphotransferase (W Plaxton, Kingston, Canada), *Amaranthus tricolor* NAD-malic enzyme (JO Berry, Buffalo, USA), and glutamate dehydrogenase (CA Loulakakis, Crete).

The antibody against glutamine synthetase 1 from *Sinapsis alba* was a gift from G Ochs (Mainz). This antibody also recognized GS2. The antiserum to PEP carboxylase was raised against the enzyme from *Amaranthus edulis* leaves and that to Rubisco against the enzyme from *Brassica napus* leaves.

Results

Marked changes occur in the abundance of many enzymes during the development of grape berries

The developmental stage of grape berries was characterized by measuring their fresh and dry weights and content of malate and soluble sugars. In common with previous studies, little soluble sugar, but substantial amounts of malate, accumulated during the first phase of development (0–50 d after full bloom). After véraison, at approximately 50 d, malate decreased, whereas approximately equimolar amounts of glucose and fructose accumulated, but little sucrose (Fig. 1). As reported previously (Coombe, 1973), véraison also marked an increase in the rate of berry growth (Fig. 1).

Immunoblotting, in conjunction with specific antisera, was used to determine how the abundance of a range of enzymes, each indicative of the operation of a key metabolic process, changed during the development of both the flesh and seed of grape. It is difficult to prepare

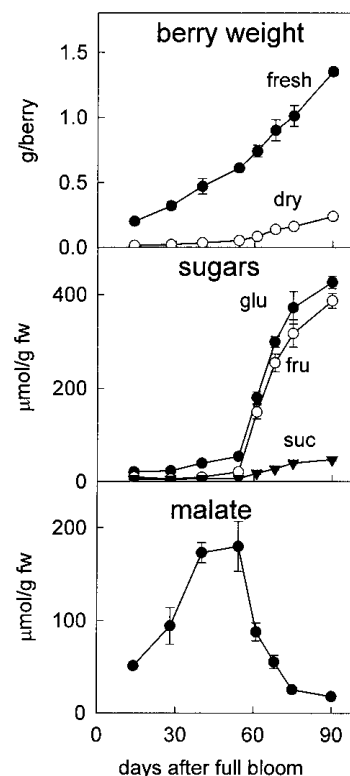


Fig. 1. Changes in the fresh and dry weight of grape flesh together with content of soluble sugars and malate during development. The values represent means \pm SE of three different samples or extracts.

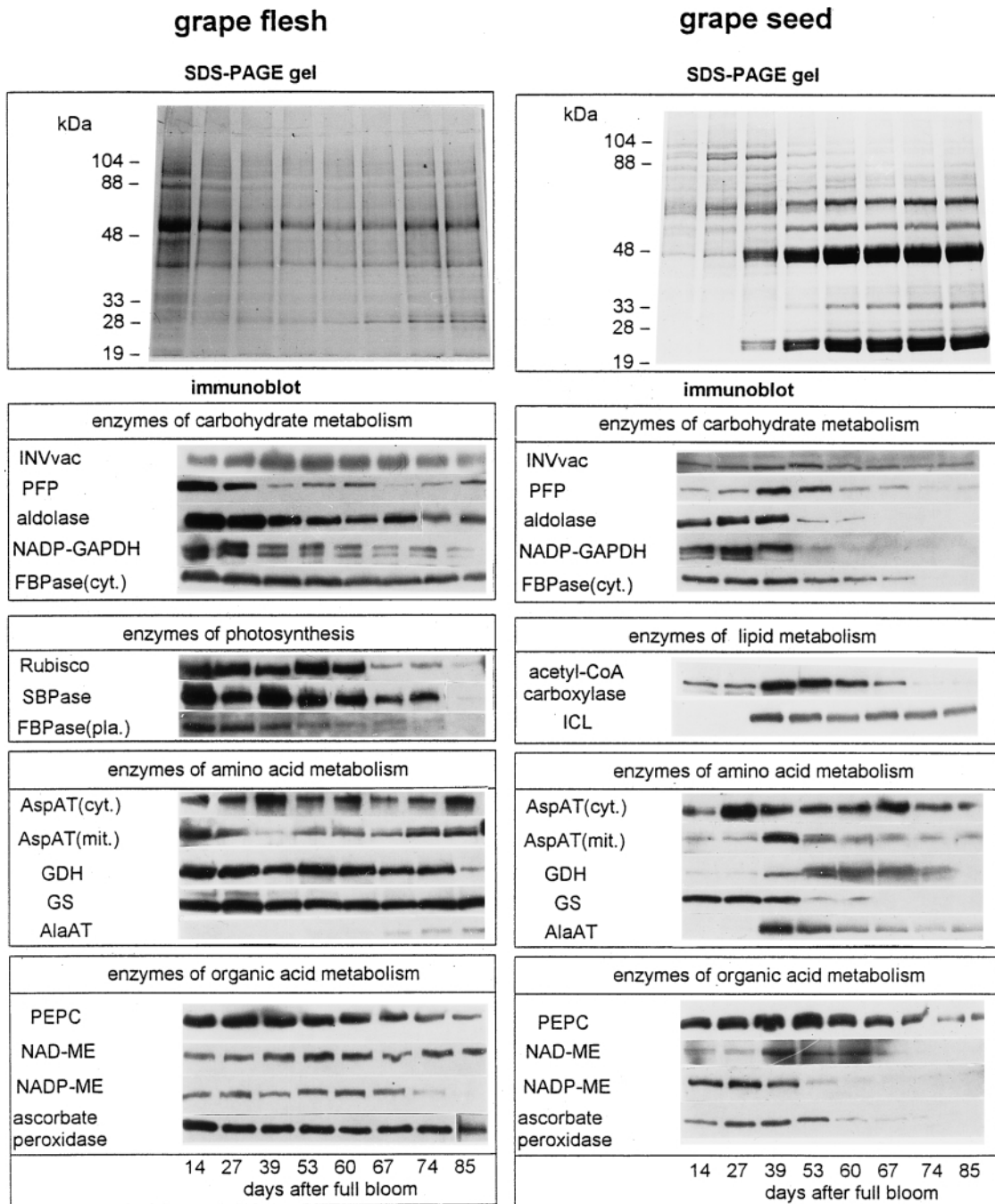


Fig. 2. The abundance of many enzymes changes during the development of grape berries. Extracts of grape pulp or seed were prepared from berries at different times after flowering and subjected to SDS-PAGE. After transfer of the fractionated polypeptides to Immobilon-P membrane enzyme protein was detected using specific antisera.

proteins for electrophoresis from grapes because of the abundance of phenolics, their high acidity and low protein content (Ruffner *et al.*, 1990; Tattersall *et al.*, 1997). Optimization of the preparation of samples for SDS-PAGE showed that it was necessary to use a large volume of extraction buffer at a high concentration to counteract the acidity of young berries, and to include PEG to mitigate the high phenolic content. Due to the low amount

of protein in grape flesh it was necessary to concentrate protein in extracts by acetone precipitation.

SDS-PAGE analysis of the flesh showed only a few major changes in polypeptide composition during development. The most prominent change was the appearance of several low molecular mass polypeptides, between 20 and 35 kDa, as the berry reached maturity (Fig. 2). The abundance of several enzymes involved in photosyn-

thesis and carbohydrate metabolism decreased during development (plastidic aldolase, NADP-glyceraldehyde 3-phosphate dehydrogenase, Rubisco, sedoheptulose 1,7-bisphosphatase [SBPase], plastidic fructose 1,6-bisphosphatase). Similarly, pyrophosphate:fructose 6-phosphate phosphotransferase, an enzyme likely to be involved in catalysing the glycolytic flux, together with PEPC and NADP-ME, which are involved in organic acid metabolism, also declined during ripening. Other enzymes such as vacuolar invertase, NAD-ME, ascorbate peroxidase, and glutamine synthetase showed little change in abundance. The abundance of several other enzymes involved in amino acid metabolism increased during the later stages of ripening (notably alanine aminotransferase and mitochondrial aspartate aminotransferase).

In contrast, SDS-PAGE analysis of the seeds showed major changes in polypeptide composition during development. In particular, several polypeptides of molecular mass 65, 55, 46, 33, and 23 kDa increased during development from undetectable amounts to become the major protein components of the seed (Fig. 2). These are likely to be storage proteins. Several enzymes likely to be involved in their synthesis (PEP, acetyl CoA carboxylase, mitochondrial aspartate aminotransferase, alanine aminotransferase, glutamate dehydrogenase, and NAD-ME) were most abundant during the period of accumulation of these proteins (Fig. 2). Another group of enzymes (aldolase, NADP-glyceraldehyde-P dehydrogenase, cytosolic FBPase, glutamine synthetase, NADP-ME, and ascorbate peroxidase) were most abundant early in seed development.

Many enzymes are localized in specific tissues and their distribution changes during development

The location of a selection of these enzymes was investigated using immunohistochemistry. The antibodies used in immunohistochemistry, the cell wall and vacuolar invertases, PEP carboxylase, NADP-malic enzyme, and glutamine synthetase, were entirely specific on Western blots of grape flesh extracts (Fig. 3). It was shown that preimmune serum, taken from the rabbit before immunization, gave no signal on immunoblots or in sections for PEP carboxylase and the invertases (data not shown). Figure 4 shows the structure of a grape berry about 28 d after full bloom, showing the pericarp, with its associated vasculature and the developing seeds within the locular cavity. PEPC was present in several tissues of a young berry (10 d after full bloom). At low magnification (Fig. 4B) PEPC can be seen in the vasculature, the parenchyma cells of the pericarp and within the developing seeds. Early in seed development PEPC was associated with the inner layer of the outer integument (Fig. 4B). In an older berry (80 d after full bloom) PEPC was particularly associated with the vasculature (Fig. 4C, D) and with cells underlying the epidermis of the pericarp (Fig. 4D).

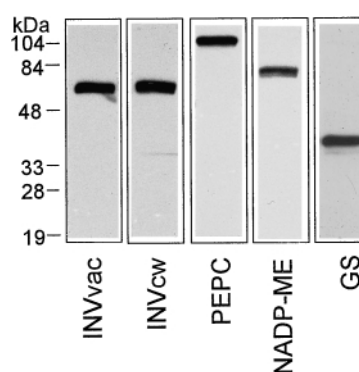


Fig. 3. Western blot of grape seed extracts probed with antisera to vacuolar and cell wall invertases, PEP carboxylase, NADP-malic enzyme and glutamine synthetase. Extracts, each containing 20 µg of protein, were subjected to SDS-PAGE and, after transfer of the fractionated polypeptides to Immobilon-P membrane, proteins were detected using a specific antiserum.

Similarly, NADP-ME was also associated with the pericarp, particularly with the vasculature (Fig. 4E), and with the seeds. Early in seed development it was associated with the inner layer of the outer integument and the inner layer of the nucellus surrounding the developing endosperm (Fig. 4F). Glutamine synthetase was also found in the vasculature in the pericarp (Fig. 4G), together with glutamate dehydrogenase, cytosolic and mitochondrial aspartate aminotransferases and the amino acid, glutamate (data not shown).

The distribution of both cell wall and vacuolar acid invertases is shown in Fig. 5. Vacuolar invertase was present in both pericarp and seeds of grape berries 10 d after full bloom. At this stage of development the structure of the pericarp was not uniform (Fig. 5A, D). Some clumps of parenchyma cells contained crystalline inclusions (c) which were particularly marked towards the epidermis (Fig. 5D). Other clumps of these cells were free of crystals (nc) and contained vacuolar invertase. Vacuolar invertase was also present in the vasculature, the cells lining the locular sac and within the young seed it was associated with the developing seed coat (Fig. 5B). On the other hand, cell wall invertase was associated with the cells containing crystals, particularly within the ring of vascular bundles (Fig. 5C, D) and with the vasculature, being present in the xylem in the pericarp in young fruits (Fig. 5C, D, E). Later in berry development (28 d after full bloom), at a stage similar to that shown in Fig. 3, cell wall invertase was also associated with the xylem (Fig. 5F) and with the layer of palisade cells which form the inner layer of the outer integument of the seed (Fig. 5G; Walker *et al.*, 1999). At later stages of development (50 d after full bloom), vacuolar invertase was present throughout the parenchyma cells of the pericarp, but was particularly abundant in a layer of cells underlying the epidermis (Fig. 5H). In contrast, cell wall invertase was localized in the layer of cells underlying the epidermis (Fig. 5I).

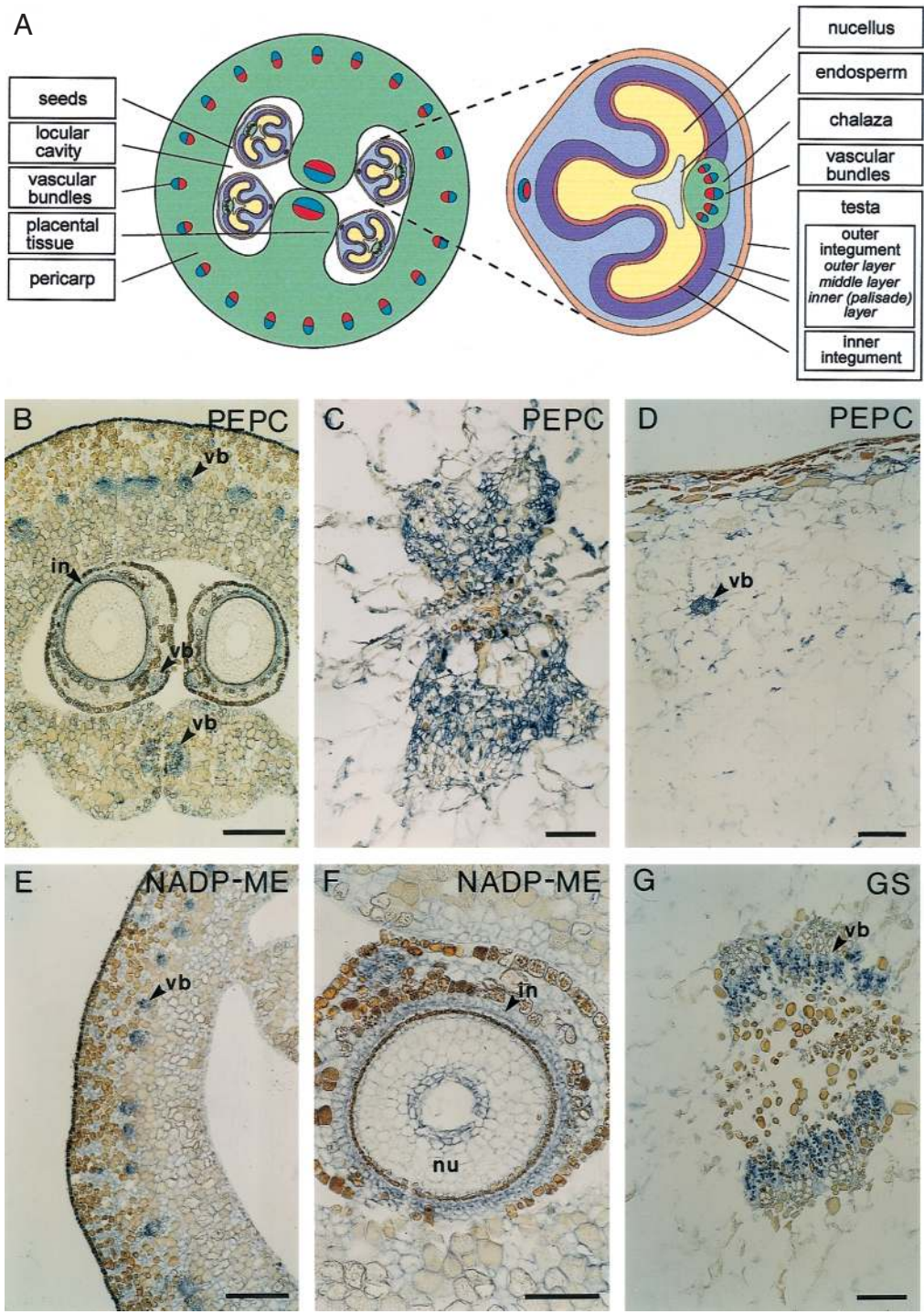


Fig. 4. Structure of grape seed and berry (A) and location of enzymes in developing grape berries (B–G). Transverse sections of fruits were cut and enzymes visualized using a specific antiserum. The blue coloration indicates the presence of the enzyme. (B, E, F) Fruits 10 d after anthesis; (C, D) fruits 80 d after anthesis; in, inner integument of developing seed; vb, vascular bundle; nu, nucellus. Scale bar = 200 μ m (B–E), 100 μ m (F), 80 μ m (G).

Discussion

The aim of this work was to investigate the importance of compartmentation of enzymes between and within different tissues in the metabolism of developing grape berries. In this study, this question was addressed by

observing the distribution of a range of enzymes that acted as markers for different metabolic processes in order to allow future studies of their function.

The only major change in the protein composition of grape flesh revealed by SDS-PAGE was the appearance of several low molecular weight polypeptides during the

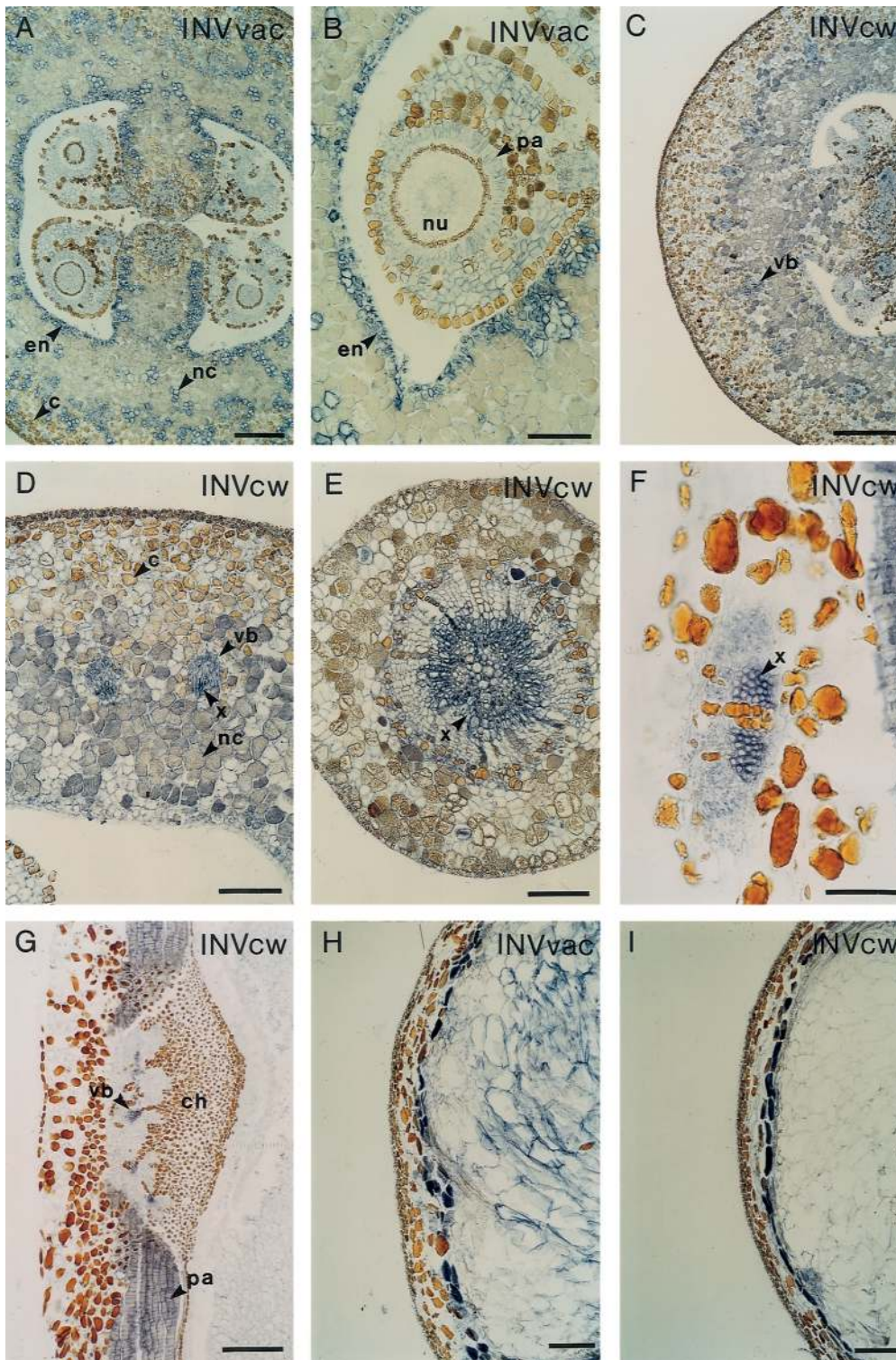


Fig. 5. Location of vacuolar (INVvac) and cell wall (INVcw) invertases in a developing grape berries. Transverse sections of fruits, 28 d after anthesis, were cut and enzymes visualized using a specific antiserum. The blue coloration indicates the presence of the enzyme. (A–E) Fruits 10 d after anthesis; (F, G) fruits 28 d after anthesis; (H, I) fruits 50 d after anthesis; ch, chalaza; nu, nucellus; pa, palisade layer of inner integument of seed; vb, vascular bundle; x, xylem; c, crystal-containing cells; nc, non-crystal-containing cells; en, endocarp. Scale bar = 100 μ m (A, C–F), 80 μ m (G), 50 μ m (B), 200 μ m (H, I).

latter stages of ripening (Fig. 2). These low molecular mass polypeptides may be a thaumatin-like protein (Tattersall *et al.*, 1997) and chitinase (Robinson *et al.*, 1997) which may play a role in the defence of the berry against pathogens. A number of enzymes involved in photosynthetic carbohydrate metabolism, such as Rubisco and plastidic fructose-1,6-bisphosphatase, declined in the flesh during development. This is consistent with previous studies showing that the photosynthetic capacity of grapes decreases as the berry develops (Pandey and Farmahan, 1977). At the onset of ripening glycolysis is inhibited (Ruffner and Hawker, 1977), in keeping with this pyrophosphate:fructose 6-phosphate phosphotransferase, an enzyme thought to play a role in catalysing the glycolytic flux (Kruger, 1997), decreased. Several enzymes involved in amino acid metabolism increased during the later stage of ripening. This is consistent with the observation that the abundance of free amino acids in grape flesh increases several-fold during the later stages of ripening (Kanellis and Roubelakis-Angelakis, 1993).

Activities of enzymes of organic acid and sugar metabolism have previously been measured in grape berries and, in agreement with these, PEPC was most abundant in the flesh early in berry development, which is consistent with a role in the synthesis of organic acids (Hawker, 1969*b*). During ripening, the organic acid content of the flesh decreased. This may be brought about by either PEPCK or by the malic enzymes. NADP malic enzyme was most abundant during the early part of ripening (Ruffner *et al.*, 1984), but at this stage of development PEPCK and NAD-ME were also present and these could also play a role in dissimilation of organic acids. The observation that NAD-ME is present in grape berries is in contrast to a previous finding that mitochondrial NAD-malic enzyme is absent from grape berries (Romieu *et al.*, 1992). However, this comparatively simple picture becomes more complex when the compartmentation of these enzymes is considered. Even within the flesh itself, there was a clear zonation at 10 d (Figs 4, 5). In the case of PEPC, the enzyme was enriched in the inner part of the flesh, within the ring of vascular bundles. In the case of NADP-malic enzyme, the enzyme was clearly present in certain groups of cells which do not contain crystals, as is PEPCK (data not shown).

Soluble acid invertase was high throughout development, which is consistent with previous studies in which its activity has been measured (Davies and Robinson, 1996), but it also indicates that it may play more than one role. This view is supported by the observation that, although the acid invertases were also associated with the parenchyma cells of the flesh, the distribution was not homogeneous. A similar situation occurs in cucumber cotyledons and petioles where both soluble and cell wall acid invertase are localized in specific cell types (Kingston-Smith *et al.*, 1999). Early in grape berry development the

parenchyma cells all possess crystalline inclusions which progressively disappear as the berry develops (except below the skin). Early in development vacuolar acid invertase was clearly confined to non-crystal containing cells whereas cell wall invertase was present in the cells that contained crystals. It could be that the crystalline inclusions located in the vacuole make it difficult for the vacuolar enzyme to function. As the berry matured, cell wall invertase disappeared from the parenchyma cells except in a layer of pigment-containing cells in the skin. In contrast the vacuolar enzyme was abundant in both these cells and the parenchyma cells of the flesh. Similarly, PEPCK is also enriched in these cells, where it might play a role in generating the PEP used in the synthesis of secondary metabolites. The observation that these enzymes are also enriched in the glandular cells of certain trichomes in tobacco and cucumber, which are known to synthesize a variety of antimicrobial secondary metabolites, and in the oil ducts of *Clusia* spp. (Leegood *et al.*, 1999; Borland *et al.*, 1998).

It is clear from the immunolocalization studies that many other enzymes showed an extremely heterogeneous distribution. PEPC, NADP-ME and glutamine synthetase were all associated with the vasculature in both the seed and the flesh. These enzymes are often associated with the vasculature in other plants such as cucumber cotyledons and in maize kernels (data not shown). The presence of cytosolic glutamine synthetase in the phloem is well documented (Edwards *et al.*, 1990). These observations suggest that extensive metabolism of assimilates occurs during their delivery to sink tissues and that these enzymes may play an important role in this process. This is further substantiated by the presence of these enzymes in the tissues associated with assimilate transfer into the seed at the appropriate time during seed development (Walker *et al.*, 1999).

In conclusion, the results of this study show that, when considering the metabolism of a complex structure, such as a fruit, or the functions of an individual enzyme within such a structure, it is important to consider compartmentation of metabolism between different types of tissues and cells. It is clear that, in grape, many of these enzymes have little to do with bulk changes of sugars and organic acids in the flesh and may be associated with the transfer of assimilates into the fruit and its developing seeds. Even within a tissue that appears anatomically homogeneous, such as the pericarp, there is striking heterogeneity in the distribution of invertases. The increasing availability of antibodies specific for different plant proteins and their use in immunohistochemical studies should allow the locations of many plant proteins to be explored in more detail and allow the compartmentation of metabolism between and within different cell types in a tissue to be more clearly understood.

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