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Sugar Accumulation in Grape Berries

Cloning of Two Putative Vacuolar Invertase cDNAs and Their Expression in Grapevine Tissues

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During grape berry (*Vitis vinifera* L.) ripening, sucrose transported from the leaves is accumulated in the berry vacuoles as glucose and fructose. To study the involvement of invertase in grape berry ripening, we have cloned two cDNAs (GIN1 and GIN2) from berries. The cDNAs encode translation products that are 62% identical to each other and both appear to be vacuolar forms of invertase. Both genes are expressed in a variety of tissues, including berries, leaves, roots, seeds, and flowers, but the two genes have distinct patterns of expression. In grape berries, hexose accumulation began 8 weeks postflowering and continued until the fruit was ripe at 16 weeks. Invertase activity increased from flowering, was maximal 8 weeks postflowering, and remained constant on a per berry basis throughout ripening. Expression of GIN1 and GIN2 in berries, which was high early in berry development, declined greatly at the commencement of hexose accumulation. The results suggest that although vacuolar invertases are involved in hexose accumulation in grape berries, the expression of the genes and the synthesis of the enzymes precedes the onset of hexose accumulation by some weeks, so other mechanisms must be involved in regulating this process.

The growth pattern of developing grape berries (*Vitis vinifera* L.) can be described as a double sigmoidal curve with an initial rapid increase in size followed by a lag period during which berry volume does not increase. The lag period is followed by a second phase of growth during which ripening occurs, and viticulturists use the French word *véraison* to describe the inception of berry ripening. During ripening, the increase in volume is accompanied by an increase in berry softness, accumulation of hexoses in the berries, and a decrease in the level of malic and tartaric acids, and in red grape varieties the skin becomes colored due to the accumulation of anthocyanins (Coombe, 1992).

The accumulation of sugar in the form of Glc and Fru within the vacuole is one of the main features of the ripening process in grape berries and is a major commercial consideration for the grape grower, winemaker, and dried fruit producer. Sugar accumulation in climacteric fruit has received considerable attention, but little is known about this process in nonclimacteric fruit such as grapes. In grapevines, Suc produced as a result of photosynthesis in the leaf is transported via the phloem to the berry (Swanson and Elshishiny, 1958), where it is cleaved to Glc and Fru, which accumulate in roughly equal amounts (Kliewer, 1965). The accumulation of Fru and Glc commences only at *véraison* and continues throughout ripening.

Invertase (β-fructosidase; EC 3.2.1.26) catalyzes the conversion of Suc to its monosaccharide constituents Glc and Fru and is probably responsible for this reaction in grape berries as it is in other species that store sugar in this way. In tomato, species that accumulate high levels of Glc and Fru, for example *Lycopersicon esculentum* Mill., have high levels of invertase activity and store little Suc (Yelle et al., 1991; Stommel, 1992). In contrast, accumulators of high levels of Suc (and low levels of Glc and Fru), such as *Lycopersicon peruvianum* and *Lycopersicon chmielewskii*, have low levels of vacuolar invertase. Further evidence for a role for vacuolar invertase in controlling the composition of sugars in tomato fruit has recently been provided by antisense (Ohyama et al., 1995) and breeding experiments (Chetelat et al., 1995).

A number of distinct invertases have been characterized in plants. Most reports deal with either soluble invertases, which have acidic pI values and acid pH optima and are located in the vacuole, or with insoluble acid invertases, which have basic pI values and are bound to the cell wall (Sturm and Chrispeels, 1990). It is thought that the vacuolar invertases are likely to be important in the regulation of hexose levels in certain tissues, e.g. in fruit tissue (Lingle and Dunlop, 1987), and in the utilization of Suc stored in the vacuoles (Leigh et al., 1979). The cell-wall forms are associated with rapidly growing tissues (Eschrich, 1980), are induced by wounding and pathogenic attack (Sturm and Chrispeels, 1990), and have been implicated in phloem unloading and source/sink regulation (Eschrich, 1980; Roitsch et al., 1995).

Hawker (1969a) found that invertase enzyme activity in Sultana berries increased immediately after flowering and that the activity peaked 6 to 7 weeks later, at *véraison*, when

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Abbreviations: °Brix, refractive index measure of total soluble solids; RACE, rapid amplification of cDNA ends.

the rapid accumulation of hexoses commenced. Another enzyme that might be involved in the breakdown of Suc, Suc synthase, also increased at *véraison*, but even at its maximal level the activity was low (200–300 times less in Sultana) compared to the level of invertase activity (Hawker, 1969a).

The proportion of soluble versus insoluble invertase activity in grape berry extracts varies depending on the composition of the extraction buffer. Most of the invertase activity present is in a soluble form provided that steps are taken to minimize the formation of insoluble protein-tannin complexes by using extraction buffers, including PEG, borate, pH 8.5, Na_2CO_3, or nonionic detergents (Hawker, 1969b; Ishikawa et al., 1989; Ruffner et al., 1995). A similar ratio of soluble to insoluble forms of invertase is found in *L. esculentum* fruit, where only about 1% of the total activity is present in the insoluble fraction (Ohyama et al., 1995). Four soluble acid invertase isoenzymes, recognized by their different separation characteristics on DEAE cellulose and concanavalin A-Sepharose, have been recognized in grape berries (Ishikawa et al., 1989). Various workers have purified soluble berry invertase to homogeneity (Nakanishi et al., 1991; Porntaveewat et al., 1994; Ruffner et al., 1995). Soluble berry invertase is glycosylated, has an acid pH optimum, and is resistant to high temperatures and acid pH conditions. The pI of a soluble invertase present in Semillon grape berry juice has been determined to be acidic (Nakanishi et al., 1991), which may indicate that this enzyme is localized in the vacuole of grape berry cells.

We have used molecular techniques to investigate the relationship between hexose accumulation in grape berries and the expression of two grape vacuolar invertase genes. The putative invertase cDNAs were cloned from Sultana berry cDNA using PCR techniques. Analysis of the sequences of these clones indicates that the proteins they encode are likely to be localized in the vacuole of grape berry cells. The patterns of expression of these two genes are consistent with invertase playing a role in hexose accumulation in grape berries during ripening, but invertase is synthesized well before hexose accumulation commences.

MATERIALS AND METHODS

Grape Tissue Sampling and Measurement of Berry Development

Tissue samples (except roots) from *Vitis vinifera* L. cv's Riesling and Shiraz were collected from John Harvey's Slate Creek vineyard (Willunga, South Australia) in the 1993/1994 and 1994/1995 seasons, respectively. Two hundred twenty-eight bunches in similar positions on 38 vines of each variety were tagged at flowering. One hundred berries were tagged individually, i.e. 2 or 3 berries on one bunch on each vine. These berries were measured each week for length, diameter, and deformability using Harpenden (British Indicators, Burgess Hill, West Sussex, UK) skin-fold callipers as described by Coombe and Bishop (1980). The volumes of Riesling berries were calculated using the formula for a sphere ($4/3\pi r^3$, where r is the radius). The volumes of Shiraz berries were calculated using the formula for an ellipsoid ($4/3\pi abc$, where a, b, and c are the semi-axes of the ellipsoid). Berries harvested for RNA extraction and enzyme studies were immediately deseeded (except for the first sample taken 2 weeks after flowering), frozen in liquid nitrogen, and stored at −80°C until required. The times of sampling are indicated in the figures. A subset of these berries (50) was kept to measure the °Brix using a Reichert (Vienna, Austria) refractometer (model 10430). Skins were removed from berries while frozen when separate flesh and skin tissues were required. To obtain root tissue, canes were rooted and young root tips were harvested for extraction.

Preparation of Grape RNA and DNA

Total RNA was extracted from grape tissue using the perchlorate method of Rezaian and Krake (1987) as modified by Rathjen and Robinson (1992) and with additional modifications. Four grams of frozen tissue were ground to a powder using a coffee grinder. The frozen powder was added to 4 volumes of extraction buffer (5 M sodium perchlorate, 0.3 M Tris-HCl, pH 8.3, 8.5% [w/v] insoluble polyvinylpolypyrrolidone, 2% [w/v] PEG 4000, 1% [w/v] SDS, 1% [v/v] β-mercaptoethanol), and the mixture was stirred at room temperature for 30 min. The slurry was then placed in two Centriflo cones (Amicon, Beverly, MA), which had been plugged with approximately 1.5 cm of glass wool, sterilized with 0.1 N sodium hydroxide, and washed with sterile water. The cones were placed in a centrifuge tube and centrifuged at 200*g* for 10 min. The eluate was mixed with 2.5 volumes of ethanol and left for at least 20 min at −20°C. Nucleic acids were sedimented by centrifugation for 15 min at 7700*g*. The resulting pellet was rinsed with 70% (v/v) ethanol, dried, and resuspended in 1 mL of TE buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA) containing 0.2% (v/v) β-mercaptoethanol. This solution was then extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). RNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, then sedimented, rinsed, and dried as described above and resuspended in 300 μL of TE buffer. RNA yields were measured by A_{260}, and concentration and quality were checked on an agarose gel. mRNA was purified from total RNA (Dry and Robinson, 1994), and cDNA was synthesized from this mRNA using the $(dT)_{17}$-adaptor primer as described by Frohman et al. (1988).

DNA was extracted from unexpanded leaves as described by Thomas et al. (1993).

Cloning and Sequencing of Berry cDNAs

The two invertase cDNAs were cloned from Sultana berry cDNA using the PCR technique. Degenerate primers were designed to conserved regions found within various plant invertase protein sequences (alignment of the sequences was done using the ClustalV program [Higgins et al., 1991]). The two forward primers used were F1, 5′-AAGAA(CT)TGGA-

TIAA(CT)GA(CT)CC-3', and F2, 5'-TA (TC)CA(TC)(TC)TIT-T(TC)TA(TC)CA(AG)TA(TC)AA(TC)-CC-3', and the reverse primer was 5'-AA(AG)TCI(AG)(GC)(AG)CA(CT)TCCCA-CATICC-3'; these correspond to the conserved amino acid sequences KNWM/INDP (residues 142–148 on the GIN2 sequence, Fig. 1), YHLFYQYNP (residues 158–166), and GM-WECP/VDF (residues 322–329), respectively.

PCR reactions (25 μL) contained 2.5 μL of cDNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM each of the primers, and 0.625 units of *Taq* polymerase (Promega). The thermocycling regime used was as follows: 2 cycles of 94°C, 1 min; 37°C, 2 min; ramp to 72°C over 2 min; 72°C, 3 min followed by 28 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 3 min; followed by a final extension period of 72°C, 7 min.

The reaction products were run on an agarose gel and the single band was isolated using Qiaex (Qiagen, Chatsworth, CA) and ligated into Bluescript SK+ plasmid (Stratagene) that had been digested with *Eco*RV and T-tailed (Marchuk et al., 1990). A number of plasmids with inserts of the correct size were sequenced, and two different classes of invertase-like sequences were found (contained within GIN1 and GIN2, Fig. 1). Specific primers were designed from these sequences to allow the 5' and 3' portions of the cDNAs to be cloned by RACE PCR (Frohman et al., 1988). 5' RACE was conducted using a 5'-AmpliFINDER RACE kit (Clontech, Palo Alto, CA), and 3' RACE was done as described by Frohman et al. (1988). Considerable overlap between the RACE and internal fragments was allowed. The primers were as follows: GIN1 5' RACE primer, 5'-GTAGAGCCAGTGGATCATGTCTC-3' (nucleotides 597–619); 3' RACE primer, 5'-TGTTCGACGGAGAA-TTGCATGGG-3' (nucleotides 981-1003); GIN2 5' race primer, 5'-GATGAAGCCACTCTATGAGGTCC-3' (nucleotides 731–753); 3' race primer, 5'-TAGAAGGGGTTCT-TCATGCTGTC-3' (nucleotides 1117–1139). The three PCR-generated fragments for each of the genes were then sequenced in both directions by creating deletion clones using restriction enzymes and exonuclease 111 (Erase-a-Base, Promega) or by using synthetic oligonucleotide primers.

Invertase Activity Assays

Invertase activity assays were carried out by the colorimetric method as described by Ruffner et al. (1995).

Sequence Analysis

The compiled sequences were aligned using the ClustalV program (Higgins et al., 1991). The unrooted phylogenetic tree was drawn from the ClustalV output using the PHYLIP package (Felsenstein, 1989). The theoretical pI values were calculated using the Isoelectric program from the University of Wisconson Genetics Computer Group (Madison, WI) package (Devereaux et al., 1984).

Northern and Southern Analysis

For northern analysis, purified total RNA (4 μg/track) was heated at 70°C for 10 min in 3 volumes of Mops buffer (20 mM Mops, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) containing 70% (v/v) formamide, 8.9% (v/v) formaldehyde and run on a 1.2% (w/v) agarose gel containing a final concentration of 7.4% (v/v) formaldehyde, with Mops buffer. DNA was transferred to a ZetaProbe membrane (Bio-Rad) by alkaline, capillary blotting as described by the manufacturer.

For Southern blot analysis, DNA (4 μg/track) was digested to completion with the relevant enzymes under conditions described by the manufacturer (Boehringer Mannheim) and run on a 0.8% (w/v) agarose gel containing TAE buffer (40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1 mM EDTA). DNA was transferred to a ZetaProbe membrane by alkaline, capillary blotting as described by the manufacturer.

Blots were UV cross-linked and then prehybridized for at least 1 h at 65°C in hybridization solution (0.25 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% [w/v] SDS). To detect the two grape invertases without the risk of cross-hybridization, radiolabeled probes were made to the unique nucleotide sequences found in the 3' untranslated region of the GIN1 and GIN2 cDNAs. Double-strand DNA probes were prepared by PCR as described by Schowalter and Sommers (1989) with modifications. Reactions (20 μL) contained 0.5 ng of template (the respective 3' end RACE clones in Bluescript SK+), 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 200 μM each of dCTP, dGTP, and TTP, 2.5 μM dATP, 0.5 μM of each of the two primers, 1.25 units of *Taq* polymerase (Applied Biosystems), and 50 μCi [α-^{32}P]dATP (3000 Ci/mmol). The thermocycles consisted of 1 cycle at 94°C for 3 min, 50°C for 30 s, and 72°C for 2 min and 30 cycles at 94°C for 40 s, 50°C for 30 s, and 72°C for 2 min. Unincorporated label was removed by passage through a Sephadex G-50 column. Probes were denatured for 10 min at 100°C and 7×10^6 cpm/mL were added to the hybridization solution. Hybridization was carried out for 16 to 20 h at 65°C. Membranes were washed twice for 5 min with 2× SSC, 0.1% (w/v) SDS at room temperature and twice for 20 min with 0.2× SSC, 0.1% (w/v) SDS at 65°C. Filters were exposed to Kodak XAE film with an intensifying screen at −80°C.

RESULTS AND DISCUSSION

Cloning and Characterization of Two Grape Invertase cDNAs

Redundant oligonucleotide primers were designed to amino acid sequences conserved in a number of plant invertases (Fig. 1). These primers were used in a PCR reaction with grape berry cDNA as template and resulted in the cloning of two similar but distinct partial invertase-like cDNAs. The complete cDNAs, named GIN1 and GIN2, were then produced using 5' and 3' RACE PCR techniques.

The complete deduced amino acid sequences of GIN1 and GIN2 are shown in Figure 1 together with sequences for vacuolar invertases from tomato, carrot, potato, and mung bean. As indicated in Figure 1, the putative grape proteins are closely related to other plant invertases. The grape clones GIN1 and GIN2 encode proteins of 642 and

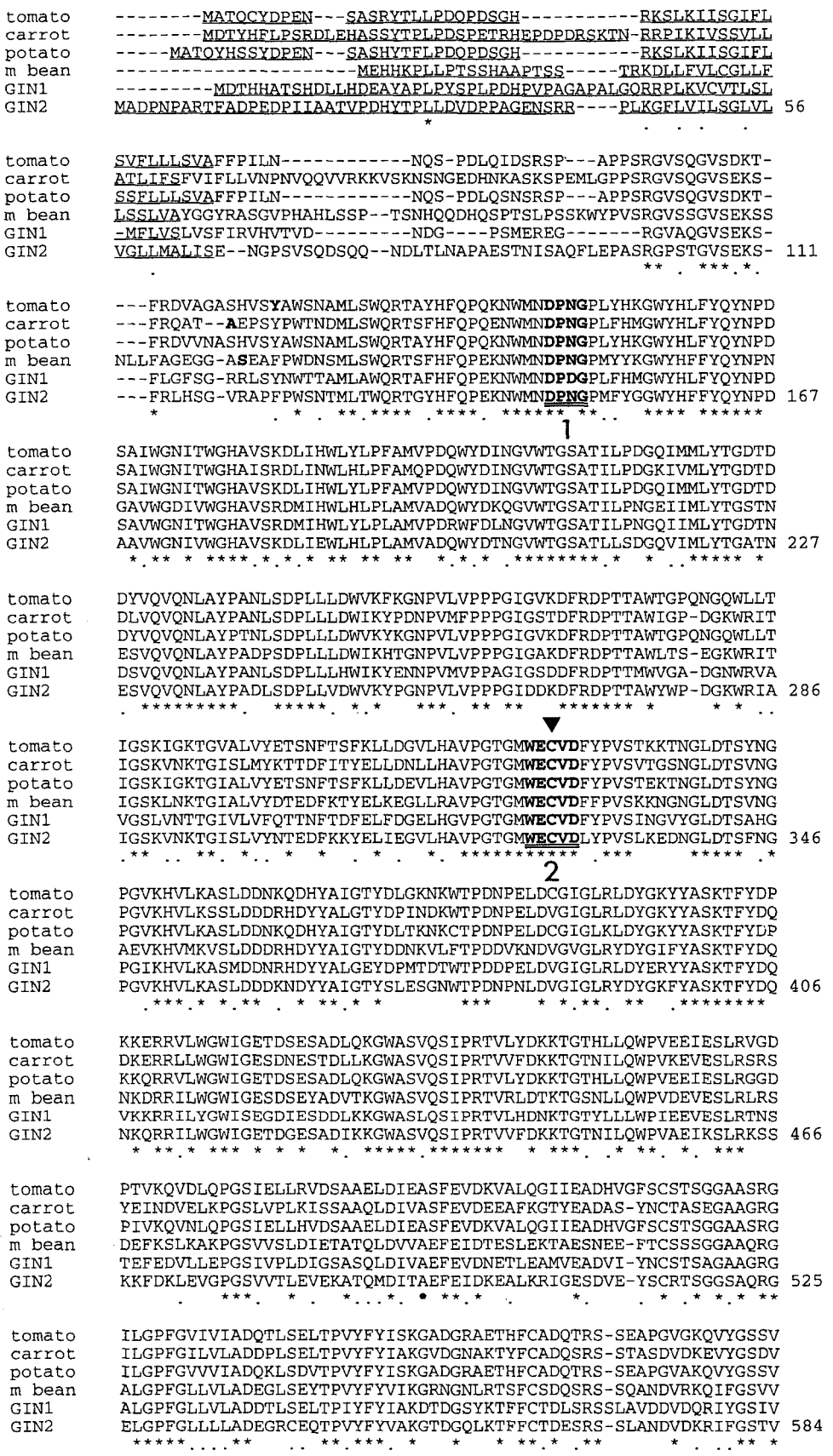

Figure 1. (Continued on facing page.)

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tomato    PVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTAS
carrot    PVLPGESLSMRLLVDHSIVESFAQGGRTVITSRVYPTRAIYSAARVFLFNNATGVSVTAS
potato    PVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNRTGASVTAS
m bean    PVLKGEKFSLRMLVDHSIVESFAQGGRTCVTSRVYPTKAIYGAARLFLFNNATEATVTAS
GIN1      PALDDEKPTMRVLVDHSIVEGFSQGGRSCITTRVYPTEAIYGAARLFLFNNATGVNVTAS
GIN2      PVIKGEKLSMRILVDHSIIESFAQGGRTCITSRVYPTKAIYGAAQLYVFNNATSASITAS 644
          * .  *  ..*.******.* *.****. .*.*.*** *.  **....*** *   .***

tomato    VKIWSLESANIQSFPLQDL---
carrot    VKAWQMASATLKPFPFDQL---
potato    VKIWSLESANIRSFPLQDL---
m bean    LKVWQMNSAFIRPFPFNPDQKS
GIN1      IKIWEMASADIHPYPLDQP---
GIN2      IQTWAMKSAYIRPYSSHQES-- 664
          .. * . ** .. .
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Figure 1. (Figure continued from facing page.) Amino acid sequences of putative grape invertases GIN1 and GIN2 aligned with vacuolar invertases from other plant species. The database accession numbers for the sequences used were: tomato, Z12025 (Elliott et al., 1993); carrot, X75353 (Unger et al., 1994); potato, L29099 (Zhou et al., 1994); and mung bean, D10265 (Arai et al., 1992); the grape sequences (GIN1 and GIN2) are from this work. Amino acids conserved in all the proteins are indicated by asterisks (*) and those similar in all proteins are indicated by dots (·). The proposed signal peptide regions are indicated by single underlining. The NH_2-terminal amino acids for the tomato (Konno et al., 1993), carrot (Unger et al., 1994), and mung bean (Arai et al., 1992) proteins are in boldface. The boldface, double-underlined regions (1 and 2) are highly conserved in invertases. Region 1 contains the "β-fructosidase" motif, and region 2 contains the Cys thought to be involved in catalysis (solid arrow) and an adjacent Val believed to be characteristic of vacuolar forms of invertase (see text for details).

664 residues, respectively, and are 62% identical at the amino acid level. Two cDNAs encoding proteins corresponding to different isoforms of vacuolar invertase have also been cloned from carrot (Unger et al., 1994), and like the putative grape proteins they are quite similar to each other (63% identical).

The GIN1 and GIN2 translation products contain sequence motifs, including the proposed active site, that are characteristic of invertase enzymes (Fig. 1). Region I contains a stretch of amino acids conserved in other β-fructosidases and region 2 contains conserved amino acids including a Cys thought to be involved in catalysis (Sturm and Chrispeels, 1990). In the invertases localized in the vacuole, the sequence included in region 2 consists of WECVDF/LYPV, whereas in the cell-wall forms this proposed catalytic site has the sequence WECPDFFPV. The substitution of Val for Pro (boldface) has been proposed as characteristic of the vacuolar invertases (Roitsch et al., 1995).

Figure 2 is an unrooted phylogenetic tree that shows how the grape proteins are related to other plant invertases. Because the locations of the NH_2 and COOH termini in the mature proteins are not known for all of these invertases, the translation products of the entire open reading frames have been used in this comparison. The invertases form two distinct groups, those of known, or proposed, cell-wall origin and those thought to be located in the vacuole. The putative grape invertases group with invertases of known, or proposed, vacuolar location. The members of this group of vacuolar invertases are characterized by having acidic pI values, whereas the cell-wall invertases have basic pI values.

On the basis of sequence homology with other plant invertases, both overall similarity and the presence of characteristic amino acid motifs, and their having acidic pI values, the two proteins deduced from the grape berry cDNA nucleic acid sequences appear to be vacuolar invertases. There is considerable evidence that most of the invertase activity present in developing grape berries is soluble (Hawker, 1969b; Ishikawa et al., 1989; Ruffner et al., 1995). Nakanishi et al. (1991) purified a soluble invertase with a low pI (3.9) from Semillon berry juice and from wine made from Semillon grapes. The amino acid composition of this protein is quite similar to that of the translation products of the GIN1 and GIN2 cDNAs (data not shown). This is especially so if the comparison is based on the assumed mature proteins encoded by GIN1 and GIN2 (see Fig. 1).

Plant vacuolar invertases appear to be synthesized with an NH_2-terminal extension, and it is possible that this

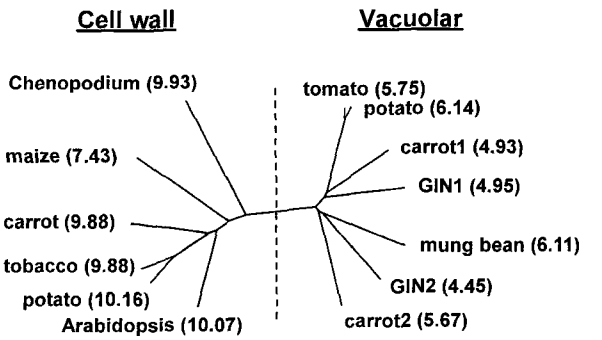

Figure 2. Unrooted phylogenetic tree of plant invertase protein sequences. The numbers in parentheses indicate the calculated theoretical pI values for the entire coding regions of the respective proteins. The database accession numbers for the cell-wall invertase sequences are: Arabidopsis, U11033 (Mercier and Gogarten, 1995); *Chenopodium*, X81792 (Roitsch et al., 1995); carrot, M58362 (Sturm and Chrispeels, 1990); maize, U17695; potato, Z22645 (Hedley et al., 1994); and tobacco, X81834. Those for the vacuolar invertases are as in Figure 1: carrot1, X75353; carrot11, X67163; mung bean, D10265; potato, L29099; and tomato, Z12025. GIN1 and GIN2 are from this work.

prosequence may be involved in targeting to the vacuole (Arai et al., 1992; Elliott et al., 1993). Assuming that the grape invertases are NH_2-terminally processed at a point similar to that where the tomato vacuolar invertase is processed (see Fig. 1), the M_r estimated for the predicted mature GIN1 and GIN2 proteins is 61,000; this is consistent with estimates for the M_r of invertases purified from grape berries, which range between 56,000 (Porntaveewat et al., 1994) and 65,000 (Nakanishi et al., 1991).

Genomic Analysis

Southern blot analysis of Shiraz leaf DNA was undertaken (Fig. 3) using gene-specific 3′ end clones of GIN1 and GIN2 as probes. The banding pattern was different for each of the cDNA probes (compare Fig. 3, A and B), indicating that the probes were specific for each gene, an important consideration when considering the northern analysis of each of the two vacuolar genes (see below). Most enzymes produced a single band for each gene, but two bands were present when DNA digested with *Bam*HI was probed with GIN1 and when *Sac*I-digested DNA was probed with GIN2. These findings are consistent with each of the two invertases being present in low copy number in the grape genome and indicate that they do not constitute part of a larger multigene family. A similar pattern was observed with the two vacuolar genes described for carrot (Unger et al., 1994).

Grape Berry Ripening: Sugar Accumulation and Invertase Enzyme Activity

Shiraz and Riesling grape berries were sampled from flowering to maturity to investigate hexose accumulation, invertase activity, and the expression of the grape invertase genes. The growth pattern of developing grape berries can be described as a double sigmoidal curve in which two periods of rapid growth are separated by a lag period of little or no growth. This pattern of development was observed in the Shiraz berries used in this work (Fig. 4A), where the lag phase between 7 to 9 weeks was followed by

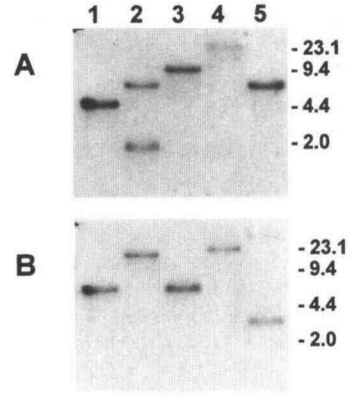

Figure 3. Southern analysis of Shiraz DNA using probes specific to the GIN1 (A) and GIN2 (B) cDNAs. Lane 1, *Eco*RI; lane 2, *Bam*HI; lane 3, *Eco*RV; lane 4, *Pst*I; lane 5, *Sac*I. The sizes of molecular mass markers (kD) are indicated at the margin.

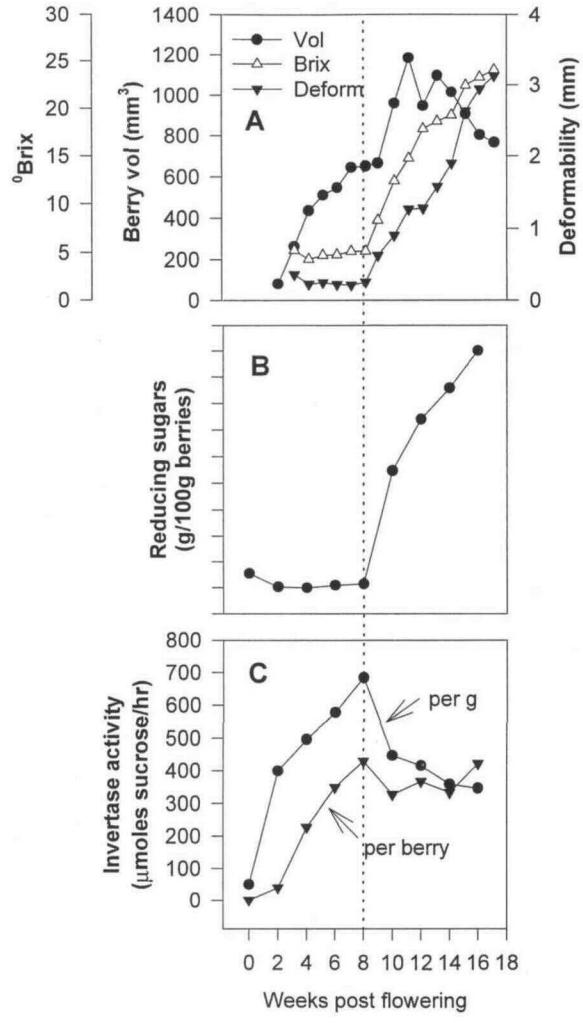

Figure 4. A, Graph of Shiraz berry development showing changes in the size, deformability, and °Brix. Berry deformability, volume, and °Brix were measured at weekly intervals, 2 to 17 weeks postflowering, as described in the text. The time of *véraison* is indicated by a dashed line. B, Reducing sugar levels in developing berries, measured using a colorimetric assay as described in the text. C, Invertase activity (μmol Suc hydrolyzed h^{-1}) in developing Shiraz berries measured at fortnightly intervals, 2 to 16 weeks postflowering. The activity is displayed on an activity per gram basis (●) and on an activity per berry basis (▼).

a period of rapid growth. Toward the end of the ripening period the average volume of the berries decreased. The increases in total soluble solids and deformability, which herald the commencement of ripening, began 8 to 9 weeks after flowering. A similar pattern of development was observed for Riesling berries (data not shown). The increase in total soluble solids measured using a refractometer corresponded to the increase in reducing sugars as measured by a colorimetric assay (Fig. 4B), because Fru and Glc constitute most of the soluble solids at this stage of development. The level of reducing sugars increased rapidly after 8 weeks and continued to rise throughout ripening, reaching a maximum of 20.0 g/100 g berry fresh weight at

Table I. *Levels of invertase enzyme activity and reducing sugars in various grapevine tissues*

Tissue Type	Invertase Activity
	μmol Suc hydrolyzed g^{-1} fresh weight h^{-1}
Root	112
Tendril	166
Young leaf	322
Mid leaf	186
Old leaf	78
Flower	49
Seed (4 weeks)	169
Berry (2 weeks)	399

16 weeks after flowering. The timing and rate of the increase in hexose concentration in separated skin and flesh was almost identical (data not shown). The pattern of reducing sugar accumulation in Riesling berries was similar to that shown for Shiraz berries, although Riesling berries accumulated less hexose than Shiraz berries. At harvest (15 weeks postflowering), Riesling berry reducing sugar concentration was 14.9 g/100 g fresh weight. The level of reducing sugars in ripe grape berries is much higher than that found in ripe *L. esculentum* (e.g. Stommel [1992] recorded 4.6 g/100 g fresh weight).

Invertase enzyme activity was assayed in a variety of Shiraz tissues (Table I), and a considerable range in values was observed. Flowers and fully expanded leaves had low levels of activity, whereas berries (see also Fig. 4C) and young (unexpanded) leaves had the highest levels. At this early stage of their development leaves would be expected to be acting as sinks for carbohydrate rather than as sources. The levels of invertase activity in grape leaves of different age are similar to those previously reported for a different grape cultivar (Ruffner et al., 1990). These authors showed that the levels of soluble invertase activity were higher than the levels of the insoluble form at all stages of leaf development and that, as in our study, the level of invertase activity decreased with leaf age, presumably as the leaves become exporters of sugars. In this paper only the activity of soluble invertase has been assayed. It is possible that the ratio of soluble to insoluble invertase varies with tissue type.

Invertase activity was also assayed in whole Shiraz berries throughout development (Fig. 4C). The level of invertase activity, on a per gram basis, was low at flowering (49 μmol Suc hydrolyzed g^{-1} fresh weight h^{-1}) but increased during the first growth phase and the lag period to reach a maximum level (684 μmol Suc hydrolyzed g^{-1} fresh weight h^{-1}) at 8 weeks postflowering. The level then declined until, at 16 weeks, it was only half (346 μmol Suc hydrolyzed g^{-1} fresh weight h^{-1}) the maximal value. On a per berry basis, invertase activity increased throughout the initial phase of berry growth, reaching a maximum 8 weeks after flowering and then remaining relatively constant throughout ripening. This suggests that the decline in activity on a per gram fresh weight basis during ripening was the result of increased berry weight (Fig. 4C). The expression of invertase activity during Shiraz berry development was also assayed in separated skin and flesh samples, and the patterns of expression were found to be virtually identical (data not shown). This finding is in agreement with the very similar pattern of hexose accumulation in skin and flesh (see above). A similar pattern of invertase activity was found in Riesling berries, although the level of maximal activity was considerably lower (232 μmol Suc hydrolyzed g^{-1} fresh weight h^{-1}), and the maximal level occurred earlier in development (6 weeks postflowering).

Grape Invertase mRNA Expression

The expression of the two grape invertase genes was investigated by northern blot analysis using the 3′ end portions of the GIN1 and GIN2 cDNAs as probes. These sequences share little sequence similarity and were used to ensure gene-specific hybridization (Fig. 3). To aid comparison between the relative levels of expression for GIN1 and GIN2, radioactive probes of the same specific activities were used for both and the film exposure times were identical for panels A and B within each figure.

The expression of the GIN1 and GIN2 mRNAs was investigated in a range of Shiraz tissues (Fig. 5). GIN1 was expressed predominantly in berry skin and flesh, but expression was also detected in young (unexpanded) leaves, partially expanded leaves, and flowers. There was little or no expression detected in roots, tendrils, fully expanded leaves, and seeds. The pattern of GIN2 expression was considerably different from that of GIN1. The highest level of expression was in flowers (the level is similar to that of GIN1 in flowers), with lower but significant hybridization present in the berry skin and flesh samples. Low levels of expression of GIN2 were found in all other tissues except fully expanded leaves, where no expression was detected. Tissue-specific expression has also been recorded for the

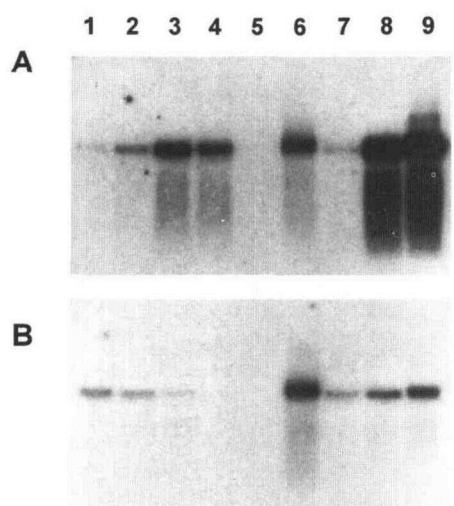

Figure 5. Northern blot analysis of RNAs extracted from various tissues and probed with the GIN1 (A) and GIN2 (B). Lane 1, Root; lane 2, tendril; lane 3, unexpanded leaf; lane 4, partly expanded leaf; lane 5, fully expanded leaf; lane 6, flower; lane 7, seed; lane 8, berry skin; lane 9, berry flesh. The berry samples were taken at 2 weeks postflowering.

two vacuolar invertase genes from carrot (Sturm et al., 1995), where one gene is expressed mainly in primary roots and the other is expressed in the developing tap roots and may control sugar storage.

Both GIN1 and GIN2 mRNAs were detected in Shiraz berry tissue, but GIN1 was expressed at a higher level than GIN2 at all stages of berry development (Fig. 6). In Shiraz, low levels of expression of both genes were detected in flowers, but the levels rapidly increased during early berry development, and at 2 weeks postflowering expression was at or near maximal levels (Fig. 6). The level of GIN1 mRNA then declined steadily until, at 12 weeks after flowering, it was no longer detectable. The GIN2 mRNA also decreased during berry development, but the initial levels were much lower than those of GIN1. The pattern of invertase mRNA expression is in accordance with the observed trend for invertase activity (Fig. 4C) and is consistent with expression being controlled at the transcriptional level. Similar results were obtained with RNA extracted from Riesling whole berries, although the overall level of expression was lower than in Shiraz berries (data not shown), in accordance with the lower level of invertase enzyme activity recorded in Riesling berries (see above). The fact that the level of invertase mRNA decreased to nondetectable levels 12 weeks postflowering while the enzyme activity (on a per berry basis) remained fairly constant suggests that the berry vacuolar invertases are stable proteins that exhibit relatively low turnover.

It is interesting to note that the increase in soluble invertase enzyme activity (Hawker, 1969a; this work) and the increase in the steady-state levels of the corresponding mRNAs occur well before the increase in the level of hexoses. This suggests that although soluble invertases may be important in the accumulation of hexoses in the vacuole, their synthesis is not the trigger for this accumulation. In fact, the rise in invertase activity considerably precedes the phase of rapid hexose accumulation. The increase in invertase activity prior to *véraison* suggests that the berry prepares for ripening by accumulating the machinery it will later require. The triggering of some later event, e.g. the downloading of Suc from the phloem, must then initiate the process of hexose accumulation.

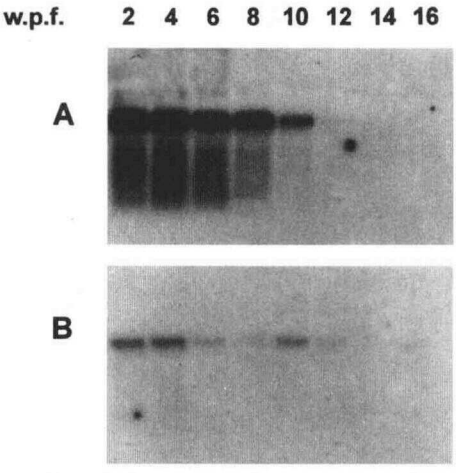

Figure 6. Northern blot analysis of RNAs extracted from berries probed with a GIN1-specific probe (A) and a GIN2-specific probe (B). Samples from 2 to 16 weeks postflowering (w.p.f.) were assayed.

The accumulation of hexoses in the grape berry vacuole may result from the action of more than one mechanism, and a number of different routes have been proposed (Coombe, 1992). It appears that the predominant invertase activity in grape berries is located in the vacuole because the invertase purified by Nakanishi et al. (1991) from Semillon grapes had an acid pI and the GIN1 and GIN2 cDNAs, which are expressed at the time of increasing invertase activity, encode proteins with acidic theoretical pI values. The most straightforward interpretation of these results is that Suc is downloaded from the phloem, transported to the vacuole, and then cleaved by vacuolar invertase to Fru and Glc. There is some evidence that this can occur in tomato fruit (Damon et al., 1988; Dali et al., 1992). Another possibility is that Suc downloading is driven by a gradient resulting from its cleavage by an extracellular invertase. Because it seems that in *L. esculentum* vacuolar invertase cleaves Suc that has been imported into the vacuole, this proposal must include provision for the resynthesis of Suc in the symplasm following its cleavage by an extracellular invertase. This could be achieved by Suc-P synthase, and this pathway may operate in tomato (Damon et al., 1988; Dali et al., 1992). Suc-P synthase activity has been shown to increase in grape berries after *véraison* (Hawker, 1969a).

The very high levels of hexoses accumulated in grape berries compared to tomato fruit may suggest that comparisons between the two should be made with caution. Grape berries may have a different or additional mode of sugar transport that accounts for their high loading. A different hypothesis for hexose accumulation in grape berries has been proposed that involves the breakdown of apoplast/symplast compartmentation. In this proposal, the plasma membrane of the pericarp cell becomes leaky during ripening and phloem sap moves into the berry due to the difference in water potential between the source and the sink (Lang and Düring, 1991). Further work needs to be done regarding the processes of phloem unloading and sugar transport to the vacuole in order to define the event(s) that trigger and control hexose accumulation in grape berries.

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