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Cloning and Expression of a Hexose Transporter Gene Expressed during the Ripening of Grape Berry[1]

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The ripening of grape (*Vitis vinifera* L.) is characterized by massive sugar import into the berries. The events triggering this process and the pathways of assimilate transport are still poorly known. A genomic clone *Vvht1* (*Vitis vinifera* hexose transporter1) and the corresponding cDNA encoding a hexose transporter whose expression is induced during berry ripening have been isolated. *Vvht1* is expressed mainly in the berries, with a first peak of expression at anthesis, and a second peak about 5 weeks after véraison (a viniculture term for the inception of ripening). *Vvht* is strictly conserved between two grape cultivars (Pinot Noir and Ugni-Blanc). The organization of the Vvht1 genomic sequence is homologous to that of the Arabidopsis hexose transporter, but differs strongly from that of the *Chlorella kessleri* hexose transporter genes. The Vvht1 promoter sequence contains several potential regulating *cis* elements, including ethylene-, abscisic acid-, and sugar-responsive boxes. Comparison of the Vvht1 promoter with the promoter of grape alcohol dehydrogenase, which is expressed at the same time during ripening, also allowed the identification of a 15-bp consensus sequence, which suggests a possible co-regulation of the expression of these genes. The expression of Vvht1 during ripening indicates that sucrose is at least partially cleaved before uptake into the flesh cells.

The grape (*Vitis vinifera* L.) is a productive plant considered as the world's premier fruit (Coombe, 1989), with nearly 9 million hectares of viticultural land in 1990 (Kanellis and Roubelakis-Angelakis, 1993). It is used for wine, distilled liquors, juice, dried fruit (raisins), fresh consumption (table grapes), and concentrate. In spite of this major economic importance, the process of grape maturation is still poorly understood (Coombe, 1992). The ripening of grapes is nonclimacteric, and the growth pattern of the berries follows a double-sigmoid curve that is usually divided into three stages (Kanellis and Roubelakis-Angelis, 1993; Tattersall et al., 1997).

Stage I, immediately following flowering, is characterized by a short period of cell division, followed by vacuolar swelling resulting from the storage of organic acids and by cell enlargement. The acidity of the berries reaches a maximum at the end of this stage. Stage II, approximately 7 to 10 weeks after flowering, is a lag phase characterized by slow growth. Stage III starts with fast softening, rapid accumulation of sugars and amino acids, decrease of acidity, and expansion of the flesh cells. The entry into stage III, which may occur within 24 h (Coombe, 1992), is called véraison and corresponds to the inception of ripening. During this stage, a decrease in organic acid content and an increase in soluble sugars induce a rapid decline of the acid/sugar balance. Just before véraison, the grape berry is hard, green, acidic, and unsweet, and contains no more than 150 mM hexose, with a Glc/Fru ratio of 2 (Findlay et al., 1987). Twenty days after véraison, the hexose concentration of the berry is close to 1 M, with a Glc/Fru ratio of 1. Due to the size increase of the berry, its hexose content is increased about 7-fold during ripening. Because Suc is the major form of translocated sugar in grape, the rapid accumulation of hexose characterizing the ripening of the berry must involve the activity of Suc, of hexose transporters located at the plasma membrane and/or tonoplast, and of invertases located in the soluble compartments.

The triggers of ripening in nonclimacteric fruits such as grape are poorly known. Davies and Robinson (1996) have cloned two cDNAs (GIN1 and GIN2) encoding vacuolar invertase from grape berries. Expression studies indicated that the rise in invertase activity considerably precedes the phase of rapid hexose accumulation. This suggests that although soluble invertases may be important for the accumulation of hexose in the vacuole, the synthesis of these enzymes does not trigger sugar accumulation in the berry (Davies and Robinson, 1996). The sugar status of the berry itself may be important for the induction of ripening-related genes. Indeed, the expression of a number of dif-

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Abbreviations: DEPC, diethylpyrocarbonate; PVPP, polyvinylpolypyrrolidone; RT, reverse transcription.

ferent genes encoding proteins with a wide range of biological functions (Jang and Sheen, 1994; Salzman et al., 1998) may be induced by the sugar status of the cells. Tattersall et al. (1997), who recently cloned a thaumatin-like protein expressed during grape ripening, suggested that the temporally and spatially defined induction of ripening-related genes may be directly or indirectly caused by the onset of sugar accumulation, possibly due to the presence of so-called "sugar boxes" identified within the promoters of these genes (Tsukaya et al., 1991).

The extent of sugar import in a sink organ depends on sugar utilization and/or compartmentation. As mentioned above, invertase activity is not tightly related to sugar accumulation in the berry (Davies and Robinson, 1996). Suc synthase activity remains at a low level throughout the maturation of berries (Hawker, 1969). This suggests that in grape berry, sugar accumulation may depend more on compartmentation than on metabolism.

The pathways of sugar unloading in grape are poorly understood (Patrick, 1997). Numerous plasmodesmata connect the flesh cells of the storage parenchyma of the berry (P. Fleurat-Lessard, unpublished data). However, at the phloem/storage parenchyma interface, sufficient plasma membrane surface area is available to support exchange with the apoplast. The high sugar concentrations found in the berry apoplast and their sensitivity to changes in phloem import rates also suggest an apoplastic step (Patrick, 1997), which makes it important to study the sugar transporters that may control this step.

Although various Suc transporters have now been cloned from plant tissues (Riesmeier et al., 1992, 1993; Gahrtz et al., 1994, 1996; Sauer et al., 1994), little is known so far about the expression and the activity of these transporters in sink tissues. AtSUC2 encodes a Suc transporter strongly expressed in the roots and seeds of Arabidopsis, which suggests that it may be involved in phloem unloading (Truernit and Sauer, 1995). Recently, Weber et al. (1997) characterized a Suc transporter clone (VfSUT1) whose expression is correlated with the differentiation of the epidermal transfer cells of broad bean cotyledons. This clone is also expressed, although at a lower level, in other parts of the plant.

Hexose transporters are encoded by a multigene family of up to 12 members in various species (for review, see Chiou and Bush, 1996; Tanner and Caspari, 1996). MST1, a cDNA clone for a monosaccharide transporter isolated from tobacco is most strongly expressed in various sink tissues, such as roots, flowers, and young leaves (Sauer and Stadler, 1993). The STP4 hexose transporter of Arabidopsis is primarily expressed in roots and flowers and is regulated by environmental stress (Truernit et al., 1996). Among the many putative hexose carriers cloned from higher plants, only a few have been functionally described by heterologous expression in yeast (Sauer et al., 1990; Sauer and Stadler, 1993; Weig et al., 1994). Some evidence also suggests that hexose transporters are involved in a hexokinase-independent signaling pathway in sugar sensing (Smeekens and Root, 1997).

For a better understanding of the unloading process and sugar accumulation, a research program aimed at the characterization of various membrane transporters in grape berry is being developed. The work described below was focused at the characterization of a hexose transporter expressed during the ripening of the berries.

MATERIALS AND METHODS

Plant Material

Tissue samples were collected from the grape (*Vitis vinifera* L.) cv Ugni-Blanc (from the Combe de Rudard in Juillac le Coq vineyard near Cognac, France and ENSAM-INRA vineyard, Le Chapitre, Montpellier, France) or from cv Pinot noir (from the Germaine vineyard, Epernay, France) in the 1995 and 1996 seasons.

RNA Extraction

Berries collected at different stages of development (as indicated in "Results") were frozen in liquid nitrogen and stored at −80°C until use. Various procedures were tested to achieve RNA isolation from the berries, which contained high levels of sugars and of phenolic compounds. Two procedures were finally selected for extracting the RNAs and studying the amounts of hexose transporter transcripts.

In the first procedure, total RNA was extracted from the samples according to a method modified from Davies and Robinson (1996). The berries were deseeded before the extraction. The frozen samples (1.5 g) were ground to a powder in liquid nitrogen and added to 10 mL of extraction buffer containing 5 M sodium perchlorate, 0.3 M Tris-HCl, pH 8.3, 8.5% (w/v) insoluble PVPP, 2% (w/v) PEG 4000, 1% (w/v) SDS, and 1% (w/v) β-mercaptoethanol. The resulting slurry was stirred for 30 min at 37°C and layered on a pierced tube (tube bottle adapter, Sorvall) containing a 1-cm layer of glass wool. This tube was inserted into a 50-mL centrifuge tube (Corning) and centrifuged at 200*g* for 10 min. After addition of 2.5 volumes of 95% ethanol, nucleic acids were precipitated for at least 20 min at −20°C, recovered by centrifugation at 7,700*g* for 15 min, and rinsed with 70% ethanol. The pellet was dried under vacuum, and resuspended in 2 mL of DEPC-treated water. The proteins were extracted twice with an equal volume of phenol: chloroform:isoamylic alcohol (25:24:1, v/v) and once with chloroform:isoamylic alcohol (24:1, v/v). After precipitation with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol, total RNA was resuspended in 0.1 mL of DEPC-treated water.

The purity and the quality of RNA were checked by gel electrophoresis and its concentration was estimated by measuring the A_{260}. This procedure was also used to extract total RNA from young leaves (2 cm long), mature leaves, petioles, tendrils, and roots of plants at the véraison stage. The yield of RNA extraction obtained with this procedure was about 75 $\mu g\ g^{-1}$ fresh weight for the berries and 100 $\mu g\ g^{-1}$ fresh weight for all other organs except the roots (40 $\mu g\ g^{-1}$ fresh weight). These RNAs were used for northern analysis as described below.

Isolation of intact RNA with a reasonable yield was also achieved by a second procedure, which was modified from that described by Tesnière and Vayda (1991). All glassware was sterilized by heating at 180°C for at least 6 h. The grinding buffer contained 200 mM Tris-HCl (pH 8.5), 300 mM LiCl, 10 mM Na_2 EDTA, 1% (w/v) sodium dexycholate, and 1% (w/v) SDS. After sterilization at 120°C for 20 min, 1 mM aurintricarboxylic acid, 5 mM thiourea, and 10 mM DTT were dissolved in the medium and 1% (v/v) Nonidet P-40 was added. The seeds were removed, the berries were crushed to a fine powder in liquid nitrogen, and 2% (w/v) PVPP was mixed with the brei. The powder was transferred to test tubes (Corning) containing the grinding buffer with 0.3 g plant material mL^{-1} buffer. After thawing, the content of the tubes was mixed by manual inversions for 10 min.

To remove intracellular debris and PVPP, the samples were centrifuged at 12,000g for 15 min. The supernatant (still containing some PVPP) was then transferred to another tube, centrifuged at 12,000*g* for 5 min, and the final supernatant was filtered on two layers of Miracloth (Calbiochem). CsCl was added (0.2 g/mL filtrate) at room temperature. After total dissolution of the salts, 20 mL of the filtrate was transferred to a tube (Ultraclear, Sorvall) containing 10 mL of a 5.7 M CsCl solution prepared in 10 mM Tris-HCl (pH 7.5). The filtrate was centrifuged on this CsCl cushion for 26 h at 20°C at 110,000*g* in a swinging rotor (model AH-629, Sorvall). The supernatant was discarded by aspiration, and the RNA pellet was resuspended in 1 mL of DEPC-treated water. The samples were kept on ice until the end of the procedure, and the centrifugations were run at 4°C. RNA was reprecipitated for 75 min at −80°C after addition of 2.5 volumes of 96% ethanol and 0.2 volume of 3 M LiCl. After centrifugation for 30 min at 12,000*g*, the RNA pellet was washed three times with 1 mL of 2.5 M sodium acetate (pH 5.5) before rinsing with 1 mL of 70% ethanol. The pellet was dried under vacuum for 5 min, and resuspended in DEPC-treated water (about 50 μL per centrifugation tube).

RNAs obtained with this extraction procedure (with yields ranging from 50 to 60 $\mu g\ g^{-1}$ fresh weight at anthesis to 20 $\mu g\ g^{-1}$ fresh weight at the latest stages of ripening) were used to estimate the amount of hexose transporter transcripts by RT-PCR at different stages of development of the berries and to prepare the cDNA library from berries at the véraison stage.

cDNA Library Construction and Screening

Total RNA was extracted from grape (cv Pinot noir) berries collected at the véraison stage using the CsCl procedure described above. Poly(A^+) RNA was isolated from RNA (2 mg) using oligo(dT) columns (Stratagene). Double-stranded cDNA was prepared using a cDNA synthesis kit, and a cDNA library was constructed using the Lambda ZAP-cDNA synthesis kit (Stratagene). The fragments were inserted at the *Eco*RI site of the Lambda ZAP II phage. The size of the DNA fragments ranked between 0.5 and 2.7 kb, with a mean size of 1.2 kb. Approximately 1.2×10^6 independent clones were obtained. The library was packaged using a gold packaging extract (Gigapack II, Stratagene).

A grape hexose transporter probe was obtained by RT-PCR starting from 15 μg of total RNA extracted from berries (cv Ugni-Blanc) harvested at the véraison stage. After denaturation of RNA for 10 min at 65°C, first-strand cDNA was synthesized by RT using a first-strand cDNA synthesis kit (Pharmacia) in a medium containing 45 mM Tris-HCl (pH 8.3), 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg mL^{-1} BSA, and 1.8 mM each dNTP. The mRNA/cDNA complexes were denaturated for 5 min at 90°C. The following degenerated oligonucleotides were used as primers for PCR: TTTGC(G/T)TGGTC(C/G)TGGGG(A/C)CC (forward primer H2) and (A/C)CC(C/T)TT(C/G/T)GTCTG(A/C/G)GGCAA (reverse primer H3). The forward primer corresponds to a FAWSWGP conserved motif and the reverse primer to a LPETKG conserved motif (Bush, 1993). PCR amplification was performed for 30 cycles (1 min at 92°C, 1.5 min at 48°C, 1 min at 72°C), and the amplified fragments were cloned in the *Eco*RV site of the pSK+ vector.

Approximately 500,000 plaques were screened with the [α-^{32}P]dATP-labeled 250-bp hexose transporter fragment obtained by RT-PCR. Seven positive plaques were obtained after three rounds of screening. The pBlueScript SK(+) (Stratagene) phagemids containing the positive cDNAs were excised from Lambda ZAP II phage in vivo with the R408 helper phage and cloned into *Escherichia coli* DH5α cells. One of the seven positive clones was lost at this stage. The size of the inserts was estimated for the six other clones using PCR with the T3 and T7 primers located on each side of the multicloning site.

Genomic Library Construction and Screening

DNA was extracted from young leaves of the grape cv Ugni-Blanc and partially digested by *Mbo*I. The resulting DNA fragments were cloned in the EMBL3 SP6/T7 phage at the *Bam*HI site. Independent clones (800,000) were obtained, with DNA fragments ranking between 9 and 22 kb. About 350,000 plaques were screened using the hexose transporter probe.

Sequence Analysis

Sequencing was run on the two DNA strands using the dideoxy method on a sequencer (ALF, Pharmacia). For long DNA fragments, progressive unidirectional deletions were made using the Erase-a-Base kit (Promega). Homology searches were done using the program BLAST (Altschul et al., 1990) and the promoter sequence was analyzed with the PLACE database using a signal scan program (http://www.dna.affrc.go.jp/htdocs/PLACE/). Sequences were also analyzed using the computer program Geneworks (Intelligenetics, Mountain View, CA).

Southern Hybridization

Genomic DNA isolation from young leaves of the grape cv Ugni-Blanc was performed according to the method of

Steenkamp et al. (1994). The DNA (15 μg) was digested with *Eco*RI, *Hin*dIII, *Bgl*II, or *Bam*HI restriction enzymes. Digested genomic DNA was separated by electrophoresis in 0.8% agarose gel, and blotted on nylon membrane (Hybond N, Amersham). Blots were hybridized to ^{32}P-labeled *Kpn*I/*Hpa*I fragment of Vvht1 (*Vitis vinifera* hexose transporter 1). Southern hybridization was performed in 6× SSPE (saline sodium phosphate EDTA buffer), 0.5% SDS, 5× Denhardt's solution, and 100 μg mL^{-1} salmon-sperm DNA for 16 h at 65°C. Filters were washed for 20 min at room temperature in 3× SSC and 0.5% SDS, then two times for 30 min at 65°C in 1× SSC and 0.1% SDS. Final washing steps were performed in 0.2× SSC and 0.1% SDS at 65°C for 45 min.

Northern Analysis

Northern analysis was according to the method of Sambrook et al. (1989) with minor modifications. Twenty micrograms of RNA was deposited on each lane. A 400-bp fragment located in the 3′ end of the Vvht1 sequence, starting from base 1,170, was prepared by digestion with *Bst*XI and *Not*I, and used for hybridization. This probe was ^{32}P labeled with the Ready-to-Go DNA labeling kit (Pharmacia). The nylon membrane was prehybridized for 4 h at 65°C in a medium containing 250 mM sodium phosphate (pH 5.2), 6.6% SDS (w/v), 1 mM EDTA (pH 8.0), and 1% (w/v) BSA. After hybridization for 16 h at 65°C, the membranes were washed with SSC (2×, 1×) and 0.1% SDS at 65°C.

The quality and the amount of RNAs were checked by hybridization with a constitutive probe (grape β-tubulin) prepared by PCR with the following primers: CTGGTATTGTTGRTAYTC and ATGAGRGARATCCTTCAC (generous gift from Nathalie Noiraud, University of Poitiers, France). The membranes were read with an imager (Instant Imager, Packard Instruments). All data were corrected with this internal standard for the amount of RNA deposited per lane.

Quantitative RT-PCR

RT was run with reverse transcriptase (Superscript II RNase H, GIBCO-BRL) on 5 μg of total RNA extracted from cv Ugni-Blanc berries harvested at different stages of development. The RNA was denaturated at 70°C for 10 min and RT was run in 20 μL for 50 min at 42°C in the presence of 0.5 μg of oligo(dT) 12 to 18 primers. One microliter of cDNA was amplified in a total volume of 25 μL containing 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 1% (w/v) Triton X-100, 0.1% (w/v) gelatin, 2 mM dNTP, and 0.2 unit of *Taq* polymerase (A.T.G.C., Noisy-Le-Grand, France). PCR was run using the degenerated primers H2 and H3 described above to allow a general amplification of all transcripts related to the hexose transporters. Twenty cycles of amplification were run in the following conditions: 92°C for 50 s, 50°C for 60 s, and 72°C for 90 s. With this number of cycles, amplification occurs in the linear range and allows good quantification of amplified products. Three independent RT-PCR reactions were performed using two different batches of RNA and yielded similar results. Amplification products were separated in a 1.5% agarose gel and transferred onto nylon membrane (Hybond N, Amersham).

Hybridization was run with two kinds of probe. One probe, specific for Vvht1, was obtained by PCR with the primers H2′ = TTGCATGGTCCTGGGGTCC, and H4′ = GTCATCAATCAATTATTTGAGAGG, which allowed amplification of a 482-bp fragment covering the 3′ noncoding sequence of Vvht1. The other probe, Vvht2, was a 250-bp fragment obtained after PCR with the H2/H3 degenerated primers and cloning in pSK+. Preliminary analysis (data not shown) indicated that although they both are highly homologous to hexose transporters, Vvht1 and Vvht2 possess significantly different sequences and do not cross-hybridize.

Blots were hybridized at 42°C overnight to the ^{32}P-labeled hexose transporter probes in 50% (w/v) formamide, 5× SSPE, 4× Denhardt's solution, 0.5% SDS, and 100 μg of salmon-sperm DNA. Filters were washed for 20 min at room temperature in 2× SSC and 0.5% SDS, for 20 min at room temperature in 1× SSC and 0.2% SDS, and twice for 40 min each at 65°C with 0.1× SSC and 0.5% SDS. Signals on the hybridization membranes were quantified by a phosphor imager (STORM, Molecular Dynamics) and the membranes were used for autoradiography.

Sugar Assays

Berries were deseeded, weighed, immediately frozen in liquid nitrogen, and stored at −80°C until use. Sugars were measured by high-performance anion-exchange chromatography as described in Ollé et al. (1996).

RESULTS

Isolation and Characterization of the VvHT1 cDNA Clone

RT of total RNA extracted from berries harvested at the véraison stage followed by amplification with the H2 and H3 primers yielded a single band about 250 bp long. The amplified DNA was purified and cloned in the ECORV site of pSK+. Restriction analysis and sequence analysis made on 20 independent clones showed the presence of two different inserts exhibiting 62% to 72% identity to the different plant hexose transporters found in the database. The DNA fragments carried by these clones were called Vvht1L and Vvht2A. The fragment Vvht1L was used to screen a cDNA library prepared from grape berries at the véraison stage. Out of nine positive signals observed after the first round of screening, seven independent clones were isolated after the third round. One clone was lost during in vivo excision. The size of the DNA insert carried by the six other clones was estimated by PCR using the T3 and T7 primers located on each side of the insertion site. Clones 2 and 3 contained a 4.3-kb insert, clones 20 and 30 contained a 2.3-kb insert, and clones 10 and 17 contained, respectively, a 1.0- and a 1.3-kb insert. Because the size of the inserts contained in clones 2 and 3 was much higher than would be expected for a hexose transporter (about 2 kb),

partial sequencing was only run for DNA inserts carried by clones 10, 17, 20, and 30. Clones 20 and 30 were identical and contained a full-length cDNA highly homologous to STP1, a hexose transporter from Arabidopsis (Sauer et al., 1990). Clone 20 was used for further analysis. Clones 10 and 17 contained partial fragments corresponding to clones 20 and 30 and were thus not further analyzed.

The insert carried by clone 20 contained a 1,560-bp ORF followed by a 217-bp sequence containing a poly(A^+) tail. The 500-bp sequence located upstream of the start codon was highly homologous to rRNA. The presence of this ribosomal sequence was likely due to contamination during the preparation of the library by RT. The ORF carried by clone 20, Vvht1, encodes a 519-amino acid (57,483 D) protein (pI = 9.19) exhibiting high homology to various monosaccharide transporters cloned from plant tissues (Fig. 1). The hydropathy pattern analyzed according to Kyte-Doolittle suggested the presence of 12 transmembrane-spanning domains, which could be resolved into two parts of six hydrophobic loops each, separated by a large central hydrophilic fragment of about 50 amino acids (Fig. 1). Alignment of VvHT1 with the five closest hexose transporter sequences: RcSCP (*Ricinus communis*, accession no. L08196; Weig et al., 1994), AtSTP1 (*Arabidopsis thaliana*, accession no. X55350; Sauer et al., 1990), NtMST1 (*Nicotiana tabacum*, accession no. X66856; Sauer and Stadler, 1993), MtuSTC (*Medicago truncatula*, accession no. U38651; Harrison, 1996), and VfSTP1 (*Vicia faba*, accession no. Z93775; Weber et al., 1997) shows the presence of three long identical stretches, between Val-29 and Met-50, between Tyr-157 and Val-185, and between Gly-400 and Ser-427.

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Vvht1    MPAVGGFDK--GTG-KAYPGNLTPYVTVTCVVAAMGGLIPGYDIGISGGVTSMAPFLQKFFPSVYRKEALDKSTNQYCKPD  78
RcSCP    MPAVGGIPPS-GGNRKVYPGNLTLYVTVTCVVAAMGGLIPGYDIGISGGVTSMDSPLKKFFPSVYRKKAKDSSNQYCQYD  80
AtSTP1   MPA-GGFVV--GDGQKAYPGKLTPFVLPTCVVAAMGGLIPGYDIGISGGVTSMPSFLKRFFPSVYRKQQEDASTNQYCQYD  78
NtMST1   M-AGGGGIGP-GNG-KEYPGNLTLYVTVTCIVAAMGGLIPGYDIGISGGVTSMDSPLSRFFPSVFRKQKADDSTNQYCKFD  78
MtuSTC   M-AGGGIPIGGGN--KEYPGNLTPFVITCIVAAMGGLIPGYDIGISGGVTSMDPPLKKFFPAVYRKKNKDKSTNQYCQYD  78
VfSTP1   MPAA-GIPIGAGN--KEYPGNLTPFVTITCVVAAMGGLIPGYDIGISGGVTSMNPPLEKFFPAVYRKKNAQHSKNQYCQYD  78
Cons     M-A--G-----G---K-YPGNLT--V--TC-VAAMGGLIPGYDIGISGGVTSM--PL--FFP-V-RK-----S-NQYC--D

Vvht1    SETLTLFTSSLYLAALLSSLVAATVTRKFPGRKLSMLFGGLLPCAGAIINGAAKAVWMLIVGRILLGPGIGFANQSVPL  156
RcSCP    SQTLTMFTSSLYLAALIASLVASTITRKFGRKLSMLFGGVLPCAGAIINGAAKAVWMLILGRILLGPGIGFANQSVPL  158
AtSTP1   SPTLTMFTSSLYLAALISSLVASTVTRKFGRRLSMLFGGILPCAGALINGFAKHVWMLIVGRILLGPGIGFANQAVPL  156
NtMST1   SQTLTMFTSSLYLAALLSSLVASTVTRKLGRRLSMLCGGVLPCAGALINGFAQNVAMLIVGRILLGPGIGFANQSVPL  156
MtuSTC   SQTLTMFTSSLYLAALLSSLVASTITRRFGRKLSMLFGGLLFLVGALINGFANHVWMLIVGRILLGPGIGFANQPVPL  156
VfSTP1   SETLTLFTSSLYLAALLSSVVASTITRRFGRKLSMLFGGLLFLVGALINGLAQNVAMLIVGRILLGPGIGFANQSVPL  156
Cons     S-TLT-FTSSLYLAAL-SS-VA-T-TR--GR-LSML-GG--F--GA-ING-A--V-MLI-GRILLGPGIGFANQ-VP-

Vvht1    YLSEMAPYKYRGALNIGPQLSITIGILVANILNYFFAKIKGGWGWRLSLGGAVVPALIITVGSLVLPDTPNSMIERGQH 235
RcSCP    YLSEMAPYKYRGALNIGPQLSITIGILVANVLNYFFAKIKGGWGWRLSLGGAMVPALIITVGSLVLPDTPNSMIERGQH 237
AtSTP1   YLSEMAPYKYRGALNIGPQLSITIGILVASVLNYFFAKIKGGWGWRLSLGGAVVPALIITIGSLVLPDTPNSMIERGQH 235
NtMST1   YLSEMAPYKYRGALNLGPQLSITIGILVANVLNYFFAKIHWGW--RLSLGGAMVPALIITIGSLFLPETPNSMIERGNH 233
MtuSTC   YLSEMAPYKYRGALNIGPQLSITIGILVANVLNYFFAKIKGGWGWRLSLGGAMVPALIITIGSLVLPDTPNSMIERGDR 235
VfSTP1   YLSEMAPYKYRGALNIGPQLSITIGILVANILNYFFAKIKGGWGWRLSLGGAMVPALIITIGSLLLPDTPNSMIERGDR 235
Cons     YLSEMAPYKYRGALNIGPQLSITIGILVA--LNYFFAKI--GW--RLSLGGA-VPALIIT-GSL-LP-TPNSMIERG--

Vvht1    EGAKTKLRRIRGVDDVEEEFNDLVVASEASKLVEHPWRNLLQRKYRPHLTMAILIPFFPQQLTGINVIMFYAPVLFKTI  313
RcSCP    EEARAHLKRVRGVEDVDEEFTDLVHASEDSKKVEHPWRNLLQRKYRPHLSMAIAIPFFPQQLTGINVIMFYAPVLFDTI  315
AtSTP1   EEAKTKLRRIRGVDDVSQEFDDLVAASKESQSIEHPWRNLLRRKYRPHLTMAVMIPFFPQQLTGINVIMFYAPVLFNTI  313
N.MST1   DEAKARLKRIRGIDDVDEEFNDLVVASEASRKIENPWRNLLQRKYRPHLTMAIMIPFFPQQLTGINVIMFYAPVLFKTI  311
MtuSTC   DGAKAQLKRIRGIEDVDEEFNDLVAASEASMQVENPWRNLLQRKYRPQLTMAVLIPFFPQQFTGINVIMFYAPVLFNSI  313
VfSTP1   DGAKAQLKRIRGVEDVDEEFNDLVAASETSMQVENPWRNLLQRKYRPQLTMAVLIPFFPQQFTGINVIMFYAPVLFNSI  313
Cons     --A---L-R-RG--DV--EF-DLV-AS--S---E-PWRNLL-RKYRP-L-MA--IPFFQQ-TGINVIMFYAPVLF--I

Vvht1    GFADDASLMSAVITGGVNVLATIVSIYGVDKWVRRRFLFLEGGTQMLICQVIVATCIGVKPGVDGEPWCLPKWYAIVV  390
RcSCP    GPGSDAALMSAVITGLVNVFATMVSIYGVDKWGRRFLFLEGGVQMLICQAIVAACIGAKPGVDGAPGDLPQWYAVVV  392
AtSTP1   GFTTDASLMSAVVTGSVNVGATLVSIYGVDRWGRRFLFLEGGTQMLICQAVVAACIGAKPGVDGTPGELPKWYAIVV  390
NtMST1   GFGADASLMSAVITGGVNVLATVVSIYYVDKLGRRFLFLEGGIQMLICQIAVSICIAIKPGVNGTPGDLPKWYAIVV  388
MtuSTC   GFKDDASLMSAVITGVVNVVATCVSIYGVDKWGRRALFLEGGAQMLICQVAVAAAIGAKPGTSGNPGNLPEWYAIVV  390
VfSTP1   GFKDDASLMSAVITGVVNVVATCVSIYGVDKWGRRALFLEGGVQMLICQVAVAVSIAAKPGTSGEPGDLPKWYAIVV  390
Cons     GF--DA-LMSAV-TG-VNV-AT-VSIY-VD---RR-LFLEGG-QMLICQ--V---I--KPG--G-P--LP-WYA-VV

Vvht1    VLFICVYVSGFAWSWGPLGWLVPSEIPLEIRSAAQSVNVSVNMFFTFIIAQIFLNMLCHMKFGLFLFAFFVVVMSF  468
RcSCP    VLFICIYVSGFAWSWGPLGWLVPSEIPLEIRSAAQSVNVSVNMFFTFVVAQVFLIMLCHLKFGLFIFPSFFVLIMSI  470
AtSTP1   VTFICIYVAGFAWSWGPLGWLVPSEIPLEIRSAAQSITVSVNMIFTFIIAQIFLTMLCHLKFGLFLVFAFFVVVMSI  468
N.MST1   VIFICVYVAGFAWSWGPLGWLVPSEIPLEIRSAAQSINVSVNMIFTFIVAQVFLTMLCHLKFGLFLFFAFFVVIMTV  466
MtuSTC   VLFICIYVAGFAWSWGPLGWLVPSEIPLEIRSAAQSVNVSVNMLFTFLVAQVFLIMLCHMKFGLFLFFAFFVLVMSI  468
VfSTP1   VLFICIYVAGFAWSWGPLGWLVPSEIPLEIRSAAQSVNVSVNMLFTFLVAQIFLTMLCHMKFGLFLFFAFFVVVMTI  468
Cons     VLFIC-YV-GFAWSWGPLGWLVPSEIPLEIRSAAQS--VSVNM-FTF--AQ-FL-MLCH-KFGLF--F-FFV--M--

Vvht1    FIYFPLPETKGIPIEEMAEVWKGHWFWSRYVNDGSYSGVE--L-V---KE-NYP-VKNV  519
RcSCP    FVTYFPLPETKGIPIEEMGQVWKQHWFWSRYVVDEDYPMG--GL--EMGKEGRIP--KNV  523
AtSTP1   FVYIPLPETKGIPIEEMGQVWRSHWFWSRFVEDGEY---GNAL--EMGKNSNQAQTKHV  522
NtMST1   FIYFPLPETKNIPIEEMVIVWKEHWFWSKFMTEVDYPGTRNGTSVEMSKGS--AGYKIV  523
MtuSTC   YVFFLLPETKGIPIEEMDRVWKSHPFWSRFVEH---GDHGNG--VEMGKGAP----KNV  518
VfSTP1   YIYTMLPETKGIPIEEMDRVWKSHPYWSRFVEH----D-DNG--VEMAKGGV----KNV  516
Cons     -----LPETK-IPIEEM--VW--H--WS--------------------K-------K-V
```

Figure 1. Features of VvHT1 protein sequence and alignment with other hexose transporter sequences, RcSCP, AtSTP1, NtMST1, MtuSTC, and VfSTP1. Putative transmembrane domains are underlined.

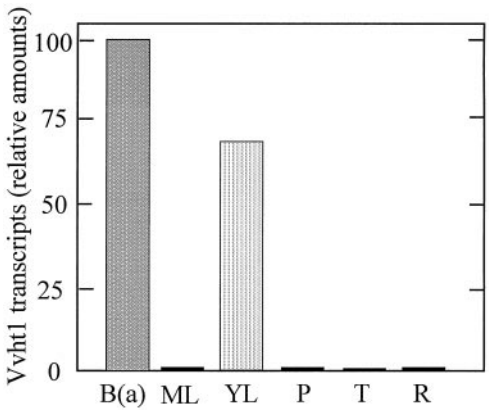

Figure 2. Northern analysis of Vvht1 expression in different organs. B(a), Berries at anthesis; ML, mature leaves; YL, young leaves; P, petiole; T, tendrils; R, roots. The nylon membranes were hybridized with a probe corresponding to the 3′ end of the Vvht1 sequence. Data were read with a phosphor imager and calibrated with a constitutive probe (grape tubulin). Intensities were expressed in percentages of the maximal value detected on the hybridization membrane.

VvHT1 contained several potential phosphorylation sites in the cytoplasmic zones, Thr-11, Ser-228, Tyr-498, and Ser-505. Of these, Ser-228 is conserved in the five hexose transporter sequences closest to VvHT1. Three N residues belong to consensus sequences Asn-X-Ser or Asn-X-Thr, allowing possible *N*-glycosylation (Asn-18, Asn-151, and Asn-429). Only Asn-151 is conserved among the five closest sequences. Asn-18 is located in a cytoplasmic loop, and Asn-151 and Asn-429 are located in the fourth and eleventh transmembrane-spanning domain, respectively. It is therefore unlikely that VvTH1 is glycosylated.

Some motifs found in hexose transporters from mammals, fungi, and bacteria are also found in VvHT1. Thus, the (R/K)XGR(R/K) motif found in position 106 through 110 in Vvht1 is also present in the GLUT1 human Glc transporter mediating facilitated diffusion (Mueckler et al., 1985), in HXT1, one of the hexose transporters from *Saccharomyces cerevisiae* (Lewis and Bisson, 1991), and in *Ara E*, a hexose transporter from *E. coli* (Maiden et al., 1987). The V/LPETK motif found in the C-terminal part of the sequence (position 474–478), at the beginning of the last hydrophilic loop is a characteristic feature of all hexose transporters cloned so far. This sequence is immediately followed by another consensus sequence, (M/V)XX(V/L)(W/Y)XXHW(F/Y)WX(R/K), which is found in all hexose transporters cloned thus far from higher plants.

Expression of Vvht1

Expression of Vvht1 in different parts of the plant was studied with RNAs extracted by the perchlorate methods from young leaves, mature leaves, tendrils, and roots of

plants at the anthesis stage (Fig. 2). Vvht1 is expressed at a significant level mainly in the berries and young leaves.

The time course of Vvht1 expression during the maturation of the berries was studied with two independent series of samples and with two different methods: northern blot (Fig. 3) and quantitative RT-PCR (Fig. 4). The first series of samples were collected on grapes grown in the Cognac region and analyzed by northern blot using a Vvht1 fragment located in the 3′ end of the cDNA sequence (Fig. 3). Vvht1 was expressed in the berries according to a biphasic mode, with a first peak at the time of anthesis, followed by a decrease in the amounts of transcripts and a second peak about 5 weeks after véraison. The expression strongly decreased during the latest stages of berry maturation and overripening. No close relationship between the total amount of sugar in the berries and the amount of Vvht1 transcripts was apparent. However, a rise in the relative rate of sugar accumulation between 12 and 14 weeks after flowering correlated with the peak of Vvht1 expression. Vvht1 expression rapidly decreased before harvest and at the time of harvest, although the sugar content of the berries still increased. Attempts to obtain readable autoradiographs from the northern blots quantified in Figures 2 and 3 were unsuccessful, suggesting that the amounts of Vvht1 transcripts do not represent a large population among the total RNA from the berries.

The results described above led us to study the expression of Vvht1 by quantitative PCR on an independent series of berries collected along the developmental cycle in Montpellier. Vvht1 was expressed at all stages of development (Fig. 4B); however, its expression also seemed to be biphasic, with a decrease between anthesis and véraison,

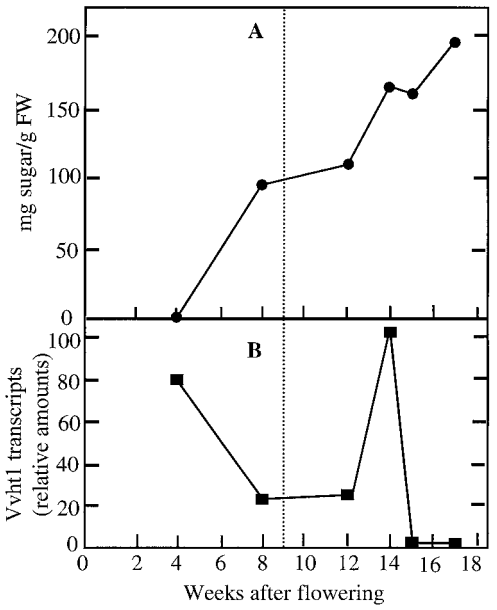

Figure 3. Northern analysis of Vvht1 expression along the developmental cycle of the berries. A, Sugar content of the berries; B, relative amounts of vvht1 transcripts. Berries from grape (cv Ugni-Blanc) were sampled in the Cognac region from the time of anthesis to the time of harvest. The dashed vertical line indicates the approximate date of véraison. Other details are as in the legend of Figure 2.

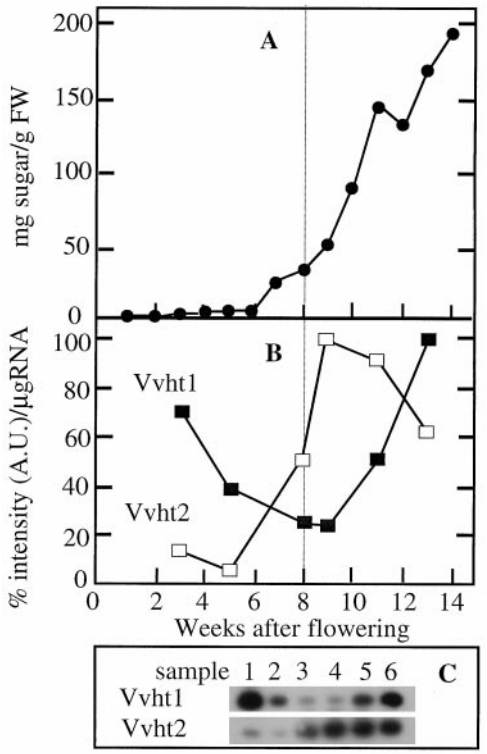

Figure 4. Quantitative RT-PCR analysis of hexose transporter expression during ripening of cv UgniBlanc grape berries. Berries were sampled from 1 to 16 weeks postflowering from grapevines located in the Montpellier area. A, Sugar content of the berries, determined on free-run juice; B, signal intensity obtained with hexose transporter probes expressed in arbitrary units (A.U.) on a per microgram of RNA basis; C, corresponding autoradiograms showing the signals obtained with the vvht1 (down) and Vvht2A (up) probes. ■, Expression pattern obtained with a probe specific for the 3′ end of the Vvht1 sequence; □, expression pattern obtained after RT-PCR and hybridization with the Vvht2A hexose transporter probe. Intensities are expressed as a percentage of the maximal value detected on the hybridization membrane. The vertical line indicates the approximate date of véraison.

and a continuous increase until 5 weeks after véraison. Although the accumulation of Vvht1 transcripts could not explain the initial rise in sugar content that accompanied véraison, it did correlate well with the accumulation of sugars occurring between 10 and 13 weeks after flowering. The overripe stages were not studied in these series. Both series of data concerning Vvht1 (Figs. 3 and 4) suggested that the expression of this gene along the development of the berries was biphasic and correlated with the phase of massive accumulation of sugars.

RT-PCR was also run with degenerated primers, allowing a more general amplification of hexose transporter transcripts, and the amplification products were hybridized with the Vvht2 probe, which does not cross-hybridize with Vvht1 (Fig. 4B). Under these conditions, a different pattern of expression was obtained, with a very low expression at 3 and 5 weeks after flowering and a transcript accumulation that started about 3 weeks before véraison and peaked shortly after véraison. These data indicate that there are several transcripts homologous to hexose trans-

porters that exhibit a different pattern of accumulation during the ripening of grape berry. Autoradiographs obtained after amplification and hybridization of the samples used in Figure 4B are shown in Figure 4C.

Isolation and Characterization of the Gene Encoding VvHT1

Out of 350,000 clones (equivalent to about 10 genomes), five positive phages were obtained after the first round of screening of the genomic library with the hexose transporter probe prepared by RT-PCR. Nine clones were still present after the third round of screening. Digestion by *Xho*I and *Sac*I showed that seven of these clones contained inserts between 13 and 15 kb. These clones were used for a maxipreparation of DNA. Restriction analysis with *Sac*I, *Xho*I, *Kpn*I, *Sa1*I, *Hin*dII, *Eco*RI, and *Bg1*II indicated that the DNA inserts could be classified into three groups: A, B, and C. The restriction patterns observed with these genomic DNA fragments were compared with the restriction patterns previously observed with Vvht1 cDNA. Both Vvht1 cDNA and the three genomic clones of group B contained only one *Kpn*I site. On the Vvht1 cDNA, this site is located 27 bp after the start codon. This feature and the restriction maps were used to select the most interesting genomic clone. Clone 4, belonging to group B, was used for further analysis. This clone contains a 3-kb genomic sequence obtained by double digestion with *Kpn*I and *Sac*I. A 2.5-kb sequence containing the promoter region was obtained by digestion with Sa1I and *Kpn*I. Upstream of this sequence, clone 4 also contained a 9-kb region that was not analyzed. Both the *Kpn*I/*Sac*I 3-kb fragment and the *Sa1*I/*Kpn*I 2.5-kb fragment were cloned in pSK+ and completely sequenced.

The coding sequence of the Vvht1 gene is 3101 bp long and comparison with the cDNA sequence allowed us to determine the site of polyadenylation and the sites of splicing. The sequence between the site of translation initiation and the site of fixation of the poly(A^+) tail is 2,821 bp long. The gene is composed of three introns and four exons. The nucleotidic sequence of the exons is completely identical with that of Vvht1 cDNA, except for three bases located in the first position of the codons corresponding to Leu-82, Arg-202, and Glu-263. Therefore, there is a high degree of conservation between different cultivars of grape, since the genomic library was prepared from cv Pinot Meunier and the cDNA library was prepared from cv Ugni-Blanc. The structure of the gene encoding Vvht1 is similar to that of AtSTP1 (data not shown), but differs from the structure of the HUP1 and HUP3 genes of *Chlorella kessleri*, which contain 14 introns (Wolf et al., 1991; Stadler et al., 1995). The splicing consensus sequences AG/GT (Breathnach and Chambon, 1981; Hanley and Schuler, 1988) are conserved for the introns of Vvht1. A TATATATA motif located in position 2,667 to 2,674 in the genomic sequence of Vvht1 is absent in the untranslated region of Vvht1 cDNA. The existence of such an octanucleotide in this position has not yet been reported in the literature and its possible function is unknown.

The promoter fragment is 2,471 bp long and is terminated by the 33 first bp after the ATG. All sequences referred to below are numbered from the first base of the ATG. The ATG is not located in one of the consensus sequences ([C/G]AANNATGG or TAAACAATGGCT) that have been described for the translation initiation site in plants (Joshi, 1987; Lütcke et al., 1987). The main boxes found in the promoter region of Vvht1 are mapped in Figure 5. A single CTATATATA sequence corresponding to the consensus sequence (C/G)TATA(T/A)A_{1-3}(C/T)A for TATA boxes in plants is found 110 bp before the ATG. Four CCAAT boxes, commonly found in the 5′ noncoding region of eukaryotic genes (Hanley and Schuler, 1988), are found in the distal region of the promoter sequence. The 1.5-kb region upstream of the ATG is rich in A/T repetitive sequences, which is a characteristic feature of the plant promoters. Among these sequences, a TATTT(A/T)AT sequence found in two positions of the Vvht1 promoter also belongs to the *cis* regions that have been previously identified as controlling the spatial or developmental specificity of gene expression (Forde et al., 1990). This motif has been found in seven genes of nodulins (Forde et al., 1990), in the promoter of the tomato vacuolar invertase (Elliott et al., 1993), and in the Arabidopsis Suc transporter AtSUC2 (Truernit and Sauer, 1995). Four repeats of the SEF4 motif (RTTTTTR), which was also identified in the β-conglycinin promoter, and one motif (CAGAAGATA) driving phloem-specific gene expression of the rice tungro bacilliform virus (Yin et al., 1997) are also present in the Vvht1 promoter.

A P-box (TGTAAAG) is present in position −650. In cereals this box is located closer to the translation initiation site (about −300) of several genes encoding seed storage proteins (Colot et al., 1987; Vicente-Carbajosa et al., 1997). Two ERELEE4 boxes (AWTTCAAA) are found in positions −1,242 and −1,236. The ERELEE box has been described as an ethylene-responsive enhancer element of a carnation glutathione-*S*-transferase gene involved in senescence

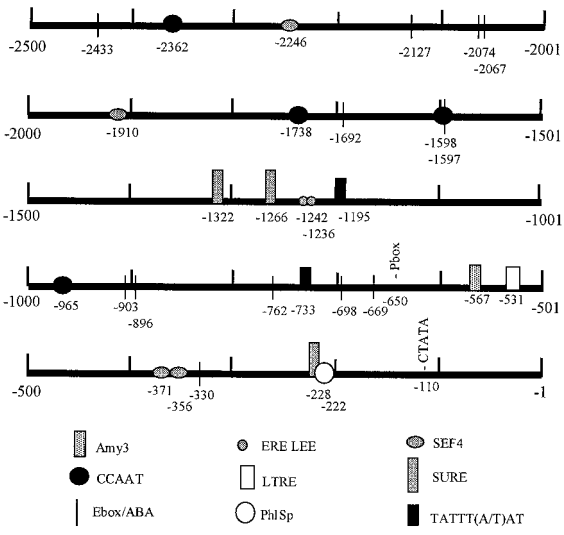

Figure 5. Map of the Vvht1 promoter. The consensus sequences corresponding to various putative *cis* elements are described in the text. Positions are numbered with respect to the first base of the translation start ATG.

Vvht1	T**AAAATCA**TGTAACA**TAA**	-1307
AmCHS	A**AAAATCA**TCTTTT**TTAA**	+482,intron
gspoB1	A**AAAATCA**CGCATC**TAAA**	-667
StPATG1	G**AAAATCA**CAGTGC**TGAA**	-340
gspoA1	CC**AAATCA**TTTCTG**TTAT**	-213
Vvht1	AT**AAATCA**GAAGATAG**AA**	-212

Figure 6. Putative Suc box B in the promoter region of Vvht1, as deduced from comparison with the promoters of various other genes in which this box was identified. AmCHS, *Antirrhinum majus* chalcone synthase (Sommer and Saedler, 1986); gspoB1, *Ipomoea batatas* sporamin clone A (Hattori et al., 1990); StPATG1, *Solanum tuberosum* patatin (Bevan et al., 1986); gspo1A, *I. batatas* sporamin clone B (Hattori et al., 1990).

(Itzahki et al., 1994) and of the E4 gene expressed during tomato ripening (Montgomery et al., 1993).

A number of repetitive CANNTG sequences identified as E-box/ABA-responsive elements (Stalberg et al., 1996) are also found in the sequence. A GCCGAC low-temperature-responsive element (Baker et al., 1994; Jiang et al., 1996) is located in position −531.

The AATAGAAAA sequence described as a Suc-responsive element (Grierson et al., 1994) is found in position −1,266, and two 15-bp regions homologous to the Suc box 3 described in the promoters of various genes including chalcone synthase and sporamine (Tsukuya et al., 1991) are located at positions −1,322 and −228 in the promoter of Vvht1 (Fig. 6). A TATCCAT motif identified as a *cis* element of the Amy3D gene of rice responsive to sugar starvation (Hwang et al., 1998) is present in position −567 of the Vvht1 promoter.

An important feature of the promoter of Vvht1 is its similarity to the promoter of the grape alcohol dehydrogenase recently cloned (Sarni-Manchado et al., 1997). Indeed, alignment of the 350-bp sequence available for the alcoholde hydrogenase promoter with the corresponding region of the Vvht1 promoter reveals 52% identity over the 270 bp flanking the ATG (Fig. 7), with regions as long as 15 nucleotides nearly identical. The two most conserved sequences are a 16-bp sequence located in position −213 (GAAAGA–ATTGAAAA) and a 14-bp sequence located in position −114 of the Vvht1 promoter, which includes the TATA box (GT/GGGCTATAT/AATA). In contrast, alignment of the Vvht1 promoter with other promoters of hexose or Suc transporters did not reveal any important identity (data not shown).

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PromVvht1   AAAAGGGGAG CA--AATTGT TAG-TG---- CAGTTAC-CT TA-TCTACTA AATAAATCAG AA GAT A GAAA  -210
PromVvAdh1  CAGAGTCTAG AAATAATTGT AAGCTGGGAA TGGTTTTGCA CAGCCTGTTG TTTTACTTGG GT GAT - GAAA  -152
Consensus   MARAGKSKAG MAATAATTGT WAGCTGGGAA YRGTTWYGCW YAGYCTRYTR WWTWAMTYRG RW GAT A GAAA

PromVvht1   GA AA ATTGAAA ATGGAAGTT GAATATTGGC TTGATTAAGA ATTAGATATT ATCGGGGACA GCTCAACAGT   -140
PromVvAdh1  GA CC ATTGAAA -TATATCCA GAA-AGAAAC TGTATCTAG- -TAACGTTTC ATC-GAAATA -ATTAAT-GA   -88
Consensus   GA MM ATTGAAA ATRRAWSYW GAATAKWRRC TKKATYWAGA ATWASRTWTY ATCGGRRAYA GMTYAAYAGW

PromVvht1   TTTCTATACA TGTTTGGAGG TGGAT- GT GGCTATA TATAC ACCCCTCTCC TTTCTATCTC ATACTTCAAT   -71
PromVvAdh1  ACT-GAAATA TTTTTCTTCC TTCTTG GG GGCTATA AATAC --------CC ---------- ----------   -46
Consensus   WYTCKAWAYA TKTTTSKWSS TKSWTG GK GGCTATA WATAC ACCCCTCTCC TTTCTATCTC ATACTTCAAT

PromVvht1   TTTATTCTCA GTTTTTCTTC TACATAGGCG TCTGTAGTTA TTTCTGTTGA TCTGTTGAAG GGAGATCAAT     -1
PromVvAdh1  --TA--CGGA G--------- GACTGAGGAG -CACCA--TA TCTTTG--GA -GTATCTAA- GAAAAT----     -1
Consensus   TTTATTCKSA GTTTTTCTTC KACWKAGGMG TCWSYAGTTA TTTYTGTTGA TSTRTYKAAG GRARATCAAT
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Figure 7. Homology between the proximal part of the Vvht1 promoter (270 bp) and that of the Adh1 grape alcohol dehydrogenase promoter (Sarni-Manchado et al., 1997). Alignments were made with Geneworks (Intelligenetics) software.

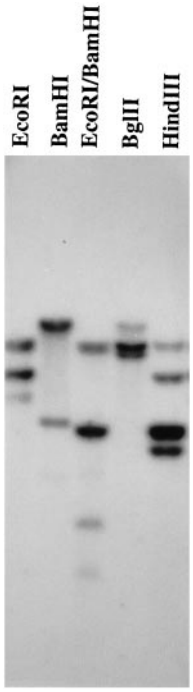

Figure 8. Southern hybridization of genomic DNA isolated from the leaves of the grape cv Ugni-Blanc. The DNA (15 μg/lane) was digested with *Eco*RI (lane 1), *Hin*dII (lane 2), *Bgl*II (lane 3), and *Bam*HI (lane 4) prior to separation in a 0.8% agarose gel. The ^{32}P-labeled *Kpn*I/*Hpa*I fragment of Vvht1 was used for hybridization.

Southern Analysis of Genomic DNA

With the VvHT1 cDNA probe used for hybridization, a complex restriction-fragment pattern was obtained from genomic DNA of grape (Fig. 8). The restriction enzyme *Eco*RI, which cuts only once within the probe, produced three fragments that hybridized to the cDNA probe, one of which had a weak intensity. *Hin*dIII, which also cuts only once in the probe produced four labeled fragments, two of which were much less labeled. Digestion by *Bam*HI, which does not cut Vvht1, resulted in two bands. These results indicate that the hexose transporter should be encoded by at least two distinct genes in the grape cv Ugni-Blanc. Comparable results were obtained with cv Shiraz (data not shown).

DISCUSSION

The ripening stage at which the berries are harvested, and in particular their sugar/acid balance at this time, play an important part in the final quality of the molt and of the resulting wines. It is therefore important to understand, characterize, and, if possible, control the maturation process. This control is more difficult for grape than for climacteric fruits, for which maturation can be triggered by ethylene treatments. In this context, the isolation and characterization of a cDNA clone and of a genomic clone en-

coding a hexose transporter expressed during the ripening of the berries provide initial clues for the understanding of this process. Sugar accumulation may also be important from the standpoint of grape resistance to phytopathogens, since accumulation of hexoses in *Vitis labruscana* berries was shown to be accompanied by a developmental-stage-specific increase of antifungal proteins (Salzman et al., 1998).

Hexose transporters cloned so far have been isolated from herbaceous species, and no data are available for woody species. The Vvht1 cDNA sequence shares a strong homology with the other hexose transporters already cloned from herbaceous species. The highest homologies were found with transporters cloned from *R. communis* (RcScP), *M. trunculata* (*not trunculata*; MtStc), *V. faba* (VfStp1), *N. tabacum* (NtMst1), and Arabidopsis (AtStp1). MtuStc (Harrison, 1996), VfStp1 (Weber et al., 1997), and NtMst1 (Sauer and Stadler, 1993) are also expressed in sink tissues. However, AtStp1 is poorly expressed in heterotrophic tissues, and strongly expressed in leaves (Sauer et al., 1990), and therefore there is no direct relationship between sequence homologies and tissue-specific expression. It is noteworthy that a perfect identity at the nucleotide level was found between the Vvht1 cDNA sequence obtained after screening of the library from cv Pinot Noir leaves and the cDNA sequence deduced from the genomic clone obtained after screening of the genomic library from cv Ugni-Blanc. Vvht1 sequence is therefore conserved between those two cultivars, and possibly in other grape cultivars.

Although the length of the central loop is strictly conserved between VvHT1 protein sequence and the five closest hexose transporter sequences, noticeable differences are found in the 20 C-terminal amino acids and in the 10 N-terminal amino acids. The presence of a conserved Ser residue in the central loop of these sequences (Ser-228 in VvHT1) raises the possibility of post-translational regulation of the transport activity by phosphorylation, which was recently suggested for a Suc transporter (Roblin et al., 1998).

Vvht1 encodes a putative hexose transporter that is expressed mainly in the berries (Fig. 2). Analysis of Vvht1 expression both by northern blot (Fig. 3) and by RT-PCR (Fig. 4) on two independent sets of berries collected at different stages of development in two distinct climatic areas confirmed that the maximal expression of Vvht1 occurs about 5 weeks after véraison. However, in both series, an earlier peak of expression was also found shortly after fecondation. High rates of sugar import may be needed to support active cell division. This biphasic pattern of expression suggests a complex regulation of the expression pattern. The only report showing a developmental pattern of expression for a hexose transporter in sink tissues was published by Weber et al. (1997), who found that VfSTP1 transcripts encoding a *V. faba* hexose transporter showed a single peak of accumulation in seed coats and cotyledons.

RT-PCR data with the Vvht2A probe (Fig. 4) also suggest the existence of another hexose transporter showing only one peak of expression that occurs shortly after véraison. The probable existence of several different hexose transporters expressed during the maturation of the berry is strengthened by the results of Southern analysis (Fig. 8) and the existence of a multigenic family encoding the plasma membrane hexose transporters in the species studied so far (Arabidopsis, Caspari et al., 1994; *R. communis*, Weig et al., 1994). The isolation of other sugar transporter clones from grape is under way in our laboratory. The exact location of Suc hydrolysis during the hexose accumulation occurring after véraison is not known. However, the fact that plasma membrane hexose transporters are expressed during this stage suggests that at least part of the Suc imported by the berry phloem is hydrolyzed prior to accumulation in the flesh cells. The fact that autoradiographic signals can be detected only after RT-PCR indicates that the vvht1 transcripts only represent a small proportion of total berry RNA and suggests that they are expressed only in a limited number of cells.

Among the numerous potential *cis* sequences found in the Vvht1 promoter, the ethylene-responsive elements, the ABA-responsive element box, the sugar boxes, and the amy3 box seem particularly relevant for further analysis. Ethylene, ABA, and water stress have been described as promoting sugar accumulation in grape (for review, see Coombe, 1992). The existence of Suc boxes in the promoter sequence of Vvht1 opens the possibility that the expression of this gene is induced by sugar level, and therefore that sugar accumulation would occur as an autocatalytic process. This fits well with earlier physiological observations in which sugar accumulation in the berry was irreversible once it was triggered (Coombe, 1992). However, this sensitivity of Vvht1 to sugar expression and, more generally, the physiological significance of the different boxes identified in the promoter should be determined by reporter gene experiments. Although grape transformation is possible (Mauro et al., 1995), about 2 years are needed to obtain the plants. Experiments using various fusions of the promoter region with GUS and GFP reporter genes, and transformation in tobacco and grape suspension cells are under way in our laboratory for functional analysis of the Vvht1 promoter (R. Atanassova, M. Leterrier, C. Gaillard, P. Coutos-Thévenot, and S. Delrot, unpublished data).

The pattern of expression of Vvht1 is paralleled by the expression of one alcohol dehydrogenase gene in grape berries (Sarni-Manchado et al., 1997). Moreover, the promoter sequence of Vvht1 and of this alcohol dehydrogenase exhibit a high level of homology (Fig. 7), while comparison of the Vvht1 promoter sequence with that of other hexose transporter promoters did not reveal any significant homology (data not shown). These data suggest that Vvth1 and alcohol dehydrogenase are co-induced during ripening, and that this co-induction may be due to the binding of a common transcription factor on the *cis* sequences that are shared by the promoters of the genes encoding these enzymes. These promoter sequences may be useful in cloning this transcription factor, which is involved at an early stage of maturation induction.

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The accession numbers for the sequences reported in the article are: AJ001061, coding sequence for *Vvht1*; AJ001062, DNA for the *Vvht1* promoter; and Y09590, mRNA for *Vvht1*.

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