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Identification and Characterization of a Fruit-Specific, Thaumatin-Like Protein That Accumulates at Very High Levels in Conjunction with the Onset of Sugar Accumulation and Berry Softening in Grapes[1]

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The protein composition of the grape (*Vitis vinifera* cv Muscat of Alexandria) berry was examined from flowering to ripeness by gel electrophoresis. A protein with an apparent molecular mass of 24 kD, which was one of the most abundant proteins in extracts of mature berries, was purified and identified by amino acid sequence to be a thaumatin-like protein. Combined cDNA sequence analysis and electrospray mass spectrometry revealed that this protein, VVTL1 (for *V. vinifera* thaumatin-like protein 1), is synthesized with a transient signal peptide as seen for apoplastic preproteins. Apart from the removal of the targeting signal and the formation of eight disulfide bonds, VVTL1 undergoes no other posttranslational modification. Southern, northern, and western analyses revealed that VVTL1 is found in the berry only and is encoded by a single gene that is expressed in conjunction with the onset of sugar accumulation and softening. The exact role of VVTL1 is unknown, but the timing of its accumulation correlates with the inability of the fungal pathogen powdery mildew (*Uncinula necator*) to initiate new infections of the berry. Western analysis revealed that the presence of thaumatin-like proteins in ripening fruit might be a widespread phenomenon.

Because of the widespread demand for wine, table grapes, and dried fruit, the common grape, *Vitis vinifera* L., is one of the most economically important fruit crops in the world. Like a small number of other fruits, the ripening of grapes is nonclimacteric and its growth pattern follows a double-sigmoid curve encompassing three stages (Coombe, 1976). Stage I, immediately following flowering, is characterized by a short period of rapid cell division, followed by marked cell enlargement (Nakagawa and Nanjo, 1965; Considine and Knox, 1979) concomitant with high rates of metabolism and rapid accumulation of acid (Kliewer, 1965). Stage II is a lag phase, approximately 7 to 10 weeks postflowering, which constitutes a short period of slow, or no, growth (Coombe, 1973). Toward the end of this period, the berries lose chlorophyll and acidity reaches a maximum. Berry softening, together with the rapid accumulation of sugars and amino acids (for review, see Kanellis and Roubelakis-Angelakis [1993]), signals entry into stage III, in which cell expansion rather than cell division is chiefly responsible for the continued growth in berry size and weight (Nakagawa and Nanjo, 1965; Considine and Knox, 1979). Viticulturists use the term veraison (from the French véraison) to describe the entry into stage III and, therefore, the inception of ripening.

Although a great deal is known about some of the chemical constituents of grape berries and the changes that occur through the three stages of berry growth, little is known about their protein composition and associated ripening physiology. The profile of soluble proteins found in the juice of ripe grapes often appears surprisingly simple, with a predominance of a few low-molecular-weight proteins (Hsu and Heatherbell, 1987; Murphey et al., 1989; Yokotsuka et al., 1991; Pueyo et al., 1993). The simplicity of this profile suggests an incomplete extraction of total cellular protein. To better understand changes in protein profiles during the development of the grape berry and other fruits, a systematic sampling throughout stages I, II, and III was performed and combined with an effective extraction procedure. Using such a strategy, we now show that one of the most abundant proteins in ripe grapes starts accumulating at the onset of berry softening.

Isolation of this protein, cloning of the corresponding cDNA, and northern, Southern, and western analyses reveal that the protein in question, VVTL1 (for *V. vinifera* TL protein 1), is a TL member of the PR-5 protein family and is encoded by a single *V. vinifera* gene, which is expressed in a berry- and ripening-specific manner. As opposed to the vast majority of other berry proteins, VVTL1 appears to be soluble in grape juice extracts, which is consistent with the recent finding by Waters et al. (1996) that the predominant proteins in white wines are chitinases and TL proteins. It is tempting to speculate that the accumulation of PR proteins in the softening and intensely sweet grape berry serves an

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Abbreviations: °Brix, refractive index measure of total soluble solids; PR, pathogenesis-related; RACE, rapid amplification of cDNA ends; SAR, systemic acquired resistance; TFA, trifluoroacetic acid; TL, thaumatin-like.

antifungal role; however, it is interesting to note that otherwise ubiquitous members of the PR-2 protein family, the 1,3-β-glucanases, have not yet been detected in the grape berry. Whatever the reason for the prominent accumulation of VVTL1, the present results indicate a common trigger for the onset of ripening and PR protein synthesis—a phenomenon that has recently also been suggested for the cherry, another example of a nonclimacteric fruit (Fils-Lycaon et al., 1996).

MATERIALS AND METHODS

All procedures were carried out at room temperature unless noted otherwise.

Berry Sampling

Berries were harvested from 26 *Vitis vinifera* cv Muscat of Alexandria vines planted in 1981 at the Alverstoke vineyard (Adelaide, South Australia). Synchronously flowering bunches were tagged and their development was followed to maturity. Ten bunches were taken from the vines every 1 to 2 weeks. No more than 10% of a particular vine's bunches were harvested. A 50-berry sample, consisting of five berries taken from the top, middle, and bottom of each bunch, was subjected to berry weight and deformability measurements as previously described by Coombe and Bishop (1980). The remaining berries were frozen in liquid N_2 and powdered in a blender (Waring) before storage at −70°C. Subsamples of the frozen berries (2–5 g) from different developmental stages were used for protein, RNA, and "free-run" juice extraction. Free-run juice was obtained by thawing powdered berries and centrifuging at 12,000*g* for 5 min. °Brix was measured with a digital refractometer (Erma, Tokyo, Japan). The pH of the juice was also measured.

Tissue Sampling and Protein Extraction

All grapevine tissues were obtained from the same vines used for berry sampling except for the roots, which were obtained from a potted vine of the same cultivar grown under shadehouse conditions. Skin, pulp, and seed tissue were obtained from mature berries harvested 17 weeks postflowering. Skin was removed from frozen berries and washed extensively in distilled water prior to protein extraction. Tissue (2–5 g) was frozen in liquid N_2 and ground to a fine powder using a mortar and pestle. Protein was extracted by the addition of 2 mL of buffer (500 mM Tris-HCl, pH 8.0, 5% [w/v] SDS, 10 mM DTT, and 10 mM sodium diethyldithiocarbamate) to 1 g of powdered tissue, incubated at 95°C for 5 min, and then centrifuged at 12,000*g* for 5 min. Protein was concentrated by precipitation with ice-cold TCA at a final concentration of 10% (w/v). After a 15-min incubation on ice the precipitated protein was collected by centrifugation for 15 min at 12,000*g* and the resulting pellet was washed twice with ice-cold ethanol:ethyl acetate (2:1, v/v). Following a brief drying, proteins were resuspended in 20 mM Tris-HCl, pH 8.0, before analysis by SDS-PAGE and immunoblotting. Juice from various fruits, which were purchased from a local fruit shop, was obtained by squeezing the pulp gently and then centrifuging at 12,000*g* for 5 min. For the measurement of 1,3-β-D-glucan endohydrolase activity, berry proteins were extracted in 50 mM sodium acetate, pH 5.0, and 2 mM DTT and assayed as described previously (Chen et al., 1993).

VVTL1 Purification

Approximately 1.2 L of commercial grape juice from cv Muscat of Alexandria berries was subjected to $(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ was added to 80% saturation and stirred overnight at 4°C. The pellet obtained after centrifugation at 10,000*g* for 30 min at 4°C was extensively dialyzed at 4°C against 20 mM Tris-HCl, pH 8.0, before loading onto a 1.5- × 16-cm Q-Sepharose column (Pharmacia) equilibrated with the above buffer. Proteins were eluted with a linear 0 to 0.35 M NaCl gradient (in equilibration buffer) over 33.3 h at a flow rate of 30 mL h^{-1}. Fractions of 8 mL were collected and those containing a prominent 24-kD component (VVTL1) as judged by SDS-PAGE were pooled, concentrated to 4 mL by ultrafiltration using a YM 10 membrane (Diaflo, Amicon, Beverly, MA), and then loaded onto a Superdex 200 26/60 column (Hiload, Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Proteins were chromatographed using a flow rate of 150 mL h^{-1}, and 4-mL fractions containing VVTL1 were identified, pooled, and concentrated as described above. The yield of essentially pure VVTL1 was about 30 mg. For antibody production VVTL1 was further purified by reverse-phase HPLC on a C_8 column (4.6 × 250 mm; Vydac, Hesperia, CA) equilibrated in buffer A (0.1% TFA) and operated with a flow rate of 0.6 mL min^{-1}. The column was washed for 5 min with buffer A, followed by a linear gradient of 0 to 100% buffer B (80% [v/v] CH_3CN, and 0.085% [v/v] TFA) developed over 60 min. VVTL1 eluting at 45% (v/v) CH_3CN was collected, freeze-dried, and resuspended in PBS.

VVTL1 Digestion, Protein Sequence Analysis, and MS

TCA-precipitated VVTL1 (approximately 400 μg) was resuspended in 50 μL of 6 M urea, 50 mM Tris-HCl, pH 8.0, and 5 mM DTT, incubated at 60°C for 30 min, and then diluted with 3 volumes of 25 mM Tris-HCl, pH 7.7, and 1 mM Na_2EDTA. Endoproteinase Glu-C (Promega) and endoproteinase Lys-C (Promega) digestions were performed at 37°C for 42 h. Prior to acid hydrolysis, purified VVTL1 was reductively alkylated as described by Høj et al. (1987). Acid hydrolysis in 70% (v/v) TFA (HPLC grade, Pierce) at 40°C was performed for 20 h and terminated by the addition of 10 volumes of water before vacuum concentration. Peptides generated by endoproteinase digestion and acid hydrolysis were purified by reverse-phase HPLC essentially as described by Chen et al. (1993) and sequenced using a protein sequencer (model G1000A, Hewlett-Packard). The mass of VVTL1 was determined on an electrospray ionization-triple quadrupole mass spectrometer (model API300, Perkin-Elmer) essentially as described by Høj et al. (1992).

SDS-PAGE and Western Transfer

Proteins were resolved by SDS-PAGE in 12% (w/v) Tris-Gly gels (Fling and Gregerson, 1986). Gels were stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes (MSI Laboratories, Westboro, MA) using a semidry transfer unit (LKB, Broma, Sweden) as described by Harlow and Lane (1988).

Western Analyses

Rabbit antibodies were produced by the Institute of Medical and Veterinary Science (Adelaide, Australia). VVTL1 (approximately 100 μg in PBS) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly. The rabbit was given two more booster injections (approximately 100 μg of VVTL1 in Freund's incomplete adjuvant) at 3-week intervals before blood samples were obtained. The IgG fraction was purified on a protein A column and stored in 30% (v/v) glycerol containing 0.02% (v/v) sodium azide at 4°C. A dilution of 1:10,000 was used for immunoblotting experiments. Immunoblots were probed with anti-VVTL1 antibodies and horseradish peroxidase-coupled anti-rabbit antibodies (Promega) before detection using ECL reagents (Amersham) and subsequent exposure to Hyperfilm-MP (Amersham).

RNA Isolation, cDNA Synthesis, and Cloning

Total RNA was extracted from grapevine tissues essentially as described by Levi et al. (1992), except that the extraction buffer contained 500 mM Tris-HCl, pH 8.0, and thiourea and aurintricarboxylic acid were excluded. Fresh or frozen tissue (−70°C) was ground to a powder in liquid N_2 before buffer was added (2 mL of buffer to 1 g of tissue). RNA concentrations and purity were determined by scanning UV spectroscopy.

The total cDNA sequence for VVTL1 was obtained by combination of the information contained in an "internal" cDNA, a 5′ RACE product, and a 3′ RACE product. First-strand cDNAs were generated with RNase H^- reverse transcriptase (Superscript II, GIBCO-BRL) according to the manufacturer's instructions in a volume of 20 μL, using 2 μg of post-veraison RNA as a template and 150 pmol of primer. A $(dT)_{15}$ primer was used in the preparation of first-strand cDNA for cloning of the internal cDNA (nucleotides 154–738, Fig. 4) and the 3′ RACE-PCR product (nucleotides 610–954, Fig. 4), and the primer TL no. 6 (described below) was used to prepare the first-strand cDNA used to generate the 5′ RACE-PCR products (nucleotides 1–254, Fig. 4).

Internal cDNA

Based on amino acid sequences obtained from VVTL1, degenerate oligonucleotides representing all possible codon usages were synthesized. The oligonucleotides and corresponding amino acid sequences are as follows: TL no. 1, 5′-TT(C/T)GA(C/T)AT(A/T/C)(C/T)TIAA(C/T)AA(A/G)TG-(T/C)ACITA-3′ (FDILNKCT); TL reverse transcription no. 2, 5′-(A/G)CA(A/G)AAIGTIAC(C/T)TT(A/G)TA(A/G)TT-3′ (NYKVTFC). Reaction mixtures typically contained 1 unit of *Taq* polymerase (GIBCO-BRL), 50 pmol of each primer, and 2 μL of the first-strand cDNA product as a template in a final volume of 25 μL, buffered according to the manufacturer's instructions. Following incubation at 94°C for 2 min, a total of 37 cycles were performed (cycles 1 and 2: 94°C for 1 min, 37°C for 2 min, and 72°C for 1 min 30 s; cycles 3–37: 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min 30 s). The reaction was then terminated by a final extension reaction at 72°C for 7 min.

RACE Products

RACE-PCR, performed essentially as described by Frohman et al. (1988), was used to obtain the 5′ and 3′ ends of the final cDNA (Fig. 4). Oligonucleotides described by Frohman et al. (1988) were used in conjunction with TL no. 5, 5′-TTGACGGTGATTGTCCAGGACTG-3′ (nucleotides 232–254, Fig. 4), and TL no. 6, 5′-CATTGGCGTCGAAGG TGC-ATGAG-3′ (nucleotides 291–313, Fig. 4) to generate the 5′ RACE-PCR product. The oligonucleotides and primers TL no. 3, 5′-AA(C/T)AA(C/T)(C/T)TIGA(C/T)TA(C/T)ATIG-A(C/T)AT-3′ (nucleotides 415–437, Fig, 4), and TL no. 7, 5′-AGCTGTGGTCCGACCACATACTC-3′ (nucleotides 610–632, Fig. 4; Frohman et al., 1988), were used to generate the 3′ RACE-PCR product.

PCR products were purified from buffered (40 mM Tris-acetate, pH 8.0, and 1 mM Na_2EDTA) agarose gels using a DNA purification system (Wizard, Promega), ligated into the pGEM-T vector (Promega), and transformed into either *Escherichia coli* XL1 blue (Stratagene) or JM109 (Promega) cells. Double-stranded plasmid templates were sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977).

Southern and Northern Analyses

For Southern analysis DNA was extracted from grape leaves by the method of Steenkamp et al. (1994). DNA (4 μg) was digested to completion with the respective restriction enzymes (Promega), resolved in a buffered (40 mM Tris-acetate, pH 8.0, and 1 mM Na_2EDTA) 0.8% (w/v) agarose gel, blotted onto a Hybond N^+ membrane (Amersham) according to the manufacturer's instructions, and then fixed with 0.4 M NaOH for 15 min. The blot was incubated at 65°C for 1 h in prehybridization solution (5× SSC, 0.5% [v/v] SDS, 5× Denhardt's reagent [Sambrook et al., 1989], and 100 μg mL^{-1} denatured and sheared salmon-sperm DNA) before the addition of a denatured DNA probe (nucleotides 154–738, Fig. 4), which was ^{32}P-labeled using a DNA-labeling system (Megaprime, Amersham) according to the manufacturer's instructions. For high-stringency screening (the Tm of the probe in 0.1× SSC was 75.2°C [Meinkoth and Wahl, 1984]), hybridization for 16 h at 65°C was followed by washes of the membrane at 65°C in 2× SSC and 0.1% (w/v) SDS for 2 × 10 min, 1× SSC and 0.1% (w/v) SDS for 15 min, and then 0.1× SSC and 0.1% (w/v) SDS for 15 min.

Labeled DNA was detected with a phospor imaging screen (Kodak) and analyzed using a phosphor imager (Storm 860, Molecular Dynamics, Sunnyvale, CA) and Im-

ageQuant software (Molecular Dynamics) before printing on Epson 720-dpi paper. Screenings at lower stringency, in which targets of greater than about 70% identity are detected (the Tm of the probe in 2× SSC was 96.8°C [Meinkoth and Wahl, 1984]), were performed exactly as for the high-stringency screens except that filters were washed only in 2× SSC at 65°C for 3 × 10 min.

For northern analysis, total RNA (10 μg) was denatured and resolved by electrophoresis through a 1.25% (w/v) agarose gel containing 6% (v/v) formaldehyde in Mops buffer, pH 7.0 (Sambrook et al., 1989). The gel was then blotted onto a Hybond N^+ membrane (Amersham), RNA was fixed with 0.04 M NaOH, hybridized, washed, and the labeled DNA was detected as described for Southern analyses under stringent conditions.

Preparation of Figures

Western blot images (Hyperfilm-MP, Amersham) and Coomassie brilliant blue-stained gels were scanned and printed on Epson 720-dpi paper without any visible loss of detail from the originals.

RESULTS

Polypeptide Pattern of Developing Grape Berries

Little is known about the changes in protein composition during development and ripening of fruits in general and in nonclimacteric berries of *V. vinifera* in particular. To gain insight into such a process in a woody perennial, development of fruit on 26 vines of cv Muscat of Alexandria was followed from flowering to berry maturity. Values obtained for berry weight, deformability, °Brix, and pH are shown in Figure 1. A double-sigmoid growth curve typical of grape berries (Coombe, 1976) was observed. The onset of veraison, signaled by the sudden increase in berry deformability and the onset of rapid accumulation of soluble solids, was clearly observed 9 weeks postflowering, shortly before the resumption of rapid berry growth.

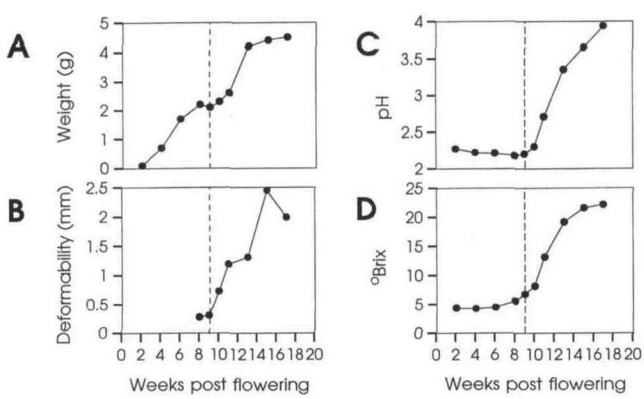

Figure 1. Physical and chemical changes in grape berries from flowering to ripeness. Berries were sampled from 2 to 17 weeks postflowering. Weight (A) and deformability (B) measurements were performed on whole berries, and pH (C) and °Brix (D) measurements were performed on free-run juice. The approximate time of veraison is indicated by a dashed line (at 9 weeks postflowering).

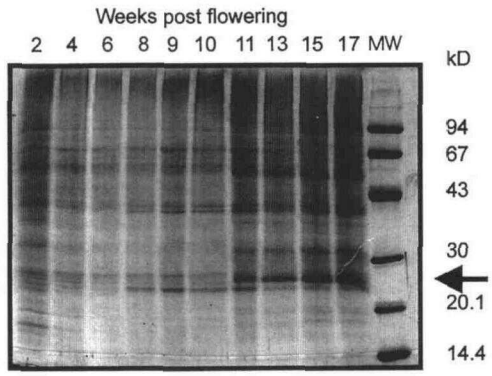

Figure 2. Protein profiles of grape berries at different stages of development. Protein extracted from whole berries (2–17 weeks postflowering) was resolved by SDS-PAGE and stained with Coomassie brilliant blue. Each lane contained protein extracted from approximately 50 mg fresh weight of tissue. The arrow indicates the position in the gel of a protein that was the subject of further study. MW, Molecular mass standard.

Extraction of berry proteins in a 5% (w/v) SDS-based buffer followed by electrophoresis revealed a relatively complex polypeptide pattern with few obvious changes during development, other than a general increase in the amount of extractable protein (Fig. 2). The most distinct feature was the continuous accumulation of two proteins with apparent masses of 24 and 32 kD, clearly distinguishable at and after 13 weeks postflowering. The apparent masses of these proteins are very similar to those of the major haze-forming proteins recently identified in a commercial white wine (Waters et al., 1996). Indeed, when clarified free-run juice, the material used for winemaking, was extracted from the same berry samples without any protective buffering agents, a very simple polypeptide pattern consisting almost exclusively of the 24- and 32-kD components was observed (data not shown). Because of the relative abundance of these proteins in juice, we decided to purify the 24-kD component from a commercial preparation of grape juice. Comparison of lanes a and b in Figure 3 clearly shows how the preparation of free-run juice constitutes a selective extraction procedure.

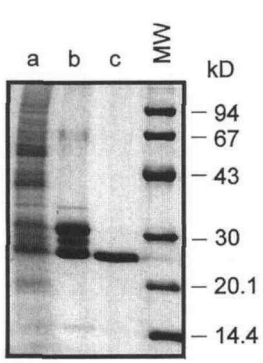

Figure 3. *Purification of VVTL1 from grape juice.* SDS-PAGE analysis of SDS-extracted whole berry protein (lane a), $(NH_4)_2SO_4$-precipitated juice protein (lane b), and purified VVTL1 (lane c). MW, Molecular mass standard.

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GGACACCGCATTAGCCCAAACCCATAAGAATTCTAAATACTTCGATTCTGATCATCTGAT 60
TCATTCAACATCAAAATGCGCTTCACCACCACCCTCCCAATTCTCATCCCTCTCCTCCTC 120
-24                M  R  F  T  T  T  L  P  I  L  I  P  L  L  L
AGCCTCCTCTTTACCTCCACCCATGCAGCCACCTTCGACATCCTCAACAAATGCACCTAC 180
 -9 S  L  L  F  T  S  T  H  A  A  T  F  D  I  L  N  K  C  T  Y
ACCGTCTGGGCAGCCGCCTCCCCCGGTGGCGGACGGAGACTTGACTCCGGCCAGTCCTGG 240
 12 T  V  W  A  A  A  S  P  G  G  G  R  R  L  D  S  G  Q  S  W
ACAATCACCGTCAACCCCGGCACCACCAATGCTCGCATCTGGGGCCGAACCTCATGCACC 300
 32 T  I  T  V  N  P  G  T  T  N  A  R  I  W  G  R  T  S  C  T
TTCGACGCCAATGGGCGTGGCAAATGCGAAACTGGTGACTGCAATGGCCTCCTCGAATGC 360
 52 F  D  A  N  G  R  G  K  C  E  T  G  D  C  N  G  L  L  E  C
CAGGGCTACGGTTCTCCCCCAAACACCCTCGCTGAATTCGCTCTAAACCAGCCCAATAAC 420
 72 Q  G  Y  G  S  P  P  N  T  L  A  E  F  A  L  N  Q  P  N  N
CTCGACTACATCGACATCTCCCTTGTCGATGGCTTCAACATCCCCATGGACTTCAGCGGC 480
 92 L  D  Y  I  D  I  S  L  V  D  G  F  N  I  P  M  D  F  S  G
TGCCGCGGCATCCAGTGCTCCGTTGACATCAATGGGCAATGCCCCAGTGAGTTGAAGGCC 540
112 C  R  G  I  Q  C  S  V  D  I  N  G  Q  C  P  S  E  L  K  A
CCCGGTGGATGCAACAACCCGTGTACAGTGTTCAAGACCAATGAGTATTGTTGCACTGAT 600
132 P  G  G  C  N  N  P  C  T  V  F  K  T  N  E  Y  C  C  T  D
GGACCTGGAAGCTGTGGTCCGACCACATACTCCAAATTCTTCAAGGACAGGTGCCCAGAT 660
152 G  P  G  S  C  G  P  T  T  Y  S  K  F  F  K  D  R  C  P  D
GCTTATAGCTACCCTCAGGATGACAAAACCAGCTTGTTCACCTGTCCTTCTGGTACCAAC 720
172 A  Y  S  Y  P  Q  D  D  K  T  S  L  F  T  C  P  S  G  T  N
TACAAGGTCACGTTTTGCCCTTGAAAGTTGGAACTCATTTTCCTTCACTTTCTTGGTGAA 780
192 Y  K  V  T  F  C  P  *
TGAAAAGAGTACAAGACACCCAGGAAAGTTTTAGTTTTCAGCGTAGAGAAGAATAAATTT 840
GTGCACGTAATGATAGTATTGTGCGCATGTGATGTGATAATCAGTCTGTAATGTGATTCC 900
AGTGAAAAATAAATGAAGAAGCCTACTTACTCAAAAAAAAAAAAAAAAAAAAAA 954
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Figure 4. Nucleotide and deduced amino acid sequence of VVTL1 cDNA. Peptide sequences obtained from purified VVTL1 are highlighted, a cleavable presequence is underlined, and a putative polyadenylation signal is boxed. Nucleotides are numbered on the right side and amino acids are numbered on the left, with the first residue of the mature protein designated +1. The translational stop codon is denoted by an asterisk (*) and an in-frame stop codon upstream of the initiation codon is shaded black. This sequence was compiled from an internal cDNA (154–738), a 5′ RACE product (1–254), and a 3′ RACE product (610–954). No sequence discrepancies were noted in the overlaps.

Identification of a Major Berry Protein as a TL Protein and Cloning of a Corresponding cDNA

Purification to apparent homogeneity of the 24-kD polypeptide from commercial grape juice (Fig. 3, lane c) was readily achieved by $(NH_4)_2SO_4$ precipitation followed by ion-exchange and gel-filtration chromatographies and HPLC. Determination of the N-terminal sequence ($_2$HN-ATFDILNKCT–) of the purified protein revealed that it is a TL protein identical to that isolated by Waters et al. (1996) from commercial wine. We called this protein VVTL1. To further characterize this protein, proteolytic digestion and sequencing of purified peptides were undertaken. Determination of the sequence of a number of peptides enabled the cloning of the corresponding cDNA by a combination of reverse transcription- and RACE-PCR. The sequence of the TL-encoding cDNA and the deduced amino acid sequence is shown in Figure 4.

Comparison of the deduced amino acid sequence and the N-terminal sequence data for the purified protein suggests that VVTL1 is synthesized as a precursor protein with a typical transient presequence (Chrispeels, 1991) of 24 residues preceding Ala-$^{+}$1 of the mature protein. The presequence, which is necessary for transport in the secretory system (Chrispeels, 1991), suggests that VVTL1 is an apoplastic protein, because it is not synthesized with a C-terminal propeptide, which may act as a vacuole-targeting signal (Melchers et al., 1993). The predicted mass of the mature protein is 21,286.45 D, whereas that obtained by MS is 21,272.90 ± 1.88 D. The discrepancy of 16 D is accounted for by the formation of eight disulfide bonds, as seen in crystallized thaumatin (Ogata et al., 1992). Thus, we conclude not only that the cDNA sequence (Fig. 4) exactly specifies the purified VVTL1 but also that the protein undergoes no posttranslational modification other than the removal of the presequence and the formation of intramolecular disulfide bonds. The predicted pI (4.6) is consistent with the protein's behavior during anion-exchange chromatography. Gel-filtration chromatography further revealed that the protein exists as a monomer in solution (data not shown).

VVTL1 Is Encoded by a Single Gene Induced at the Onset of Berry Softening and the Accumulation of Sugars

The availability of the cDNA encoding VVTL1 allows us to determine exactly when and in which tissue expression of the gene encoding TL is induced. In preparation for such studies it is useful to ascertain how many closely related VVTL1-encoding genes are contained in the genome of *V. vinifera*. Southern analysis of restriction endonuclease-digested genomic DNA under highly stringent conditions (0.1× SSC, 65°C), in which only targets of greater than about 90% identity will be detected, revealed that VVTL1 is encoded by a single gene (Fig. 5A). Hybridization under less-stringent conditions (2× SSC, 65°C), in which only targets of greater than about 70% identity will be detected, revealed additional hybridizing DNA fragments (Fig. 5B). Therefore, other genes encoding distinct proteins with

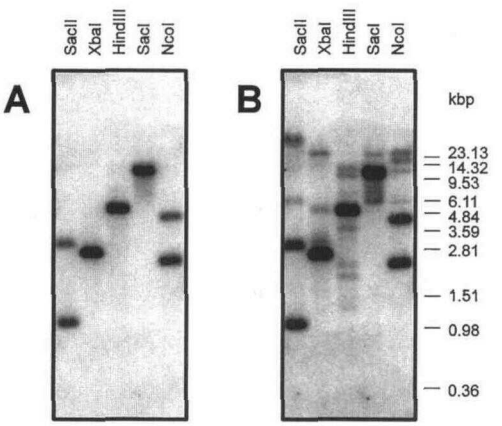

Figure 5. Genomic Southern analysis of grape DNA indicates that VVTL1 is encoded by a single gene and that related genes exist. Grape DNA was digested with the restriction enzymes *Sac*II, *Xba*I, *Hin*dIII, *Sac*I, and *Nco*I, blotted onto a nylon membrane, and probed with VVTL1 cDNA. Membranes were then screened under high-stringency (A) and low-stringency (B) conditions, as detailed in "Materials and Methods." Note: *Sac*II and *Nco*I cut the VVTL1-encoding cDNA.

some degree of sequence similarity to VVTL1 may exist within the grape genome. Indeed, Waters et al. (1996) detected a minor protein component in wine with homology to VVTL1. However, under stringent conditions of hybridization these would clearly not interfere with the analysis of VVTL1 gene expression.

When equal amounts of RNA extracted from fruits at different stages of development were subjected to northern analysis (Fig. 6), a correlation between the onset of veraison at 9 weeks postflowering and substantial accumulation of the VVTL1 message was observed. Despite the very high levels of VVTL1 mRNA found in berry homogenates, very low levels or no VVTL1 message is present in either flowers or leaves, suggesting a highly tissue-specific pattern of expression of the VVTL1 gene.

VVTL1 Is Specific to Ripening Fruit of *V. vinifera*

The data described above suggest a tight temporal and site-specific control on the expression of the gene encoding VVTL1, indicating that the berry may be the sole site of VVTL1 accumulation. To investigate this aspect further, polyclonal antibodies were raised to VVTL1 purified from juice, and western analysis was performed. Western analysis of total berry protein extracts (Fig. 7) and of free-run juice samples (data not shown) clearly identified the 24-kD protein accumulating in ripening fruit as a TL protein. High levels of the VVTL1 message appeared 10 weeks postflowering and remained high throughout stage III of ripening. VVTL1 was readily detectable 11 weeks postflowering and, although its level appeared to increase throughout ripening, the relatively slow rate of accumulation from weeks 13 to 17 postflowering could, given the high mRNA levels, indicate that some protein turnover or posttranscriptional control is taking place, as was previously observed for a tobacco osmotin (LaRosa et al., 1992).

The availability of the highly specific antibodies also allowed us to search for TL protein in other tissues of the grapevine. Equal amounts of protein extracted from berry skins, berry pulp, seeds, roots, flowers, tendrils, and young and mature leaves were electrophoresed and subjected to western analysis. We repeatedly found that only the berry pulp and skin extracts contained detectable amounts of VVTL1 (Fig. 8). The level of VVTL1 in the pulp extract prepared as described here was many times higher than the levels found in the skin. Indeed, we cannot rule out that the low amount of VVTL1 detected in the skin extract is derived from small amounts of pulp adhering to the skin preparation. Conversely, we cannot rule out that VVTL1 was lost from the skin during its preparation, which included washing in distilled water to remove the majority of adhering pulp.

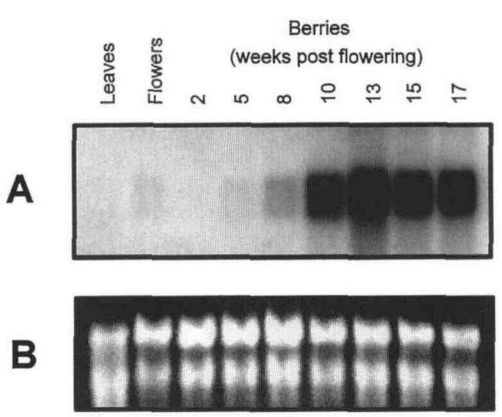

Figure 6. Northern analysis of RNA extracted from young grape leaves, flowers, and berries indicates an increasing level of VVTL1 mRNA during berry ripening. A, Total RNA (10 μg) was loaded in each lane, blotted onto a nylon membrane, and probed with VVTL1 cDNA. B, To demonstrate that similar amounts of RNA were loaded in each lane of the northern blot, a replica gel was run and stained with ethidium bromide.

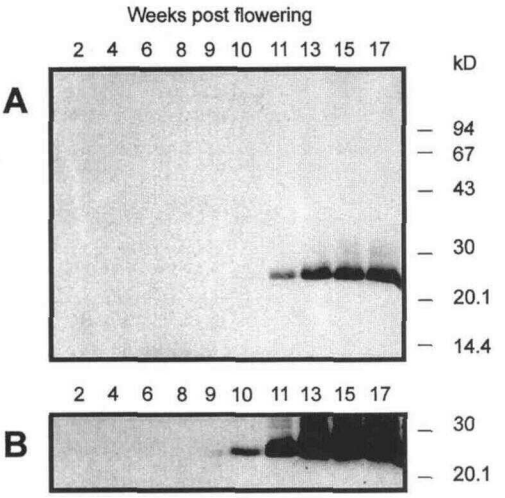

Figure 7. VVTL1 accumulates as the grape matures. A, Proteins in a replica gel to that shown in Figure 2 were transferred to a nitrocellulose membrane and probed with rabbit anti-VVTL1 antibodies and a horseradish peroxidase-conjugated secondary antibody. B, A longer exposure of the immunoblot shown in A.

TL Proteins Are Major Protein Species in a Range of Fruits

The muscat family of grapes is renowned in the wine industry for the high levels of protein that may be found in the wines prepared from them, with concomitant problems of precipitation during product storage and so-called haze formation. However, extraction of free-run juice from the ripe berries of six different grape cultivars shows that the accumulation of a TL protein as a major protein is widespread (Fig. 9). The highly conserved accumulation of VVTL1 in the fruits of *V. vinifera* raises the question of how ubiquitous this phenomenon is. To address this question a variety of commercially available fruits with no apparent disease symptoms were obtained, and the presence of VVTL1 homologs in their juice was determined by western analysis (Fig. 10). In some fruits (banana and kiwifruit) large amounts of TL proteins accumulated, whereas others appeared to contain little or no TL protein. Thus, the accumulation of TL proteins in fruits may be widespread but is possibly not universal. The antibodies raised against VVTL1 may not recognize TL homologs from all species, since significant sequence diversity may exist. For example,

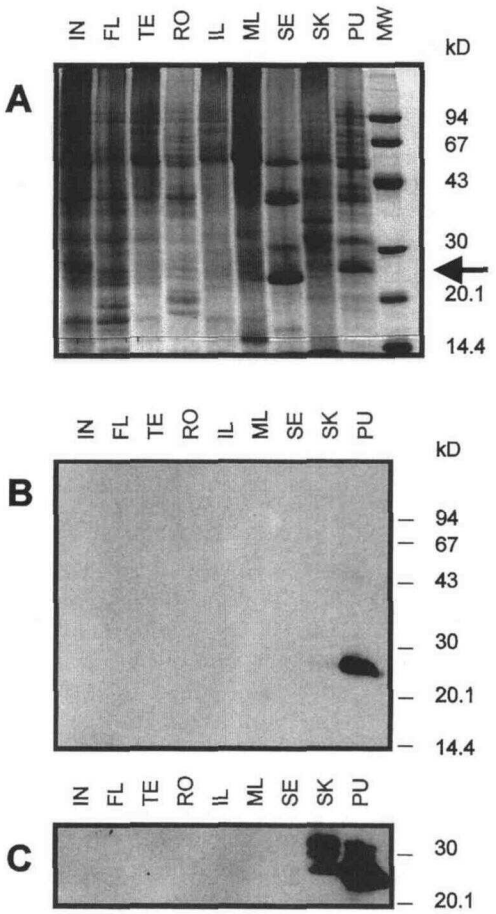

Figure 8. VVTL1 is specific to the grape berry and is present in high levels in the pulp. A, Protein extracted from inflorescences (IN), flowers (FL), tendrils (TE), roots (RO), immature leaves (IL), mature leaves (ML), seeds (SE), skins (SK), and berry pulp (PU) were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The arrow indicates the position in the gel of VVTL1. B, As for A except proteins were transferred to a nitrocellulose membrane and VVTL1 was detected by western analysis. C, A longer exposure of the immunoblot shown in B. MW, Molecular mass standard.

a TL protein purified from cherry exhibits only 45% positional identity with VVTL1, whereas a tobacco TL protein exhibits 86% positional identity with VVTL1 (Fig. 11). Whether the onset of TL protein synthesis in banana and kiwifruit is tightly coupled to ripening is yet to be established, as is the nature of the regulatory signals controlling the promoters of these highly inducible genes.

DISCUSSION

Fruit ripening in climacteric fruit has been studied extensively and is recognized as a highly regulated developmental process in which activation of specific genes takes place (Picton et al., 1995). By contrast, relatively little is known about the triggers of ripening in nonclimacteric fruits such as grapes. To gain insight into the ripening process in grapes, we attempted to describe changes in berry protein profiles during berry development, with emphasis on the identification of proteins, the synthesis of which coincides with the onset of veraison in the grape cv Muscat of Alexandria. VVTL1, which constitutes a moderate amount of the total protein in extracts and an overwhelming part of the free-run juice of grapes, was purified and identified as a TL protein. Southern and western analyses revealed that the protein is encoded by a single gene in grape and that it accumulates exclusively in the ripening berry. Somewhat surprisingly in view of studies of other plant species (Cornelissen et al., 1986; Vigers et al., 1991), no VVTL1 or close homolog thereof was detected in leaf tissue or seeds.

Thaumatin was originally found in the arils of ripe fruits from *Thaumatococcus daniellii* (van der Wel and Loeve, 1972) and, subsequently, a TL protein was found at very low levels in persimmon (*Diospyros texana*; Vu and Huynh, 1994). More recently, the presence of a TL protein has also been noted in ripe cherries, but its organ specificity and exact timing of onset of accumulation were not established (Fils-Lycaon, 1996). In this study we monitored carefully the ripening sequence pattern of grape berry samples. Northern analysis of samples taken throughout the growing season revealed that accumulation of the VVTL1 transcript and, potentially, transcriptional activation of the corresponding gene coincide with the transition of berries from the stage II lag phase, leading to the onset of sugar accumulation and the initialization of berry softening (veraison). Future isolation and characterization of the VVTL1 gene may therefore assist in elucidation of some of the factors triggering the onset of ripening.

The trigger at veraison for the onset of VVTL1 synthesis and its accumulation throughout the remainder of maturation is unknown. In a variety of plants a number of other TL proteins, like the majority of PR proteins, are induced in

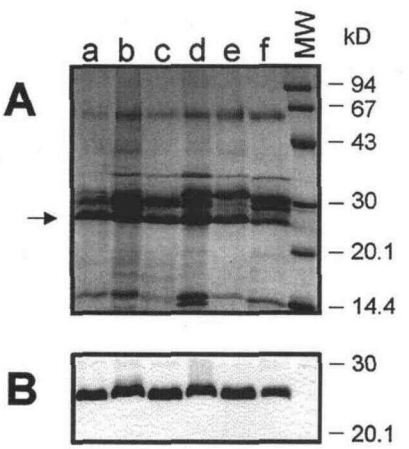

Figure 9. VVTL1 homologs are present in the free-run juice of several grape cultivars. A, Proteins from the juices (°Brix in parentheses) of the following cultivars were subjected to SDS-PAGE and the gel was stained with Coomassie brilliant blue: Lane a, Chardonnay (23.8); lane b, Pinot noir (27.8); lane c, Palomino (18.5); lane d, Shiraz (23.4); lane e, Semillon (26.3); and lane f, Sultana (also known as Thompson seedless, 21.7). The arrow represents the position of VVTL1 homologs. B, Proteins in a replica gel to that shown in A were subjected to western analysis using VVTL1 antibodies. MW, Molecular mass standard.

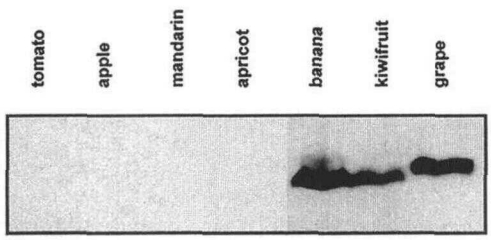

Figure 10. VVTL1 homologs are present in the juices of other fleshy fruit. Juice (20 μL) from the indicated plants was resolved by SDS-PAGE and the protein was transferred to a nitrocellulose membrane. The blot was probed with rabbit anti-VVTL1 antibodies and a horseradish peroxidase-conjugated secondary antibody. The fruits (°Brix of juices in parentheses) are as follows: tomato (4.1), apple (12.9), mandarin (13.4), apricot (18.3), banana (20.9), kiwifruit (14.1), and grapes (22.2).

response to a suite of signals including pathogen attack, wounding, and signaling molecules such as salicylic acid and ABA (van de Rhee et al., 1994). It is tempting to speculate that the accumulation of VVTL1 occurred in the berries sampled as a result of pathogen attack. However, the absence of any β-glucanase activity in our extracts, coupled with the ubiquitous presence of VVTL1 in apparently healthy grape samples from different varieties (Fig. 9) and other ripe, fleshy fruits (Fig. 10), is evidence against this notion.

Homologs of TL proteins include the osmotins, a group of proteins that was originally purified from salt-stressed tobacco cells (Singh et al., 1987) and accumulate at levels directly related to the degree of osmotic stress. Lowering of osmotic potential is expected to occur very rapidly in the ripening berry at the beginning of stage III as the total soluble solids increase from about 5% of fresh weight at 8 weeks postflowering to about 22% of fresh weight at 17 weeks postflowering. The majority of these solutes are contributed by Fru and Glc, which each accumulate to near molar levels in grape juice (Kliewer, 1965). Such levels of free hexoses have now been shown to repress the synthesis of chlorophyll *a*/*b*-binding proteins (Sheen, 1990) and induce the synthesis of a number of defense-related genes such as proteinase inhibitor II (Johnson and Ryan, 1990), chalcone synthase (Tsukaya et al., 1991), and four PR proteins (PAR-1, PR-1b, PR-Q, and SAR 8.2) in the leaves of transgenic tobacco plants (Herbers et al., 1995).

Such photoassimilate-induced gene expression occurs only above a certain threshold of hexose concentration, and the situation is therefore reminiscent of that seen when the onset of stage III (veraison) occurs. We therefore suspect that the temporally and spatially defined induction of not only VVTL1, but perhaps a majority of ripening-related genes as well, is directly or indirectly caused by the onset of sugar accumulation, possibly due to the presence of regulatory "sugar boxes" within the promoters of these genes (Tsukaya et al., 1991). The mechanism by which such hypothetical "sugar sensing" and intermediate signals operate remains unclear. It has recently been demonstrated that vacuolar but not cytosolic expression of invertase in tobacco leaves leads to an SAR effect (Herbers et al., 1996a) and that hexose sensing therefore appears to take place in the secretory pathway. In this context it is important to note that vacuolar grape invertases are synthesized well in advance of veraison (Davies and Robinson, 1996), with no apparent VVTL1 gene expression as a result, possibly because the free hexoses generated do not reach critical

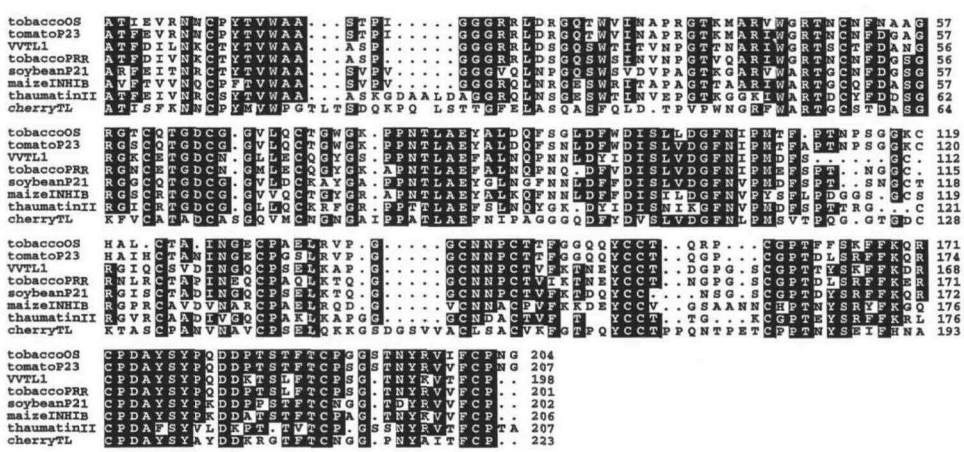

Figure 11. Multiple amino acid sequence alignments of some PR-5 protein family members using the PILEUP program (Genetics Computer Group, Madison, WI). Residues that are identical in at least five of the eight sequences are highlighted. Actual or deduced mature amino acid sequences were obtained from the following sources: TobaccoOS (cDNA isolated from NaCl-stressed tobacco culture cells encoding an osmotin [Singh et al., 1989]), tomatoP23 (citrus exocortis viroid-induced cDNA from tomato leaves encoding the vacuolar PR protein P23 [Rodrigo et al., 1993]), tobaccoPRR (cDNA encoding PR protein R [major form]) isolated from tobacco leaves infected with tobacco mosaic virus [Payne et al., 1988]), soybeanP21 (protein purified from healthy soybean leaves [Graham et al., 1992]), maizeINHIB (α-amylase/trypsin inhibitor protein purified from maize seed [Richardson et al., 1987]), thaumatinII (cDNA encoding for thaumatin, an intensely sweet protein from the seed arils of the West African shrub *T. daniellii* [Edens et al., 1982]), and cherryTL (cDNA encoding a protein purified from ripe cherry fruit [Fils-Lycaon et al., 1996]). Amino-terminal presequences and transient carboxy-terminal propeptides are not included.

threshold levels at early stages of berry development due to a multitude of competing metabolic demands.

A final consideration in regard to what triggers VVTL1 gene expression at veraison is the role of phytohormones. Salicylic acid is a product of the phenylpropanoid pathway (Yalpani et al., 1993) that is induced in grape berries at veraison (Boss et al., 1996). The concentration of salicylic acid during berry ripening remains unknown, although the absence of any detectable PR-2 protein (1,3-β-glucanase) synthesis suggests that it does not accumulate to significant levels. Therefore, other salicylic acid-independent mechanisms for the induction of TL proteins must be considered. Indeed, it has now been demonstrated that the sugar-dependent induction of PR proteins discussed previously can occur independently of salicylic acid (Herbers et al., 1996b). Also, other members of the PR-5 protein family have been shown to be induced by ABA (for review, see van de Rhee et al. [1994]), a hormone that accumulates to high levels during grape berry ripening (Coombe, 1976).

Whatever the exact mechanism for the berry-specific accumulation of VVTL1, our findings have implications for the interpretation of previous research on the effect of fruit maturity on the ability of powdery mildew (*Uncinula necator*) to establish an infection. Delp (1954) noted that new powdery mildew infections had never been observed on ripe grape berries in the field and further established that they do not occur on berries from the varieties Muscat of Alexandria, Tokay, Sultana (also known as Thompson seedless), and Carignane at a level of maturity greater than about 8 °Brix. In contrast, berries at a more immature stage, at or below 6 °Brix, were consistently susceptible. A more recent study (Chellemi and Marois, 1992) confirmed these observations and demonstrated that grape berries become resistant to new infections of powdery mildew above 7 °Brix.

The concentration of soluble solids at which berries are resistant or susceptible to new infections correlates well with the presence or absence, respectively, of VVTL1 gene expression, suggesting that VVTL1 may play a role in the phenomena observed by Delp (1954) and Chellemi and Marois (1992). TL proteins and the related osmotins, like many other proteins, have been demonstrated to have antifungal properties (Roberts and Selitrennikoff, 1990; Vigers et al., 1991; Hejgaard et al., 1991; Woloshuk et al., 1991). It is interesting to note that the disease susceptibility of plants for decades has been recognized to be correlated with the sugar status in leaves (for review, see Vanderplanck [1984]). From this the concept of "low-" and "high-sugar diseases" was developed (low-sugar diseases, for example, are those in which resistance is increased as leaf sugar content increases). Our results suggest a link among high sugar concentration, PR protein expression, and disease resistance in grape berries. In view of the demonstrated expression of TL proteins in other fruits at maturity, it would be interesting to examine the relative resistance or susceptibility of these fruits to new infections of known pathogens throughout their maturation to determine how universal this link may be. In this respect, it is interesting to note that *Botrytis cinerea*, a fungus that readily infects a number of fruits, including grape berries at maturity, has been found to be relatively insensitive to TL proteins when challenged in vitro (Abad et al., 1996; Fils-Lycaon et al., 1996).

Although it is tempting to speculate that a TL protein is present in ripening berries for protective reasons, it cannot be ruled out that it serves other functions. Despite their wide distribution in plants and their general classification as PR proteins (van de Rhee et al., 1994), the exact functions of the TL proteins remain unclear. Zeamatin, a TL protein from maize seeds, and tobacco osmotin are believed to exert antifungal action through a membrane-permeabilizing activity (Roberts and Selitrennikoff, 1990; Abad et al., 1996); however, a recent study in which radiographic crystallography of zeamatin was used (Batalia et al., 1996) does not offer any clues as to how it may exert such an effect. Progress in this area is likely to require several lines of research. With the discovery that a TL protein is synthesized at a specific time of fruit development, and with the recent development of transformation systems for grape (Mauro et al., 1995; Perl et al., 1996; Scorza et al., 1996), important clues to the functions of VVTL1 may now be gained from attenuation of its expression in grapes with parallel studies in other ripening fruits in which TL proteins have been shown to accumulate to high levels.

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