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Environmental Conditions during Vegetative Growth Determine the Major Proteins That Accumulate in Mature Grapes

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Despite recent reports in the literature that chitinases comprise ~50% of the soluble proteins present in mature *Vitis vinifera* L. (cv. Moscatel) grapes, with the other major proteins being thaumatin-like proteins, a careful inspection of the published data reveals inconsistencies as to which proteins actually accumulate in mature grapes. Mature Moscatel grapes were harvested in the same vineyard in two consecutive years, 1999 and 2000. The grapes exhibited widely distinct polypeptide patterns when analyzed by either FPLC cation exchange chromatography or two-dimensional electrophoresis: whrereas the 2000 grapes possessed a much higher protein content (1.96 versus 1.11 mg g^{-1} of fresh weight), the 1999 grapes contained a greater heterogeneity of polypeptides. In addition, highly specific antibodies that recognize the pathogenesis-related proteins present in the grapes demonstrated that the 2000 harvest grapes had a wider variety of pathogenesis-related polypeptides. N-Terminal sequencing of the major polypeptides revealed differences in the relative abundance and amino acid sequence of thaumatin-like and osmotin-like polypeptides present in the 1999 and 2000 harvest Moscatel grapes and did not detect the presence of chitinase. As a whole, the data indicate that the expression and accumulation of the major proteins in grapes is essentially determined by the environmental and/or pathological conditions prevailing during grape development and maturation. The potential physiological and technological implications are discussed. The results of the present work suggest that it is not possible to base varietal differentiation of grapes on the profile of the pathogenesis-related polypeptides present in the mature berries.

KEYWORDS: Chitinase; grapes; osmotin; proteins; thaumatin; *Vitis vinifera*

INTRODUCTION

Plants activate several different defense mechanisms against both biotic and abiotic stresses. Among these, synthesis of an appropriate protein complement has been demonstrated to be essential for survival. For example, the synthesis of heat shock proteins for thermo tolerance acquisition is very well documented (*1*). On the other hand, fungal infections are usually deterred by the synthesis of a number of pathogenesis-related (PR) proteins (*2*). Typically, these antifungal proteins are expressed constitutively at low levels in cells but accumulate in response to fungal attack or other inducers (*3*).

Expression of genes encoding a number of PR proteins increases dramatically in grapes during ripening. Furthermore, there is a considerable increase in total protein content after veraison, but only a small number of proteins are synthesized in significant amounts during ripening (*4*). The two most prominent soluble proteins accumulated in grapes during ripening have apparent molecular masses of 32 and 24 kDa and have been identified as chitinase and thaumatin-like proteins, respectively (*5*). The constitutive expression and accumulation of class IV chitinases in grapes was followed during ripening, but no β-1,3-glucanase activity was detected (*6*). N-Terminal amino acid sequence analysis and/or function allowed the identification of the major proteins that showed a developmental stage-specific increase in grapes during ripening as grape osmotin, a lipid-transfer protein and a basic and an acidic chitinase (*7*). When differential screening was used to isolate ripening-associated cDNAs from grapes, accumulation in a developmental manner of stress response proteins was observed (*8*).

The grapevine PR proteins can be induced by fungal attack. Induction of PR proteins in ripening grape berries by wounding, salicylic acid, or *Botrytis cinerea* was reported (*9*, *10*). Observation of the leaves from 21 different grapevine genotypes led to

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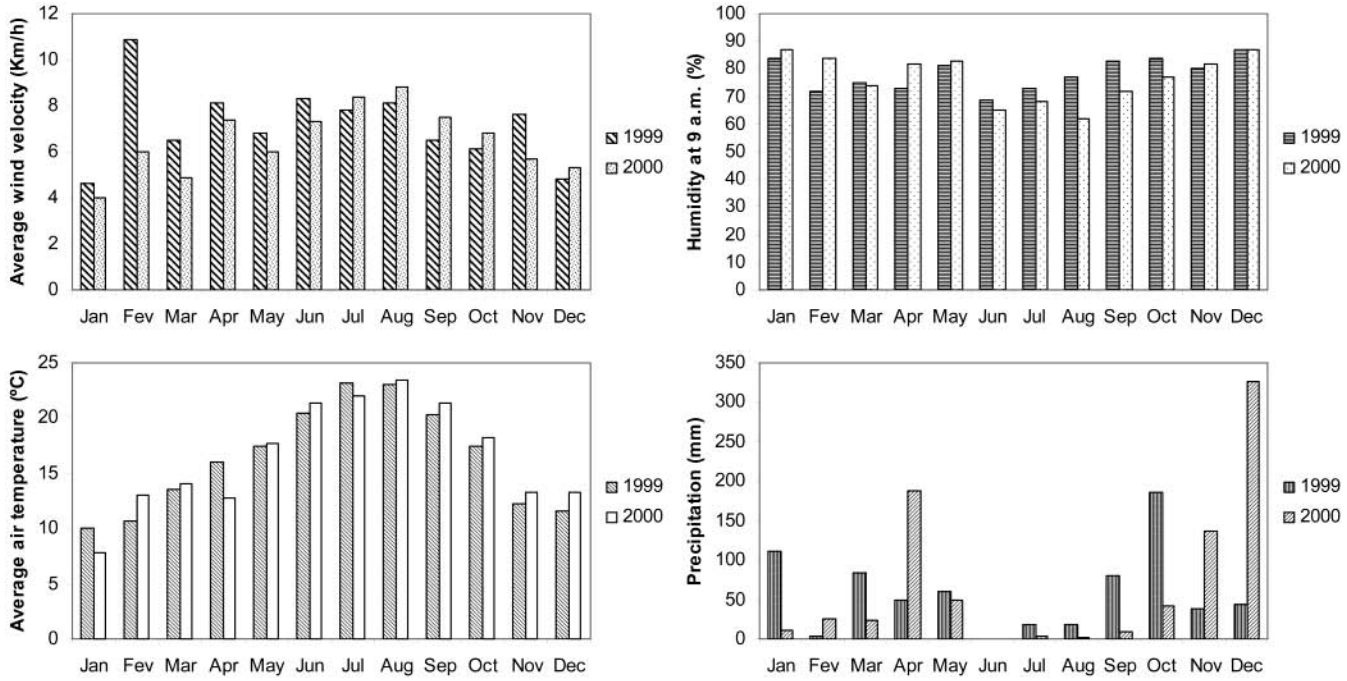

Figure 1. Meteorological data [average wind velocity (km/h), humidity at 9:00 a.m. (%), average air temperature (°C), and precipitation (mm)] for the years 1999 and 2000 in Palmela (Portugal).

a correlation between resistance to *Uncinula necator* rating and the levels of activity of chitinase and β-1,3-glucanase (*11*). Elevated activities of chitinase and β-1,3-glucanase in the leaves and berries of susceptible grapevine cultivars were detected, and the induction of the PR genes coding for class III chitinase, class I glucanase, and a thaumatin-like protein in response to infection by *U. necator* was observed (*12*). When the rice class I chitinase gene was introduced into the somatic embryos of grapevine, it was observed that some of the transgenic plants obtained exhibited enhanced resistance against *U. necator* (*13*).

In summary, multigene families in various plant species typically encode PR proteins. In addition, they may be grouped into many protein families, some of which are expressed constitutively, whereas others are tissue-specific and expressed during development or subjected to differential induction by both biotic and abiotic stresses (*14*, *15*).

Comparison of the N-terminal sequences of the polypeptides isolated from Moscatel wine with proteins from other sources revealed a very high degree of homology to PR proteins (*16*).

Proteins belonging to the PR family are typically stable at acidic pH values and highly resistant to proteolysis (*17*). This resistance to proteolysis and stability at acid pH means that wine-making is a selective extraction procedure for grape berry PR proteins (*18*). Combination of low pH with the proteolytic pool present during fermentation ensures that only proteins resistant to these conditions, such as PR proteins, survive the wine-making process. The three most abundant proteins present in a wine vinified from cv. Moscatel were identified by N-terminal sequencing: two of them as chitinases and a third as a thaumatin-like protein (*18*). These authors concluded that the PR proteins present in wines are technologically important in the sense that they cause haze formation, lowering the commercial value of these beverages. The use of sequence and mass spectral analysis (*19*) showed that chitinases account for ~50% of the soluble proteins in Moscatel berries, with thaumatin-like proteins comprising the other major proteins. The authors observed that both of these groups of proteins persist through the vinification process and cause hazes and sediments in bottled wines.

There are discrepancies in the literature as to which individual proteins accumulate predominantly in mature grapes (which is of physiological relevance) and hence in wines (which is of technological importance). On the other hand, the observation that some of the proteins present in the berries are expressed in response to biotic and/or abiotic stresses suggests that the actual pattern of proteins present in mature grapes may depend on the precise environmental and pathological conditions that occurred during vegetative growth. To address this question, mature grapes (cv. Moscatel) were harvested in the same vineyard in two consecutive years; their polypeptide patterns were analyzed and the major proteins identified using a combination of FPLC ion exchange chromatography, two-dimensional electrophoresis, immunological detection, and N-terminal sequencing.

MATERIALS AND METHODS

Biological Material. Mature grapes (*Vitis vinifera* L. cv. Moscatel) were harvested in 1999 (harvest started on September 13) and 2000 (harvest started on October 5) at José Maria da Fonseca, Palmela, Portugal. **Figure 1** shows the average wind velocity (km/h), humidity at 9:00 a.m. (%), average air temperature (°C), and precipitation (mm) for the years 1999 and 2000 in Palmela. Precipitation was much higher in 1999 than in 2000. In 1999 it started raining in mid-September, and the grapes were harvested under rain, which led to a wine with a lower content in alcohol (1999 harvest, 48180 kg of grapes/10 ha, 18.6 °Brix, and wine with 10.96% alcohol; 2000 harvest, 56950 kg of grapes/10 ha, 22 °Brix, and wine with 12.42% alcohol).

The white wine used in the preparation of the antibodies was prepared from the single grape variety Assario. Ripened Assario grapes were harvested in 1994 in the Dão region, Portugal, and processed into wine by a conventional microvinification procedure, according to the classical white wine technology. Wine and grapes were stored frozen at −80 °C until required.

Isolation of the Soluble Proteins from *Lemna minor*. *L. minor* L. was grown autotrophically at 25 °C under continuous light in a completely sterile culture medium as described previously (*20*). Lemna

fronds were frozen in liquid nitrogen and ground to a fine powder, and the total soluble protein was extracted (2 mL g^{-1} of fresh weight) in 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered through two layers of cheesecloth and centrifuged at 40000*g* for 10 min at 4 °C, and the supernatant was desalted at 2 °C on a PD-10 prepacked Sephadex G-25M column previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

Purification of a Wine Polypeptide To Be Used as an Antigen. Wine aliquots (75 mL) were thawed and centrifuged at 15800*g* for 5 min, and the supernatant was desalted at 4 °C on a PD-10 column previously equilibrated with water. The protein sample (105 mL) was subsequently lyophilized and the dried residue resuspended and solubilized in 9 mL of 20 mM citrate−NaOH buffer, pH 2.5. A sample (2 mL) containing the wine total proteins was fractionated by FPLC cation exchange chromatography on the Mono S HR5/5 column previously equilibrated in 20 mM citrate−NaOH buffer, pH 2.5. The flow rate was 1.5 mL min^{-1}, and the bound proteins were eluted with a continuous gradient (0−1 M) of NaCl. To avoid cross-contaminations, a major polypeptide present in one of the main protein peaks was further purified by preparative SDS-PAGE. The acrylamide band corresponding to the polypeptide was sliced, ground in a mortar, and used for antibody production.

Preparation of Antibodies Specific for a Wine Polypeptide. New Zealand female rabbits were immunized with the purified antigen (400 μg) in complete Freund's adjuvant. To obtain a high titer, three booster injections of 400 μg of antigen each were given every 2 weeks in complete Freund's adjuvant diluted 1:10 with incomplete adjuvant. Total blood was taken from the heart 12 days after the third booster injection. Blood samples were allowed to clot, and the serum was collected, centrifuged at 650*g* for 5 min at 4 °C, frozen in liquid nitrogen, and stored at −80 °C.

Extraction of the Total Protein from Grapes. Two different extraction procedures were followed to isolate the proteins from mature grapes.

In the experiments involving one-dimensional electrophoresis and FPLC cation exchange chromatography, the grape proteins were extracted following a modification of a methodology suitable to extract proteins from plant tissues rich in phenolic compounds (*21*). The pulps from mature grapes (100 g) were ground to a fine powder under liquid nitrogen and homogenized in 250 mL of ice cold, 250 mM HEPES/200 mM Tris buffer, pH 7.7, containing 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM sodium diethyldithiocarbamate, 0.1% (v/v) Tween 80, and 6 g of PVPP. The solution was mixed in a blender and centrifuged at 10000*g* for 40 min at 4 °C. The supernatant was passed through filter paper, desalted on a PD-10 column pre-equilibrated with water (pH adjusted to 7.5), frozen, and lyophilized. The dried residue was resuspended and solubilized in 20 mM citrate−NaOH buffer, pH 2.5.

In the experiments involving two-dimensional electrophoresis, a different extraction procedure was used (*4*). Grape pulps (4 g) were ground to a fine powder under liquid nitrogen and homogenized in 8 mL of 500 mM Tris-HCl buffer, pH 8.0, containing 5% (w/v) SDS, 10 mM dithiothreitol, and 10 mM sodium diethyldithiocarbamate. The homogenate was incubated at 95 °C for 5 min and centrifuged at 12000*g* for 5min. The total protein was precipitated with trichloroacetic acid (10% w/v final), incubated for 15 min at 0 °C, and centrifuged at 12000*g* for 15 min. The resulting pellet was washed twice with an ice-cold solution of ethanol/ethyl acetate (2:1, v/v). The pellet was dried under nitrogen, resuspended in a solution containing 7 M urea, 2 M thiourea, 2% (v/v) NP-40, and 1% (w/v) dithiothreitol, solubilized in a sonicator, and desalted in NAP-10 columns previously equilibrated in water. After lyophilization, the dried residue was solubilized in the same solution added with 0.5% (v/v) IPG buffer, pH 3−10 (Amersham Pharmacia).

Fractionation of the Grape Proteins by FPLC Cation Exchange Chromatography. The solution containing the grape proteins, dissolved in 20 mM citrate−NaOH buffer, pH 2.5, was cleared by passage through a carboxymethylcellulose column equilibrated in the same buffer. The bound proteins were eluted with buffer containing 1 M NaCl, desalted in 20 mM citrate−NaOH buffer, pH 2.5 and loaded into the FPLC Mono S HR5/5 column previously equilibrated in the same buffer. The flow rate was 1.5 mL min^{-1}, and the bound proteins were eluted with a continuous gradient (0−1 M) of NaCl.

Electrophoresis and Immunoblotting (One-Dimensional). SDS-PAGE electrophoresis and immunoblotting were performed as described before (*22*).

Electrophoresis and Immunoblotting (Two-Dimensional). Isoelectric focusing was performed using the IPGphor system (Amersham Pharmacia). Immobiline Drystrip gel strips (IPG strips) (13 cm, pH 3−10) were obtained from Amersham Pharmacia. IPG strips were rehydrated with 250 μL of a solution containing 0.5% (v/v) IPG buffer, pH 3−10, 7 M urea, 2 M thiourea, 2% (v/v) NP-40, 1% (v/v) dithiothreitol, and protein sample in the IPGphor strip holders. The program used for isoelectric focusing included the following steps: rehydration, 30 V, 12 h; step 1, 200 V, 1 h; step 2, 500 V, 2 h; step 3, 1000 V, 2 h; step 4, 8000 V, 3.5 h. After focusing, the gel strips were immediately frozen at −80 °C.

SDS-PAGE (second dimension) was performed as described above except that the gel contained only the separating gel. The gel strips were thawed and equilibrated for 15 min, with agitation, in 50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 26% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) dithiothreitol. The strips were subsequently equilibrated for another 15 min, with agitation, in a similar solution that contained 2.5% (w/v) iodoacetamide instead of the dithiothreitol, placed on top of the SDS-PAGE gel, sealed with 0.5% (w/v) agarose, and electrophoresed (220 V, 15 mA for 15 min followed by 220 V and 30 mA).

Total protein, in gels, was either stained with Coomassie Brilliant Blue R-250 or silver stained (*23*).

The preparation of immunoblots from the two-dimensional gels was performed as described above.

Purification of Polypeptides for N-Terminal Sequencing. The grape protein fraction was fractionated by FPLC cation exchange chromatography (Mono S column) as explained above. The major protein peaks were desalted into 20 mM Tris-HCl buffer, pH 7.5, and individually loaded into the FPLC anion exchange Mono Q column previously equilibrated in the same buffer.

The major proteins were then subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and stained with Ponceau S before being sliced for N-terminal sequencing.

N-Terminal Sequencing. All the solutions used in the gels and the electrophoresis buffers were freshly prepared and filtered (Whatman 3MM). The SDS was used either ultrapure or twice recrystallized from ethanol and water (*24*). To prevent blockage of the amino terminals of the polypeptides, the gels were subjected to a pre-electrophoresis (30 min at 200 V) in 350 μL of 10 mM glutathione per 70 mL of the upper buffer (to avoid degradation of tryptophan and methionine residues). Electrophoresis was then performed by adding 70 μL of 100 mM thioglycolic acid to 70 mL of the upper buffer (*25*). These precautions are meant to remove charged impurities, noncharged reactive species such as acrylamide monomers, and other reactive substances and to reduce peroxides and residual radicals (*26*). The membrane used for electroblotting (*27*) was a ProBlot poly(vinylidene difluoride) polymer (PVDF) from Applied Biosystems (*28*). The buffer used for electroblotting was a 10 mM solution of 3-(cyclohexamide)-1-propanosulfonic acid (CAPS) in 10% (v/v) methanol (*28*, *29*). Electroblotting was performed for 50 min at 80 V and 4 °C in a TransBlot Semi-Dry Transfer Cell from Bio-Rad. The membranes were stained with Ponceau S (*28*). The polypeptides immobilized in the membranes were then sequenced (Edman degradation) in a protein sequencer from Perkin-Elmer−Applied Biosystems (model 477A) on line with an HPLC analyzer (model 120 A).

Protein Determination. Protein concentrations were measured according to a modification of the Lowry method (*30*).

RESULTS AND DISCUSSION

As grapes ripen there is a decrease in the variety of proteins present and an increase in the content of PR proteins, which are the ones responsible for haze in wines (*18*). Wine proteins are typically composed of a very large number of distinct polypeptides that belong to a reduced number of PR protein

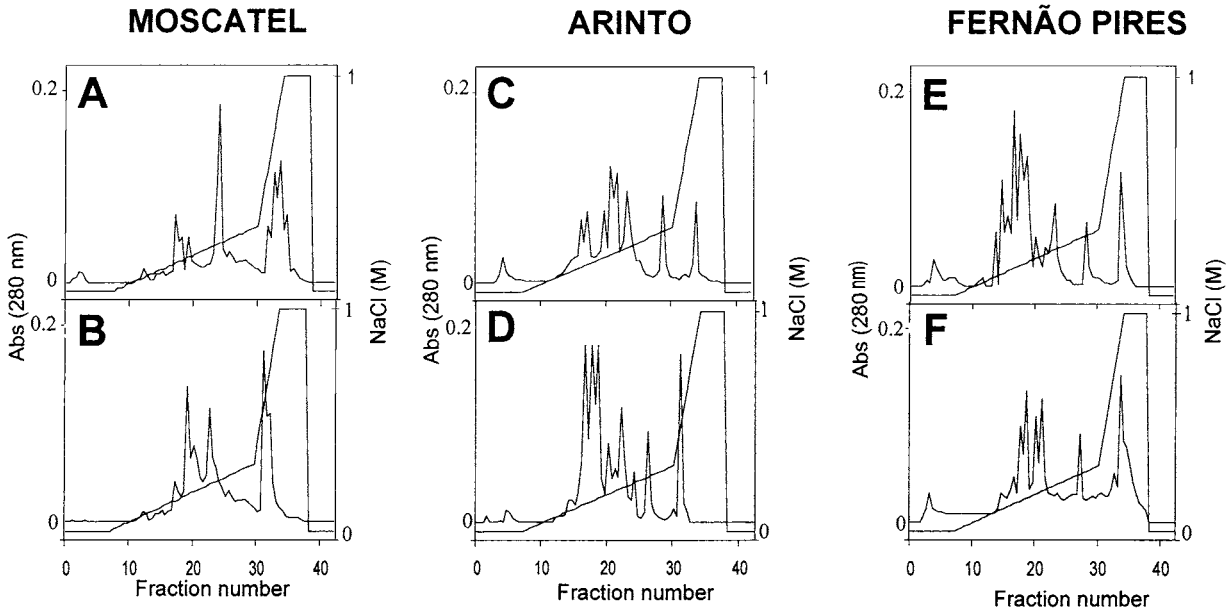

Figure 2. Patterns of proteins isolated from mature Moscatel, Arinto, and Fernão Pires grapes fractionated by FPLC cation exchange chromatography. The total soluble protein was isolated from Moscatel grapes harvested in 1999 (A) or 2000 (B), Arinto grapes harvested in 1996 (C) or 1998 (D), and Fernão Pires grapes harvested in 1996 (E) or 1998 (F) and fractionated in the Mono S column as described under Materials and Methods.

Table 1. Protein Content of Wines Prepared from Grapes Harvested in Different Years Using the Same Microvinification Technology

wine	[protein], mg/L
Moscatel 1996	334.3 ± 44.8
Moscatel 1998	146.6 ± 18.2
Fernão Pires 1996	277.0 ± 1.8
Fernão Pires 1998	146.0 ± 4.2
Arinto 1996	87.0 ± 18.0
Arinto 1998	227.8 ± 30.3

families (*16*, *18*, *19*). Although some grape varieties are, constitutively, richer in protein, PR proteins are induced. Experiments performed in this laboratory with wines prepared using the same microvinification technology from single grape varieties of Moscatel, Fernão Pires, and Arinto grapes, harvested in different years, showed the protein concentrations listed in **Table 1** and the FPLC cation exchange chromatography profiles presented in **Figure 2C−F**. This previous work suggested that wines prepared from grapes harvested in the same vineyard but in different years may possess different proportions of polypeptides or even different proportions of PR protein families. This observation, which is technologically relevant, may result from the differential effects originated during vinification procedures or from the physiologically important hypothesis that different environmental conditions (including both biotic and abiotic stresses) may lead to a differential expression and accumulation of polypeptides in mature grapes.

To test the latter hypothesis, a series of experiments were performed on mature grapes (*V. vinifera* L. cv. Moscatel) harvested from the same vineyard in two consecutive years (1999 and 2000). However, to address this question, suitable tools had previously to be developed.

Polyclonal antibodies were produced in rabbits using a polypeptide that was isolated from Assario wine. This polypeptide has been shown to derive from the grape pulp (*22*). The experiment illustrated in **Figure 3** indicates that the antibodies obtained are highly specific. The SDS gel presented in **Figure 3A** shows the polypeptide patterns of *L. minor* total soluble protein (lane 1) and of the total Assario wine protein. In this

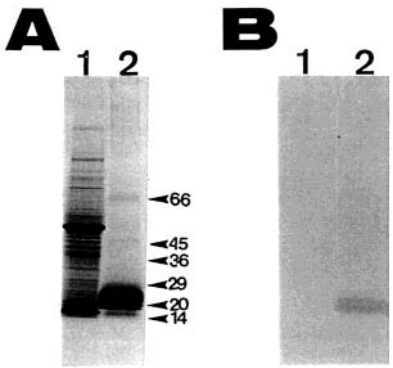

Figure 3. Specificity of the antibodies. *L. minor* total soluble protein [lane 1, 40 μL of extract in (A) or 20 μL in (B)] or the total Assario wine protein [lane 2, 80 μg of protein in (A) or 25 μg in (B)] were subjected to SDS-PAGE and the total polypeptides stained with Coomassie Brilliant Blue R (A) or transferred onto a nitrocellulose membrane and subjected to immunoblotting and probed with the anti-wine polypeptide antibodies (B). Molecular masses of markers are indicated in kDa.

experiment, *L. minor* was used as a control. This aquatic and simple higher plant is routinely used in our laboratories. The advantage of using this plant to test the specificity of antibodies results not only from the presence in *Lemna* cells of a very wide range of different polypeptides but also from the presence of two extremely abundant polypeptides—the large (52 kDa) and small (14.5 kDa) subunits of ribulose bisphosphate carboxylase (*31*). When the total polypeptides contained in the gel presented in **Figure 3A** were transferred onto a nitrocellulose membrane and the resulting blot probed with the anti-wine polypeptide antibodies, the immunoblot presented in **Figure 3B** was obtained. The antibody produced no signal with the *Lemna* proteins, despite the extremely large number of different structures (i.e., polypeptides) present and the extraordinary abundance of some of them. This result clearly indicates that the antibody produced exhibits a very high specificity toward the wine polypeptide selected. However, a closer inspection of **Figure 3** also reveals that the antibodies produced against a single polypeptide recognize specifically the vast majority of

Table 2. N-Terminal Sequencing of the Wine Polypeptides Presented in **Figure 4** and Comparison with the Sequences of Proteins from Other Sources[a]

Protein	Source	Alignment		identity
Fig.4, lane 1	This work	1 A T F N I Q N H H S Y T V V A A A V P G G G M Q L 25	——	——
Osmotin-like precursor	*Vitis vinifera* (grape)	1 A T F N I Q N H C S Y T V W A A A V P G G G M Q L 49	a	92.00
Fig.4, lane 2	This work	1 A T F D I L N K K T Y T V V A A A A 18	——	——
Thaumatin-like protein	*Vitis vinifera* (grape)	25 A T F D I L N K C T Y T V W A A A S 42	b	83.33

[a] The databases consulted were Swiss-Prot/TrEMBL (www.expasy.ch/cgi-bin/) and EMBL Outsation, European Bioinformatics Institute (www2.ebi.ac.uk). References: (a) Loulakakis and Roubelakis-Angelakis (1996), AN P51118 (TrEMBL); (b) Tattersall et al. (1997), AN Q04708 (TrEMBL).

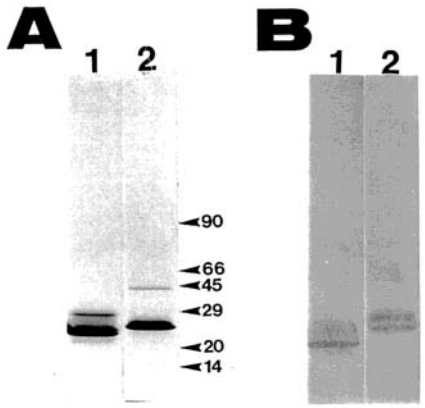

Figure 4. Identification of the polypeptides recognized by the antibodies. Selected 1999 harvest Moscatel wine polypeptides (lanes 1 and 2) were purified by a combination of FPLC cation/anion exchange chromatographies, subjected to SDS-PAGE, and either stained for total protein with Coomassie Brilliant Blue R (A) or probed with the antibodies (B). Molecular masses of markers are indicated in kDa. The protein amounts loaded in each lane were 100 μg in (A) and 40 μg in (B).

the wine polypeptides. Given the very high specificity of the antibodies, this observation suggests that the main wine polypeptides are structurally related. Indeed, similar or identical primary amino acid sequences have been reported for most of the protein fractions present in wines (*16*, *18*). The antibodies produced against the single wine polypeptide were also shown to recognize the most abundant polypeptides present in other wines and in mature grapes irrespective of the cultivar, year, or region where berries were produced (*22*).

After the specificity of the antibodies had been assessed, emphasis was directed toward the identification of the structurally related polypeptides that are recognized by the antibody. To this end, selected wine polypeptides were purified by a combination of FPLC cation/anion exchange chromatographies. The individual polypeptides were then submitted to SDS-PAGE (**Figure 4A**) and probed with the anti-wine polypeptide antibodies (**Figure 4B**) or subjected to N-terminal sequencing (**Table 2**). The results illustrated in **Figure 4B** indicate that both selected polypeptides were recognized by the antibodies. The data presented in **Table 2** allow the identification of the above polypeptides as grape osmotin and grape thaumatin.

Having established that the antibodies exhibit a very high degree of specificity, that they recognize the major proteins present in wines, and that they bind to grape osmotin and thaumatin, a series of experiments were preformed to investigate the expression and accumulation of proteins in mature grapes as affected by environmental and/or pathological conditions. Two different samples were used: mature Moscatel grapes were collected in the same vineyard in 1999 and in 2000. Measurements of protein concentration indicated that the 2000 harvest grapes (1.96 ± 0.17 mg of protein g^{-1} of fresh weight) are far richer in protein than the 1999 grapes (1.11 ± 0.22 mg of protein g^{-1} of fresh weight). In the experiment exhibited in **Figure 2**, the grape proteins were analyzed by FPLC cation exchange chromatography. This technique has been utilized previously to characterize the proteins from wines (*32*, *33*). The chromatograms presented in **Figure 2** show that the total protein fractions isolated from mature Moscatel grapes harvested in 1999 (**Figure 2A**) and 2000 (**Figure 2B**) produce very different profiles when analyzed by cation exchange chromatography. In particular, the proteins from 1999 harvest Moscatel grapes exhibit greater heterogeneity as evidenced by the larger number of A_{280} peaks obtained. It is important to note that these chromatograms are extremely reproducible as indicated by the very large number (>10) of replications performed. Therefore, the 2000 harvest grapes contain a higher amount of protein but a lower heterogeneity of protein fractions.

The proteins from 1999 and 2000 harvest Moscatel grapes were subsequently analyzed by two-dimensional electrophoresis. When the resulting gels were stained for total protein, it became evident that the 1999 harvest Moscatel grapes (**Figure 5A**) contain a much wider variety of different polypeptides than the 2000 harvest Moscatel grapes (**Figure 5B**), confirming the results shown in **Figure 2**. Informatics analysis (Program ImageMaster 2D, Pharmacia; this program subtracts the background) of the gels shown in **Figure 5** gave the following information: (a) the grapes from the 1999 harvest contain 376 polypeptides with p*I* values from 3.61 to 9.382 and MW from 13.42 to 65.74 kDa; (b) the grapes from the 2000 harvest contain 162 polypeptides with p*I* values from 3.049 to 9.595 and MW from 13.51 to 73.00 kDa; (c) the program provided additional information by comparing the two gels and found the number of matched spots [54 (33.33% similarity)].

Considering that the grapes were harvested in the same vineyard in two consecutive years, it seems somewhat surprising that environmental and/or pathological conditions are responsible for such differences in the polypeptide patterns.

When the polypeptides present in the two-dimensional gels were blotted onto a nitrocellulose membrane and probed with the antibodies, a different result was obtained. The proteins recognized by the antibody (and therefore those that are likely to appear in the wine) are now far more heterogeneous in the 2000 harvest Moscatel grapes (**Figure 5D**) than in the 1999 harvest Moscatel grapes (**Figure 5C**). In other words, the grapes

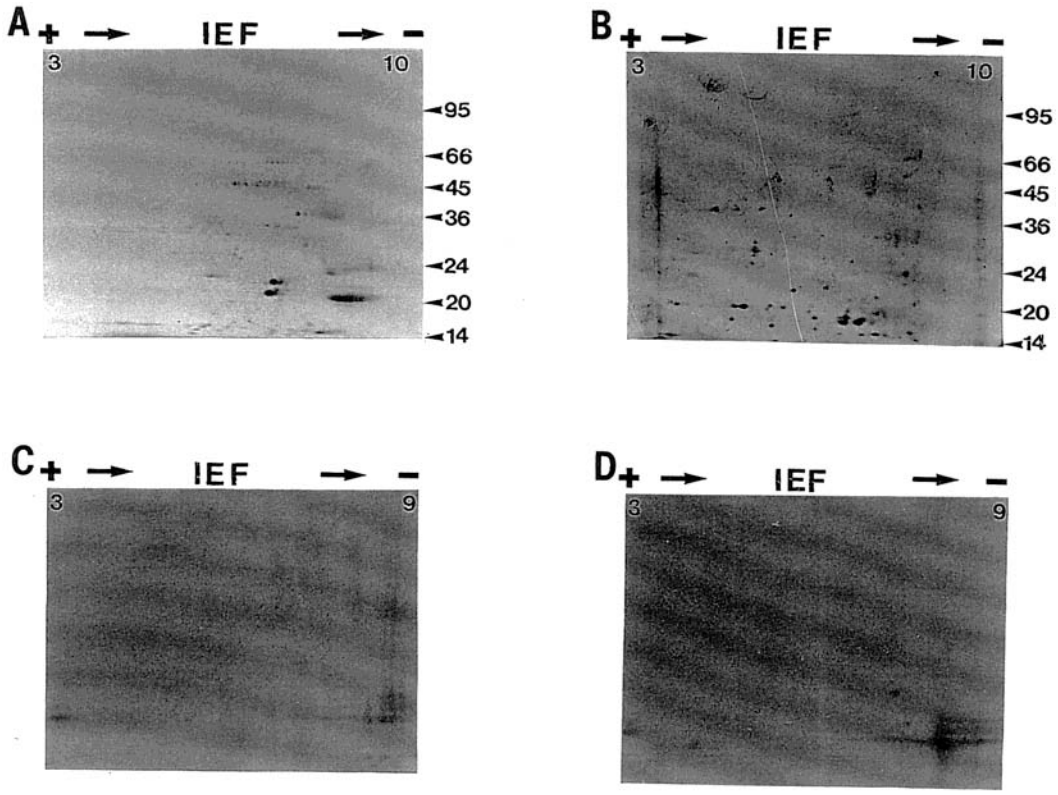

Figure 5. Two-dimensional electrophoresis of the proteins isolated from mature Moscatel grapes harvested in 1999 (A, C) and in 2000 (B, D). The grape proteins were isolated, subjected to two-dimensional electrophoresis, and stained for total protein with Coomassie Brilliant Blue (A) and silver stained (B) or blotted onto a nitrocellulose membrane and probed with antibodies (C, D) as explained under Materials and Methods. The pH gradient formed during the first dimension is indicated on top of the gels and blots, and the molecular mass markers are given in kDa. The protein amounts loaded were 250 μg in (A) and (B) and 100 μg in (C) and (D).

with a higher protein content (Moscatel 2000) possess a lower variety of total polypeptides but a higher heterogeneity of pathogenesis-related polypeptides that become nuisance proteins in the wine than do the grapes with a lower protein content (Moscatel 1999).

The major polypeptides present in mature Moscatel grapes harvested in 1999 and 2000 were isolated and subjected to N-terminal sequencing. Eleven polypeptides were selected from the 1999 harvest grapes and four from the 2000 harvest grapes. Their molecular masses, as estimated by SDS-PAGE using suitable markers, varied from 23 to 31 kDa. The results obtained are presented in **Table 3**. Among the 11 polypeptides from the 1999 grapes, 1 (from which 11 amino acid residues were identified) possesses high homology with segments of 4 other proteins, apparently unrelated: a homology of 75% with glutamine synthetase from *V. vinifera* grapes (*34*), a homology of 75% with phospho-2-dehydro-3-deoxyheptonate aldolase from yeast (*35*), a homology of 70% with a gas vesicle protein present in aquatic bacteria (*36*, *37*), and a homology of 70% with bacterial ribosome recycling factor (*38*). Two other polypeptides, with 18 amino acid residues sequenced each, exhibit a high degree of homology with segments of PR proteins and also with the other 8 polypeptides sequenced; they show an 83.33% homology with a thaumatin-like protein, VVTL1, from mature grapes of *V. vinifera* (*3*), 77.77 and 72.22% homologies, respectively, with a segment of PR proteins R1 and R2 from *Nicotiana tabacum* (*39*), and a 66.67% homology with an osmotin-like protein from *Arabidopsis thaliana* (*40*, *41*). Finally, 8 other polypeptides, each with 25 amino acid residues sequenced, show a 92% homology to an osmotin-like protein precursor from *V. vinifera* grape (*42*), a 76% homology to a banana ripening-associated protein fragment (*43*), a 68% homology with fragments of basic chitinase from *Citrus sinensis* (*44*), a 68% homology with a thaumatin-like protein from *Actinidia deliciosa* (*45*), and a 68% homology with PRR2 tobac from *N. tabacum* (*39*).

Among the four main polypeptides sequenced from the 2000 harvest grapes, one with 14 amino acid residues sequenced originates a 100% homology with the osmotin-like protein precursor from *V. vinifera* grape; a second polypeptide, with 11 amino acid residues sequenced, exhibits a 81.82% homology with the osmotin-like protein precursor from *V. vinifera* grape; two other polypeptides, from which 25 and 24 amino acid residues were sequenced, produced homologies of 81.82 and 87.50%, respectively, with the VVTL1 protein.

As a whole, the data indicated in **Table 3** show that there is a differential expression of the major proteins present in the mature Moscatel grapes from 1999 and 2000. It is important to note that there is no 100% homology even when the polypeptides from the 1999 harvest grapes are compared with the homologous polypeptides from the 2000 harvest grapes. The majority of the polypeptides sequenced from the 1999 harvest grapes are highly homologous to the sequence reported for the osmotin-like protein precursor from *V. vinifera* grapes, with two polypeptides showing a high homology with the thaumatin-like protein also from *V. vinifera* grapes. Among the four major polypeptides sequenced from the 2000 harvest grapes, two exhibited high homologies with the osmotin-like and two with the thaumatin-like proteins from *V. vinifera* grapes. In clear contrast with the results reported in this work are the results published in ref *19* which claim that chitinases account for ~50% of the soluble proteins present in *V. vinifera* cv. Moscatel

Table 3. N-Terminal Sequences of the Major 1999 and 2000 Moscatel Grape Polypeptides and Comparison with the Sequences of Proteins from Other Sources[a]

PROTEIN	SOURCE		ALIGNMENT		PERCENT IDENTITY
-	Moscatel 1999	1	Y D D T T A M F A A Q	11	-
GLUTAMINE SYNTHETASE	*Vitis vinifera* (grape) (a)	53	Y D D T T V V F	60	75.00
PUTATIVE PHOSPHO-2-D	*Schizosac. pombe* (b)	348	W D D T T A V F	355	75.00
GAS VESICLE PROTEIN	*Halobac.salinarium* (c)	184	F D D T A A S F A A	193	70.00
RIBOSOME RECYCLING F	*Strept. coelicolor* (d)	161	D D T T A K Y V A Q	170	70.00
-	Moscatel 1999	1	A T F D I L N K K T Y T V V A A A A	18	-
-	Moscatel 1999	1	A T F D I L N K K T Y T V V A A A A	18	-
VVTL1	*Vitis vinifera* (grape) (e)	25	A T F D I L N K C T Y T V W A A A S	42	83.33
PRR1 TOBAC	*Nicotiana tabacum* (f1)	26	A T F D I V N K C T Y T V W A A A S	43	77.77
PRR2 TOBAC	*Nicotiana tabacum* (f2)	26	A T F D I V N Q C T Y T V W A A A S	43	72.22
OSMOTIN PRECURSOR	*Arabidopsis thaliana* (g)	23	A T F E I L N Q C S Y T V W A A A S	40	66.67
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
-	Moscatel 1999	1	A T F N I Q N H H S Y T V V A A A V P G G G M Q L	25	-
OSMOTIN-LIKE PROTEIN PREC.	*Vitis vinifera* (grape) (h)	25	A T F N I Q N H C S Y T V W A A A V P G G G M Q L	49	92.00
RIPENING-ASSOCIATED PROTEIN	*Musa acuminata (i)*	27	A T F X I V N R C S Y T V W A A A V P G G G R Q L	51	76.00
BASIC CHITINASE	*Citrus sinensis* (j)	1	A T F D I R N N X P Y T V W A A A V P G G G R R L	25	68.00
THAUMATIN-LIKE PROTEIN	*Actinidia deliciosa* (l)	1	A T F N I I N N C P F T V W A A A V P G G G K R L	25	68.00
PRR2 TOBAC	*Nicotiana tabacum* (f2)	26	A T F D I V N Q C T Y T V W A A A S P G G G R Q L	50	68.00
-	Moscatel 2000	1	A T F N I Q N H C S Y T V W	14	-
OSMOTIN-LIKE PROTEIN PREC.	*Vitis vinifera* (grape) (h)	25	A T F N I Q N H C S Y T V W	38	100.00
-	Moscatel 2000	1	A T F N I Q N H H S Y	11	-
OSMOTIN-LIKE PROTEIN PREC.	*Vitis vinifera* (grape) (h)	25	A T F N I Q N H C S Y	35	81.82
-	Moscatel 2000	1	A T F N I L N K K T Y Y V V A G A A P G G G A L N	25	-
VVTL1	*Vitis vinifera* (grape) (e)	25	A T F N I Q H H C S Y	35	81.82
-	Moscatel 2000	1	A T F D I L N K K T Y T V V A A A S P G G G R R	24	-
VVTL1	*Vitis vinifera* (grape) (e)	25	A T F D I L N K - C T Y T V W A A A S P G G G R	47	87.50

[a] The databases consulted were Swiss-Prot/TrEMBL (www.expasy.ch/cgi-bin/) and EMBL Outsation, European Bioinformatics Institute (www2.ebi.ac.uk). Sources: (a) Loulakakis and Roubelakis-Angelakis (1996), AN P51118 (TrEMBL); (b) Barrell et al. (1995), AN PQ09755 (TrEMBL); (c) Jones et al. (1991), AN P08958 (TrEMBL); (d) Murphy et al. (1998), AN O86770 (TrEMBL); (e) Tattersall et al. (1997), AN Q04708 (TrEMBL); (f1) Cornelissen et al. (1986), AN P13046; (f2) AN P07052 (TrEMBL); (g) Takeda et al. (1991), AN CAB39936 (EMBL); (h) Loulakis (1997), AN P93621 (TrEMBL); (i) Clendennen and May (1997), AN O22322 (TrEMBL); (j) Mayer et al. (1996), AN G1881844 (TrEMBL); (l) Wurms et al. (1998), AN P81370 (TrEMBL).

grapes, with the other major proteins being thaumatin-like proteins (*5*). This discrepancy, as well as the data reported in this work, may be explained by a different pattern of synthesis and accumulation of proteins in the grapes as affected by environmental and/or pathologic conditions during vegetative growth (*14*). This assumes greater relevance because it is known that many of the proteins synthesized during grape growth are inducible (*9*−*12*).

Comparing the data provided in **Figure 1** for the years 1999 and 2000, one observes a much higher precipitation in the year 1999, which does not favor the development of powdery mildew (the pathogen most frequent in the region of Palmela). The grapes harvested in 1999 showed, consequently, lower protein content. Because this harvest took place under rain, the grapes had a lower sugar content (18.6 °Brix) and led to a wine with a lower percentage of alcohol (10.96%), as previously mentioned. In the year 2000, climacteric conditions (higher humidity and generally higher temperature during spring) favored the development of powdery mildew and, in fact, that year's grapes had a higher content of PR proteins. The 2000 harvest took place under better climacteric conditions than the one in the previous year, and the grapes were richer in sugar (22 °Brix) so the wine obtained had 12.42% alcohol.

As a whole, the data in **Table 3** and **Figures 2** and **5** show that wines prepared from the same vineyard and subjected to the same vinification procedures may contain totally different protein contents when prepared from grapes harvested in different years.

The results obtained in the present work raise serious questions about the applicability of the method reported by Waters and collaborators (*46*), who proposed to base varietal differentiation of grapes on the PR polypeptide profile present in the mature berries.

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