JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 11773–11785 **11773**

Ripening and Genotype Control Stilbene Accumulation in Healthy Grapes

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In grapes, stilbene synthesis occurs in the skin, and it is induced by biotic and abiotic stresses. To date, experimental evidence of a constitutive production of resveratrols in healthy grape is scarce and not conclusive. The aim of the present work was to investigate stilbene biosynthesis in healthy grapes both at biochemical and molecular levels. By measuring the concentration of resveratrols in ripe berries of 78 *Vitis vinifera* varieties for 3 years, we could identify significant differences among genotypes, providing the first tentative varietal classification based on resveratrol content. Furthermore, an increasing stilbene accumulation from véraison to ripening phase was also observed. Using real-time reverse transcription polymerase chain reaction and a berry-specific cDNA array, gene expression analysis was carried out on two distinct pools of berries belonging to the high and low resveratrol producers and on three berry developmental stages. The stilbene synthase, phenylalanine ammonia-lyase, and 4-coumarate-CoA ligase expression profiles showed an increasing concentration of these transcripts from véraison to maturity and a higher accumulation in the grape of high resveratrol producers. Macroarray data analysis revealed that high resveratrol levels are also accompanied by the up-regulation of genes involved in plant defense and the concomitant underexpression of genes related to the ripening process and to indole alkaloid synthesis.

KEYWORDS: ***Vitis vinifera*; grape; resveratrol; piceid; stilbene synthase; gene expression; cDNA array**

INTRODUCTION

Stilbenes are polyphenolic secondary metabolites occurring in a few plant families, including *Vitaceae* (*1*). Grapevine stilbenes have been the subject of numerous studies due to their function as phytoalexins with a broad spectrum activity. In addition, several members of the stilbene family including resveratrol (3,5,4′-trihydroxystilbene) are considered to have beneficial effects on human health (*2*). Stilbenes are formed via the phenylpropanoid and acetate-malonate pathway. The stilbene synthase (StSy) is the key enzyme that catalyzes the formation of *trans*-resveratrol via condensation of 4-coumaroyl-CoA and malonyl-CoA. Simple stilbenes such as *trans*-pterostilbene, a dimethylated resveratrol derivative (*3*), and *trans*- and *cis*-piceid, a 3-*O*-β-D-glucoside of resveratrol (*4*), have been identified. Oxidative dimerization of resveratrol units by means of peroxidase activity leads to several oligomers collectively termed viniferins (*5*).

The synthesis of stilbenes can be constitutive or induced by biotic and abiotic elicitors. Monomers and oligomers of resveratrol are present as constitutive substances in the lignified organs of grapevine, such as the roots, stems, canes, and grape seeds. Upon fungal infection in leaf, *trans*-resveratrol is synthesized and converted into more toxic derivatives (viniferins) in leaf (*1*).

In the berry, stilbene synthesis occurs in the skin (*6, 7*) and has been primarily ascribed to fungal infection, environmental stress, or mechanical injury (*8*−*10*). The capacity of grape berries to actively synthesize stilbene compounds was shown to vary along development. Early studies indicated that in *Vitis vinifera* and interspecific varieties, the resveratrol content of UV-irradiated grape berries decreased from the green stage to complete maturity, being close to zero in ripe

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10.1021/jf8017707 CCC: $40.75
Published on Web 11/25/2008

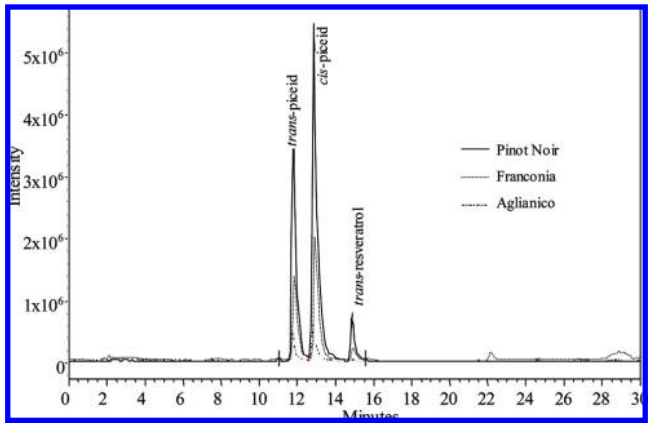

Figure 1. Example of an HPLC-MS chromatogram (MS trace at 229.0 *m*/*z*) of resveratrols in ripe Pinot Noir, Franconia, and Aglianico grapes.

fruit (*6, 7, 11*). Other studies reported that the accumulation of resveratrol in the berry upon UV irradiation can still be remarkably high at ripeness, and the postharvest use of UV irradiation has been shown to improve the content of resveratrol in grapes and wines (*12, 13*). A decline in the inducible stilbene level in developing grape berries was observed following exposure to *Botrytis cinerea* (*14*). The competition between stilbene synthase and chalcone synthase for the same substrates was suggested to be the reason for the decrease in resveratrol accumulation after the onset of fruit ripening, when flavonoid levels are increasing (*15*). Conversely, Mattivi et al. (*16*) found that stilbene content, especially both *cis*- and *trans*-piceid, increases from véraison to harvesting in the absence of fungal infection. In agreement with these findings, Versari et al. (*17*) reported that the total resveratrol content in healthy grape cluster cv. Corvina (*V. vinifera* L.) rose during berry ripening. It thus appears unclear from the cited literature to what extent resveratrol concentration is influenced by the berry developmental stage or by biotic or abiotic stresses. A comprehensive analysis of the constitutive accumulation of resveratrols in healthy grape is still lacking to date. Three open questions have been thoroughly investigated in the present study: (i) the role of the genotype in determining the concentration of resveratrols in the ripe berry, (ii) the influence of the ripening stage on the concentration of resveratrols in healthy grapes, and (iii) the molecular regulation of resveratrol synthesis during berry development. The analysis on a large number of grape genotypes repeated on three vintages allowed us to provide a first tentative classification of grape varieties on the basis of their average resveratrol concentration. The berry transcriptional profiles of varieties identified as high and low resveratrol producers as compared during development provided new insights into the molecular regulation of the resveratrol pathway and its correlation with other metabolic and physiological processes.

MATERIALS AND METHODS

Plant Materials. Seventy-eight *V. vinifera* L. varieties of the Istituto Agrario San Michele all'Adige (IASMA) ampelographic collection were harvested at ripeness in the years 2000, 2001, and 2004. All accessions of this collection were of certain origin, checked and named in agreement with existing literature, and cultivated using a standardized system, with Guyot trellising. The grapes belonging to 48 red-skinned varieties (**Figure 2A**) and 27 white-skinned plus three pink varieties (**Figure 2B**) were sampled at technological maturity, defined as a content of soluble solids in the must corresponding to 18 °Brix, and immediately frozen at −30 °C for resveratrol analysis.

To investigate resveratrol accumulation along berry ripening, grape samples from a subset of 21 of the varieties described above were harvested in the year 2003 at véraison, ripening, and postripening phase. This sampling included one group of "high producers" (11 varieties: Franconia, Malvasia Puntinata, Marsanne, Pinot Gris, Pinot Noir, Pinot Tete de Negre, Refosco, Rousanne, Schioppettino, Tarrango, and Xarello) and a group of "low producers" (10 varieties: Aglianico, Greco di Tufo, Montagna, Nebbiolo, Ortrugo, Petit Manseng, Primitivo di Gioia, Schiava Grossa, Teroldego, and Verduzzo Trevigiano). The véraison phase was defined as the time point when berries began to soften and to develop their characteristic color; the ripening phase (technological ripeness) was defined via measurements of total soluble solids (18−22 °Brix) in the berries using a Reichert hand-held refractometer model; and the postripening phase was defined as the time point 20 days after the ripening phase and corresponded to physiological maturity for late varieties or to over-ripening for early varieties. All grape samples were of sound fruit with no detectable symptoms of disease or damage. Berry clusters of each variety were immediately frozen in liquid nitrogen in the field and stored at −80 °C for both resveratrol analysis and transcriptional profiling experiments.

For the subtractive cDNA library construction, grape berries of four *V. vinifera* L. varieties (cv. Pinot Noir, Moscato Bianco, Teroldego, and Merzling) and leaves and roots of cv. Pinot Noir were used. The berries were harvested in summer 2000 from plants grown in the IASMA collections at the véraison phase judged on the base of the color change and deformability. Leaves at the juvenile stage and young roots were sampled from 1 year old plants grown in the greenhouse. After harvesting, all of the samples were immediately frozen in liquid nitrogen and stored at −80 °C.

Analysis of Stilbene Content. *Chemicals.* Acetonitrile, methanol, and acetic acid were high-performance liquid chromatography (HPLC) grade and were purchased from Carlo Erba (Italy), ethyl acetate was from BDH, and phosphoric acid was from Merck. The *trans*-resveratrol was purchased from Sigma, *cis*-resveratrol was prepared from the standard of *trans*-resveratrol by photoisomerization, and *trans*-piceid (*trans*-resveratrol-3-*O*-β-D-glucopyranoside) was a pure standard isolated from the roots of *Polygonum cuspidatum*. The purity of each stilbene was controlled by HPLC−diode array detection−mass spectrometry (DAD-MS), and the identity was confirmed according to Mattivi et al. (*4*).

From each of the *V. vinifera* L. varieties, a sample of 200 g of berries, randomly picked to obtain a representative sample, was frozen and homogenized in the presence of an internal standard (*trans*-4-hydroxystilbene, 200 mg/L in ethanol, 2 mL added) and of excess ascorbic acid (2 g) and SO_2 (2 g of Na_2SO_3) to prevent oxidation. An aliquot of 50 g of homogenate was transferred into a 250 mL separatory funnel and extracted three times with ethyl acetate (3 × 150 mL). The combined extract (450 mL) was washed once with 50 mL of aqueous $NaHCO_3$ (3%) and twice with water, treated with anhydrous Na_2SO_4, and evaporated under reduced pressure at 38 °C. The residue was immediately redissolved into methanol (5 mL), filtered through a 0.22 μm PVDF filter (Millipore, Bedford, MA) into a HPLC vial, and analyzed by HPLC with photodiode array and mass spectrometric detection.

HPLC-DAD-MS conditions. Mass spectrometric analysis was carried out on a Micromass ZQ LC-MS system (Micromass, Manchester, United Kingdom), equipped with a Waters 2690 HPLC system and a Waters 996 DAD detector (Waters Corp., Miliford, MA) and Empower (Waters Corp., Miliford, MA). Separation was performed using a Zorbax column (SB-Aq, 3.5 μm, 2.1 mm × 150 mm, Agilent Technologies, Palo Alto, CA) and a Zorbax precolumn (SB-Aq, 3.5 μm, 2.1 mm × 12.5 mm, Agilent Technologies). The mobile phases consisted of 0.1% acetic acid in H_2O (A) and acetonitrile (B). Separation was carried out at 40 °C in 27 min, under the following conditions: linear gradients starting at 5% B, to 70% B in 25 min, to 95% B in 0.1 min, 95% B for 2 min, back to 5% B in 0.1 min. The column was equilibrated for 7 min prior to each analysis. The flow rate was 0.25 mL/min, and the injection volume was 4 μL. Under these conditions, the retention times were as follows: *trans*-piceid, 11.6 min; *cis*-piceid, 12.7 min; *trans*-resveratrol, 14.7 min; *cis*-resveratrol, 15.4 min; and *trans*-4-hydroxystilbene, 23.5 min.

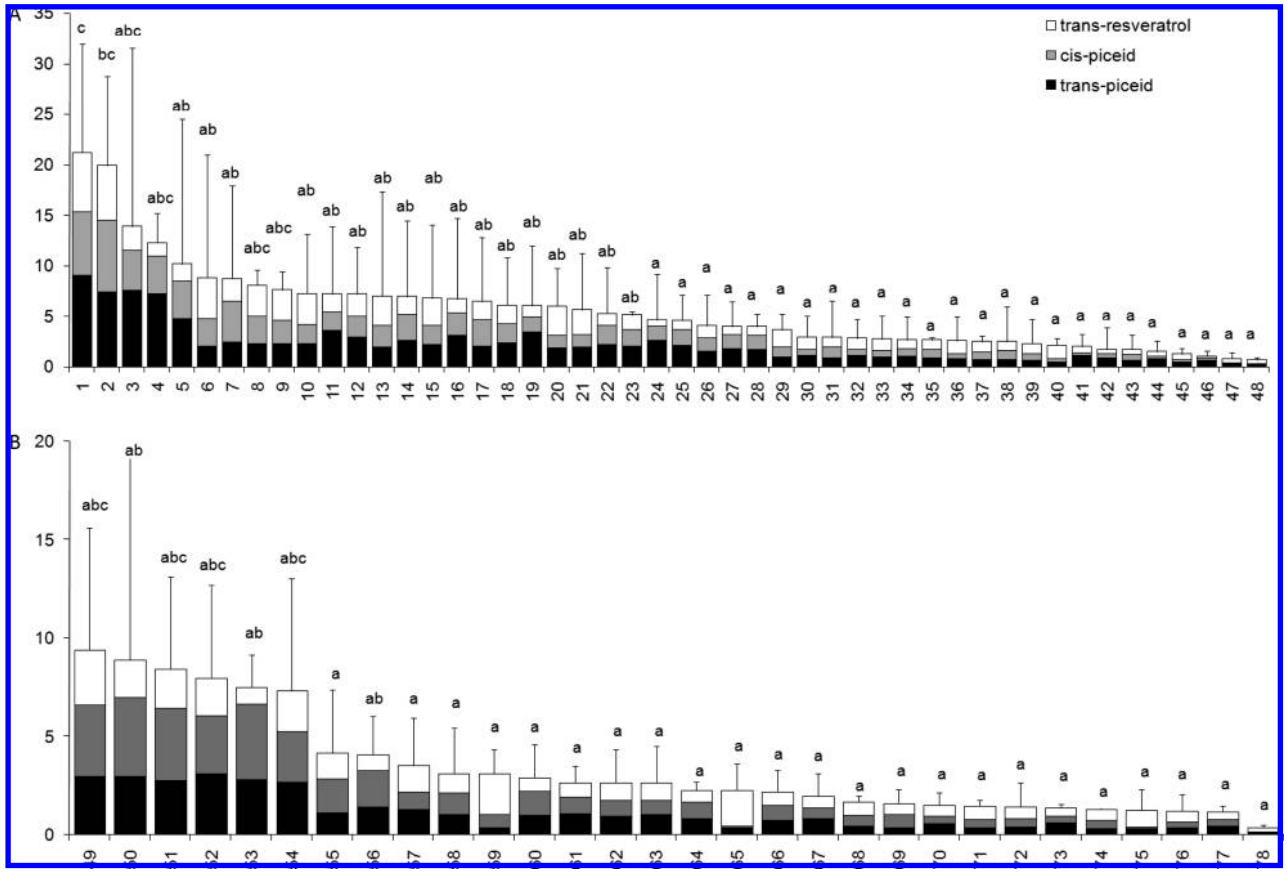

Figure 2. Stilbene content of *V. vinifera* red (**A**) and white or pink (**B**) grape varieties harvested during vintages 2000, 2001, and 2004. The height of the bars refers to the average of the total resveratrol content as a sum of *trans*- and *cis*-piceid and *trans*-resveratrol of 3 years expressed as mg/kg of fresh weight of total grape berry. The standard deviation is also reported. The letters indicate groups of means within which there are no statistically significant differences (p value ≤ 0.05), according to Tukey's honestly significant difference (HSD) procedure applied to within-year normalized data. The numbers correspond to the variety names as follows: Pinot Noir (1), Pinot Tete de Negre (2), Tarrango (3), Franconia (4), Alicante Bouquette (5), Carmenere (6), Schioppettino (7), Ancellotta (8), Galioppo (9), Nero (10), Bovale Sardo (11), Lambrusco Salamino (12), Schiava Lombarda (13), Merlot (14), Pinotage (15), Dolcetto (16), Lambrusco Oliva (17), Negro Amaro (18), Tempranillo (19), Primitivo (20), Frappato (21), Zweigelt (22), Montepulciano (23), Cesanese (24), Uva di Troia (25), Cigliegiolo (26), Sangiovese (27), Pavana (28), Sagrantino (29), Primitivo di Gioia (30), Enantio (31), Cannonau (32), Croatina (33), Raboso del Piave (34), Casetta (35), Moscato Rosa (36), Grignolino (37), Marzemino (38), Tannat (39), Aleatico (40), Cabernet Sauvignon (41), Nera dei Baisi (42), Aglianico (43), Cabernet Franc (44), Schiava Grossa (45), Nebbiolo (46), Teroldego (47), Rebo (48), Marsanne (49), Pinot Gris (50), Rousanne (51), Italia (52), Malvasia Puntinata (53), Inzolia (54), Xinomavro (55), Garganega (56), Muscat Rouge de Madere (57), Madeleine Angevine (58), Sauvignon Blanc (59), Peverella (60), Malvasia di Candia Aromatica (61), Verdicchio (62), Viogner (63), Grechetto (64), Riesling (65), Verduzzo Friuliano (66), Kozma Palne Muskotali (67), Prosecco (68), Petit Manseng (69), Fiano (70), Chardonnay (71), Ribolla Gialla (72), Nosiola (73), Verduzzo Trevigiano (74), Gewuerztraminer (75), Pignoletto (76), Ortrugo (77), and Greco di Tufo (78). The stilbene contents of Xarello (79), Refosco (80), and Montagna (81) measured for 2 years only are reported in the Supporting Information, Table 4.

The UV−vis spectra were recorded from 220 to 400 nm, and UV signal at 310 nm was used for quantification of internal standard and estimate of the recovery factor. The MS conditions were as follows: capillary voltage, 3000 V; cone voltage, 40 V; extractor voltage, 5 V; source temperature, 105 °C; desolvation temperature, 200 °C; cone gas flow (N_2), 30 L/h; and desolvation gas flow (N_2), 450 L/h. The outlet of the HPLC system was split (9:1) to the electrospray ionization (ESI) interface of the mass analyzer. Electrospray mass spectra ranging from m/z 100 to 800 were taken in positive mode with a dwell time of 0.1. The stilbenes *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, and *cis*-piceid were identified on the basis of their retention times and UV and MS spectra and quantified with MS in SIM mode (m/z 229.0) using the external standard method. An example of the HPLC-MS chromatogram is given in **Figure 1**.

Statistical Analysis of Biochemical Data. Statistical analysis was carried out using the Statistica data analysis software, version 6 (StatSoft, Tulsa, OK). Significant differences in the total concentration of resveratrols between varieties were assessed by one-way analysis of variance (ANOVA) and Kruskal−Wallis test. In addition, a multiple comparison procedure was applied to determine which means were significantly different from which others, using the Tukey's HSD method. Before the Tukey HSD method was applied, the data were normalized within each year by subtracting the total resveratrol mean of all varieties for a given year to each value and then by dividing by the standard deviation. Significant differences in the total concentration of resveratrols between varieties grouped in high and low resveratrol producers were assessed also by nested ANOVA.

Total RNA Extraction. For the subtractive cDNA library construction, five berries were selected from berry clusters of four *V. vinifera* L. varieties (cv. Pinot Noir, Moscato bianco, Teroldego, and Merzling) and pooled together. For the transcript profiling experiments, the berries from the high and low resveratrol producers were pooled in two separate groups for each developmental stage. Three berries (six berries for Petit Manseng) were taken from different parts of the cluster of each selected variety. Pedicels and seeds were removed. Total RNA was isolated from berry pools according to Moser et al. (*18*) and from leaves and roots as described by Kiefer et al. (*19*). Remaining traces of DNA were removed with RQ1-RNase free DNase (Promega, Madison, WI) according to the manufacturer's procedure. RNA purity and quality were checked by both agarose-gel electrophoresis and spectrophotometry.

Construction of a Subtractive cDNA Library. A 0.6 μg amount of total RNA from berry, leaf, and root tissues were reverse transcribed

in double-stranded cDNA using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA), as described by the manufacturer. A berry subtractive cDNA library was constructed using the PCR-Select cDNA Subtraction Kit (Clontech), following the manufacturer's instructions. To obtain berry-specific transcripts, tester cDNA from berry tissue was hybridized in two rounds with driver cDNA consisting of leaf and root cDNAs in equal parts. The berry-specific cDNA was inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into One Shot TOP10 electrocompetent *Escherichia coli* (Invitrogen). Positive transformants based on blue/white color selection were grown at 37 °C in 384 well plates containing LB medium supplemented with 50 μg/mL kanamycin and 10% glycerol and then stored at −80 °C.

Production of High-Density cDNA Arrays. The cDNA inserts of 4224 clones of the berry subtractive cDNA library were polymerase chain reaction (PCR) amplified. In a final volume of 40 μL, the concentrations of the PCR reagents were 10 mM Tris-HCl (pH 8.3), 2.25 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 1.5 M betaine, 0.25 μM M13 universe primer (5′-GTTGTAAAACGACGGCCAGT-3′), 0.25 μM M13 reverse primer (5′-ACACAGGAAACAGCTATGAC-3′), 0.1 mM Cresol Red (SIGMA, Munich, Germany), 1.5 U Taq polymerase, and 1 μL of bacterial culture (*20*). Reaction samples were first denatured at 95 °C for 1 min and then subjected to 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. The volume of the amplification products was reduced to a quarter by evaporation for 24 h at 30 °C in an air-ventilated incubator and then adjusted to 20 μL, by adding 10 μL of PCR grade water to each PCR sample (*20*). The concentrated amplification products were transferred into Genetix 384 well microarray plates (Genetix, New Milton, United Kingdom) and were printed in duplicate onto 7.3 cm × 11.5 cm Hybond-N+ nylon membranes (Amersham Biosciences, Piscataway, NJ), using a 5 × 5 gridding pattern. The spotting was automated with a Q-Pix biorobot (Genetix), using a 384 pin gravity gridding head with 0.4 mm pin diameter. Before spotting, membranes were placed in denaturation buffer (0.5 M NaOH; 1.5 M NaCl) and then placed on Whatmann paper saturated with denaturation buffer until they became matt. After spotting, nylon filters were positioned face up for 10 min onto Whatman paper soaked with denaturation buffer, followed by 10 min of neutralization buffer (1.5 NaCl; 0.5 M Tris-HCl, pH 7.2), then dried overnight on Whatman paper, and UV cross-linked at 50 mJoule in a UV cross-linker (GS Gene Linker UV Chamber, Bio-Rad, Hercules, CA).

Target Preparation and Filter Array Hybridization. For target preparation, 50 μg of total RNA from berry tissue was used to synthesize cDNA, using the LabelStarArray kit (Qiagen, Valencia, CA) with [^{33}P]-α-dCTP as a radioactive label. Cleanup of labeled cDNA from unincorporated [^{33}P]-α-dCTP was performed using MinElute spin columns according to the LabelStar Array Cleanup module (Qiagen). [^{33}P]-α-dCTP incorporation was quantified via liquid scintillation counting (Beckmann LS6500, Beckman Coulter, Fullerton, CA). The nylon filters were initially prehybridized with 20 mL of Church buffer [0.5 M sodium phosphate buffer, pH 7.2; 7% sodium dodecyl sulfate (SDS); and 1 mM EDTA] including 200 μL of denatured salmon sperm DNA (10 mg/mL) at 65 °C for at least 4 h. The hybridization was started with 20 mL of fresh Church buffer including 200 μL of denatured salmon sperm DNA (10 mg/mL) and the denatured radioactive-labeled cDNAs. The hybridization was perfomed at 65 °C overnight. The nylon filters were washed twice with 100 mL of washing buffer (40 mM sodium phosphate, pH 7.2; 0.1% SDS) at 65 °C for 30 min. The nylon filters were placed on a support and sealed with plastic wrap. The nylon filters were then exposed to phosphor screen imaging plates (Fujifilm) for 20 h. To remove hybridization signals after scanning, the nylon filters were incubated twice in 200 mL of stripping solution (0.4 NaOH; 0.1% SDS) for 30 min at 65 °C in a shaking waterbath. The nylon filters were then washed twice with 300 mL of wash solution (0.2 M Tris-HCl, pH 8; 1X SSC; and 0.1% SDS) for 10 min at room temperature. For each experimental condition, three independent (technical) hybridizations were performed.

Image Acquisition, Data Processing, and Statistical Analysis. Imaging plates were scanned with a FLA-3000 phosphorimager (Fujifilm) at 50 μm resolution. Detection and quantification of the signals, representing the hybridizing DNA, were performed using the ArrayVision 8.0 imaging software (Imaging Research Inc., Haverhill, United Kingdom). Data processing and statistical analysis were performed using R (*21*), (http://www.r-project.org/) and Bioconductor software (*22*) (http://www.bioconductor.org). Before normalization, intensity values were log2 transformed. Quantile normalization (*23*) was performed to normalize filter arrays within the same berry developmental stage. The median intensity of all of the filter arrays was then centered around zero, and the resulting normalized intensities were used for statistical analysis. Significant changes in gene expression between the high and the low resveratrol berry pools for each berry developmental stage were identified by SAM (Significance Analysis of Microarrays) (*24*) analysis. To compare the two experimental groups, a two-class unpaired test was performed with a modified t statistic, assuming equal group variances. The number of permutations was set to 900, and the missing values were replaced by a gene-wise mean. In each hybridization experiment, both duplicates of the same clone were treated separately. Hence, six replications of each data point were considered. δ values were adjusted to achieve a false discovery rate of 3% for each comparison. Only cDNA clones with a fold change parameter greater than or equal to 1.5 between the two experimental groups were selected.

Sequencing and Sequence Analysis. The inserts of selected cDNA clones were PCR amplified and subjected to cycle sequencing using the BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA) and M13 universe primer. After purification, the reaction products were run on a ABI Prism 3730 Genetic Analyzer (Applied Biosystems). After low-quality read regions and repeats were trimmed, a clustering process was performed on the EST sequences to obtain nonredundant sets of consensus sequences using CAP3 (*25*). Sequences sharing more than 90% identity over 40 or more contiguous bases and with unmatched overhangs shorter than 20 bases were placed in the same cluster. The annotation of the consensus sequences was performed by similarity search using blast (*26*) against the UniProt database (http://www.uniprot.org). The consensus sequences, for which no significant sequence similarity was found, were designated as "no hit found". The sequences were manually assigned to 10 GO ontology categories (http://www.geneontology.org), representing the major cellular metabolisms.

Real-Time Reverse Transcription (RT)-PCR. To verify the reliability of the macroarray results, the expression patterns of 15 genes were analyzed by real-time RT-PCR. Total RNA was isolated from new berry pools as described in the Total RNA Extraction section. After DNA traces were removed with RQ1-RNase free DNase (Promega), 1 μg of RNA samples was reverse transcribed and amplified using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen), following the two-step qRT-PCR protocol recommended by the manufacturer with some modifications. PCR reactions were set in triplicate in 12.5 μL volume containing 1 μL of 1:25 diluted first-strand cDNA synthesis reaction, 1X Platinum SYBR Green qPCR SuperMix-UDG, 1X ROX Reference Dye, and a 300 nM concentration of each primer. PCR cycling was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following thermal cycling protocol: one step at 50 °C for 2 min, one step at 95 °C for 10 min, 40 cycles with a denaturation step at 95 °C for 15 s, and an annealing/extension step at 58 °C for 1 min. A negative control (no cDNA template) was run with every assay to assess the primers specificity. The primers used in real-time RT-PCR analysis (see Supporting Information, Table 1) were designed with Primer Express v. 2.0 software (Applied Biosystems) on the sequences of the selected genes and on sequences of actin, StSy, PAL, C4H, and C4L genes deposited in public databases (DFCI Grape Gene Index, release 6). Because PAL and StSy genes belong to large gene familes in grape (*27*) and no specific information on the expression pattern of their isogenes in grape berry was available, the primers for the real-time RT-PCR analysis were designed in the coding regions showing high sequence similarity among the different isogenes. For CHS gene, published primers for the isogene Chs2 were used (*28*). Among the three main isogenes, Chs2 has been reported to accumulate in both white and red cultivars (*29*). The PCR efficiency for each gene was calculated using the linear regression of the log-transformed, background-subtracted relative fluorescence values (LinRegPCR program, Version

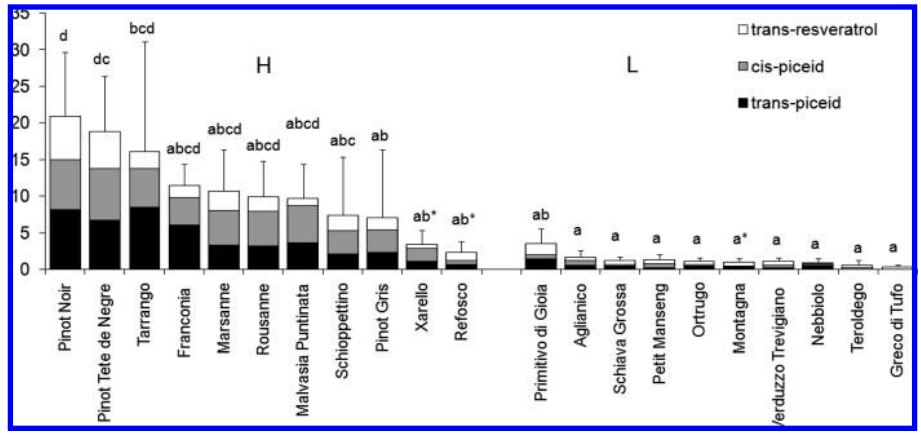

Figure 3. Stilbene content of *V. vinifera* grape varieties (vintages 2000, 2001, 2003, and 2004) classified as high ("H") and low ("L") resveratrol producers. The height of the bars refers to the average of total resveratrol content as a sum of *trans*- and *cis*-piceid and *trans*-resveratrol of 4 years expressed as mg/kg of fresh weight of total grape berry. The standard deviation is also reported. The letters indicate groups of means within which there are no statistically significant differences (p value ≤ 0.05), according to the Tukey's HSD procedure applied to within-year normalized data. *For these varieties, the average of total resveratrol was calculated on 3 years of data.

7.5) (*30*). The expression of actin was used to normalize the results, and the relative quantification was carried out according to the mathematical model developed by Pfaffl (*31*), where target is the gene of interest and reference is actin. The overall standard error of the mean normalized expression was obtained by applying the error calculation based on Taylor's series as developed for REST software (*32*).

RESULTS

Resveratrol Content of Different *V. vinifera* Varieties. The average content of resveratrols in grapes of 78 different red and white/pink varieties, resulting from a 3 year survey, is reported in **Figure 2**. The one-way ANOVA analysis on the total concentration of resveratrols in the 78 varieties demonstrated that the effect of the variety was highly significant (p value = 0.0004; for Kruskall−Wallis test, p value = 0.012). The red varieties (**Figure 2A**) had on average a higher content than the white/pink ones (**Figure 2B**). The differences among the varieties were higher than 1 order of magnitude, reaching a factor of 30 among the red varieties (Pinot Noir vs Rebo) and of 26 among the white or pink varieties (Marsanne vs Greco di Tufo). Because of the expected large variability within each variety, the differences among the total content of resveratrols of the varieties were evaluated with the 95.0% Tukey HSD test on data normalized separately within each year. Significant differences for the varieties were labeled with a different letter in **Figure 2**. The experimental observation that the three genetically close varieties belonging to the family of Pinots (Pinot Noir, Pinot Tete de Negre, and Pinot Gris) ranked at the top of the respective list of the red or white/pink varieties strongly suggests a major role of the genotype.

High vs Low Producers of Resveratrol. Two subgroups of about 10 *V. vinifera* varieties each were selected on the base of their berry resveratrol content determined on 78 grape varieties harvested in the 2000 vintage. The plants were divided in high resveratrol producers, which accumulated between 2.3 and 33.5 mg of total resveratrol per kg of grape berries (these values are significantly above the mean calculated on the 78 analyzed varieties), and low resveratrol producers, which accumulated between 0.2 and 1.8 mg of total resveratrol per kg of grape berries (these values are significantly under the mean calculated on the analyzed varieties). The comparison of the average total content of resveratrols at ripeness in the berries of these two groups of varieties (average of the years 2000, 2001, 2003, and 2004) is shown in **Figure 3**. The one-way ANOVA analysis on the total concentration of resveratrols in the 21 varieties confirmed that the effect of the variety was highly significant (p value = 0.0000; for the Kruskall−Wallis test, p value = 0.000006). The significance of the differences among the mean total values registered was evaluated with the 95.0% Tukey HSD test on data normalized separately within each year. The 21 grape varieties assigned to the two groups had significantly different concentrations of resveratrols for the varieties labeled with a different letter in **Figure 3**, with a single and partial misclassification of the variety Primitivo di Gioia. The mean values found in the berries of high producers (*trans*-resveratrol = 2.37, *cis*-piceid = 4.19, *trans*-piceid = 4.18, and total resveratrols = 10.74 mg/kg) were 1 order of magnitude higher than those registered in the low producers (*trans*-resveratrol = 0.53, *cis*-piceid = 0.29, *trans*-piceid = 0.48, and total resveratrols = 1.30 mg/kg), the difference between groups being larger for the glucosides than for the free *trans*-resveratrol. As a consequence, the difference of the total concentration of resveratrols for the two groups was highly significant according to nested ANOVA (p value < 0.005), thus demonstrating for the first time that the genotype plays a very important role in determining the concentration of resveratrols in ripe grape.

Influence of the Ripening Stage on the Concentration of Resveratrols in Grapes. In the case of the varieties listed in **Figure 3** and collected during 2003, the berry stilbene content was determined at three key developmental stages: véraison, ripening, and postripening. Measurements of *trans*-piceid, *cis*-piceid, and *trans*-resveratrol levels (**Figure 4**) confirmed the classification in two groups observed in the 2000 vintage and showed the progressive accumulation of resveratrol during berry development in both groups. High resveratrol producers contained between 0.2 and 3.7 mg of total resveratrol per kg of grape berries at véraison (**Figure 4A**), except for Malvasia Puntinata, Refosco, and Roussane, which were below the detection limit at this stage. The total resveratrol level in this group increased at the ripening phase being between 0.8 and 22.5 mg per kg of grape berries and at the postripening phase with values between 1.2 and 22.5 mg per kg of grape berries. In comparison with the high resveratrol producers, the low producers showed lower total resveratrol contents at each berry developmental stage. This group contained between 0.01 and 0.4 mg of total resveratrol per kg of grape berries at véraison (**Figure 4B**), except for Nebbiolo, Montagna, Teroldego, and

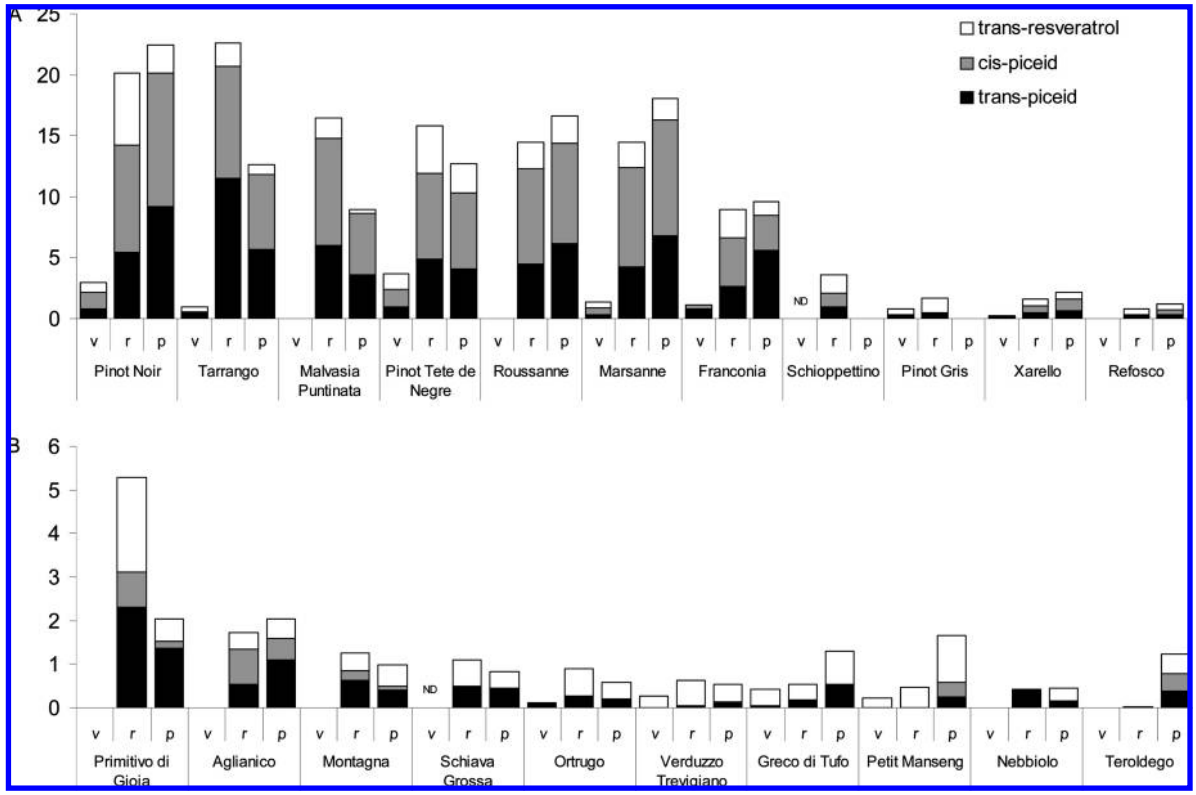

Figure 4. Stilbene content of *V. vinifera* grape varieties (vintage 2003) classified as high (**A**) and low (**B**) resveratrol producers. The grape varieties were collected at véraison (v), ripening (r), and postripening (p) phases. The height of the bars refers to the average of the total resveratrol content as a sum of *trans*- and *cis*-piceid and *trans*-resveratrol expressed as mg/kg of fresh weight of total grape berry. ND means "not determined".

Aglianico, which did not show detectable resveratrol levels at this stage. At the ripening stage, the total resveratrol content ranged mainly from 0.03 to 1.7 mg per kg of grape berries with the exception of Primitivo di Gioia variety, which showed a strong accumulation up to 5.3 mg of total resveratrol per kg of grape berries. At the postripening phase, the resveratrol levels slightly increased, between 0.4 and 2 mg per kg of grape berries. The *cis*-resveratrol was detected only in Franconia at the ripening phase at a concentration of 0.37 mg per kg of grape berries (data not shown). In general, the mean total resveratrol content of the high producers was 10-fold higher than the value observed in the low producers at each sampling time, and it increased around 10-fold from véraison to the ripening phase in both groups. The biochemical analysis (**Figure 4**) also showed that the high producers did accumulate resveratrol preferentially in the glucosylated forms, *trans*- and *cis*-piceid, while the low resveratrol producers contained mainly the sugar-free form of resveratrol.

Gene Expression Analysis. The gene expression studies, which are the matter of the following sections, were carried out on berry pools obtained by grouping berries from each variety of the 21 ones described above and for each berry developmental stage on the base of classification in high or low resveratrol producers. The rationale for the pooling was to highlight the molecular mechanisms behind resveratrol accumulation and at the same time to minimize the single genotype-related differences that would make it difficult to relate gene expression differences to the resveratrol content.

Transcriptional Characterization of the Main Genes Involved in the Phenylpropanoid Pathway. By real-time RT-PCR, we investigated in berries of high and low resveratrol producers the expression profiles of five genes of the phenylpropanoid pathway known to be involved in stilbene biosynthesis: phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), stilbene synthase (StSy), and chalcone synthase (CHS). While the first three structural genes code for enzymes of the early steps of the phenylpropanoid pathway, the StSy and CHS genes code for the enzymes, which, via malonyl-CoA and 4-coumaroyl-CoA condensation, lead to the biosynthesis of stilbenes and flavonoids, respectively. In **Figure 5**, the gene expression profile of PAL, 4CL, StSy, and CHS genes in the three sampling times is reported. PAL, 4CL, and StSy genes are expressed at much higher levels in the high resveratrol producers as compared to the low ones. In particular, StSy transcript abundance is 32 times greater in the high resveratrol producers with respect to the low ones at véraison, and it remains higher, although at lower ratios, in the ripening and postripening phases. PAL and 4CL genes also showed a significantly higher steady-state concentration (about two times) in the high resveratrol producers at each berry developmental stage. For the C4H (data not shown) and CHS genes, no significant difference in gene expression was observed.

Furthermore, in the high resveratrol producers, a strong increase in the expression of the PAL, 4CL, and StSy genes was observed from véraison to ripening phase, and no significant changes were measured thereafter. In the low resveratrol producers, only the StSy gene appeared to be significantly modulated during berry ripening, showing an increase of ~33-fold from véraison to the postripening phase. An expression change of about 2-fold from véraison to ripening phase was also observed for the CHS gene in both high and low resveratrol producers.

Identification of Differentially Expressed and Berry-Specific Genes in High and Low Resveratrol Producers. To profile gene expression in grape varieties with high and low stilbene content during berry development, a cDNA array containing berry-specific genes was constructed, by spotting ~4200 cDNA clones of a cDNA library enriched in berry-specific transcripts onto nylon filters. The cDNA library was obtained by suppression subtractive

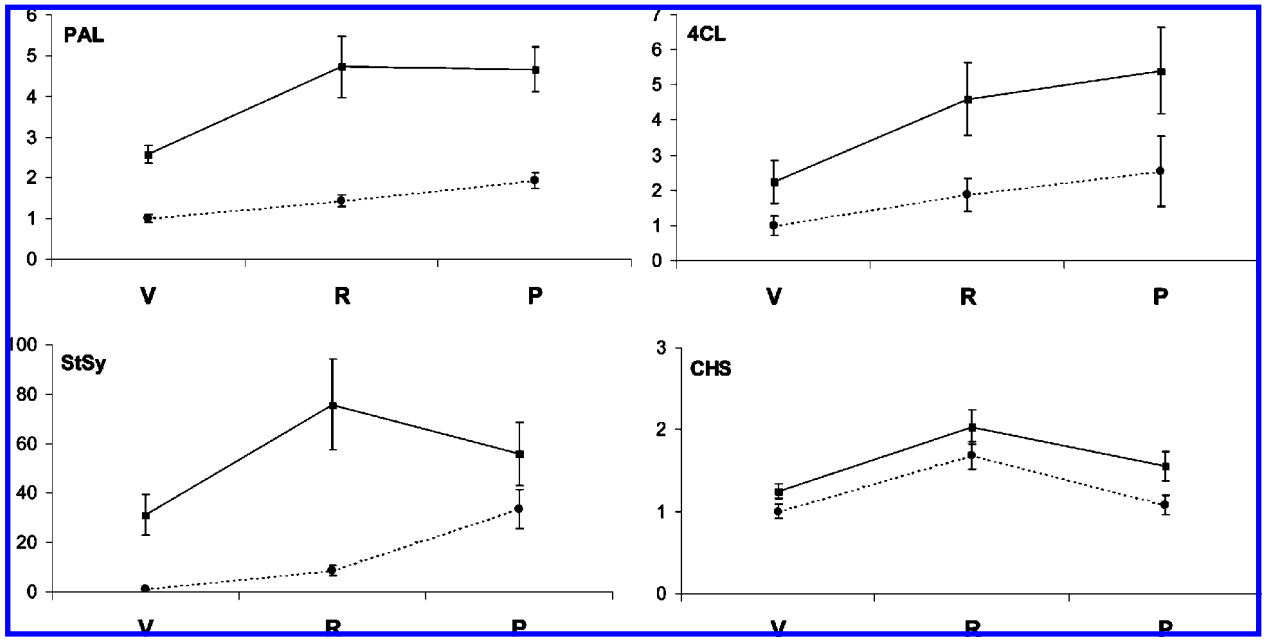

Figure 5. Real-time RT-PCR analysis of genes encoding phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS, isogene 2), and stilbene synthase (StSy) in berries of high (black squares and solid lines) and low resveratrol (black circles and dotted lines) producers at véraison (V), ripening (R), and postripening (P) phases. The expression of the target gene (on the *y*-axis) is expressed relatively to the expression measured in the low resveratrol producers at the véraison phase taking actin as a reference gene. SE (standard error) is calculated on three technical replicates.

hybridization using cDNA from berry tissue as the tester and cDNA from root and leaf tissues as the driver. Before cDNA array construction, the tissue-specific cDNA library was accurately characterized to check for subtraction efficiency, average insert size, and sequence redundancy (data not shown). The printed cDNA arrays were next hybridized with ^{33}P-labeled probes prepared from the total RNA from grape berry pools as described in the Total RNA Extraction section. After normalization of signal intensities for each experimental condition, calculation of the determination coefficient revealed good reproducibility among replica filters of the same experiments (see the Supporting Information, Figure 1A,B) and lower coefficient values when comparing different samples or different developmental stages (see the Supporting Information, Figure 1C,D) as expected since this reflects gene expression differences due to biological diversity. The statistical significance of the RNA changes observed between high and low producers was assessed by SAM analysis (Significance Analysis of Microarrays) (*24*). SAM run at a median FDR (false discovery rate) of 3% identified a set of differentially expressed cDNA clones in the high resveratrol producers as compared to the low ones at each developmental stage. Setting a cutoff value of 1.5-fold change, 285, 154, and 289 cDNA clones were selected as being differentially expressed between high and low resveratrol producers at the véraison, ripening, and postripening phase, respectively. Out of this group of modulated cDNA clones, 451 high quality ESTs were obtained and clustered in 186 gene sequences (see the Supporting Information, Table 2). In **Table 1**, the number of differentially expressed genes between high and low resveratrol producers at véraison, ripening, and postripening phase is reported. In general, more genes were found to be down-regulated in the high resveratrol producers with respect to the low ones.

Functional Annotation of Differentially Expressed and Berry-Specific Genes in High and Low Resveratrol Producers. A putative function was assigned to 138 out of 186 gene sequences (75%). To relate the spectrum of presumed gene functions to the comprehensive physiological activity of the cell, the annotated sequences were categorized according to the biological process that they perform using the gene ontology (GO) vocabulary (see the Supporting Information, Figure 2). The majority of the genes differentially expressed between high and low resveratrol producers fell in the GO category "response to stimulus" (22%), a broad functional category that comprises genes involved in the detection of and response to many different stimuli such as biotic and abiotic stimuli, external and endogenous stimuli, and stress and hormone stimuli. Other functional classes including at least seven genes were "cell wall organization" (9%), "transport" (8%), "transcription" (5%) "carbohydrate metabolic process" (5%), "protein metabolic process" (4%), "signal transduction" (5%), "developmental process" (4%), and "secondary metabolic process" (4%). The sequences matching with proteins with unknown function or not belonging to the above-mentioned metabolisms were grouped in the main category "biological process" (19%).

Table 1. Results of SAM Analysis after EST Analysis and Clustering[a]

SAM analysis	no. of genes (EST) up-regulated	no. of genes (EST) down-regulated	no. of total genes (EST) differentially expressed
veraison	33 (84)	63 (140)	96 (224)
ripening phase	26 (75)	26 (60)	52 (135)
postripening phase	26 (77)	72 (128)	98 (205)

[a] The number of differentially expressed genes and ESTs (between squares) found in the high resveratrol producers relative to the low ones at vèraison, ripening, and postripening phases is shown.

Looking in more details into each functional category (**Table 2**) revealed that high and low resveratrol producers modulate different set of genes. In the category "response to stimulus", the genes up-regulated in the high resveratrol producers were mainly involved in plant defense against fungal pathogens and in the general response to biotic stress. Example are class I (CLU0172) and IV chitinases (CLU0031, CLU0085, CLU0345, CLU0445, and CLU0461), thaumatin-like protein VVTL1 (CLU0294, CLU0295), VVTL2 (CLU0365), VVTL3 (CLU0419), endo-1,3-1,4-β-D-glucanase (CLU0234),

Table 2. List of the Differentially Expressed Unique Sequences Assigned to the Main Categories of GO Biological Process[a]

CLU ID	Annotation	e-value	V	R	P
response to stimulus					
0411	Auxin-induced protein 22A (*Phaseolus aureus*)	4E-17			
0396	BURP domain-containing protein (*Bruguiera gymnorrhiza*)	2E-08			
0393	Catalase (*Vitis vinifera*)	1E-95			
0172	Chitinase Ib (*Castanea sativa*)	7E-33			
0031	Class IV endochitinase (*Vitis vinifera*)	2E-41			
0085	Class IV endochitinase (*Vitis vinifera*)	6E-43			
0270	Class IV chitinase (*Vitis vinifera*)	3E-115			
0345	Class IV chitinase (*Vitis vinifera*)	4E-53			
0445	Class IV endochitinase (*Vitis vinifera*)	3E-48			
0461	Class IV endochitinase (*Vitis vinifera*)	3E-21			
0397	Cyclophilin (*Euphorbia esula*)	8E-18			
0088	DC1.2 homologue (*Nicotiana tabacum*)	2E-41			
0234	Putative endo-1,3-1,4-beta-D-glucanase (*Arabidopsis thaliana*)	1E-87			
0178	Early-responsive to dehydration stress protein, ERD3 (*Arabidopsis thaliana*)	3E-85			
0262	Ethylene response factor 1, ERF1 (*Lycopersicon esculentum*)	1E-07			
0038	Putative ripening-related protein, GRIP 21 (*Vitis vinifera*)	6E-57			
0051	Putative ripening-related protein, GRIP 22 (*Vitis vinifera*)	3E-36			
0083	Putative ripening-related protein, GRIP 22 (*Vitis vinifera*)	2E-25			
0277	Putative ripening-related protein, GRIP 22 (*Vitis vinifera*)	4E-73			
0491	Putative ripening-related protein, GRIP 22 (*Vitis vinifera*)	9E-54			
0086	Putative ripening-related protein, GRIP 61 (*Vitis vinifera*)	1E-58			
0060	Leucine rich repeat protein (*Cicer arietinum*)	4E-43			
0070	Leucine rich repeat protein (*Cicer arietinum*)	8.E-110			
0018	Pathogenesis-related protein 10, PR-10 (*Vitis vinifera*)	7E-16			
0059	Phase-change related protein (*Quercus robur*)	4E-32			
0074	Phase-change related protein (*Quercus robur*)	2.E-31			
0076	Phase-change related protein (*Quercus robur*)	9E-76			
0101	Phase-change related protein (*Quercus robur*)	1E-91			
0188	Phase-change related protein (*Quercus robur*)	3E-131			
0488	Phase-change related protein (*Quercus robur*)	2E-07			
0241	Pit2 protein (*Nicotiana tabacum*)	2E-15			
0194	Pathogenesis-related protein 4, PR-4 type protein (*Vitis vinifera*)	1E-68			
0455	Putative acyl-CoA synthetase (*Capsicum annuum*)	1E-24			
0458	Putative Pop3 protein (*Arabidopsis thaliana*)	6E-29			
0294	Putative thaumatin-like protein, TL1 (*Vitis vinifera*)	1E-20			
0295	Putative thaumatin-like protein, TL1 (*Vitis vinifera*)	8E-81			
0365	Putative thaumatin-like protein, TL2 (*Vitis vinifera*)	2E-74			
0405	Putative thaumatin-like protein, SCUTL2 (*Vitis vinifera*)	1E-46			
0419	Putative thaumatin-like protein, TL3 (*Vitis vinifera*)	3E-25			
0493	RD22-like protein (*Vitis vinifera*)	5E-40			
0320	Seed maturation protein PM31 (*Glycine max*)	8E-20			
0473	Stress-related protein (*Vitis riparia*)	1E-07			
0460	Stress-related protein (*Vitis riparia*)	6E-53			
transcription					
0411	Auxin-induced protein 22A (*Phaseolus aureus*)	4E-17			
0252	MYC1 (*Catharanthus roseus*)	4E-17			
0030	BTB and TAZ domain protein (*Arabidopsis thaliana*)	1E-27			
0137	G-box binding factor bZIP transcription factor (*Catharanthus roseus*)	3E-43			
0262	Ethylene response factor 1, ERF1 (*Lycopersicon esculentum*)	1E-07			
0054	MADS-box transcripion factor 1 (*Vitis vinifera*)	2E-09			
0383	MADs-box protein, GDEF1 (*Gerbera* hybrid cv. 'Terra Regina')	3E-40			
0104	MADS-box protein 4 (*Vitis vinifera*)	5E-52			
0138	Putative RNA-binding protein (*Arabidopsis thaliana*)	2E-50			
0105	MYB-like DNA-binding protein (*Catharanthus roseus*)	2E-53			
transport					
0287	Aquaporin, PIP2-1 (*Vitis vinifera*)	4E-88			
0290	Aquaporin, PIP2-1 (*Vitis vinifera*)	6E-33			
0284	Aquaporin, PIP2-1 (*Vitis vinifera*)	2E-52			
0089	(Putative) aquaporin PIP1-3 (*Vitis berlandieri* x *Vitis rupestris*)	1E-154			
0269	Glutamate/malate translocator (*Nicotiana tabacum*)	5E-38			
0327	Hypothetical protein At2g41190 (Amino acid transport) (*Arabidopsis thaliana*)	2E-87			
0332	Hypothetical protein At3g23550 (Multidrug transport) (*Arabidopsis thaliana*)	3E-41			
0079	MRP-like ABC transporter, MRP3 (*Oryza sativa*)	2E-54			
0045	Multidrug-resistance related protein, MRP6 (*Arabidopsis thaliana*)	4E-129			
0364	Multidrug-resistance associated protein (*Vitis vinifera*)	5E-15			
0354	Probable membrane transporter (*Oryza sativa*)	2E-33			
0187	Putative nuclear transport factor Ntf2p (*Oryza sativa*)	2E-26			
0228	Putative potassium transporter (*Oryza sativa*)	4E-01			
0168	Sulfate transporter 3.1 (*Arabidopsis thaliana*)	2E-104			
0050	Sulphate transporter 3.5 (*Brassica oleracea*)	8E-57			
developmental process					
0411	Auxin-induced protein 22A (*Phaseolus aureus*)	4E-17			
0262	Ethylene response factor 1, ERF1 (*Lycopersicon esculentum*)	1E-07			
0271	GA 20-oxidase (*Pisum sativum*)	2E-67			
0054	MADS-box transcripion factor 1 (*Vitis vinifera*)	2E-09			
0104	MADS-box protein 4 (*Vitis vinifera*)	5E-52			
0383	MADs-box protein, GDEF1 (*Gerbera* hybrid cv. 'Terra Regina')	3E-40			
0199	Senescence-associated protein, SAG29 (*Arabidopsis thaliana*)	6E-37			

Table 2. Continued

CLU ID	Annotation	e-value	V	R	P
protein metabolic process					
0026	Aspartyl aminopeptidase (*Arabidopsis thaliana*)	2E-61			
0482	Carboxyl terminal protease-like protein (*Arabidopsis thaliana*)	8E-100			
0248	Chloroplast protease, ClpP (*Arabidopsis thaliana*)	1E-22			
0475	PSII D1 protein processing enzyme (*Arabidopsis thaliana*)	2E-150			
0397	Cyclophilin (*Euphorbia esula*)	8E-18			
0250	Putative serine protease (*Populus canadensis*)	1E-113			
0479	SBT1 protein (Subtilisin-like protease) (*Lycopersicon esculentum*)	7E-70			
0320	Seed maturation protein PM31 (*Glycine max*)	8E-20			
carbohydrate metabolic process					
0474	Glucosidase II alpha subunit (*Arabidopsis thaliana*)	1E-40			
0047	Phosphoenolpyruvate carboxykinase (*Lycopersicon esculentum*)	1E-68			
0155	Phosphoenolpyruvate carboxykinase (*Flaveria trinervia*)	8E-82			
0129	Phosphoglucan, water dikinase (*Arabidopsis thaliana*)	7E-53			
0263	Potential 6-phosphogluconolactonase (*Oryza sativa*)	2E-17			
0198	Sucrose-phosphate synthase 1 (*Vitis vinifera*)	5E-41			
0260	Sucrose-phosphate synthase 1 (*Vitis vinifera*)	1E-37			
0409	Sucrose-phosphate synthase 1 (*Citrus unshiu*)	1E-24			
0204	UDP-glucose pyrophosphorylase (*Amorpha fruticosa*)	3E-32			
cell wall organization					
0382	Alpha galactosyltransferase (*Trigonella foenum-graecum*)	3E-13			
0185	Putative ripening-related protein, GRIP 28 (*Vitis vinifera*)	2E-75			
0349	Putative ripening-related protein, GRIP28 (*Vitis vinifera*)	7E-15			
0251	Putative proline-rich cell wall protein, GRIP 3 (*Vitis vinifera*)	8E-20			
0282	Putative ripening-related protein, GRIP 31 (*Vitis vinifera*)	6E-184			
0163	Mannan endo-1,4-beta-mannosidase (*Arabidopsis thaliana*)	6E-77			
0107	Pectate lyase (*Vitis vinifera*)	2E-125			
0267	Pectate lyase (*Fragaria ananassa*)	0E+00			
0489	Merlot proline-rich protein 2, MRP2 (*Vitis vinifera*)	2E-14			
0387	Pectin methylesterase PME1 (*Vitis vinifera*)	4E-46			
0421	Pectin methylesterase PME1 (*Vitis vinifera*)	4E-15			
0057	Polygalacturonase PG1 (*Vitis vinifera*)	7E-29			
0049	Polygalacturonase PG1 (*Vitis vinifera*)	0E+00			
0376	Polygalacturonase PG1 (*Vitis vinifera*)	3E-137			
0095	Putative beta-galactosidase BG1 (*Vitis vinifera*)	7E-64			
0189	Xyloglucan endo-transglycosylase (*Vitis vinifera*)	2E-90			
0384	Xyloglucan endo-transglycosylase (*Vitis labrusca x Vitis vinifera*)	6E-49			
signal transduction					
0123	CDK5 activator-binding protein-like (*Oryza sativa*)	1E-12			
0070	Leucine rich repeat protein (*Vitis vinifera*)	8E-110			
0060	Leucine rich repeat protein precursor (*Cicer arietinum*)	4E-43			
0285	Putative G-protein-coupled receptor (*Solanum chacoense*)	1E-13			
0053	Receptor-protein kinase (*Arabidopsis thaliana*)	5E-72			
0276	Response regulator receiver; CCT (*Medicago truncatula*)	5E-22			
0347	SOS2-like protein kinase (*Glycine max*)	2E-80			
0357	SOS2-like protein kinase (*Glycine max*)	2E-141			
0417	SOS2-like protein kinase (*Glycine max*)	2E-15			
secondary metabolic process					
0019	Cycloartenol synthase (*Betula platyphylla*)	3E-39			
0271	GA 20-oxidase (*Pisum sativum*)	2E-67			
0092	LYTB-like protein 1 (*Brassica rapa*)	1E-95			
0242	Sinapyl alcohol dehydrogenase, sad1 (*Plantago major*)	9E-30			
0455	Putative acyl-CoA synthetase (*Capsicum annuum*)	1E-24			
0367	Squalene synthase (*Glycine max*)	1E-50			
0145	UDP-glucose glucosyltransferase-like protein (*Arabidopsis thaliana*)	3E-46			
biological process					
0097	Hypothetical protein (*Ricinus communis*)	9E-60			
0098	MtN3-like protein (*Arabidopsis thaliana*)	2E-23			
0110	Hypothetical protein F22D16.19 (*Arabidopsis thaliana*)	2E-18			
0132	Leucine-rich repeat-like protein (*Oryza sativa*)	2E-36			
0169	Hypothetical protein (*Ricinus communis*)	2E-54			
0191	Hypothetical protein At4g36850 (*Arabidopsis thaliana*)	7E-72			
0224	Hypothetical protein P0431A02.7 (*Oryza sativa*)	5E-71			
0255	DREPP2 protein (*Nicotiana tabacum*)	3E-36			
0323	Hypothetical protein T29A15.210 (*Arabidopsis thaliana*)	4E-28			
0358	Kelch repeat-containing F-box-like (*Arabidopsis thaliana*)	8E-51			
0401	Hypothetical protein (*Ricinus communis*)	4E-23			
0437	Unidentified precursor (*Medicago sativa*)	6E-13			
0442	Hypothetical protein (*Oryza sativa*)	3E-82			
0465	At5g57780 (*Arabidopsis thaliana*)	7E-26			
0487	Hypothetical protein F5M6.14 (*Arabidopsis thaliana*)	4E-43			
0436	Aldehyde dehydrogenase (NAD+) (**primary metabolism**) (*Nicotiana tabacum*)	8E-87			
0133	7S globulin (**nutrient reservoir activity**) (*Sesamum indicum*)	3E-26			
0048	Centromere/kinetochore protein zw10 homolog (**cell cycle; cell division**) (*Arabidopsis thaliana*)	4E-37			
0335	Chloroplast ferritin (**iron ion homeostasis**) (*Vigna angularis*)	7E-29			
0400	Chloroplast ferritin (**iron ion homeostasis**) (*Vigna angularis*)	6E-25			
0087	Digalactosyldiacylglycerol synthase 1(**glycerolipid metabolism**) (*Glycine max*)	3E-122			
0457	Digalactosyldiacylglycerol synthase 1(**glycerolipid metabolism**) (*Glycine max*)	9E-70			
0380	Early nodulin 93 (**nodulation**) (*Glycine max*)	1E-27			
0062	Hypothetical protein At2g30610 (**cell adhesion**) (*Arabidopsis thaliana*)	4E-35			
0403	Kinesin-related protein KRP120-2 (**microtubule-based movement**) (*Daucus carota*)	6E-67			
0486	NADH dehydrogenase subunit F (**ATP synthesis coupled electron transport**) (*Nyssa ogeche*)	2E-35			
0331	Oxidoreductase (**electron transport**)(*Arabidopsis thaliana*)	6E-88			
0449	Potential sarcosine oxidase (**amino acid metabolism**) (*Arabidopsis thaliana*)	4E-25			
0412	Putative AMP-binding protein (**metabolism**) (*Oryza sativa*)	4E-58			
0366	Putative retroelement pol polyprotein-like (**DNA recombination**) (*Solanum tuberosum*)	2E-07			
0182	Putative short-chain type alcohol dehydrogenase (**primary metabolism**) (*Solanum tuberosum*)	5E-47			
0034	Reductase 1(**cellular metabolism**) (*Hydrangea macrophylla*)	1E-28			
0440	Sts15 protein (**pollination**) (*Solanum tuberosum*)	2E-07			
0377	Sts15 protein (**pollination**) (*Solanum tuberosum*)	5E-53			
0483	Submergence induced protein 2-like (**amino acid metabolism**) (*Vitis vinifera*)	8E-36			
0167	T23E18.10 (**pollen germination**) (*Arabidopsis thaliana*)	2E-20			
0336	Vacuolar ATP synthase (**ATP synthesis coupled electron transport**) (*Nicotiana tabacum*)	1E-04			

[a] For each gene, the putative function, *e*-value, and transcriptional profiles at véraison (V), ripening (R), and postripening (P) phases are reported. A white box means not differentially expressed between high and low resveratrol producers, and black and gray boxes indicate up-regulation and down-regulation in the high resveratrol producers relative to the low ones, respectively.

leucine-rich repeat proteins (CLU0060, CLU0070), pathogenesis-related proteins such as PR-4 (CLU0194) and PR-10 (CLU00018), and phase-change related proteins (CLU0488, CLU0074, CLU0101, CLU0065, CLU0076, CLU0059, and CLU0188). Unlike, the genes down-regulated in the high resveratrol producers were mainly related to abiotic stresses such as GRIP 21 (CLU0038), GRIP 22 (CLU0277), GRIP 61 (CLU0086), DC1.2 homologue (CLU0088), POP3 protein (CLU 0458), ERD3 protein (CLU0178), RD22-like protein (CLU0493), and a BURP domain-containing protein (CLU0396).

In the category "transcription", a gene homologous to CrMYC1 (CLU0252) was found to be up-regulated in the high resveratrol producers with respect to the low ones. CrMYC1 is an elicitor- and jasmonate-responsive bHLH transcription factor that has been isolated in *Catharanthus roseus*, and it binds the G-box element of the strictosidine synthase gene promoter (*33*).

Other DNA binding protein encoding genes were instead found to be down-regulated in the high resveratrol producers such as bZIP transcription factor (CLU0137), ERF1 (CLU0262), MADS-box proteins (CLU0054, CLU0104, and CLU0383), and a MYB-like DNA-binding protein (CLU0105). The bZIP transcription factor, CrGBF-3, binds to the G-box element of the strictosidine synthase promoter of *C. roseus* (*34*). The MYB-like DNA-binding protein was identified in *C. roseus* (CrBPF-1) and specifically binds to the BA element of the strictosidine synthase promoter and is induced by elicitor via a JA-independent signal transduction pathway (*35*).

Concerning the "transport" category, four genes (CLU0045, CLU0079, CLU0364, and CLU0332) coding for ATP-binding cassette (ABC) transporters belonging to the multidrug resistance-associated proteins (MRP) subfamily were found to be down-regulated in the high resveratrol producers as compared to the low ones. In particular, a gene (CLU 0045) homologous to MRP6 is also similar to the maize ABC transporter, ZmMRP3 (*e*-value: 9E-84), which is localized in the tonoplast and is required for anthocyanin transport (*36*). A gene (CLU0079) is homologous to the *Arabidopsis* MRP3, which is involved in the transport of glutathione conjugates and chlorophylls (*37*). All of the genes falling in the category "carbohydrate metabolic process" turned out to be down-regulated in the high resveratrol producers as compared to the low ones. Among the others, the genes CLU0198, CLU0260, and CLU0409 code for the enzyme sucrose-phosphate synthase 1, which reversibly catalyzes the synthesis of sucrose 6-phosphate from UDP-glucose and fructose 6-phosphate in the cytosol. Interestingly, a gene (CLU 0204) is homologous to the UDP-glucose pyrophosphorylase (UGPase) enzyme, which reversibly converts glucose 1-phosphate to UDP-glucose, which supplies the substrate for the sucrose phosphate synthase (*38*).

Transcripts encoding cell wall-modifying enzymes related to grape berry softening were found to be mainly down-regulated in the high resveratrol producers throughout berry development. They are GRIP 3 (CLU0251), GRIP 31 (CLU0282), putative pectate lyase (CLU 0107, CLU267), pectin methylesterase 1 (CLU0387, CLU0421), polygalacturonase 1 (CLU0049, CLU0057, and CLU0376), and β-galactosidase (CLU0095).

Among the genes falling into the category "signal transduction", three genes (CLU0347, CLU0357, and CLU0417) coding for SOS2 (salt overlay sensitive 2) protein were found to be down-regulated in the high resveratrol group. SOS2 is a serine/threonine protein kinase identified in *Arabidopsis* and is required for potassium and sodium ion homeostasis and plant salt tolerance (*39*).

In the category "secondary metabolic process", four genes involved in the gibberellin biosynthesis and downstream pathways biosynthesis turned out to be down-regulated in the high resveratrol as compared to the low ones: gibberellin (GA) 20-oxidase (CLU0271), LtyB-like protein 1 (CLU 0092), squalene synthase (CLU0367), and cycloartenol synthase (CLU0019). In a study with the flowering plant *Adonis aestivalis*, LytB gene has been proposed to encode an enzyme that catalyzes the terminal branching step of the methylerythritol (MEP) pathway to form isopentenyl diphosphate (IPP) and dimethylallyl diphospate (DMAPP) (*40*).

The absence of the structural genes of the phenylpropanoid pathway is probably due to subtraction of their transcripts in the subtractive hybridization step during construction of the berry-specific cDNA library. However, their expression profile was analyzed by real-time RT-PCR as reported in the above section.

Real-Time RT-PCR. Some cDNA array data were validated also by real-time RT-PCR experiments, using sequence-specific primers (see the Supporting Information, Table 1). Because for the macroarray experiments no biological replicates were used, we decided to make new berry pools to confirm the differential expression ratios of 15 genes in the high resveratrol producers of the same sampling times considered in the array experiments. Expression data from macroarray and real-time RT-PCR were compared (see the Supporting Information, Table 3). For most of the genes, a similar expression pattern was obtained. The discrepancy between array and real-time RT-PCR data observed for some genes might be due to cross-hybridization problems that may have occurred in the macroarray experiments.

DISCUSSION

The objective of this work was to survey stilbene biosynthesis in grape berry during ripening, looking at both metabolites and transcripts. Previous studies indicated that resveratrol production in grape berries exclusively occurs upon pathogen attack, UV elicitation, or mechanical damage. Furthermore, it has been reported that berry potential to synthesize stilbene compounds decreases from véraison to complete maturity, providing a hint for the increased susceptibility to *Botritys cinerea* observed in the late stages of maturation (*6, 7, 11*). Increasing total resveratrol contents in grape berry during ripening have been explained by a latent pathogen attack or UV irradiation (*17, 41*). In a preliminary study, however, Mattivi et al. (*16*) reported an increase in stilbene content in grape berries from véraison to ripeness, in the absence of visible *Plasmopara viticola* infection. In light of these controversial results, the stilbene content of a group of *V. vinifera* varieties was monitored at three key developmental stages: véraison, ripening, and postripening phase. The biochemical analysis revealed a large variability in stilbene accumulation among these varieties but a common increasing trend from véraison to ripening phase. Furthermore, unlike in the low resveratrol producers, in the high ones, a preferential accumulation of the glucosylated forms of resveratrol, *trans* and *cis*-piceid, was observed. These results point out that resveratrol glucosides are preferentially expressed constitutively, while *trans*-resveratrol is the inducible form. Romero-Pérez et al. (*10*) reported, indeed, that grape berries with no apparent fungal infection contained similar amounts of *trans*-resveratrol and *trans*- and *cis*-piceid, but highly infected grape berries showed a much higher *trans*-resveratrol proportion. Three possible roles for resveratrol glucosides have been suggested as follows: storage of resveratrol, transport from cytoplasm to apoplasm, and protection of *trans*-resveratrol from

peroxidative degradation (*42*). The discrepant results reported in the past studies focusing on resveratrol biosynthesis during berry ripening are possibly due to the fact that some of them (*6, 7, 11*) were monitoring just the inducible form of resveratrol, *trans*-resveratrol, upon UV irradiation or fungal infection, while others (*16, 17*) were looking also at the constitutive forms of resveratrol and *trans*- and *cis*-piceid. This consideration is in agreement with the results reported by Larronde et al. (*43*) who observed that the berry capacity to synthesize *trans*-resveratrol naturally or in response to UV exposure declines markedly during fruit ripening, while piceid synthesis increases in the same period and is not influenced by UV light. In addition, the absence of resveratrol production in nonelicited berries (*6, 11*) seems also to depend on the studied grape genotype, as shown by our results. Generally, we observed higher accumulation of resveratrols in the red varieties than in the white/pink ones. A large difference within each group was also highlighted, allowing to define a subset of varieties as "high resveratrol" producers and another set as "low resveratrol" producers. The influence of the genotype has been further confirmed by the gene expression analysis carried out in this study.

The higher accumulation of the StSy mRNA in the high resveratrol producers with respect to the low ones well correlates with the different resveratrol contents highlighted in the two groups by biochemical analysis. Interestingly, the difference in StSy mRNA concentration between high and low resveratrol producers decreased throughout berry development, although the total resveratrol level remained 10-fold higher in the high resveratrol producers at each developmental phase. These results suggest that the steady state concentration in the berry is mainly governed by the StSy gene transcription, but other factors also play a role.

StSy gene expression seems to be highly coordinated with the expression of two genes acting upstream in the phenylpropanoid pathway: PAL and 4CL. These two genes appeared indeed to be up-regulated in the high resveratrol group, although to a minor extent than measured for the StSy gene. Thus, StSy, PAL, and 4CL genes seem to be essential points of regulation of this biosynthetic pathway, as suggested by Bais et al. (*11*), which observed a coordinated induction of StSy and PAL genes in UV-elicited berries of four *V. vinifera* varieties. The similar expression patterns of the CHS gene in high and low resveratrol producers were not unexpected, since the berry pooling was based on the stilbene content and not on the flavonoid content.

To get a broader view on the genes involved in resveratrol expression and their expression profiles during fruit ripening, comprehensive cDNA array analysis experiments were performed. Macroarray data analysis demonstrated that the plant defense and the ripening process are mostly different at the transcript level when comparing high and low resveratrol producing genotypes.

In high resveratrol-accumulating plants, genes involved mainly in defense responses to pathogen attacks, such as class IV chitinase, PR-proteins, and thaumatin-like proteins, appeared to be up-regulated, while genes playing a role in plant response to abiotic stress, such as GRIPs, ERD protein, RD22-like proteins, and SOS2-like protein kinase, were down-regulated. These observations might indicate that the accumulation of defense proteins and stilbenes in healthy berries is a constitutive protectional mechanism during berry development. Early studies postulated that defense proteins like, class IV chitinase and thaumatin-like proteins, may have a role in normal growth and development of grape berry, as being highly expressed at the onset of ripening and thereafter (*44*−*46*).

The high resveratrol accumulation appeared also to be correlated with a down-regulation of genes playing a role in the berry ripening process, such as the transcription factors ERF1 and members of the MADS-box class of transcription factors. MADS-box transcription factors have been studied in plants for their role in floral development, but they have also been suggested to play a role in fruit development after fertilization in grapevine (*47*). Ethylene has been suggested to play a role in grape berry development and ripening, as well influencing berry expansion, the acidity drop, and anthocyanin accumulation (*48*).

The onset of ripening is characterized by the induction of genes involved in sugar transport and accumulation and of genes responsible for the profound changes in the properties of the mesocarp and pericarp cell walls that accompany berry enlargement (*46, 49*). Our macroarray analysis data revealed that many genes encoding proteins and enzymes related to these two metabolisms were expressed at lower levels in the high resveratrol producers with respect to the low ones along fruit maturation.

A down-regulation of some MRP type ABC transporters in the high resveratrol producers was observed as well. The multidrug resistance-associated proteins (MRPs) constitute a large family of proteins that use the energy generated by the hydrolysis of ATP to facilitate the transmembrane movement of a variety of small molecules, including compounds conjugated to glutathione, sugar, or amino acids. Recently, a MRP, ZmMRP3, has been demonstrated to be involved in anthocyanin transport in *Zea mays* (*36*). Similarly, a secondary transporter-like protein belonging to the MATE family has been proposed to be responsible for the vacuolar transport of anthocyanin in *Arabidopsis* (*50*). We could thus speculate that the MRP type ABC transporters down-regulated in the high resveratrol producers are not likely involved in the transport of stilbenes.

The induction of stilbene biosynthesis seemed to be also correlated with a down-regulation of the isoprenoid pathway and the downstream pathways like carotenoids, gibberellin, terpenoids, and brassinosteroids. Genes encoding LytB (*40*), GA 20-oxidase, squalene synthase, and cycloartenol synthase were down-regulated in the high resveratrol producers as compared to the low ones. Gibberellins and brassinosteroids are hormones with a potential role in fruit development (*51, 52*).

Three of the transcription factors differentially expressed between high and low resveratrol producers, homologous to MYC1 (CLU0252), a bZIP transcription factor (CLU0137), and a MYB-like DNA-binding protein (CLU0105), appeared to be involved in the regulation of strictosidine synthase (Str). This enzyme catalyzes the condensation of the amino acid derivative tryptamine and the terpenoid secologanin, resulting in the formation of strictosidine, which is the universal precursor of the terpenoid indole alkaloids (TIAs) (*53*). TIAs are produced by several plant species such as *C. roseus.* In grape, the presence of indole alkaloids and indoleamines was reported (*54*). Indole alkaloids and stilbene biosynthesis might be in competition, since the amino acidic precursors of these secondary metabolites are tryptophan and phenylalanine, respectively, which both originate from the shikimate pathway end product chorismate. Three transcription factors have been shown to bind specific elements of strictosidine synthase promoter in *C. roseus*: CrMYC, CrGBF-3, and CrBPF-1. CrBPF-1 appeared to positively regulate Str gene (*35*), while the role of CrMYC and CrGBF-3 on the Str gene expression has not been yet

clarified (*33, 34*). The down-regulation in the high resveratrol producers of the two genes CLU0137 and CLU0105, which are homologous to CrGBF-3 and CrBPF-1, respectively, might thus suggest that the induction of stilbene biosynthesis is accompanied by the down-regulation of the indole alkaloid biosynthesis. This hypothesis is also to be supported by expression data showing the concurrent down-regulation of several genes of the isoprenoid and terpenoid pathway, which leads to the synthesis of monoterpenes possibly channelled into terpenoid indole alkaloid pathway.

In conclusion, we present biochemical and transcriptional evidence that grape berry accumulates resveratrol also in the absence of biotic and abiotic stimuli and progressively during the ripening process. We also demonstrate the role of the genotype in the resveratrol accumulation in the berry. This piece of information might be relevant when designing grape breeding programs aimed to improve the healthy properties of both table grape and wine. Furthermore, the accumulation of resveratrol in the berry appeared to be in competition with the indole alkaloid synthesis and accompanied by the under-expression of many genes related to the ripening process. In perspective, it remains to be further investigated, as our gene expression data suggest, if the constitutive accumulation of resveratrol corresponds to a lower susceptibilty toward fungi and other microorganisms.

ACKNOWLEDGMENT

We thank Domenico Masuero for assistance with biochemical analysis, Isabella Endrizzi for assistance with statistical analysis of biochemical data, and Alessandro Cestaro for bioinformatic support.

Supporting Information Available: Details concerning primer sequences (Table 1), quality estimation of cDNA array data (Figure 1), fold change of the differentially expressed ESTs and EST clustering (Table 2), classification in GO categories of differentially expressed genes (Figure 2), real-time RT-PCR data (Table 3), and stilbene content of three *V. vinifera* grape varieties regarding to two vintages (Table 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review June 9, 2008. Revised manuscript received October 24, 2008. Accepted October 27, 2008. This work was supported by the Project "Resveratrol" funded by the Fondo Unico of the Provincia Autonoma di Trento.

JF8017707