

# Gene expression variation in grapevine species *Vitis vinifera* L. and *Vitis aestivalis* Michx.

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**Abstract** Comparative microarray (*Vitis* GeneChip) analysis in young leaves revealed considerable variation in gene expression between *Vitis vinifera* L. and *Vitis aestivalis* Michx. Approximately 12% of the genes were differentially expressed in the two grapevine species ( $P < 0.001$ ). Over 200 probe sets were identified which consistently detected transcripts in one grapevine species, but not in the other. We were unable to identify any broad functional category in which transcript abundance was overall different in any one species. Of the genes expressed only in *V. aestivalis* leaves, we identified a class IV chitinase which was previously shown by others to have a flower- and fruit-specific expression in *V. vinifera*. Among the transcripts which were differentially expressed ( $P < 0.001$ ) in both species, we identified genes encoding key enzymes in flavonoid, mono-

lignol, and proanthocyanidin biosynthesis. Statistical exploration of the data suggested that sequence divergence between the predominantly *V. vinifera*-derived GeneChip probes and the *V. aestivalis* cRNA did not confound the hybridization data and that the reliability of the microarray results was similar in the two grapevine species.

**Keywords** Chitinase · Grapevine · Gene expression · Microarray · Phenylpropanoid metabolism · Species-specificity · Transcriptome · *Vitis vinifera*

## Introduction

Wild grapevines represent important genetic resources. During the past century, a broad range of wild bunch grape species (subgenus *Euvitis*) from North America and East Asia have been used to introgress genes for pest and pathogen resistance and environmental stress tolerance into cultivated scion and rootstock varieties (Alleweldt et al. 1990). Current trends toward reducing chemical input and relying more on biological disease resistance in grape cultivation makes the genetic diversity of the wild grapevines even more relevant (Bisson et al. 2002). Understanding the molecular basis of this diversity will accelerate progress towards harnessing the biological resources in the *Vitis* genus.

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*Vitis* is a highly heterozygous genus (Reisch and Pratt 1996). At the molecular level, heterozygosity manifests itself in DNA sequence divergence among the different species and between haplotypes of *V. vinifera*, as evidenced by results from molecular marker-based genotyping studies and from sequencing of allelic variants of genes and bacterial artificial chromosome inserts (Aradhya et al. 2003; Salmaso et al. 2004; Adam-Blondon et al. 2005). Grapevine heterozygosity is also expected to be evident in the assortment of genes expressed and the level at which they are transcribed. Microarray studies conducted in fungi, plants, and animals have identified substantial variation in mRNA abundance among different lineages of the same species (Townsend et al. 2003; Oleksiak et al. 2002; Vuylsteke 2005; Nuzhdin et al. 2004). To our knowledge, genome-scale transcript level variation has not been examined in different *Vitis* species or different *V. vinifera* genotypes. An assessment of gene expression variation among grapevine species is important, because it provides information about the role transcriptional regulation plays in phenotypic variation and adaptation to abiotic and biotic stress.

If transcriptional regulation plays a role in agronomically important phenotypes, then future breeding efforts can focus on improving grape varieties by the use of regulatory genes as functional molecular markers. Emerging technologies, such as expression quantitative trait loci (eQTL) analysis and physical mapping, will enable grape breeders to identify genes for environmental sensing proteins, signal transducing proteins, and transcription factors (Varshney et al. 2005). The use of such genes as functional markers is expected to enhance the effectiveness of crop breeding. Recent findings that resistance gene analog-based genetic markers co-localize with a single dominant locus for powdery mildew resistance and with a QTL for downy mildew resistance in grapevine (Barker et al. 2005; Zyprian et al. 2005) lend support to the premise that genes that are positioned high in the hierarchy of signal transduction or transcriptional regulation are likely to be powerful factors in crop improvement. Validated regulatory genes can also be introduced into grapevine by a transgenic approach.

In the present work, we conducted comparative mRNA abundance measurements in two grapevine species using the Affymetrix *Vitis* GeneChip array. The *Vitis* GeneChip is based on oligomicroarray technology and is composed of 16,436 grapevine-derived probe sets, which correspond to 13,028 unigene entries. The unigene entries were derived from known genes, predicted genes, and numerous EST clusters. The two grapevine genotypes which we used in this study were *V. aestivalis* ‘Norton’ and *V. vinifera* ‘Cabernet Sauvignon’. The former is a cultivated variety of North American origin. It is a typical representative of North American grapevines in that it is highly resistant to economically important diseases such as black rot, powdery mildew, and downy mildew (Reisch et al. 1993). *V. vinifera* ‘Cabernet Sauvignon’, on the other hand, is a Eurasian variety which is highly susceptible to many fungal diseases. The two species differ in a number of other traits, including morphological and physiological features and fruit characteristics.

## Materials and methods

### Plants and culture conditions

*Vitis vinifera* “Cabernet sauvignon” plants were obtained as certified virus-free propagation material in the form of dormant canes from Sunridge Nurseries (Bakersfield, California). *V. aestivalis* ‘Norton’ plants were collected as dormant canes from symptomless vines at the experimental field of the Missouri State Fruit Experiment Station (Mountain Grove, Missouri). Currently, no certified virus-free propagation material is available for this cultivar. The canes were rooted in Promix BX horticultural medium (Premier Horticulture, Inc., Quakertown, PA). Shoot growth was induced by transferring rooted plants into 3.78 l pots and by placing them in a greenhouse. Young plants were transferred to growth chambers at least 2 weeks prior to sample collection. All plants were arranged in the growth chamber in a randomized manner. Environmental conditions were adjusted to 25°C temperature, 85% relative humidity, and light intensity of 500  $\mu\text{mol m}^{-2}$  with a 14/10 h day/night cycle. The fourth unfolded

leaf from the shoot apex was harvested from each of ten vines, and the ten leaves were combined to represent one replicate. Six independent replicates were collected for each species. Samples were collected approximately 2 h after the daily light cycle began.

#### RNA extraction

Total RNA was extracted from each replicate sample individually. The leaves were ground in liquid nitrogen and homogenized in extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1% sodium dodecyl sulfate, 2.5 M NaCl, 0.5 M Tris, 50 mM EDTA, 5%  $\beta$ -mercaptoethanol, and 3% polyvinyl poly-pyrrolidone). Following a 30-min incubation at  $-80^{\circ}\text{C}$ , the samples were thawed and centrifuged twice at 7,000g for 30 min at  $4^{\circ}\text{C}$ . The supernatant was supplemented with 1/30 volume of 3 M sodium acetate (pH 5.2) and 1/10 volume of 100% ethanol, incubated on ice for 10 min and centrifuged at 5,000g for 20 min at  $4^{\circ}\text{C}$ . The supernatant was supplemented with 1/9 volume of 3 M sodium acetate (pH 5.2) and iso-propanol to a final concentration of 33%. Following a 60-min incubation at  $-20^{\circ}\text{C}$ , the RNA was pelleted by centrifugation at 5,000g for 30 min at  $4^{\circ}\text{C}$  and resuspended in 1 ml of diethyl pyrocarbonate-treated (DEPC) water. The RNA was then precipitated with 1/3 volume of 8 M LiCl, incubated overnight at  $4^{\circ}\text{C}$ , and centrifuged at 12,000g for 30 min at  $4^{\circ}\text{C}$ . The RNA pellet was then washed with 75% ethanol and resuspended in 40  $\mu\text{l}$  of DEPC water.

#### Array hybridizations, data processing, and data quality assessment

Total RNA samples (4  $\mu\text{g}$ ) were used as template for cDNA synthesis and linear RNA amplification by in vitro transcription. The resulting cRNA targets were hybridized to Affymetrix GeneChip *V. vinifera* Genome Arrays. Complementary DNA synthesis and anti-sense cRNA amplification/biotin labeling were performed using the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, California). The hybridization and washing steps were completed by a Fluidic

Station 450 script, and the resulting fluorescent image was captured with high resolution scanning using GSC3000 laser scanner protocols (Affymetrix 2005).

Raw intensity data were processed using the GeneChip Operating Software Version 1.2 (GCOS 1.2, Affymetrix 2001). Background correction and expression value calculation were performed as described by Affymetrix (2002). Normalization was done by global scaling, with a target intensity value of 150 (Affymetrix 2001). Probe sets with missing values across all replicates and species were deleted to allow comparisons among data sets. Detection calls (present, absent, or marginal transcripts) were determined using the GCOS 1.2 software. Stringency parameters of call generation were set at default values, (Affymetrix 2004). To assess the reliability of the data, the concordance of hybridization and labeling control signals were determined within each pair of the three biological replicates. Hybridization quality control parameters for all 12 datasets were as follows: cRNA yield ranged from 20.10  $\mu\text{g}$  to 65.05  $\mu\text{g}$ , noise (RawQ) ranged from 1.69 to 2.54, scaling factor values ranged from 0.282 to 1.022, and  $\beta$ -actin 3'/5' ratio ranged from 2.23 to 3.83.

#### Statistical analysis

The scaled hybridization data were  $\log_2$ -transformed and subjected to exploratory analysis. The examination of box plots and numerical descriptors, such as mean, median, quartiles, range, and standard deviation, did not reveal irregularities in the data. These observations indicated that no further normalization was necessary. The species-to-species comparison of the hybridization data was performed using the three-factor cross-nested mixed effects linear ANOVA model  $Y = \mu + \alpha + \beta + \gamma(\alpha) + \alpha\beta + (\beta\gamma)(\alpha) + \varepsilon$ , where  $Y$  is the  $\log_2$ -transformed signal value of genes,  $\mu$  is the overall grand mean,  $\alpha$  is the fixed effect of the species,  $\beta$  is the fixed effect of the sampling time,  $\gamma(\alpha)$  is the random effect of biological replicate which is nested within the species,  $(\alpha\beta)$  is the fixed interaction effect of species and sampling time,  $(\beta\gamma)(\alpha)$  is the random interaction effect of sampling time and biological replicate which is nested within the species, and  $\varepsilon$  is the random

observational error. Interaction effects such as  $(\alpha\gamma)$  and  $(\alpha\beta\gamma)$  were not considered since the effect  $\gamma$  of biological replicate was nested within the effect  $\alpha$  of species.

#### Annotation and sequence alignment

Annotation of gene sequences was performed by searching the NCBI protein database (<http://www.ncbi.nih.gov/>) for homology to translated query sequences using the Basic Local Alignment Search Tool X (BLASTx) algorithm (Altschul et al. 1990). Homology between query and database sequences was considered informative only if the  $E$  value was below  $1e^{-10}$ . Nucleotide and amino acid sequence alignments were performed using the ClustalW algorithm (Higgins et al. 1996) provided by the European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/>).

## Results

#### Reliability of the hybridization data

The Affymetrix *Vitis* GeneChip was designed with predominantly *V. vinifera* DNA sequences, with 14,509 of the 16,436 probe sets derived from *V. vinifera* cDNAs. Consequently, it is likely that a certain level of sequence divergence exists between many of the probes and the *V. aestivalis* cRNA. This sequence divergence could potentially increase the random noise in the hybridization data. If sequence divergence substantially influenced hybridization, then random noise would be greatly elevated and a lower correlation would be expected between the *V. aestivalis* replicate data than between the *V. vinifera* replicate data. We found no evidence for differences in the correlation between replicates for each species. Average Spearman's rank correlation coefficients ( $r$ ) between *V. vinifera* replicates were 0.9781 (range: 0.9572–0.9909), which was comparable to the average  $r$  of 0.9688 (range: 0.9279–0.9871) between the *V. aestivalis* replicates. Substantially large random noise may also affect the intensity distribution of the  $\log_2$ -transformed data. The intensity distributions of the data were normal-like and similar in the datasets

derived from the two species (data not shown). The similar  $r$  values and similar intensity distributions indicated that the intra-class correlation of the results in the two species was similar.

If sequence divergence prevented efficient hybridization, the overall hybridization intensity may be lower in *V. aestivalis* than in *V. vinifera*. This, however, was not the case. The average  $\log_2$ -transformed abundance values for consistently present transcripts were  $7.35 \pm 1.56$  and  $7.21 \pm 1.75$  (across-replicate average  $\pm$  SD) in *V. aestivalis* and *V. vinifera*, respectively. Further, if sequence divergence influenced hybridization, more genes would be expected to be assigned absent calls in *V. aestivalis* than in *V. vinifera*. However, the number of probe sets which were assigned an absent call was 2,983 in *V. aestivalis*, and 3,555 in *V. vinifera* (Table 1). The similar overall hybridization intensity levels and the fewer absent calls lent support to the reliability of the *V. aestivalis* microarray results. These observations strengthen the conclusion that the magnitude of sequence divergence-based hybridization error was similar in the two grapevine species.

#### Species-specific transcripts

The number of transcripts consistently present or absent in all six replicates are shown in Table 1. In addition to those shown in Table 1, 2,442 transcripts in *V. aestivalis* and 2,993 transcripts in *V. vinifera* were detected present, absent, or marginal in some but not all six replicates. By excluding transcripts which received inconsistent calls, we focused on only those genes which provided the most informative expression data. Of the 16,436 probe sets on the *Vitis* Genechip, 9,621 detected transcripts in both species (Table 1). Of the remaining probe sets, 183 consistently detected transcripts in *V. aestivalis* leaves, but not in *V. vinifera* leaves. On the other hand, 39 probe sets consistently detected transcripts in *V. vinifera* leaves, but not in *V. aestivalis* leaves. The 20 *V. aestivalis*-specific genes and the 20 *V. vinifera*-specific genes which were most highly expressed are shown in Tables 2 and 3, respectively. Interestingly, both lists of species-specific transcripts include putative stress-associated

**Table 1** Number of transcripts detected as present or absent in leaves of *V. vinifera*, *V. aestivalis*, or both

Detection call	Transcripts (probe sets) <sup>a</sup>		
	<i>V. vinifera</i>	<i>V. aestivalis</i>	Shared by both
Present	9,888	11,011	9,621
Absent	3,555	2,983	2,439

<sup>a</sup>Only those transcripts are indicted which were consistently present or absent across the six replicates

genes. For example, *V. vinifera* leaves expressed a Class 1 pathogenesis-related protein precursor (Table 3), which was absent from the *V. aestivalis* transcriptome. In *V. aestivalis*, transcripts for a class IV chitinase and a dirigent-like protein were present (Table 2). In addition to the genes listed in Table 2, *V. aestivalis* leaves also expressed a member of the  $\beta$ -1,3-glucanase gene family (Affymetrix probe set ID: 1615595\_at; relative expression level:  $111 \pm 54$ ) which was absent from the *V. vinifera* leaf transcriptome. The expression of these defense-related genes in the

absence of biotic stress (see “Materials and methods”) was unexpected, and prompted us to explore transcript abundance for some of these gene families more extensively.

Chitinases and  $\beta$ -1,3-glucanases were previously hypothesized to play a role in the disease resistance of American grapevines and their hybrids (Giannakis et al. 1998). Giannakis et al. (1998) reported a positive correlation between levels of chitinase and  $\beta$ -1,3-glucanase activity and resistance to fungal pathogens in grapevine. We searched the microarray data, and identified fifteen  $\beta$ -1,3-glucanase and 12 chitinase transcripts which were consistently detected in at least one of the grapevine species. Of the  $\beta$ -1,3-glucanase probe sets, only one (1615595\_at) exhibited *V. aestivalis*-specific transcripts, and the others detected similar transcript levels in the two species (data not shown). In contrast, the chitinase probe sets detected strikingly dissimilar transcript levels in the two species. Figure 1 shows the expression level of those eight chitinase genes whose relative hybridization signal intensity

**Table 2** Transcripts detected as present in *V. aestivalis* leaves, but absent in *V. vinifera* leaves

Affymetrix probe set ID <sup>a</sup>	Predicted function [organism in which nearest ortholog was found] <sup>b</sup>	Expression level in <i>V. aestivalis</i> <sup>c</sup>
1616201_at	No homology in databases	1,145 $\pm$ 195
1606635_at	Thioredoxin-dependent peroxidase [ <i>Plantago major</i> ]	1,043 $\pm$ 194
1618113_at	No homology in databases	950 $\pm$ 274
1607557_at	Class IV chitinase [ <i>Zea mays</i> subsp. <i>parviglumis</i> ]	699 $\pm$ 165
1618907_x_at	No homology in databases	482 $\pm$ 179
1619266_at	Weakly similar to cystatin-like protein [ <i>Actinidia deliciosa</i> ]	439 $\pm$ 155
1622430_x_at	Similar to proteins containing universal stress protein A (USPA) domain [ <i>Oryza sativa</i> ]	425 $\pm$ 49
1606770_s_at	Dirigent-like protein [ <i>Podophyllum peltatum</i> ]	402 $\pm$ 110
1616968_at	Ribosomal protein L11 family protein [ <i>Arabidopsis thaliana</i> ]	351 $\pm$ 19
1622633_at	No homology in databases	310 $\pm$ 103
1614404_x_at	No homology in databases	287 $\pm$ 87
1622834_at	Protein kinase MK5 [ <i>Mesembryanthemum crystallinum</i> ]	282 $\pm$ 94
1615949_at	No homology in databases	267 $\pm$ 65
1621024_at	Cyclase-like protein [ <i>Vitis pseudoreticulata</i> ]	236 $\pm$ 67
1620723_at	Steroid membrane binding protein [ <i>Oryza sativa</i> ]	223 $\pm$ 19
1621812_x_at	Ribosomal protein [ <i>Petunia <math>\times</math> hybrida</i> ]	208 $\pm$ 35
1607438_at	Putative ripening-related protein [ <i>Vitis vinifera</i> ]	191 $\pm$ 66
1622055_x_at	No homology in databases	178 $\pm$ 91
1609256_s_at	No homology in databases	151 $\pm$ 41
1621218_at	No homology in databases	141 $\pm$ 38

<sup>a</sup> The 20 highest-level expressed *V. aestivalis*-specific transcripts of the total of 183

<sup>b</sup> Based on BLAST search results conducted on 25 May 2006

<sup>c</sup> Average normalized hybridization intensity values  $\pm$  standard deviation

**Table 3** Transcripts detected as present in *V. vinifera* leaves, but absent in *V. aestivalis* leaves

Affymetrix probe set ID <sup>a</sup>	Predicted function [organism in which nearest ortholog was found] <sup>b</sup>	Expression level in <i>V. vinifera</i> <sup>c</sup>
1608579_at	Homologous to UDP-glucose:cinnamate glucosyltransferase [ <i>Fragaria × ananassa</i> ]	1,326 ± 139
1607645_at	Homologous to pathogen-induced protein of unknown function [ <i>Cucumis sativus</i> ]	628 ± 169
1610415_at	Geraniol 10-hydroxylase [ <i>Catharanthus roseus</i> ]	305 ± 78
1620400_at	Class 1 pathogenesis related protein precursor [ <i>Nicotiana tabacum</i> ]	240 ± 143
1617336_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	210 ± 50
1619773_at	Homologous to protein of unknown function [ <i>Medicago truncatula</i> ]	204 ± 37
1611821_at	Glutathione S-transferase GST 20 [ <i>Glycine max</i> ]	171 ± 17
1618893_at	NADPH-dependent oxidoreductase [ <i>Oryza sativa</i> ]	145 ± 72
1618775_at	Homologous to pathogen-induced protein of unknown function [ <i>Hordeum vulgare</i> subsp. <i>vulgare</i> ]	120 ± 55
1607655_at	Photosystem I reaction center subunit III [ <i>Vigna radiate</i> ]	82 ± 36
1611752_at	MYB transcription factor [ <i>Arabidopsis thaliana</i> ]	78 ± 17
1622459_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	76 ± 13
1618662_at	RPM1 disease resistance protein-like [ <i>Arabidopsis lyrata</i> ]	76 ± 14
1610283_at	Homologous to protein of unknown function [ <i>Zea mays</i> ]	63 ± 49
1616574_at	Branched-chain alpha keto-acid dehydrogenase E1 alpha subunit [ <i>Oryza sativa</i> ]	60 ± 31
1606891_at	Cytochrome P450 [ <i>Glycine max</i> ]	54 ± 26
1606603_at	Homologous to protein of unknown function [ <i>Oryza sativa</i> ]	54 ± 11
1615626_at	Subtilisin-like protein [ <i>Narcissus pseudonarcissus</i> ]	38 ± 9
1621436_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	37 ± 12
1617553_at	Weakly homologous to DNA binding/transcription factor-like protein [ <i>Arabidopsis thaliana</i> ]	35 ± 6

<sup>a</sup> The twenty highest-level expressed *V. vinifera*-specific transcripts of the total of 39

<sup>b</sup> Based on BLAST search results conducted on 25 May 2006

<sup>c</sup> Average normalized hybridization intensity values ± standard deviation

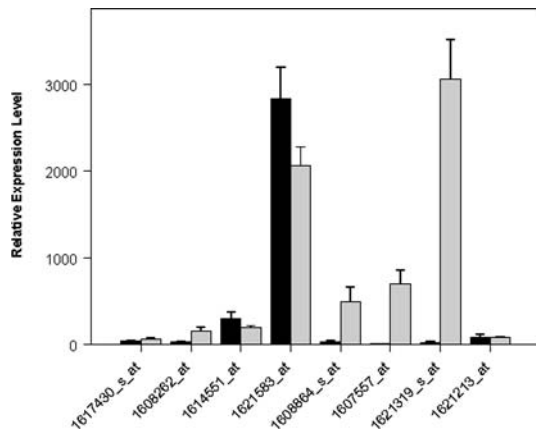
was above 50. Four of these chitinase genes were expressed in both grapevine species, but another four were represented only in the *V. aestivalis* leaf transcriptome. Two of the *V. aestivalis*-specific transcripts encoded distinct class IV chitinase enzymes (1607557\_at and 1621319\_s\_at in Fig. 1), one encoded a class I (1617430\_s\_at), and one encoded a class III enzyme (1608864\_s\_at). The detection of class IV chitinase transcripts in grapevine leaves was unexpected, because these genes were previously shown to be expressed in a flower- and fruit-specific manner in *V. vinifera* (Robinson et al. 1997).

The *V. vinifera*-specific gene that was most highly expressed had sequence homology to a strawberry UDP-glucose:cinnamate glucosyltransferase (Table 3). This enzyme was recently demonstrated to form esters with cinnamic and several other phenolic acids in strawberry fruit (Lunkenbein et al. 2006). In a consensus tree of aligned glucosyltransferase (GT) sequences, the

*V. vinifera*-specific UDP-glucose:cinnamate glucosyltransferase clustered with ester-forming GTs from other plant species. We searched the microarray data, and identified 14 GT probe sets, which consistently detected transcripts in at least one of the grapevine species. Our data indicated that most of these 14 probe sets exhibited similar expression levels, except for 1620624\_at and 1618112\_at. Both of these detected higher transcript abundance in *V. vinifera* at a significance level of 0.001. In clustalW sequence alignments, the gene sequence for 1620624\_at clustered with UDP-glucose:anthocyanin glucosyltransferases, whereas the gene sequence for 1618112\_at clustered with UDP-rhamnose:rhamnosyltransferases (data not shown).

#### Differentially expressed genes

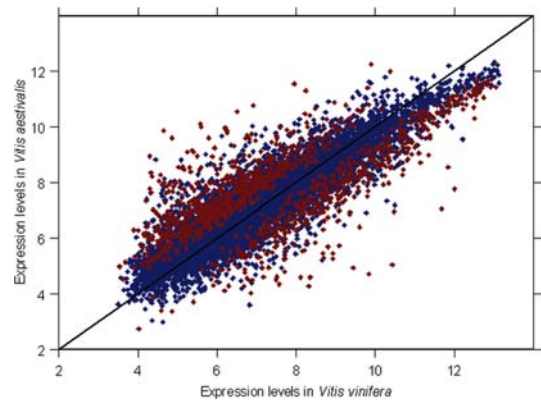
To identify genes which were expressed at different levels, we compared the abundance of the



**Fig. 1** Expression of eight chitinase genes in *Vitis vinifera* and *Vitis aestivalis* leaves as measured on the Affymetrix Vitis GeneChip. Black and gray bars indicate the level of expression in *V. vinifera* and *V. aestivalis*, respectively; error bars indicate standard deviation among six replicates. Probe sets measure transcript abundance in the following chitinase classes: Class I: 1617430\_s\_at, 1608262\_at, 1614551\_at, and 1621583\_at; Class III: 1608864\_s\_at; Class IV: 1607557\_at and 1621319\_s\_at; Class V: 1621213\_at

9,621 transcripts which were consistently present in the leaves of both grapevine species. We compared gene expression levels by using a three-factor cross-nested mixed effects linear ANOVA model. We identified 37, 236, and 1,126 genes that were differentially expressed between the two species at significance levels of 0.00001, 0.0001, and 0.001, respectively. A graph showing log<sub>2</sub>-transformed relative expression values from *V. aestivalis* plotted against those from *V. vinifera* is shown in Fig. 2.

We annotated gene sequences for those 236 probe sets, which detected different transcript levels at the 0.0001 significance level. These genes belonged to a wide variety of functional categories, which included intracellular signal transduction, transcription, secondary metabolism, amino acid synthesis, and protein synthesis/degradation. Table 4 lists those 40 genes whose expression level varied to the greatest extent between the two species. We did not find any broad functional category in which genes were overall differentially expressed in either grapevine species. Instead, certain genes of a functional category were expressed differently in one species, whereas



**Fig. 2** Comparison of gene expression levels in two grapevine species. Log<sub>2</sub>-transformed average transcript abundance values in *Vitis aestivalis* plotted against those from *Vitis vinifera*. Expression values are shown for only those 9,621 probe sets which consistently detected transcripts in all six replicates in both species. Data points shown in red and blue indicate genes whose transcript levels differed in the two species at the significance levels of <0.001 and ≥0.001, respectively

other genes of the same category were expressed differently in the other species. For example, within the category of protein degradation, an aminopeptidase, a serine carboxypeptidase, and a zinc ion-binding ubiquitin ligase were expressed at higher levels in *V. vinifera*, whereas a glutamate carboxypeptidase and an ubiquitin protein ligase were expressed at higher levels in *V. aestivalis*. In the category of lignin synthesis, a cinnamyl-CoA reductase and a ferulate 5-hydroxylase gene were expressed at a significantly higher level in *V. vinifera*, and a cinnamyl alcohol dehydrogenase (CAD) and a caffeic acid *O*-methyltransferase (COMT) gene were expressed higher in *V. aestivalis*. The differential expression of the lignin biosynthesis genes prompted us to systematically examine transcript abundance for all probe sets involved in phenylpropanoid metabolism. We did not find differentially abundant transcripts in the central phenylpropanoid pathway, but we did find a leucoanthocyanidin reductase (Affymetrix ID: 1615174\_s\_at) gene expressed in *V. vinifera* at a level approximately 4 times higher relative to *V. aestivalis* ( $P < 0.001$ ).

**Table 4** Genes expressed differentially in leaves of *V. aestivalis* and *V. vinifera* at a significance level of 0.0001

Affymetrix probe set ID	Predicted function [organism in which nearest ortholog was found] <sup>a</sup>	Expression level ratio <sup>b</sup>
Transcripts of highest <i>V. aestivalis</i> to <i>V. vinifera</i> expression ratio		
1608007_x_at	No significant homology in databases	38.79
1611166_at	Leucine-rich repeat protein [ <i>Oryza sativa</i> ]	25.95
1609973_at	Putative ABC transporter [ <i>Oryza sativa</i> ]	22.34
1607236_at	Eukaryotic translation initiation factor 5A [ <i>Hevea brasiliensis</i> ]	16.19
1607127_s_at	Homologous to pathogen-induced protein [ <i>Cucumis sativus</i> ]	15.06
1608196_at	$\beta$ -amylase [ <i>Arabidopsis thaliana</i> ]	14.83
1607262_at	Weakly similar to EDS1 protein [ <i>Lycopersicon esculentum</i> ]	13.39
1620371_at	No significant homology in databases	13.33
1617397_x_at	No significant homology in databases	12.28
1607609_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	11.57
1613692_at	No significant homology in databases	11.52
1612124_at	Caffeic acid <i>O</i> -methyltransferase [ <i>Vitis vinifera</i> ]	10.48
1609712_at	Cytochrome P450 [ <i>Populus × canescens</i> ]	9.51
1613175_at	Insulin degrading enzyme-like protein [ <i>Oryza sativa</i> ]	9.12
1607069_at	CsIE cellulose synthase-like protein [ <i>Nicotiana tabacum</i> ]	8.25
1616789_at	Ubiquitin-conjugating enzyme-like protein [ <i>Solanum tuberosum</i> ]	7.79
1620502_at	Serine/threonine kinases-like protein [ <i>Arabidopsis thaliana</i> ]	7.66
1606455_at	Weakly similar to Lr10 rust resistance kinase [ <i>Oryza sativa</i> ]	7.41
1615565_x_at	Ubiquitin [ <i>Antirrhinum majus</i> ]	7.36
1615226_at	Glutaredoxin-related protein [ <i>Arabidopsis thaliana</i> ]	7.17
Transcripts of highest <i>V. vinifera</i> to <i>V. aestivalis</i> expression ratio		
1615048_at	Homologous to protein of unknown function [ <i>Fragaria × ananassa</i> ]	42.06
1622368_at	Putative dioxygenase [ <i>Solanum demissum</i> ]	36.01
1611841_at	Subtilisin-like protease C1 [ <i>Glycine max</i> ]	25.12
1612409_at	Homologous to protein of unknown function [ <i>Fragaria × ananassa</i> ]	23.78
1619608_at	No significant homology in databases	19.16
1611891_at	Serine carboxypeptidase [ <i>Medicago truncatula</i> ]	11.21
1612848_x_at	Ribulose-1,5-biphosphate carboxylase [ <i>Vitis pseudoreticulata</i> ]	9.95
1608048_at	Cinnamoyl-CoA reductase [ <i>Oryza sativa</i> ]	9.66
1613881_at	No significant homology in databases	8.22
1610670_at	Nodulin-like protein [ <i>Arabidopsis thaliana</i> ]	7.35
1610503_at	No significant homology in databases	6.23
1610801_at	Glutamate decarboxylase [ <i>Arabidopsis thaliana</i> ]	5.60
1611367_at	SecA-type chloroplast protein transport factor [ <i>Arabidopsis thaliana</i> ]	5.56
1611508_at	Acyl CoA thioesterase [ <i>Arabidopsis thaliana</i> ]	5.33
1614524_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	5.23
1614014_at	Serine acetyltransferase [ <i>Nicotiana tabacum</i> ]	4.83
1619064_at	No significant homology in databases	4.25
1609560_at	Homologous to protein of unknown function [ <i>Arabidopsis thaliana</i> ]	4.24
1615202_at	No significant homology in databases	4.21
1614903_at	Acyl carrier protein [ <i>Brassica oleracea</i> ]	4.20

<sup>a</sup> Based on results of BLAST searches conducted on 25 May 2006

<sup>b</sup> Computed as the ratio of the average normalized hybridization intensity values

## Discussion

### Reliability of microarray results

The *Vitis* GeneChip was designed primarily for transcriptome analysis in *Vitis vinifera* with most of its probe sets derived with the coding sequences from that species. Transcriptome

analysis is also important in non-*vinifera* members of the *Vitis* genus, as those plants represent agronomically valuable genetic resources for grapevine improvement (Reisch and Pratt 1996). Sequence differences between the GeneChip probes and non-*vinifera* grapevine cRNA may confound gene expression data which, in turn, may influence the reliability of results

when working with non-vinifera species. We addressed this question by comparing the characteristics of the hybridization data generated with cRNA from leaf tissues from *V. vinifera* and *V. aestivalis*.

Our observations suggested that the random noise in the hybridization data from *V. aestivalis* were similar to that from *V. vinifera*. Results from various replicates correlated well in both species. Furthermore, the level of overall hybridization intensity and the number of absent calls were similar across the two grapevines. The likely reason the number of absent calls did not reflect sequence differences is probably due to the robustness of the Affymetrix system. Present, absent, and marginal detection calls are generated by a GCOS algorithm, which incorporates hybridization values from 16 perfect-match probes and the corresponding 16 mismatch probes for each gene assayed. Based on the 16 pairs of measurements, the algorithm calculates a discrimination score for which a detection *P*-value is assigned using a Wilcoxon signed rank test. By determining the detection *P*-value on the basis of 16 measurements, the algorithm attenuates the effect of a mismatch at only a few probes.

The similar intra-class correlation of the results in the two grapevines suggested that a similar level of variation occurs when their cRNA samples are hybridized to the Vitis GeneChip. This could be explained by the fact that the intra-specific polymorphism between haplotypes is nearly as large as the inter-specific sequence divergence between two grape species. Salmaso et al. (2004) reported an average level of single nucleotide polymorphism of 0.863 for every 100 nucleotides among *V. vinifera* cultivars, 1.366 between *V. vinifera* and *V. riparia*, and 1.090 between *V. vinifera* and a hybrid grapevine. Furthermore, the Vitis GeneChip also contains non-vinifera-based probe sequences: 1,336 and 591 probe sets are derived from inter-species hybrids and North American species, respectively. These probe sets are likely to contribute to random noise even when hybridization is performed with *V. vinifera*-derived cRNA.

The comparative exploration of young leaf transcriptome revealed substantial variation in gene expression between *V. vinifera* and

*V. aestivalis*. This variation was apparent across all functional categories. Detailed exploration of species-specific and differential transcription was carried out in the glucosyltransferase, chitinase, and  $\beta$ -1,3-glucanase gene families, and in the genes involved in the central phenylpropanoid pathway and in the monolignol and flavonoid synthesis pathways.

#### Expression of class IV chitinase and $\beta$ -1,3-glucanase genes

*Vitis aestivalis* has been extensively used in grape breeding programs as a source of resistance against fungal pathogens (Reisch and Pratt 1996). Further, the cultivated *V. aestivalis* variety 'Norton' is the only bunch grape cultivar which is able to produce healthy fruit with minimal or no fungicide application in the humid regions of temperate North America. It was an important finding, therefore, that two Class IV chitinase genes were expressed at high levels in the leaves of this plant, but not in the leaves of *V. vinifera*. In addition, a Class I and a Class IV chitinase gene also were expressed in a species-specific manner in *V. aestivalis* leaves (Table 2). Robinson et al. (1997) showed that Class IV chitinase transcription occurs in flowers and berries, but not in leaves or other organs in *V. vinifera*. This organ-specific expression was recently confirmed using EST frequency analysis by Goes da Silva et al. (2005). Our microarray data were in agreement with these observations in that class IV chitinase probe sets did not detect above-background transcript levels in *V. vinifera* leaves. In contrast, class IV chitinases were expressed at high levels in leaves of *V. aestivalis*, suggesting distinctly different transcriptional regulation of these genes in that species. In *V. vinifera*, class IV chitinases were proposed to play a specific role in defense of reproductive organs against fungal pathogens (Robinson et al. 1997). Their up-regulation in the ripening grape berry is well documented from several other laboratories and is considered to be an example of developmentally regulated defense mechanism (Robinson et al. 1997; Derckel et al. 1996; Pockock et al. 2000).

In addition to class IV chitinases, we found that one member of the  $\beta$ -1,3-glucanase gene family

also was expressed in a species-specific manner in *V. aestivalis*. Other  $\beta$ -1,3-glucanase genes had similar expression levels in the two grapevines. The differential expression of a  $\beta$ -1,3-glucanase gene is interesting. It is a pathogenesis-related protein, which was shown to act in synergistic manner with chitinase. Mauch et al. (1988) described the enhanced combined effectiveness of pea tissue-purified  $\beta$ -1,3-glucanase and chitinase enzymes in degrading fungal cell walls. The different tissue specificity of expression of these genes suggests that their transcriptional control evolved divergently in the two grapevine species. It is tempting to speculate that this differential regulation might result from adaptation to the disparate climatic conditions at the geographic origin of these grapevine species. The warm and humid climate of temperate North America, where *V. aestivalis* evolved, created more favorable environmental conditions for fungal pathogens than the semi-arid climate of central Asia from where *V. vinifera* originated. Expression of anti-fungal genes in the leaves of *V. aestivalis* may therefore be the result of adaptation to humid, high disease pressure conditions.

#### Expression of glucosyltransferases and genes of phenylpropanoid metabolism

In *V. vinifera*, anthocyanins occur as monoglucosides, whereas in American grapevine species, they are predominantly present in the form of diglucosides (Ribereau-Gayon et al. 2000). Therefore, we expected to find *V. aestivalis*-specific or differentially expressed GTs which are involved in the glucosylation of anthocyanidins at their carbon-5 position (Vogt and Jones 2000). Contrary to our expectations, we identified a *V. vinifera*-specific gene which was closely related to a UDP-glucose:cinnamate GT and other phenolic acid glucose esterases. In addition, we identified two other grapevine glycosyltransferases which also were expressed at higher levels in *V. vinifera* than in *V. aestivalis*. Interestingly, the grapevine UDP-glucose:flavonoid 3-GT was expressed at similar levels in the two grapevine species (data not shown). This gene, which was previously cloned from leaves and berries (Sparvoli et al. 1994; Ford et al. 1998), was found to be

an important regulator of flavonoid synthesis (Kobayashi et al. 2001).

Sequence alignment-based clustering of the deduced GT protein with known GT enzymes suggested that the genes that were specifically and differentially expressed in *V. vinifera* may catalyze anthocyanin and phenolic acid glycosylation and, thus, are involved in the stabilizing and further processing of phenylpropanoid-derived secondary products. We observed additional differentially expressed genes in various pathways of the phenylpropanoid metabolic grid. A leucoanthocyanidin reductase, for example, was represented with higher transcript levels in *V. vinifera* than in *V. aestivalis*. Leucoanthocyanidin reductase is a key enzyme in the synthesis of proanthocyanidins which are precursors for condensed tannins. Further examples of differentially expressed phenylpropanoid metabolism genes were CAD and COMT of the monolignol synthesis pathways. Both of these genes were expressed at higher levels in *V. aestivalis* than in *V. vinifera*. High expression of CAD could enhance the rate of lignin synthesis (Lapierre et al. 1999), whereas high expression of COMT may cause an elevated syringyl:guaiacyl monolignol ratio in the lignin of *V. aestivalis*, as shown by results from transgenic poplar and maize studies in which these genes were down-regulated by anti-sense technology (Lapierre et al. 1999; Piquemal et al. 2002).

Differential expression of genes encoding glycosyltransferases and enzymes of the phenylpropanoid metabolism may have important phenotypic and physiological consequences. Phenolic metabolites, such as tannins, are believed to play a role in providing defense against herbivores (Winkel-Shirley 2002; Dixon et al. 2005). In *Arabidopsis*, there is experimental evidence suggesting that flavonoids protect photosynthetic tissues from solar UV-B radiation (Landry et al. 1995; Bieza and Lois 2001). Lignin provides mechanical support for tissues and plays a role in defense against pathogens, as was demonstrated in *Arabidopsis* mutants with dramatically altered lignin composition (Jones et al. 2001; Franke et al. 2002). One class of COMT genes was previously shown to be transcriptionally up-regulated in response to environmental stress, viral infection, and treatment with pathogen-derived elicitors in tobacco and

*Zinnia* (Pellegrini et al. 1993; Ye et al. 1994). We speculate, therefore, that the species-specific transcript level variation in these secondary metabolism genes might manifest different strategies employed by these two grapevines to cope with biotic and environmental stress.

It is important to point out that growth conditions used in these studies included 85% relative humidity. This particular environmental condition is characteristic of the continental North American climate, and was chosen, because its impact on grapevines is of interest to the viticulture industry in the eastern and mid-western United States. Such high relative humidity levels are natural in the native habitat for *V. aestivalis*, but not for *V. vinifera*. Thus, it is possible that the gene expression features described here mirror a state of environmental stress for *V. vinifera*, but a more-or-less normal physiological state for *V. aestivalis*. Further microarray studies using humidity or other environmental factors as independent variables could provide insight into the physiological state of a grape genotype under certain climatic conditions.

Microarray data, nevertheless, have limitations in that they only provide correlative information on how phenotypic features might be influenced by the observed gene expression patterns. Without biochemical follow-up studies on the catalytic capacity of the individual enzymes and the availability of substrates, the extrapolation to possible phenotypes remains hypothetical. Furthermore, for each of the genes examined here, there may be several other members of the gene family in the grapevine, which were not represented on the *Vitis* GeneChip. Such non-represented family members might be differently expressed from the ones which are assayed here. Microarray analysis can be greatly leveraged by combining it with QTL mapping (Varshney et al. 2005). This novel approach, termed expression eQTL analysis, is able to map regions of the genome that influence transcript levels. The integration of the eQTL data with the genetic or physical map positions of the transcribed genes enables one to determine if transcript level variation is the property of the transcribed gene itself (*cis*-acting), or if it is determined by a *trans*-acting regulatory factor (Kirst et al. 2005). With

the availability of the physical map for grapevine in the near future (Adam-Blondon 2006), such complex approaches can soon be applied to map and identify candidate genes, which are key regulators of transcription in grapevine. The use of regulatory genes as functional markers is expected to greatly increase the precision and effectiveness of grapevine breeding (Varshney et al. 2005).

## Conclusions

This comparative analysis opened a new window into the variation in gene expression between two economically important grapevines. In light of these results, we propose that genomic differences among grapevine species are not limited to coding sequence divergence or genotype-specific genes, but extend to transcriptional regulation. This highlights the role regulatory protein complexes and promoter and enhancer sequences play in determining the phenotypic features in grapevine.

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