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RESEARCH PAPER

Transcriptional and metabolic profiling of grape (*Vitis vinifera* L.) leaves unravel possible innate resistance against pathogenic fungi

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Abstract

Grapevine species (*Vitis* sp.) are prone to several diseases, fungi being the major pathogens compromising its cultivation and economic profit around the world. Knowledge of the complexity of mechanisms responsible for resistance to fungus infection of cultivars, such as Regent, is necessary for strategies to be defined which will improve resistance in highly susceptible crop species. Transcript and metabolic profiles of the *Vitis vinifera* cultivars Regent and Trincadeira (resistant and susceptible to fungi, respectively) were analysed by cDNA microarray, quantitative real-time PCR, and nuclear magnetic resonance spectroscopy. The integration of datasets obtained through transcriptome and metabolome analysis revealed differences in transcripts and metabolites between both cultivars. These differences are probably associated with the innate resistance of Regent towards the mildews. Several transcripts related to stress and defence, namely a subtilisin-like protease, phenylalanine ammonia lyase, *S*-adenosylmethionine synthase, WD-repeat protein like, and J2P, were up-regulated in Regent suggesting an intrinsic resistance capability of this cultivar. A metabolic profile revealed an accumulation of compounds such as inositol and caffeic acid, which are known to confer resistance to fungi. The differences in transcripts and metabolites detected are discussed in terms of the metabolic pathways and their possible role in plant defence against pathogen attack, as well as their potential interest to discriminate among resistant and susceptible grapevine cultivars.

Key words: cDNA microarrays, NMR-based metabolomics, Regent, *Vitis vinifera*.

Introduction

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world. It is prone to several diseases mainly caused by fungal pathogens. During the 19th century, pathogens such as powdery mildew [*Uncinula necator* (Schw.) Burr] and downy mildew [*Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni] were introduced in Europe from North America and became major risk factors for viticulture. Since *Vitis vinifera* does not carry any resistance to mildew fungi, multiple fungicide applications per growing season became indispensable for traditional cultivars. The best way to overcome these problems is to develop resistant cultivars producing high quality fruit.

The grapevine variety Regent was bred at the Institute for Grapevine Breeding Geilweilerhof and combines high wine quality and resistance to the two most important pathogens (downy and powdery mildew agents) worldwide (Anonymous, 2000). Its resistance traits were achieved by multiple crosses involving wild species such as *Vitis rupestris*, *V. riparia*, and *V. lincecumii*, that are known sources of resistance to both pathogens, and susceptible traditional *V. vinifera* cultivars like Diana and

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Chambourcin (Eibach and Toepfer, 2003). Resistance QTLs were detected for both powdery and downy mildew (Fisher *et al*., 2004; Welter *et al*., 2007).

The origin of the resistance traits encountered in Regent is not yet known, but cultivars that were bred as Regent, Orion and Phoenix, present a defence response that seems to be based on biochemical reactions rather than on hypersensitive reactions (Kortekamp and Zyprian, 2003). A better understanding of the complexity of the resistance capability of *Vitis vinifera* cultivars, such as Regent, is necessary for an overall understanding of the resistance mechanism and for the definition of improvement strategies for highly susceptible crop species like Trincadeira (a traditional Portuguese cultivar, widely used for wine manufacture and highly susceptible to both downy and powdery mildew). To understand the possible innate resistance capabilities of the cultivar Regent, both transcript and metabolome profiling were accessed. Microarray technology allows monitoring the expression level of thousands of genes simultaneously in one single experiment (Shena *et al*., 2002; Meyers *et al*., 2004). It has been used to study the grapevine transcriptome related to berry development (Terrier *et al*., 2005; Waters *et al*., 2005; Ageorges *et al*., 2006), modulation of endogenous grapevine gene expression (Franks *et al*., 2006), water and salinity stress (Cramer *et al*., 2007), and virus infection (Espinoza *et al*., 2007) responses.

Metabolome analyses describe the chemical profile or fingerprint of the metabolites in whole tissues (Choi *et al*., 2004; Krishnan *et al*., 2005; Ward *et al*., 2007). Nuclear magnetic resonance, in particular ^{1}H NMR, yields a comprehensive fingerprint of all hydrogen-bearing metabolites. It can also provide direct structural information regarding individual metabolites in the mixture, with the help of two-dimensional techniques (Defernez and Colquhoun, 2003; Kikuchi *et al*., 2004). The data obtained by NMR can be analysed by multivariate or pattern recognition techniques such as principal component analysis (PCA) (Choi *et al*., 2004). Hence, metabolic profiling, especially when integrated with transcript profiling, has a high potential to elucidate gene function and to establish data networks.

As far as is known, the only published data regarding the comparison of resistant and susceptible grapevine plants involves plant–pathogen interaction. The aim of the present work was to discriminate between grape cultivars, which vary in resistance to mildews, using transcript and metabolic profiles, and to discuss these profiles with possible known resistance mechanisms.

Materials and methods

Plant material

Young leaves (second to fourth insertion from the apex) of *Vitis vinifera* L. cvs Regent and Trincadeira were randomly collected from 15 plants with no evidence of disease or stress symptoms, prior to flowering (PLANSEL Vineyards, Montemor, Portugal), late in the morning in two years (2004 and 2006), and stored after shock freezing with liquid nitrogen at –80 °C. Regent was bred at the Institute for Grapevine Breeding Geilweilerhof and shows resistance to both powdery (*Uncinula necator*) and downy mildew (*Plasmopara viticola*) (Anonymous, 2000). Trincadeira is a traditional fungus-susceptible *Vitis vinifera* cultivar.

RNA extraction

Total RNA was isolated from young leaves of Regent and Trincadeira cultivars essentially as described by Wan and Wilkins (1994). Briefly, 2 g of ground plant material were added to 20 ml of extraction buffer (0.2 M sodium borate, 30 mM EDTA, 1% SDS, 1% sodium deoxicolate, 10 mM DTT, 1% Nonidet P-40, 2% PVP-40) previously heated to 90 °C. Proteinase K (1.2 mg ml^{-1}) (Gibco BRL, Life Technologies) was added and samples were kept for 90 min at 42 °C with agitation. Proteins were selectively precipitated with 160 mM KCl by incubating at 4 °C for 30 min. Supernatant was recovered by centrifugation at 9500 *g* (Biofuge Stratos, Heraeus Instruments) for 30 min and nucleic acids were precipitated overnight with 2 M LiCl. The pellet was washed three times with cooled 2 M LiCl and resuspended in 10 mM TRIS–HCl, pH 7.5. After 10 min centrifugation at 12 000 *g*, supernatant was collected and polysaccharides were precipitated with 0.2 M KAc, pH 5.5, by incubating at 4 °C for 30 min. Supernatant was collected and RNA was precipitated by addition of 2.5 volumes of 100% ethanol. The pellet was washed with 70% ethanol. RNA purification was carried out with DNase (Invitrogen, San Diego, CA, USA) and with the RNeasy Plant Mini kit according to the supplier's instructions (Qiagen, Valencia, CA, USA). All solutions used were treated with diethyl pyrocarbonate (Sigma).

Construction of grapevine cDNA microarray

A cDNA library representative of the two *Vitis vinifera* cultivars was constructed with leaves collected in 2004 according to Figueiredo *et al.* (2007). Randomly chosen clones were PCR amplified using M13fw (5′-TGCAACATTTTGCTGCC-3′) and M13rev (5′-CAGGAAACAGCTATGAC-3′) primers, and inserts longer than 400 bp were selected (2712 cDNAs).

Amplified cDNA inserts were purified with MultiScreen-PCR plate (Millipore, Bedford, MA, USA), transferred to printing plates (Microtiter V plates, Sigma), resuspended in 50% dimethylsulphoxide and 0.2% SSC, and spotted in duplicate onto poly-L-lysine-treated glass slides using VersArray ChipWriter Compact® (Bio-Rad Lab., Hercules, CA, USA). ESTs (expression sequence tags) coding for a chitinase class III (DQ406693), actin (AY847627), stilbene synthase (DQ406692), allene oxide cyclase (DQ406694), 26S ribosomal RNA (AY847628), and AUX like protein (AY849386) were included as controls.

Technical details of spotting are provided as MIAME (Brazma *et al*., 2001). Briefly cDNA clones were printed on poly-L-lysine-coated slides, using a 12-pin configured ChipWriter Compact System (Biorad). The general design of the microarray was 12 subgrids, each subgrid with 24 columns and 23 rows, with each clone replicated within the same subgrid. After printing, cross-linking was performed with heat and UV according to the protocol from Vodkin Laboratory (http://soybeangenomics.cropsci.uiuc.edu). Microarray quality was accessed by GelStar nucleic acid stain (FMC, Rockland, ME, USA).

Cy-labelling and hybridization

RNA used for hybridizations was obtained from three sets of leaves from 15 different plants collected in 2004, yielding three biological

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replicates. Four hybridizations were carried out corresponding to three biological replicates and one dye-swap. Self–self hybridizations were also carried out to evaluate dye bias. Seventy micrograms of RNA from each biological replicate were used for cDNA synthesis with an RT primer (oligo dT) and labeled with either Cy3 or Cy5 dye molecules (Amersham, Bucks, UK). Following cDNA synthesis, RNA was removed with RNase (Fermentas) and labelled targets were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Prior to hybridization, slides were treated with 1% BSA, 5× SSC, and 0.1% SDS (w/v) for 30 min at 50 °C. Briefly, the hybridization mix consisted of 30 µl of labelled cDNAs, 7.5 µg Cot-1 DNA (Invitrogen), 15 µg poly(A)$^+$, 7.5 µg of salmon sperm DNA, 1.5 µl of 50× Denhardt's solution, 7.9 µl 20× SSC, and 1.5 µl 10% SDS (w/v). The mixture was denatured at 97 °C for 2 min, kept on ice, and then 1 µl of 10×DIG blocking buffer was added (Roche Diagnostic GmbH, Mannheim, Germany). Hybridization was carried out for 17 h at 65 °C in ArrayIt chambers (Telechem International, Sunnyvale, CA, USA) placed in a water bath. Slides were then washed once in 0.5× SSC and 0.1% SDS (w/v), then in 0.5× SSC and 0.01% SDS (w/v) and twice in 0.06× SSC.

Microarray analysis

Slides were scanned using a VersArray ChipReader®. Spot and background intensities were quantified using VersArray Analyser software (Bio-Rad Lab.). Background was calculated as the trimmed mean of pixel intensity on local corners of spots. Low intensity signal spots (trimmed mean of raw intensity/trimmed mean of background <1.5), uneven background (trimmed mean of raw intensity/standard deviation of background <2.5), uneven spots (trimmed mean of raw intensity of background/standard deviation of raw intensity <1), and not validated spots (flags) were removed from analysis before normalization. Data files were imported into GEPAS (http://gepas.bioinfo.cipf.es) and $\log_2$-transformed prior to normalization (DNMAD). Print-tip Lowess (Yang *et al*., 2002) was used for within-slide normalization considering background subtraction. Statistical analysis was performed using the Rank Products (RP) method (Breitling *et al*., 2004). The RP values for each gene were compared with the RPs of 1000 random permutations, with the same number of replicates and genes as the real experiment. ESTs with a false discovery rate <0.05 and a fold change of 1.6 were considered differentially expressed. This rank-based test statistic is a non-parametric method shown to generate accurate results with biological datasets, particularly with small numbers of replicates (Breitling and Herzyk, 2005). This method has already been used to analyse transcriptional profiling in plants (Armengaud *et al*., 2004).

Sequencing and sequence analysis

Differentially expressed ESTs were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems, Inc.) with an ABI Prism 310 genetic analyser (Applied Biosystems, Inc.) according to the manufacturer's instructions. Homology searches were performed at NCBI databases, using the blastx and megablast algorithms (Altschul *et al*., 1997). Sequences with an e-value $\leqslant 10^{-4}$ were considered to identify known genes or have partial similarity to known genes. Assembly of the individual ESTs into groups of tentative consensus sequences, representing unique transcripts, was performed using the SeqManII program (DNAStar) (90% similarity over 40 nt length).

Quantitative real-time (qRT)-PCR

To evaluate the accumulation pattern of ESTs considered to be directly or indirectly related to Regent's innate resistance capabilities, six differentially expressed ESTs were analysed by qRT-PCR.

Five micrograms of total RNA from each biological replicate (leaves collected in 2006) were used to synthesize cDNA. Complementary DNAs were quantified using a spectrofluorimeter (Anthos Zenyth 3100, Anthos Labtec Instruments, Austria) and brought to equal concentration. Quantitative RT-PCR reactions were performed with the LightCycler Fast Start ReactionMix MasterPLUS SYBR Green I (Roche, Mannheim, Germany) on a LightCycler® detection system (Roche) according to the manufacturer's instructions. Gene-specific oligonucleotide sequences are shown in Table 1. The transcript concentration for each sample was calculated based on a standard calibration curve obtained from serial dilutions of plasmid DNA containing the insert to be analysed. A negative control reaction without a template was always included for each primer combination. Two to three biological replicates and one technical replicate were performed for each time point. The average of three or four qRT-PCR reactions is presented for each time point.

Metabolic profiling using ^{1}H NMR, J-resolved, COSY, and multivariate analysis

Plant material collected in 2006 was frozen, ground in liquid nitrogen, and lyophilized for at least 72 h at –40 °C. Twenty-five

Table 1. S*equence of the oligonucleotides used for the qRT-PCR transcript profile*

Target sequence identification, oligonucleotide sequence, sequence length (bp), and T_m (°C) are shown.

Target sequence	Genebank ID	Primer sequence (5′–3′)	Length (bp)/T_m (°C)
Subtilisin-like protease	ES880786	F: CTCTTTCCAGCCTCACCAA R: CACCATCATCCCACACCAA	242/64
Phenylalanine ammonia lyase	EC907689	F: GGAAGGAGTTTGATAAGGTG R: TACACAAACATGTGAGAAGTG	223/64
WD-repeat protein like	EC907666	F: ACTATTGCAATGGAGGAAC R: TGTCAGATTGAGATGAGGA	116/60
S-Adenosylmethionine synthetase	EC907686	F: GTGTCTTATGCTATTGGTGTG R: TTGAGGTCAAGGTTGATGGT	148/68
Cinnamyl alcohol dehydrogenase	EC907662	F: GAAGCTAGTTATGGTTGGT R: TCTGCCGTTATGTTGTGTT	155/68
J2P	EC907669	F: TATGAAGAGGATGAGGATA R: AGGACACACAGAGAGAGA	182/64

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milligrams of material were used from each sample extraction according to Choi *et al*. (2006) and Abdel-Farid *et al*. (2007). Potassium phosphate (KH_2PO_4) was added to D_2O (99.9%; Cambridge Isotope Laboratories, Miami, FL, USA) as a buffering agent. The pH of the D_2O for NMR measurements was adjusted to 6.0 with 1 N NaOD solution (Cortec, Paris, France). Samples were dissolved in 750 µl of KH_2PO_4 with 0.1% trimethyl silane propionic acid sodium salt (standard purchased from Merck, Darmstadt, Germany), and 750 µl of methanol-*d4* (99.9%, Cambridge Isotope Laboratories). Then, samples were briefly vortexed, sonicated for 10–20 min, and centrifuged for 10 min at 17 000 *g*.

The supernatant (800 µl) was then used for analysis. ^{1}H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer operating at a proton NMR frequency of 500.13 MHz. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10.26 min measuring time with the following parameters: 0.16 Hz/point, pulse width 30°, acquisition time 3.17 s, and relaxation delay 1.5 s. A presaturation sequence was used to suppress the residual water signal at δ 4.91 with low power selective irradiation at the water frequency during the recycle delay. Free induction decays were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected and calibrated to TSP at δ 0.0, all using XWIN NMR (version 3.5, Bruker). The ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (version 3.8, Bruker Biospin). Spectral intensities were scaled to TSP and to reference and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region δ 0.40–10.00. The region δ 3.28–3.34 was excluded from the analysis due to the residual signal of water and methanol-*d4*. PCA analysis was carried out with the SIMCA-P software (version 11.0; Umetrics, Umea, Sweden). PCA is an unsupervised pattern recognition method requiring no knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (Goodacre *et al*., 2000). The Pareto scaling method was used as it gives each variable a variance numerically equal to its standard deviation.

Two dimensional (2D) J-resolved ^{1}H NMR spectra were acquired using eight scans per 128 increments that were collected into 8 k and 128 data points for the F2 and F1 axes, respectively, using spectral widths of 5 kHz in F2 (chemical shift axis) and 66 Hz in F1 (spin-spin coupling constant axis). A 1.49 s relaxation delay was employed, giving a total acquisition time of 56.7 min. Both dimensions were multiplied by sine-bell functions prior to double complex FT. J-resolved spectra were tilted by 45°, symmetrized about F1, and then calibrated, all using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H-correlated spectroscopy (COSY) spectra were acquired with 1.0 s relaxation delay, 5 kHz spectral width in both dimensions. Microsoft® Excel files containing spectral intensities reduced to integrated regions of equal width (0.04 ppm) were used for Wilcoxon rank sum test to evaluate metabolites present in samples in significantly different amounts (P <0.05). The Wilcoxon rank sum test is the classical non-parametric alternative to the *t*-test when comparing two samples, being more powerful in the case of non-normal data distribution.

Results

Genes differentially expressed in Vitis vinifera *Regent and Trincadeira cultivars*

The differential transcript expression of both cultivars was analysed by cDNA microarrays, allowing the identification of 121 transcripts as being differentially expressed, representing 29 unique gene sequences. The identified unigenes were classified into eight primary functional categories, according to the putative function of their homologous genes in databases (National Center for Biotechnology Information) as follows: stress and defence, transcription regulation, regulation of chloroplast gene expression, photosynthesis, energy, signal transduction, protein modulation, and unknown function. The most redundant contigs included: sequences homologous to genes coding for a transcript that presented no homology with the database (59 clones); a cDNA coding for a subtilisin-like protease (ES880786) (13 clones); a cDNA coding for a chlorophyll *a*/*b* binding protein (EC907687) (15 clones); a cDNA coding for a phenylalanine ammonia lyase (PAL; EC907689) (four clones); and cDNAs coding for Lil3 protein (EC907663), catalase (EC907667), and *S*-adenosyl methionine synthase (EC907686) (two clones each). The remaining differentially expressed cDNA clones were unique sequences.

The ratios (fold change of normalized signals) of the clones found within the same contig were averaged and counted as one gene. Unigenes are shown in Table 2.

Complementary DNA encoding a subtilisin-like protease, PAL, WD-repeat protein like, *S*-adenosylmethionine synthase, cinnamyl alcohol dehydrogenase (CAD), and J2P were independently quantified by qRT-PCR in both cultivars presenting similar expression patterns as the ones obtained by microarray analysis (Fig. 1).

Metabolic profiling

One-dimensional and 2D NMR techniques enabled the recognition of a broad metabolome allowing the identification of several compounds such as amino acids, carbohydrates, organic acids, and phenolic compounds. 2D NMR spectroscopy, such as J-resolved and COSY techniques, was applied to identify metabolites from the congested ^{1}H NMR signals (Choi *et al*., 2004). Amino acids were identified at δ 2.46 (m) and δ 2.14 (m) as glutamine; at δ 2.39 (m) and δ 2.04 (m) as glutamate; at δ 2.18 (s) as methionine; δ 1.48 (d, J=7.5 Hz) as alanine; δ 1.34 (d, J=6.5 Hz) as threonine and at δ 1.06 (d, J=6.6 Hz), and δ 1.01 (d, J=7 Hz) as valine. At δ 2.35 (m) trace amounts of proline were detected. Anomeric protons of sucrose were detected at δ 5.40 (d, J = 3.5 Hz), α-glucose at δ 5.18 (d, J=3.8 Hz), β-glucose at δ 4.58 (d, J=7.8 Hz), and fructofuran moiety of sucrose at δ 4.17 (d, J=8.5 Hz). In addition to these compounds, inositol δ 4.03 (t, J=2.8 Hz), succinic acid δ 2.54 (s), and trace amounts of α-linolenic acid δ 0.95 (*t*) were identified (Fig. 2). Signals at δ 5.7–9.0 correspond to the phenolic compound region (Fig. 3).

The 2D techniques, J-resolved and COSY (Fig. 4), were used to identify metabolites in the phenolic compound region. The combination of the 2D techniques and the comparison with libraries of spectra

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Table 2. *Differentially expressed genes identified when comparing* Vitis vinifera *Regent and Trincadeira transcriptomes*

From 121 transcripts identified as differentially expressed, 29 unigenes were sorted into groups according to their putative physiological role estimated according to the highest Blast hits (blastn and blastx). Negative and positive values indicate down- and up-regulation, respectively.

Gene bank acc. no.	e-value	Annotation gene Annotation [species[	Fold change	Clone ID/contig
Photosynthesis				
EC907687	6e-22	Chlorophyll *a*/*b* binding protein CP29 [*Vigna radiata*]	–2.59	VvTR30
EC907664	6e-12	Ferredoxin A	–1.95	VvTR351
EC907678	2e-44	TMP14 [*Arabidopsis thaliana*]	–1.62	VvTR2309
EC907682	5e-76	Rubisco activase [*Zantedeschia aethiopica*]	–1.80	VvTR2655
EC907671	8e-67	Ribulose-1,5-bisphophate carboxylase/ oxygenase small subunit mRNA [*Vitis pseudoreticulata*]	–2.00	VvTR1293
EC907663	7e-26	Putative Lil3 protein [*Oryza sativa* (japonica cultivar-group)]	1.86	VvTR305
EC907680	5e-22	Chlorophyll *a*/*b* binding protein CP24 precursor [*Vigna radiata*]	–1.62	VvTR2470
Energy				
EC907683	4e-51	[*Vitis vinifera*] NAD-malate dehydrogenase (Mdhg) mRNA*	–1.62	VvTR2657
EC907685	2e-29	[*Gossypium mustelinum*] NADH dehydrogenase subunitA*	–1.80	VvTR2901
Stress and defence				
ES880786	1e-29	Subtilisin-like protease C1 [*Glycine max*]	3.45	VvTR65
EC907674	3e-27	Putative glutathione *S*-transferase T4 [*Lycopersicon esculentum*]	–1.85	VvTR1578
EC907686	2e-55	*S*-Adenosylmethionine synthetase [*Litchi chinensis*]	2.00	VvTR3044
EC907689	6e-11	Phenylalanine ammonia lyase [*Arabidopsis thaliana*]	1.82	VvTR1889
EC907667	5e-26	Catalase [*Vitis vinifera*]	–3.32	VvTR789
EC907662	4e-50	Cinnamyl alcohol dehydrogenase [*Fragaria*×*ananassa*]	–1.62	VvTR12
Transcription regulation				
EC907668	2e-9	Putative replication factor A [*Capsium chinense*]	1.78	VvTR804
EC907681	1e-25	Histone H4 [*Hyacinthus orientalis*]	2.14	VvTR2601
EC907672	7e-107	*Vitis vinifera* putative transcription factor mRNA*	–2.03	VvTR1356
EC907666	4e-13	Nucleotide binding/WD-repeat protein like [*Arabidopsis thaliana*]	3.01	VvTR642
EC907665	2e-32	Putative LepA protein [*Oryza sativa* (*japonica* cultivar group)]	–1.62	VvTR396
Regulation of chloroplast gene expression				
EC907684	3e-30	29 kDaA ribonucleoprotein [*Nicotiana sylvestris*]/29 kDa ribonucleoprotein A, chloroplast precurssor (CP29A)/RNA binding protein 30 [*Nicotiana plumbaginifolia*]	1.95	VvTR2707
Cellular transport				
EC907677	2e-15	Tonoplast intrinsic protein [*Panax ginseng*]	1.75	VvTR1962
Protein modulation				
EC907669	2e-4	JP2 [*Daucus carota*]	2.46	VvTR912
No homology with database				
EC907673			2.01	VvTR1485
EC907679			2.24	VvTR2328
EC907680			3.16	VvTR2470
EC907675			–2.02	VvTR1711
EC907676			–1.80	VvTR1911

corresponding to pure compounds enabled the identification of caffeic acid, quercetin-3-*O*-glucoside, and shikimic acid (Table 3.)

Two principal components (PC1 and PC2) were considered as they explain 76.2% of the variation in the entire data set: PC1 accounts for 41.2% of the variation while PC2 accounts for 35.0%. PC2 discriminates both Regent and Trincadeira cultivars as it is shown by the score scatter plot (Fig. 5). The Wilcoxon Rank Sum test (non-parametric test) was performed in order to identify the metabolites that discriminate between both cultivars using spectral intensities at different chemical shifts (δ 0.4–10.0). The metabolites that were found to be statistically different between both cultivars (P <0.05) are shown in Table 4. Regent was characterized by a higher amount of inositol, alanine, glutamine, glutamate, and caffeic acid and Trincadeira by a higher amount of α-glucose, succinic acid, and shikimic acid.

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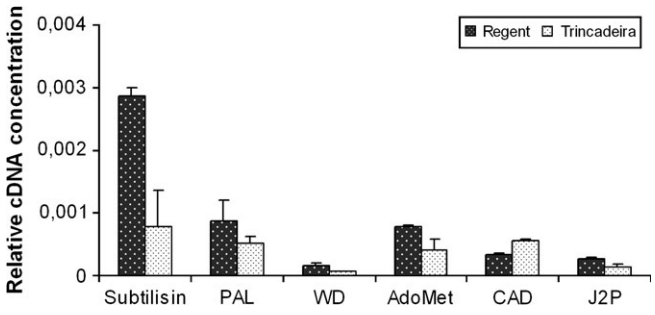

Fig. 1. Quantitative RT-PCR carried out with cDNAs encoding a subtilisin-like protease C1, phenylalanine ammonia lyase, WD-repeat protein like, *S*-adenosylmethionine sythetase, cinnamyl alcohol dehydrogenase, and J2P. Data were normalized by cDNA concentration (spectrofluorimeter Anthos Zenyth 3100). Values presented are the mean of three experiments; bars represent standard deviation. Graphs are plotted against relative cDNA concentration (*y*-axis) assessed by comparison with a standard curve.

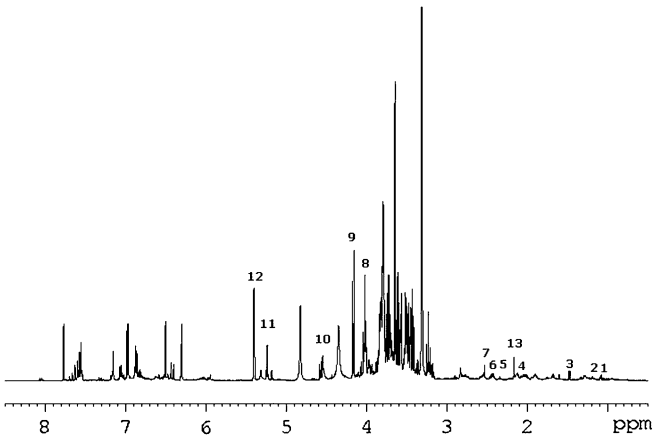

Fig. 2. ^{1}H NMR spectrum of Regent leaves in the range δ 0–8.5. 1, linoleic acid; 2, valine; 3, alanine; 4, glutamate; 5, proline; 6, glutamine; 7, succinic acid; 8, inositol; 9, H-1 of β-glucose; 10, frutofuran moiety of sucrose; 11, H-1 of α-glucose; 12, sucrose; 13, methionine.

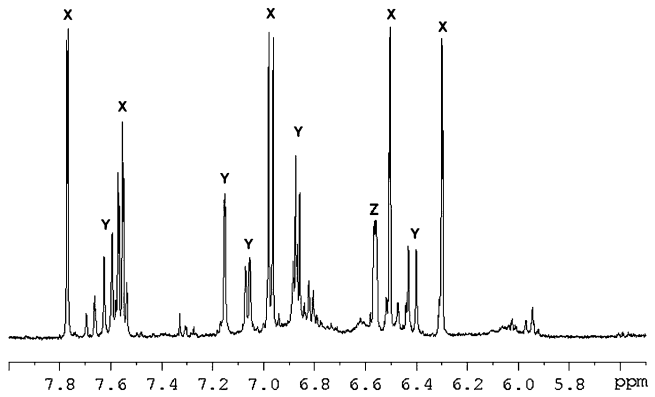

Fig. 3. ^{1}H NMR spectrum in the range δ 5.5–8.0. Y, Caffeic acid; X, quercetin-3-*O*-glucoside; Z, peak (δ 6.56) corresponding to shikimic acid. The chemical shift (ppm) is shown.

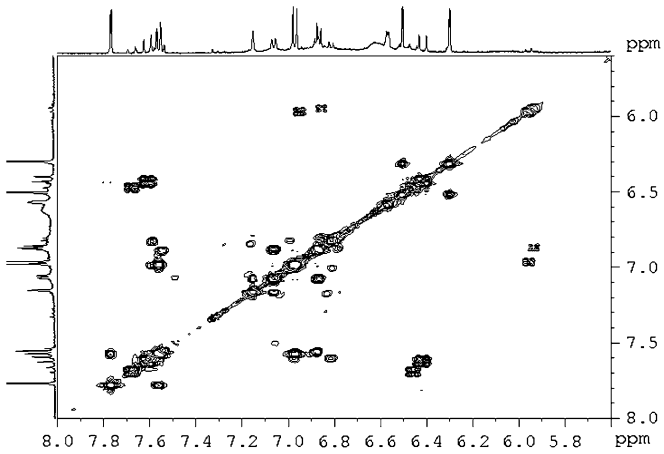

Fig. 4. COSY spectrum in the range δ 5.6–8.0. This 2D spectrum allows the determination of peak correlation. The chemical shift (ppm) is shown. As an example, the peak at δ 7.61 correlates with δ 6.42. Correlations between δ 7.16, δ 7.06, and δ 6.86; δ 7.77 and δ 7. 56; 7.56 and 6.97; 6.51 and 6.30 were also detected.

Discussion

With the progress of genomic initiatives to profile genome and gene expression in the context of plant–pathogen interaction, metabolic profiling is increasingly needed for a better understanding of plant defence mechanisms against various environmental stresses, including pathogen stress. In this context, knowledge of the defence capabilities of resistant cultivars such as Regent is gaining higher importance.

The combined metabolomic and transcriptomic approaches made it possible to identify transcripts and metabolites which could discriminate between Regent and Trincadeira (resistant and susceptible to mildews, respectively) without inoculation with a pathogen.

The rank products statistic (RP) was used in order to determine which transcripts are statistically relevant. Absolute fold change does not provide a significant estimate for the observed changes and the normal cut-off values (2-fold) are essentially arbitrary. According to Breitling *et al*. (2004), the difference between RP and fold change criteria is very important because RP is more robust against outliers. At the same time it provides a simple and straightforward procedure to determine the significance of an observed change. This way, transcripts statistically relevant by the rank product method and presenting a fold change lower that 2-fold were also considered.

Regent presents several up-regulated transcripts that are known to be associated with defence capabilities against stress, such as subtilisin-like protease, *S*-adenosyl methionine synthase, PAL, WD-repeat protein like, and J2P protein (Table 2).

Subtilisin-like proteases can play important roles in the regulation of biological processes such as the recognition

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Table 3. *Identification of the metabolites in the phenolic compound region: caffeic acid, quercetin-3-*O*-glucoside, and shikimic acid*

Chemical shift (δ), multiplicity, coupling constant, and ^{1}H–^{1}H correlation determined by 2D spectrum (COSY) analysis are shown.

Compound	Chemical shift	Multiplicity/coupling constant	Correlation ^{1}H–^{1}H
Caffeic acid			
	δ 7.61	(d, J= 16 Hz)	δ 6.42
	δ 7.16	(d, J=2 Hz)	δ 7.06, δ 6.86
	δ 7.06	(dd, J= 2 Hz, J= 8 Hz)	
	δ 6.86	(d, J=8 Hz)	
	δ 6.42	(d, J=16 Hz)	
Quercetin-3-*O*-glucoside			
	δ 7.77	(d, J=2 Hz)	δ 7.56
	δ 7.56	(dd, J=2 Hz, 8 Hz)	δ 6.97
	δ 6.97	(d, J=8.5 Hz)	
	δ 6.51	(d, J=2 Hz)	δ 6.30
	δ 6.30	(d, J= 2 Hz)	
Shikimic acid			
	δ 6.56	td (t, J=4 Hz; dd, J=1 Hz, 4 Hz)	δ 4.38
	δ 4.38	(t, J=4.5 Hz)	δ 3.67
	δ 3.97 (m)		δ 2.75, δ 2.18
	δ 3.67	(dd, J=4 Hz, 10 Hz)	
	δ 2.75	(dt, J=5 Hz, 18 Hz)	δ 3.97, δ 2.18
	δ 2.18	(dt, J=7 Hz, 18 Hz)	δ 2.75, δ 3.97

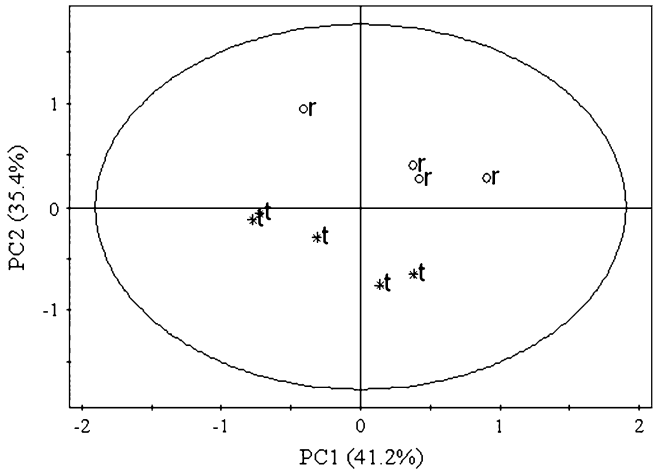

Fig. 5. Score scatter plot discriminating Regent (r) and Trincadeira (t) cultivars by using metabolic profiling coupled to principal component analysis. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm). The ellipse represents the Hotelling T2 with 95% confidence in score plots. PC2 discriminates between r and t, and explains 35% of the variance.

Table 4. *Identification of the metabolites that discriminate between* Vitis vinifera *Regent and Trincadeira cultivars*

+ represents the cultivar where significantly more of the compound had accumulated (P <0.05). Both chemical shift and P-value are shown.

Compound	Chemical shift (ppm)	*Vitis vinifera*		P-value
		Regent	Trincadeira	
α-Glucose	δ=5.18		+	0.0159
Inositol	δ=4.02	+		0.0159
Succinic acid	δ=2.54		+	0.0317
Alanine	δ=1.48	+		0.0159
Glutamine	δ=2.45; δ=2.13	+		0.0159
Glutamate	δ=2.04; δ=2.39	+		0.0159
Caffeic acid	δ=7.16; δ=6.42	+		0.0317
	δ=7.06; δ=6.86			0.0158
Shikimic acid	δ=6.56; δ=2.18; δ=2.75		+	0.0158
	δ=4.38			0.0317

of pathogens with consequent induction of defence responses (van der Hoorn and Jones, 2004). These proteases have been shown to accumulate after a pathogen attack (Tornero *et al*., 1996, 1997; Jorda *et al*., 1999; Golldack *et al*., 2003; Coffeen and Wolpert, 2004). As the plant material used in this study was not infected, we hypothesize that accumulation of subtilisin-like protease transcripts in Regent could participate in pathogen recognition upon infection and subsequent induction of the defence responses.

S-Adenosyl-L methionine synthase (AdoMet synthetase, EC 2.5.1.6) plays an important role in the production of *S*-adenosyl methionine which acts as precursor for the biosynthesis of several compounds involved in defence responses namely phenylpropanoids and polyamines (Roje, 2006). Phenylpropanoid compounds derived from the shikimic acid pathway are precursors of several phenolics, such as lignan, flavonoids, isoflavonoids, coumarins, and stilbenes. The deamination of phenylalanine by PAL (EC 4.3.1.5) is the branch point between primary metabolism (shikimate pathway; Herrmann, 1995) and secondary metabolism (phenylpropanoid synthesis) (Rohde *et al*., 2004). *Vitis vinifera* cv. Regent presents up-regulation of the transcripts coding for PAL while the transcripts coding for CAD are down-regulated.

CAD (EC 1.1.1.195) is a key enzyme in lignin biosynthesis (Blanco-Portales *et al*., 2002). Metabolome analysis revealed a significant (P <0.05) increased content

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of caffeic acid in Regent. All together, these data suggest that secondary metabolite production in Regent may be more activated than in Trincadeira (Fig. 6). Moreover, the down-regulation of CAD in Regent suggests that phenolic compounds are not being polymerized to lignin, thus, the general phenylpropanoid pathway leading to the accumulation of phenolic compounds may be more active than the monolignol-specific pathway (Fig. 6).

Compounds occurring constitutively in healthy plants are likely to represent chemical barriers to infection and may protect plants against attack by a wide range of potential pathogens (Creasy, 1999; Morrissey and Osbourn, 1999). Constitutive phenols are known to directly or indirectly confer resistance through activation of post-infection responses in the host (Dixon, 2001; Lavania *et al*., 2006). Analysis of transgenic tobacco plants with reduced levels of constitutive phenolic compounds resulting from sense inhibition of PAL expression revealed an important role of preformed phenolic compounds in limiting the extent of infection by a fungal pathogen (Maher *et al*., 1994). Tobacco plants overexpressing L-phenylalanine ammonia-lyase (PAL+) were shown to produce high levels of chlorogenic acid and to exhibit reduced susceptibility to infection with a fungal pathogen (Shadle *et al*., 2003). PAL activity has also been related to the accumulation of caffeic acid derivatives in *Echinacea purpurea* hairy root cultures (Abbasi *et al*., 2007). Also, the accumulation in Regent of caffeic acid, a compound presenting antimicrobial activity (Dixon, 2001; Iwaki *et al*., 2006) which inhibits the germination of zoospores of the pathogenic fungi *Phytophthora* spp.

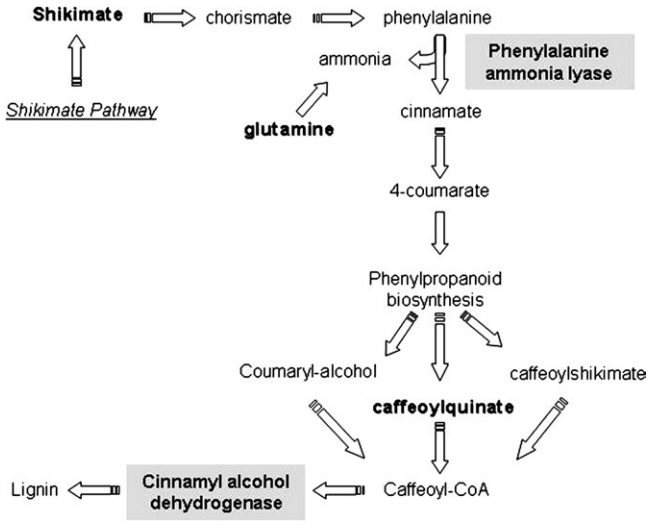

Fig. 6. Metabolic pathways leading to the synthesis of different phenylpropanoid compounds. The Metacyc database was used to elucidate metabolic networks (http://metacyc.org). The enzymes that presented different expressions in the transcriptomic approach are shown: phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase. Metabolites that accumulated in Regent are shown in bold. For simplicity, all reactions are shown as unidirectional.

(Widmer and Laurent, 2006), could be related to the inherent resistance of this cultivar to *Plasmopara viticola*. The constitutive up-regulation of PAL in Regent, together with the accumulation of caffeic acid, may be responsible for the higher protection of this grapevine cultivar to a possible pathogen attack.

WD repeat protein-like and J2P transcripts overexpressed in Regent may also be involved in the resistance of this cultivar. According to Park *et al*. (2004), the WD repeat protein-like may be involved in the regulation of secondary metabolism. J2P (a member of the J-protein family) is a molecular chaperone that regulates the activity of Hsp70 proteins (Walsh *et al*., 2004). J2P has been suggested to be related to the intrinsic resistance of a *Beta vulgaris* genotype (Puthoff and Smigocki, 2007).

Regent presents a constitutive accumulation of alanine. Although the precise function of stress-induced alanine in the cell is still unknown, it has been shown that added alanine stimulates a gene encoding for stress-protein synthesis in mammalian kidney, protecting cells against injury damage (Monselise *et al*., 2003). The constitutive accumulation of alanine in Regent may allow a faster accumulation of this compound upon fungus attack, contributing to an increased resistance to the pathogen. Moreover, the constitutive accumulation of inositol in resistant cultivars may be related to a higher signal perception and transduction capacity, enabling a rapid response to pathogen attack as suggested by Hamzehzarghani *et al*. (2005). Indeed, plants with higher levels of resistance to diseases present overproduction of inositol (Smart and Flores, 1997; Pritsch *et al*., 2000).

Regent also presents higher accumulation of glutamine and glutamate. Glutamine helps the plant cell to recycle ammonia ions liberated from phenylalanine through the action of PAL. Glutamine accumulation in Regent, together with the up-regulation of PAL, may be considered as evidence for a more active PAL pathway, which is corroborated by the accumulation of caffeic acid in this cultivar. Glutamate is a precursor of stress-related molecules (Lam *et al*., 1995; Hothorn *et al*., 2006) and, therefore, a higher pool of glutamate may lead to a faster production of these molecules upon a pathogen attack.

The metabolic profile of spikelets of the wheat cultivars Roblin and Sumai3, respectively susceptible and resistant to *Fusarium* head blight, revealed a constitutively higher production of inositol, glutamine, and phenolic compounds in a resistant cultivar of wheat (Hamzehzarghani *et al*., 2005). This corroborates the results obtained for *Vitis vinifera* cv. Regent. Upon infection, the resistant cultivar of wheat showed a higher inositol and glutamine accumulation and increase of cinnamic acid and phenylpropanoids compared with the susceptible cultivar which did not present these compounds constitutively (Hamzehzarghani *et al*., 2005). It is hypothesized that the same would probably occur in Regent after pathogen attack, since this

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V. vinifera cultivar also presents a higher constitutive abundance of inositol, glutamine, and caffeic acid.

Regent also presents a down-regulation of a catalase and a glutathione *S*-transferase. RT-PCR analyses of the plant material harvested in 2006 have shown a different regulation of these transcripts (data not shown) when compared with the material harvested in 2004, so it is hypothesized that catalase and glutathione *S*-transferase are not related to the innate resistance capabilities of Regent against pathogenic fungi, being probably related to environmental conditions like light intensity at the harvesting times. This means that their down-regulation can be related to a new redox balance generated through photosynthesis.

The comparison between these grapevine cultivars was accomplished without plant inoculation with a pathogen, thus the transcript and metabolite differences encountered cannot be related exclusively to the innate resistance capabilities of Regent, and probably other reasons may account for these differences. Several metabolites and transcripts identified in Regent are known to be associated with defence mechanisms. This way, the hypothesis that they may be associated with the resistance mechanism of Regent cannot be discarded. It is tempting to speculate that, as the majority of the genetic background of both cultivars is the same, the transcripts and metabolites that discriminate Regent from Trincadeira could be responsible for a faster response against pathogen attack, thus contributing to the higher resistance of Regent against downy and powdery mildew.

Conclusion

As far as is known, the work hereby reported on the distinction between resistant and susceptible grapevine cultivars not involving interaction between plant and pathogen, through a combination of both cDNA microarrays and ^{1}H NMR, has no precedent. The higher transcription of genes related to defence, such as PAL, *S*-adenosyl methionine synthase, subtilisin-like protease, WD-repeat protein like, and J2P protein and the accumulation of inositol, glutamine, glutamate, alanine, and caffeic acid in *V. vinifera* cv. Regent suggest that this cultivar presents a higher capacity to resist a pathogen attack. It also suggests that susceptible varieties, such as Trincadeira, possess the genetic information required to initiate a defence response. However, the perception and transduction pathways, probably being very slow, may not be able to switch-on in time for the defence mechanisms to cope with invasion of fungi. These preliminary results allow us to hypothesize that the resistant cultivar, Regent, may be able to activate the defence response in a rapid and more intensive manner. The hypotheses presented here have to be confirmed through studies in which both cultivars are inoculated with powdery or downy mildew. These studies have already been initiated.

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