Plant Pathology (2009) **58**, 170–180 Doi: 10.1111/j.1365-3059.2008.01904.x

Induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field

M. Hren[a]*, M. Ravnikar[a], J. Brzin[a], P. Ermacora[b], L. Carraro[b], P. A. Bianco[c], P. Casati[c], M. Borgo[d], E. Angelini[d], A. Rotter[a] and K. Gruden[a]

[a]*National Institute of Biology, Department of Biotechnology and Systems Biology, Večna pot 111, 1000 Ljubljana, Slovenia;* [b]*Università degli Studi di Udine, Dipartimento di Biologia Applicata alla Difesa delle Piante, Via delle Scienze 208, 33100 Udine;* [c]*Istituto di Patologia Vegetale, Via Celoria 2, 20133 Milano; and* [d]*C.R.A. Istituto Sperimentale per la Viticoltura, Sezione Biologia e Difesa, Viale XXVIII Aprile 26, 31015 Conegliano, Italy*

Interaction between phytoplasma and grapevine at the physiological level is still poorly understood, as are plant defence mechanisms against the pathogen. This study investigates the level of gene expression of three selected genes in a large number of grapevine plants belonging to six disease/cultivar groups (healthy Chardonnay, Bois noir-infected Chardonnay, Flavescence dorée-infected Barbera and Prosecco, and recovered Barbera and Prosecco). All plants were grown in vineyards in uncontrolled conditions in order to represent the physiology of disease as accurately as possible. Sucrose synthase was significantly upregulated in infected plants of all cultivars with the lowest *P*-values in cvs Chardonnay and Prosecco ($P < 0{\cdot}001$) and median fold-change around 2. This clearly indicates that carbohydrate metabolism changed in infected compared to healthy or recovered plants. Alcohol dehydrogenase I was significantly upregulated in infected relative to healthy Chardonnay plants ($P < 0{\cdot}05$) indicating that alcoholic fermentation, a sign of hypoxic conditions, was induced in infected plants. Heat shock protein 70 was upregulated in infected compared to recovered plants only in cv. Prosecco. Linear discriminant analysis showed that classification of samples into disease status groups based on gene expression was highly accurate (82%), indicating that the response of field-grown plants to phytoplasma infection at the level of expression of selected genes was so intensive and uniform that it was possible to detect it in grapevine plants regardless of natural variables.

Keywords: Bois noir, field study, Flavescence dorée, grapevine yellows, heat shock protein 70, linear discriminant analysis, real-time PCR

Introduction

Phytoplasmas are bacteria lacking a cell wall that live and reproduce in phloem sieve elements in plants (Lee *et al*., 2000; Garnier *et al*., 2001; Christensen *et al*., 2005) and in phloem-sucking homopterous insects (Webb *et al*., 1999; Christensen *et al*., 2005). The phytoplasmas associated with Bois noir (BN, stolbur group, 16SrXII-A, '*Candidatus* Phytoplasma *solani*') and Flavescence dorée (FD, elm yellows group, 16SrV, '*Candidatus* Phytoplasma *vitis*') diseases investigated in this study are the main cause in Europe of grapevine yellows (GY), a group of diseases caused by different phytoplasmas (Boudon-Padieu, 2003). GY are detrimental to infected plants, affecting vitality, reducing yields and decreasing the quality of vines, with high acid and low sugar content of clusters. Furthermore, control of the disease is so far limited only to control of insect vectors. Some phytoplasma-infected plants, however, cease to show the symptoms after several years of infection and no phytoplasma can be detected in their shoots. This phenomenon has been described as recovery, a presumed development of resistance to phytoplasma infection (Osler *et al*., 1993).

In general, experiments to study the interaction of phytoplasmas are difficult to set up, especially with woody plants like grapevine. The plant's response to phytoplasma infection has been studied mainly at the metabolite level, showing that phytoplasma infection affects sugar metabolism (Lepka *et al*., 1999; Maust *et al*., 2003; Choi *et al*., 2004). Only a few studies have been conducted at the level of gene expression, mainly on *Catharanthus roseus*, maize and fruit trees, showing that

*E-mail: matjaz.hren@nib.si

Published online 9 July 2008

genes involved in photosynthesis, and even response to stress, are differentially expressed in phytoplasma-infected plants (Jagoueix-Eveillard *et al.*, 2001; Carginale *et al.*, 2004). No gene expression data are so far available for grapevine – phytoplasma interaction.

Most studies of the plant stress response have been conducted under controlled conditions, as it was not clear whether the impact of individual stress would be discernible in the complexity of signalling networks that mediate the plant's response to environmental changes. When a population of field grown plants is subjected to plant pathogens or herbivores, the response of individual plants at the level of gene expression is a combination of different factors and is more diverse than in a population of plants grown under controlled conditions. This represents the situation in nature more realistically and comprehensively, which may also depend on overall fitness of the infected plant and not just on the stress under study. With the development of new technologies, such as high throughput molecular biological methods, it has been possible to investigate plant eco-physiological behaviour at the level of gene expression (Schmidt *et al.*, 2007).

In order to study the infection of grapevine plants with phytoplasma, three genes were selected for comparing infected, healthy and recovered plants at the level of gene expression: sucrose synthase (*SuSy*), alcohol dehydrogenase I (*Adh1*) and heat shock protein 70 (*Hsp70*). *SuSy* is involved in the metabolism of sucrose in the phloem (including breakdown into monosaccharides, glucose and fructose), the most common carbohydrate for the transport of photosynthates (Taiz & Zaiger, 2002). This gene is presumably affected by phytoplasma infection in an indirect manner: monosaccharides are the main source of energy for phytoplasmas but they lack the enzymes for sucrose degradation (Oshima *et al.*, 2004), and therefore presumably utilize the host plant *SuSy*. The accumulation of carbohydrates in the leaves and the reduced carbohydrate transport to the roots lead to several metabolic changes (inhibition of photosynthesis, decreased respiration in roots) which in the end contribute to the disease symptoms. *Adh1*, involved in alcoholic fermentation, was selected based on the hypothesis that phytoplasma infection causes, or at least intensifies, the already present hypoxic conditions (Dolferus *et al.*, 1994; van Dongen *et al.*, 2003) in the phloem of leaves, and thus switches the plant metabolism towards fermentative. *Hsp70* has been shown to be involved in the plant response to many abiotic stresses (Sung *et al.*, 2001a; Wang *et al.*, 2004), and was found to be differentially expressed in phytoplasma-infected *Prunus armeniaca* (Carginale *et al.*, 2004).

The expression profiles of the three genes were exploited to: i) determine how phytoplasmas affect the sugar metabolism and the stress response of infected plants; ii) determine whether the plants' response to phytoplasma infection can be distinguished from natural variations within the plant population caused by individual responses to microclimate, the extent of phytoplasma infection and grapevine cultivar; and iii) characterize the differences between and within populations of healthy, infected and recovered plants.

Materials and methods

Plant samples

Field-grown grapevine samples with different sanitary status regarding phytoplasma infection were collected in Goriška Brda, Slovenia (cv. Chardonnay), in Montebelluna, Italy (cv. Prosecco) and in Milano, Italy (cv. Barbera) during summer 2004. The sanitary status of Prosecco and Barbera plants had been monitored since 2000 and of cv. Chardonnay since 2004 by visual inspections (for fungal, bacterial and viral infections). In the case of Prosecco plants, absence of viral infections were additionally screened with ELISA (Agritest or Bioreba kits). All sampled plants were monitored for infection with other pathogens and no symptoms typical for viral diseases were observed.

Leaf midribs with 1–2 mm of leaf lamina on each side of the rib were cut in the field and immediately stored in liquid nitrogen. One shoot per plant was sampled except in the case of 10 Chardonnay plants where two shoots per plant were sampled (Table 3). In four Chardonnay plants both shoots were symptomless (Plant IDs 3, 4, 12 and 14), in the next four Chardonnay plants both shoots expressed symptoms (Plant IDs 19, 21, 27, 28 and 29) and in case of two Chardonnay plants one shoot had symptoms and the other was symptomless (Plant IDs 15 and 29). All samples of a particular cultivar were collected from 1–2 m above ground within one or two hours. The number and description of samples are summarized in Table 3. Chardonnay was infected with BN, and cvs Prosecco and Barbera with FD. Unfortunately, healthy Prosecco and Barbera samples were not available for this study.

RNA extraction and reverse transcription (RT)

Samples were ground with a pestle and mortar in liquid nitrogen. RNeasy Plant Mini Kit (Qiagen) with a modified protocol was used for total RNA extraction. One millilitre of RLC buffer (heated to 56°C), containing 10 μL mL^{-1} β-mercapto-ethanol (Sigma) and 10 mg mL^{-1} PVP Mw 40 000 (Sigma), was added to 100 to 150 mg of ground frozen plant material, vortexed vigorously, incubated for 3 min at 56°C and centrifuged 30 s at 10 000 g. The subsequent steps were performed according to the manufacturer's instructions, using only the supernatant. RNA was eluted twice using 50 μL of RNase free water (heated to 65°C) each time, with 5 min incubation at room temperature in between.

Sixty microlitres of total RNA were treated with 1 μL of DNase I (Invitrogen, Amplification Grade) in 80 μL reactions and quantified on 1% agarose E-Gels (Invitrogen) using GelPro Analyzer (Media Cybernetics) and Mass-Ruler Mix^{TM} (Fermentas) as mass standard. RNA was additionally quantified using a Nanodrop spectrophotometer (NanoDrop Technologies).

RNA was reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems) in 50 μL reactions with 2·5 μL MultiScribe RT enzyme (50 U mL^{-1}). RT mix included 2 μL of RNase Inhibitor (20 U μL^{-1}, Applied

Biosystems) and 50 pg of Luciferase control mRNA (Promega). Based on the quantity of RNA, samples were divided into three groups: 6–80 ng μL^{-1}, 81–160 ng μL^{-1} and 161–240 ng μL^{-1}, and adequate volumes of RNA samples were added to the RT reaction in order not to exceed the capacity of the RT kit (0·1–10 μg total RNA). RNA was denatured at 80°C for 5 min prior to the RT reaction, which was carried out according to the kit's specifications in GenAmp 9700 (Perkin Elmer).

PCR and RFLP analysis of field-grown grapevine plants

In order to confirm the disease status of the plants from which the samples were collected and to identify the phytoplasma type in infected plants, each shoot was tested using a conventional PCR method (Chardonnay samples) or with nested PCR-RFLP (Prosecco and Barbera samples).

In the case of BN-infected Chardonnay, the DNA was extracted and conventional PCR reactions (Daire *et al.*, 1997; Clair *et al.*, 2003) were conducted as described in Hren *et al.* (2007). Extractions of FD-infected samples and nested PCR-RFLP assays were performed according to Angelini *et al.* (2001).

Publicly available sequences of transcripts from GenBank® and DFCI Grape Gene Index (formerly known as VvGI at TIGR) databases were analyzed in the set-up of quantitative real-time PCR reactions (qPCR), with SYBRGreen I chemistry for three target genes, *Adh1*, *SuSy* and *Hsp70*. Primer Express© software (Applied Biosystems) was used for the design of primer pairs. Specificities of the designed amplicons were tested *in silico* with a Basic Local Alignment Search Tool (BLAST) search of public databases.

All real-time PCR reactions were performed on an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) in 384-well plate format using universal cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, 9600 Emulation) which allowed all reactions to be run on the same plate. Real-time PCR was performed in a final reaction volume of 10 μL containing 2 μL of sample DNA, 900 nM of each primer and 1x SYBR® Green PCR Master Mix (Applied Biosystems), which includes ROX™ as a passive reference dye. Each sample DNA was tested with: i) three target genes: *Adh1*, *SuSy* and *Hsp70*; ii) two endogenous controls (reference genes): cytochrome oxidase (*cox*, Weller *et al.*, 2000) and *18S* (Eukaryotic 18S rRNA TaqMan endogenous control, Applied Biosystems) used in the normalization process; and iii) exogenous luciferase control (*luc*, Toplak *et al.*, 2004) to check the efficiency of the RT reaction. The last three amplicons were based on TaqMan® chemistry. Each reaction was performed in two replicate wells in two dilutions on the same 384-well plate. An automated liquid handling system (Multiprobe® II PLUS EX, PerkinElmer) was used to prepare cDNA dilutions and to pipette large numbers of cDNA samples and master mixes onto the 384-well plates. The dilution factors were determined individually for each amplicon on a subset of samples, in order for the cycle threshold (Ct) values to be in the range 22–34.

The software SDS 2·1 (Applied Biosystems) was used for fluorescence acquisition and Ct calculation. For this calculation, the baseline was set automatically and the fluorescence threshold set manually (0·08) to intersect with the linear part of the amplification curves of all amplicons in all runs.

Melting curve analysis was performed with SDS 2·1 software after each run for each of the designed amplicons, in order to detect unspecific amplification (in wells with samples) and primer-dimer formation (in non-template control wells): samples were heated to 95°C then cooled to 60°C and heated again to 95°C. During the last step of heating the change in fluorescence was recorded. Dissociation curves (plots of change of fluorescence against temperature) were analyzed and melting temperatures of peaks (T_m) were determined.

The relative quantification approach was used basically as described in Pfaffl (2001). The amplification efficiency and linear range of amplification were followed for each amplicon on each plate by analyzing one randomly chosen sample in five dilution steps of cDNA with two replicate wells per dilution step (range of dilution factors: 10 to 10^5). Each sample was analyzed in two dilutions and two replicates per dilution step. Only samples where the ΔCt between two dilutions of target gene did not deviate by more than 0·5 from ΔCt of the reference gene were used for relative quantification. Additionally, the ΔCt values between the two dilutions of cDNA were calculated for each sample and used to calculate approximate amplification efficiencies ($E = 10^{[1/\Delta Ct]}$) for each sample individually. These sample-specific efficiencies were used in the relative quantification formula.

The information obtained with the *luc* amplicon was used only to verify the success of RT reaction and not in the normalization process. The geometric mean of Ct values of *18S* and *cox* was used as the final reference (Vandesompele *et al.*, 2002). A relative expression ratio (eqn 1) was calculated separately for each dilution of each sample and averaged to yield the final relative expression ratio for the sample. The ratio was then $\log_2$ transformed. For comparison, the relative expression was additionally calculated using *cox* and *18S* as separate reference genes. All mathematical operations were performed in Microsoft Excel.

$$R = \frac{E_{target}^{\Delta Ct_{target}(control-sample)}}{E_{reference}^{\Delta Ct_{reference}(control-sample)}} \quad \text{(eqn 1)}$$

Equation 1 provides a mathematical model of relative expression ratio in real-time PCR: E_{target} is the real-time PCR efficiency of the target gene transcript (Adh1, SuSy or *Hsp70*) calculated for individual samples; $E_{reference}$ is the real-time PCR efficiency of the reference gene transcript (*18S*/*cox*) calculated for individual samples; ΔCt_{target} (control-sample) is the difference in Ct values of target gene transcripts between control (healthy Chardonnay sample 5) and 'unknown' samples; $\Delta Ct_{reference}$(control-sample) is the difference in Ct values of reference gene transcripts between the control and 'unknown' sample.

Each sample was analyzed for the presence of phytoplasmas and quantified by real-time PCR: phytoplasmas

associated with BN were detected with BNgen amplicon (amplifying genomic phytoplasmal DNA) and phytoplasmas acssociated with FD with UniRNA amplicon (amplifying ribosomal phytoplasmal DNA) (Hren *et al*., 2007). Quantification of phytoplasmas was performed in the same way as for *Adh1*, *SuSy* and *Hsp70*, using *18S/cox* as reference genes and samples 39 and 73 as control samples for phytoplasmas associated with BN and FD, respectively. The relative quantities of phytoplasmas associated with BN and FD were then scaled.

Statistical analysis of gene expression data

The Welch two sample *t*-test was used to determine statistically significant differences between relative expression ratios of infected and healthy samples (Chardonnay) and between infected and recovered samples (Prosecco and Barbera), with a $P = 0{\cdot}05$ as the limit for statistical significance.

For further analysis, expression data from *Adh1*, *SuSy*, *Hsp70* and phytoplasma quantity were centred and scaled to ensure a similar distribution of data for all quantitative results. *Scale* function in R software environment (R Development Core Team, 2005) was used to perform the operation.

Linear discriminant analysis (LDA, Quinn & Keough, 2002) was used to test the appropriateness of the selected three variables (relative gene expression of *Adh1*, *SuSy* and *Hsp70*) for classification of individual grapevine samples within the pre-determined groups (supervised classification). In the first LDA setting three pre-determined groups were formed based on disease status: healthy, infected and recovered. In the second setting six pre-determined groups were formed based on the combination of disease status and cultivar: healthy Chardonnay (Hc), infected Chardonnay (Ic), recovered Barbera (Rb), infected Barbera (Ib), recovered Prosecco (Rp) and infected Prosecco samples (Ip). Discriminant function scores for each grapevine sample were plotted on an LDA plot and positions of samples on plots were analyzed. LDA was performed in a bioconductor package MASS using *lda* function in R (Venables & Ripley, 2002).

Results

Real-time PCR design

Amplicons *Adh1*, *SuSy* and *Hsp70* were designed using the nucleotide sequences available in the public nucleotide database GenBank® (*Adh1*) and DFCI Grape Gene Index (*SuSy* and *Hsp70*). Primer pairs were designed close to the 3′ end of the transcripts to ensure better specificity (Table 1).

BLAST search of public databases showed no unspecific hits with high similarity to the amplicon sequences ($E < 0{\cdot}04$). Melting curve analysis for all the designed amplicons detected no unspecific products or primer-dimer formation in amplification of samples or in the no-template-control (NTC) reactions (results not shown).

All amplicons were validated on serial dilutions of seven samples (in duplicate wells, dilutions ranging from 10-fold to 10^5-fold). Each sample was run on a separate 384-well plate. Performance characteristics (slope, efficiency and correlation coefficient between duplicate samples) were appropriate for reliable quantitative analysis (Table 2). Validation data was used to select the most appropriate dilutions of cDNA samples for each gene in order to obtain Ct values ranging from 22 to 34.

Table 1 Characteristics of primer pairs for real-time PCR amplification of *Adh1*, *SuSy* and *Hsp70*

Gene	Orientation	Sequence (5′–3′)	Amplicon length	Reference sequence used in design process and position of amplicon
Adh1	Forward	AAG GTG ATC TTG GGT GAC TTT CA	72 bp	AF194173 (1220–1291 bp)
	Reverse	CAA CCA GAC AGA TGC TCT CTT TCA		
SuSy	Forward	TGT TAA GGC TCC TGG ATT TCA ATT A	71 bp	TC38393 (2741–2817 bp)
	Reverse	AGC CAA ATC TTG GCA AGC A		
Hsp70	Forward	CGG AGA AAT GCG GCT GAT A	71 bp	TC38947 (2175–2245 bp)
	Reverse	TCC CTT TAC TTC CAC CGC TAG A		

Table 2 Performance characteristics of *Adh1*, *SuSy*, *Hsp70*, *18S* and *cox* genes. The data for *18S*/*cox* were calculated from the geometric mean of corresponding *cox* and *18S* Ct values. Mean values of seven samples (each run in duplicate wells) are represented with standard deviations. r^2: correlation coefficient; *E*: efficiency of amplification

Amplicon	Linear regression			Ct range	Sample dilution factors used for quantification quantification
	Slope	r^2	*E*		
Adh1	–3·473 ± 0·194	0·993 ± 0·005	0·945 ± 0·075	22·4–33·6	10^2–10^3
SuSy	–3·410 ± 0·140	0·967 ± 0·041	0·967 ± 0·054	23·4–34·6	10^2–10^3
Hsp70	–3·386 ± 0·086	0·995 ± 0·005	0·975 ± 0·034	23·4–33·5	10^2–10^3
18S	–3·588 ± 0·079	0·999 ± 0·001	0·887 ± 0·027	15·8–24·5	10^3–10^4
cox	–3·549 ± 0·086	0·998 ± 0·003	0·914 ± 0·030	26·1–34·0	10^3–10^4
18S/*cox*	–3·381 ± 0·286	0·993 ± 0·013	0·987 ± 0·127	19·7–27·8	/

Table 3 Details of grapevine cultivar, disease status, phytoplasma type detected by PCR/PCR-RFLP, within plant replica (Plant ID), relative expression values for *Adh1*, *SuSy* and *Hsp70* genes, and information on phytoplasma activity (qPCR result). All qPCR data are $\log_2$ transformed and scaled. Hc: healthy Chardonnay; Ic: infected Chardonnay; Ib: infected Barbera; Rb: recovered Barbera; Ip: infected Prosecco; Rp: recovered Prosecco

Sample[a] ID	Plant ID	Phytoplasma type[b]	Sample status[c]	Last year of infection	Infected since	qPCR (relative expression value, $\log_2$ transformed, scaled)			Phytoplasma infection (activity)
						Adh1	*SuSy*	*Hsp70*	
1	1	/	Hc			0·588	–0·918	0·549	/
3	2	stolbur	Ic			–0·565	–0·105	–0·858	19·916
5	3	/	Hc			–1·453	–1·740	0·641	/
6	3	/	Hc			–1·009	–1·021	–0·902	/
7	4	/	Hc			–0·287	–0·425	1·570	/
8	4	/	Hc			–0·984	–1·683	0·273	/
14	7	/	Hc			–0·754	–0·811	0·841	/
16	8	/	Hc			–1·152	–0·991	–0·270	/
17	9	/	Hc			–1·565	–1·321	0·449	/
19	10	/	Hc			–0·285	–0·645	1·842	/
23	12	/	Hc			–0·685	–1·069	1·016	/
24	12	/	Hc			–0·515	–1·770	1·004	/
26	13	/	Hc			–1·129	–1·075	1·060	/
27	14	/	Hc			–1·128	–1·208	1·278	/
28	14	/	Hc			–0·143	–0·748	0·452	/
29	15	/	Hc			–0·428	–0·618	1·881	/
30	15	stolbur	Ic			0·450	0·058	–0·218	20·395
32	16	/	Hc			–0·731	–0·339	0·919	/
36	18	stolbur	Ic			3·281	2·166	0·573	1·643
37	19	stolbur	Ic			3·049	1·555	–1·670	52·648
38	19	stolbur	Ic			2·686	1·966	–0·310	27·568
39	20	stolbur	Ic			–0·027	0·086	1·527	0·000[e]
41	21	stolbur	Ic			–0·412	–0·035	0·861	1·998
42	21	stolbur	Ic			–0·349	–0·127	1·270	5·000
43	22	stolbur	Ic			0·732	0·548	0·966	2·728
45	23	stolbur	Ic			0·084	0·550	1·169	3·421
47	24	stolbur	Ic			–0·333	0·047	0·498	/
49	25	/	Hc			–0·035	–0·192	–0·013	/
52	26	/	Hc			–0·738	–0·280	1·246	/
53	27	stolbur	Ic			–0·559	0·304	0·511	0·075
54	27	stolbur	Ic			–1·008	0·523	0·330	0·235
55	28	stolbur	Ic			0·095	0·331	1·614	/
56	28	stolbur	Ic			–0·321	–0·196	1·336	0·633
57	29	stolbur	Ic			–1·168	–0·850	–0·763	2·924
58	29	/	Hc			–0·887	–1·351	–0·721	/
59	30	stolbur	Ic			–0·634	–0·190	0·989	1·570
63	31	FD-D	Ib		2000	–0·277	1·021	–0·206	/
64	32	FD-D	Ib		2000	1·139	1·473	0·533	149·619
69	33	FD-D	Ib		2000	–0·172	1·656	–0·059	[f]
70	34	FD-D	Ib		2000	1·161	1·112	0·598	95·296
73	35	/	Rb	2002	2000	0·944	–0·739	–0·599	0·000[e]
74	36	/	Rb	2002	2000	0·683	0·453	0·817	/
75	37	/	Rb	2001	2000	0·089	0·446	0·474	/
77	38	/	Rb	2002	2000	–0·545	–0·520	–0·550	/
78	39	/	Rb	2002	2000	–0·544	–1·205	–1·514	/
80	40	/	Rb	2003[d]	2003	1·013	1·201	0·557	/
82	41	FD-C	Ip		2001	0·114	0·432	–0·303	/
83	42	FD-C	Ip		2004	0·492	1·140	–1·029	/
84	43	FD-C	Ip		2004	0·306	0·595	–0·735	61·952
86	44	FD-C	Ip		2004	1·241	0·870	–0·947	96·938
87	45	FD-C	Ip		2001	1·447	2·055	–0·564	99·649
88	46	FD-C	Ip		2000	0·336	0·708	–1·006	80·408
90	47	FD-C	Ip		2000	0·807	0·594	–1·102	81·239
91	48	FD-C	Ip		2000	0·998	1·726	–0·803	111·089
92	49	FD-C	Ip		2004	1·239	2·014	–0·112	91·549
93	50	/	Rp	2001	2000	0·601	–0·813	–1·296	/

Table 3 *Continued*

Sample[a] ID	Plant ID	Phytoplasma type[b]	Sample status[c]	Last year of infection	Infected since	qPCR (relative expression value, log_2 transformed, scaled)			Phytoplasma infection (activity)
						Adh1	*SuSy*	*Hsp70*	
94	51	/	Rp	2001	2000	–0·063	–0·585	–0·464	/
95	52	/	Rp	2002	2000	0·908	–1·127	–1·557	/
96	53	/	Rp	2001	2000	0·256	0·172	–1·304	/
98	54	/	Rp	2001	2000	0·568	–0·944	–1·399	/
99	55	/	Rp	2001	2000	1·035	–0·601	–1·713	/

[a]Sample IDs are not continuous because some samples were omitted (samples where ΔCt between the two dilutions of the target gene deviated by more than 0·5 from the ΔCt between the two dilutions of the reference gene).
[b]Identified by PCR/RFLP.
[c]Hc: healthy Chardonnay; Ic: infected Chardonnay; Ib: infected Barbera; Rb: recovered Barbera; Ip: infected Prosecco; Rp: recovered Prosecco.
[d]Only mild symptoms in 2003, no symptoms before or after.
[e]Samples 39 and 73 had the lowest phytoplasma content and were therefore used as calibrator samples in the process of normalisation of the phytoplasma content. After scaling, the phytoplasma content in those two samples appeared as 0.
[f]Sample contained phytoplasmas but due to signal in only one dilution (average Ct 35·2) the presence of phytoplasmas could not be quantified.

Presence of phytoplasma in analyzed samples

All plants sampled for experiments were analyzed for the presence of phytoplasmas associated with BN and FD using the PCR or PCR-RFLP identification system. The disease status (symptoms and PCR-RFLP detection of phytoplasmas) of Prosecco and Barbera plants that were included in the study had been carefully monitored since the year 2000, and the history of the disease, which is especially important in the case of recovered plants, is thus known (Table 3). Phytoplasma associated with BN was identified in cv. Chardonnay with symptoms, while two phytoplasma isolates associated with FD (Martini *et al*., 2002) were found in the other two varieties: FD-D in all the affected Barbera plants and FD-C in all the Prosecco plants showing symptoms (Table 3). In addition, each cDNA sample collected for gene expression analysis was analyzed for local presence and quantity of phytoplasmas using qPCR. This allowed the determination of the quantity of phytoplasma in exactly the same sample subjected to gene expression analysis. RNA-based detection of phytoplasmas also provided the confirmation of physiological activity of phytoplasmas so that samples possibly harbouring nonviable pathogens could be eliminated (Table 3).

Phytoplasmas were detected by qPCR in all Chardonnay samples showing symptoms, in all but one Barbera samples (63) and in all but two Prosecco samples (82, 83). Phytoplasmas were absent from all healthy symptomless and recovered samples. Sample 73 (recovered Barbera) was an exception, containing a barely detectable level of phytoplasmas. Prosecco plants from which these two samples originated showed symptoms and were phytoplasma-positive using the PCR-RFLP assay (Table 3), indicating that plants were infected and that phytoplasmas were most probably unevenly distributed within the plants. Another argument supporting this is the fact that these two samples behaved like the rest of infected Prosecco samples based on gene expression analysis and were therefore treated as such throughout the analysis.

Expression of the selected genes

Detailed expression results for all samples are presented in Table 3. Welch two sample *t*-test on relative expression values for these samples showed that *Adh1* and *SuSy* were significantly up-regulated in samples from infected Chardonnay plants relative to samples from healthy plants (Fig. 1a). However, *Hsp70* showed no significant difference in expression. Three infected samples (36, 37 and 38) appeared as outliers in *Adh1* and *SuSy* boxplots of Chardonnay samples, the last two originating from the same plant. Even if the three outliers were removed from analysis, *Adh1* and *SuSy* still showed significant difference in gene expression (data not shown). Sample 6 appeared as an outlier in *Hsp70* boxplot.

In the case of recovered versus infected samples of cvs Prosecco and Barbera, *SuSy* was differentially expressed (up-regulated in infected samples, Fig. 1b), a similar response to that observed between healthy and infected Chardonnay samples. In the case of recovered Prosecco, samples 96 and 94 appeared as outliers for *SuSy* and *Hsp70*, respectively (Fig. 1c). *Hsp70* was only upregulated in infected Prosecco samples.

Linear discriminant analysis of gene expression data

Linear discriminant analysis (LDA) was performed in two different settings. First, the information on cultivars was discarded and the samples were grouped into three pre-determined groups according to their disease status (healthy, infected and recovered; Fig. 2). Secondly, the information on cultivars was added, producing six pre-determined groups of samples (Hc, Ic, Rb, Ib, Rp and Ip; Fig. 3). Expression data of *Adh1*, *SuSy* and *Hsp70* genes were used as variables in order to assign the samples into the pre-determined groups. LDA results were visualized by plotting scores of discriminant function 1 (LD1, X axis) *versus* scores of discriminant function 2 (LD2, Y axis). The proportion of the variance explained by LD1

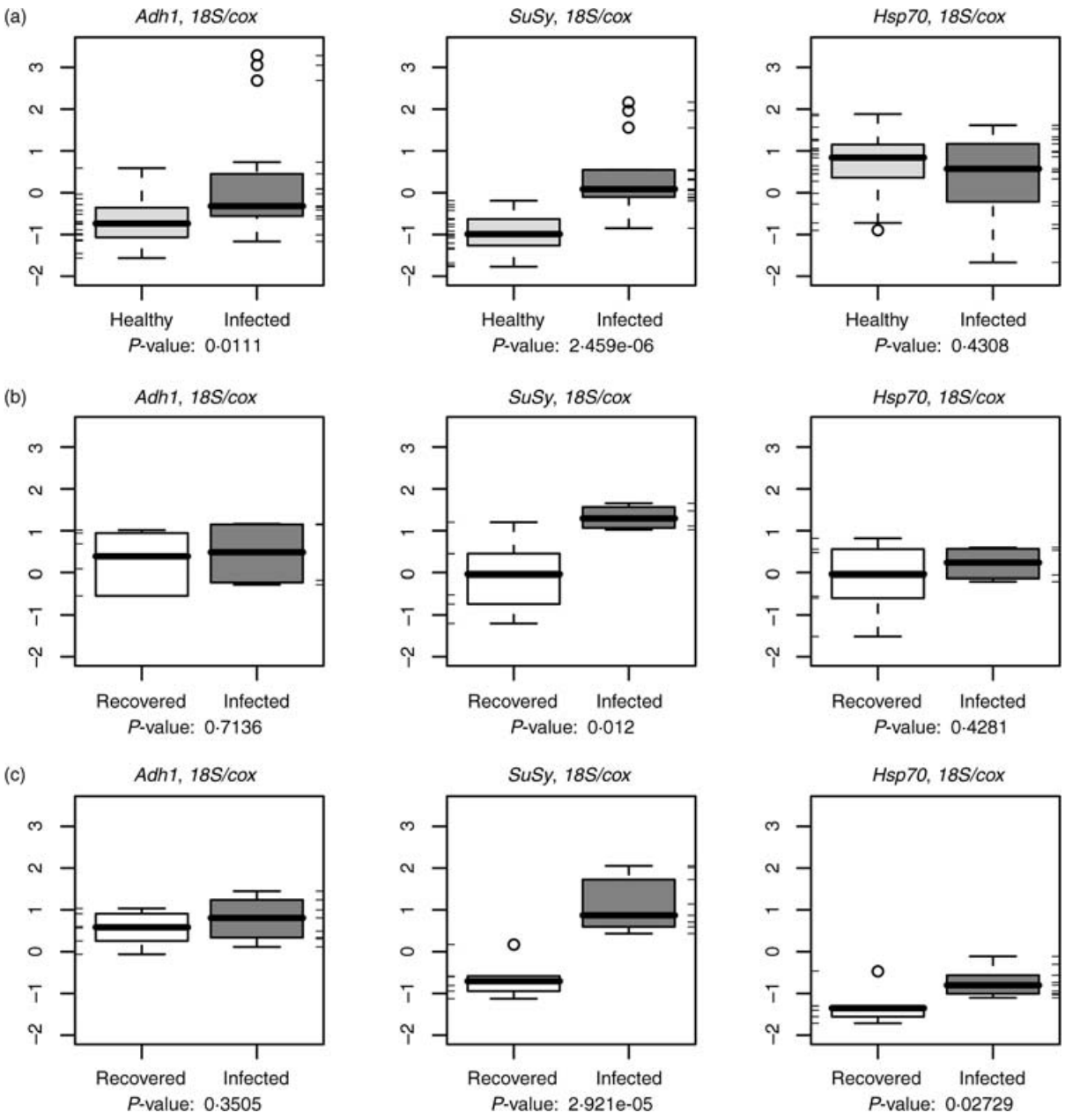

Figure 1 Boxplots showing relative expression ratios (log2 transformed) of *Adh1*, *SuSy* and *Hsp70* genes normalized using *18S*/*cox* (geometric mean of *18S* and *cox* Ct values) in: healthy and phytoplasma-infected Chardonnay samples (a), infected and recovered Barbera (b) and Prosecco samples (c). Corresponding *P*-values (Welch two sample *t*-test) are plotted under each comparison. Individual data points are represented on the y-axis as tick marks.

was very high, 78·2% in the first LDA (no information on cultivars included) and 74·3% in the second LDA setting (information on cultivars included). Proportions of variance explained by LD2 were 21·8 % and 24·3% in the first and second LDA settings, respectively.

LDA was also used to predict the classification of samples into pre-determined groups based on the input data. The predicted classification regions with linear boundaries were calculated and included in the plots (coloured regions in LDA plots). Therefore the degree of correlation between real and predicted data can be assessed visually. Samples belonging to the three pre-determined groups (healthy, infected and recovered) appear in the plot as clusters. Infected samples grouped to the right side, healthy samples to the left and recovered samples to the bottom of the plot (Fig. 2). The percentage of samples correctly positioned in the predicted region (i.e. correctly classified samples) was 82·0%. Inclusion of information on the cultivars led to only slight changes in the position of the samples on the plot (Fig. 3). Samples

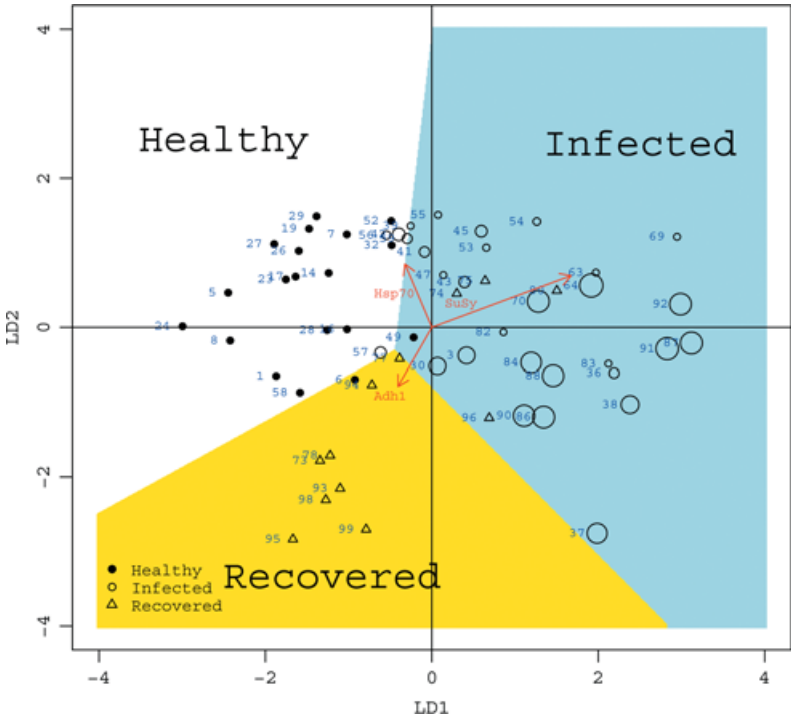

Figure 2 Linear discriminante analysis (LDA) plots showing expression data with *18S*/*cox* (geometric mean) used as a reference gene. LDA with three variables (expression values of *Adh1*, *SuSy* and *Hsp70*) and three pre-determined grapevine-groups (Healthy, Infected and Recovered) were included. Expression values were scaled and $\log^2$ transformed. The information on the rate of phytoplasma infection is displayed as the size of the plotting characters (the bigger the plotting character the more severe infection). The coefficients of linear discriminants for expression of all three genes are plotted as arrows, pointing towards the coefficients' coordinates. The length and direction of arrows (vectors) indicate the contribution of a particular gene to the variability in the data. Coloured areas denote predicted classification regions for determining disease status.

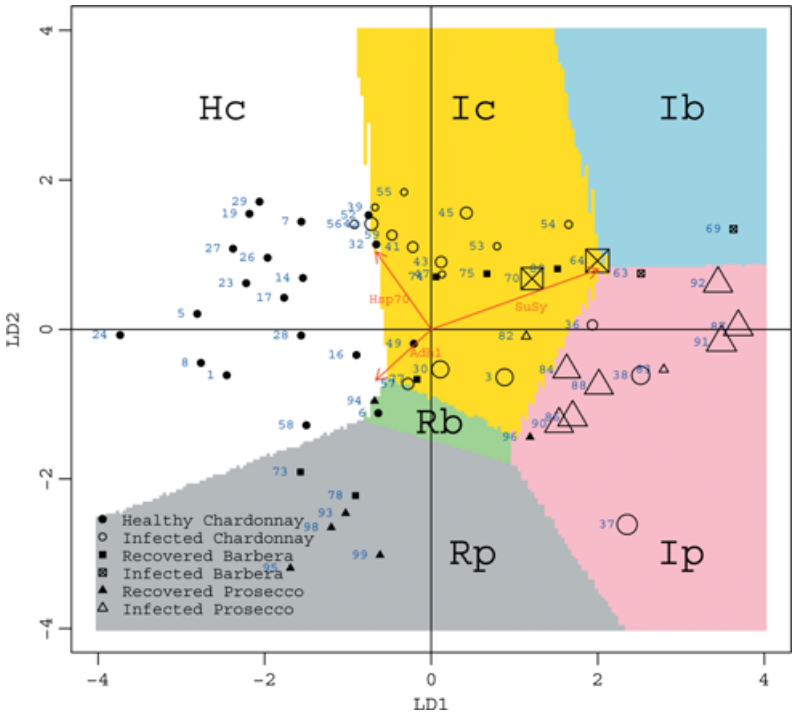

Figure 3 Linear discriminant analysis (LDA) plots with expression data with *18S*/*cox* (geometric mean) used as a reference gene. LDA with three variables (expression values of *Adh1*, *SuSy* and *Hsp70*) and six pre-determined grapevine groups (Hc, Ic, Rb, Ib, Rp and Ip) – the information on cultivar were included. Expression values are scaled and $\log^2$ transformed. The information on the rate of phytoplasma infection is displayed as the size of the plotting characters (the bigger the plotting character the more severe infection). The coefficients of linear discriminants for expression of all three genes are plotted as arrows, pointing towards the coefficients' coordinates. The length and direction of arrows (vectors) indicate the contribution of a particular gene to the variability in the data. Coloured areas denote predicted classification regions for determining disease status and cultivar.

appeared to be slightly more dispersed, those belonging to a certain disease status being additionally split according to the cultivar. This could be observed quite clearly when the predicted regions were co-plotted. The percentage of correctly classified samples still remained high but was decreased to 72·1%.

By plotting the coefficients of linear discriminants for expression of the three genes as arrows pointing towards the coefficients' coordinates on LDA plots, the influence of each component on the distribution of samples was visualized. It appears that the component with the greatest influence is *SuSy*, pointing towards the region of infected

samples (Figs 2 and 3), which indicates that this gene is most responsible for distinguishing between infected and healthy/recovered samples (Figs 2 and 3, X-axis), as was already suggested by low *P*-values in *t*-tests. The vector showing the contribution of *Hsp70*, almost perpendicular to that for *SuSy*, is the component mostly responsible for distinguishing the healthy samples from the other disease statuses (Figs 2 and 3, Y-axis). *Adh1* was found to contribute to distinguishing recovered samples from infected and recovered samples.

The extent of phytoplasma infection also plays a role in the position of samples in LDA plots. Although this information was not directly included as a variable into LDA, the samples with the greatest infection rate are seen to appear more to the right side of the plot, along the vector that represents the contribution of the *SuSy* component. Thus the extent of phytoplasma infection affects the position of samples in the LDA plot through the higher expression of the three genes.

Most samples were classified correctly. Only three Chardonnay samples (6, 49, 56) and two Prosecco samples (94, 96) were positioned outside predicted regions. Three Ic samples (36, 37 and 38) had extreme expression values of *SuSy* and low expression values of *Hsp70*, which positioned them deep into the region of infected samples, among infected Prosecco samples (they also appeared as outliers in boxplots in Fig. 1a). In the case of cv. Barbera samples greater biological variation was observed, especially in recovered samples. However, all infected samples were positioned within the infected region in the plot, although two were positioned close to infected Chardonnay samples (64, 70). More conclusive results for this cultivar would probably be obtained by including a greater number of Barbera samples. This is supported by the increase in percentage of correctly classified samples when Barbera samples are excluded from LDA (88·2% without information on cultivars and 80·4% when information on cultivars is included; LDA plots not shown).

Differences within plants in expression of selected genes

Two samples per plant were collected for eight Chardonnay plants, each from a separate shoot, to assess within plant variability in the expression of selected genes (four plants with and four plants without symptoms). Variability in expression of the analyzed genes between two shoots of the same healthy plant was similar to the variability between plants (samples 5–6, 7–8, 23–24, 27–28), as sample pairs do not cluster any closer than other healthy samples (Figs 2 and 3). However, infected sample pairs (samples 37–38, 41–42, 53–54, 55–56) have more similar expression patterns as they cluster more closely together (Figures 2 and 3). Both conclusions were confirmed by two hierarchical clustering analyses, one performed only with Hc and the second only with Ic samples (data not shown).

In the case of two BN-infected plants, (one shoot with, and the other shoot without symptoms) the presence of active phytoplasmas was only confirmed in the shoot with symptoms (Table 3). Samples 29–30 are positioned correctly within the predicted classes in the LDA plot, while samples 57–58 appear closer together, 58 correctly classified and 57 at the border, with the recovered samples having low expression of both *SuSy* and *Adh1*.

Discussion

Phytoplasmas are mostly abundant in phloem sieve elements, therefore central leaf veins were sampled in order to limit the analysis to the tissue where the physiological impact of the pathogen is expected to be the greatest. When phytoplasmas colonize phloem sieve cells they act as an additional sink for photosynthesized carbohydrates and block sugar transport from leaves to sink tissues (the plant's response to infection) (Maust *et al*., 2003). Therefore, soluble sugars and starch start to accumulate in infected source leaves and are depleted in roots (Lepka *et al*., 1999; Choi *et al*., 2004). Because phytoplasmas lack enzymes for sucrose utilisation (Oshima *et al*., 2004) they might use fructose or glucose as a source of energy. It was shown that, after phytoplasma infection, carbohydrate metabolism changed at the transcription level. Sucrose synthase (*SuSy*) gene expression was greater in leaf veins of infected than in healthy Chardonnay plants, and greater in infected than in recovered Barbera and Prosecco plants (Fig. 1). This confirms the hypothesis that the increase in demand for simple sugars is probably compensated by the upregulation of plant *SuSy* in infected tissue.

Phloem is a tissue in which hypoxic conditions are normally present (van Dongen *et al*., 2003). The hypothesis was that hypoxic conditions become even more severe after phytoplasma infection. This would be expected to result first from inhibition of photosynthesis and secondly, from increased energy consumption because of an additional phytoplasmic carbohydrate sink and because of increased consumption of energy in the biosynthesis of many metabolites which are part of the plant's response to the infection (Choi *et al*., 2004). Hypoxic conditions have at least two effects: i) they stimulate activity and transcription of *SuSy*, which controls the energetically less demanding degradation of sucrose to hexoses (Zeng *et al*., 1998; Geigenberger 2003), and ii) they cause plants to switch to fermentative metabolism (alcoholic fermentation), in which alcohol dehydrogenase I plays a major role (Dolferus *et al*., 1994; Gibbs *et al*., 2000; Taiz & Zaiger, 2002). Besides upregulation of *SuSy*, higher expression of *Adh1* has also been shown in infected compared to healthy Chardonnay plants, which supports the view that alcoholic fermentation is activated in infected tissue. Interestingly, the expression levels of *Adh1* in recovered and infected samples of Prosecco and Barbera were the same (Fig. 1b,c), indicating that anaerobic metabolism was still active in recovered plants. However, expression of *SuSy* was lower in recovered plants making them similar to healthy plants.

Although Hsp70 proteins are not directly involved in the plant's response to pathogen infection, they were

regulated in certain cases of biotic stress. A member of an *Hsp70* family was shown to be induced in phytoplasma infected leaves of *P. armeniaca* (Carginale *et al*., 2004). *Hsp70* is a quite highly conserved multi-gene family whose members are expressed under different stress conditions and can be divided into at least four subgroups (Sung *et al*., 2001a). It is known that members of different subgroups have different, sometimes even opposite, responses to the same stress conditions (Sung *et al*., 2001a,b). In this study, however, changes in gene expression of the selected *Hsp70* differed significantly only in infected compared to recovered samples of cv. Prosecco but not in other cultivars. Several explanations are possible. The particular *Hsp70* family member investigated in this study might not have been involved in the plant response to phytoplasma infection, or its regulation might have been limited to a specific group of cells which could not be detected using the selected approach.

Using expression of the individual analyzed genes did not allow the disease statuses to be distinguished completely (Fig. 1). However, a combination of the expression profiles for all three genes, as used in the LDA, produced additional valuable information. Analysis of the coefficients of linear discriminants showed that the expression of each of the three genes was essential. With LDA analysis it was possible to identify groups of samples according to their disease status (Fig. 2) with great accuracy (82·0% correctly classified samples), and also further cultivar-related subgroups (Fig. 3) with slightly lower accuracy (72·1% correctly classified samples). When Barbera samples, the most varying of all samples tested, were omitted from LDA analysis, both percentages of correctly classified samples increased (to 88·2 and 80·4%), although the difference between them remained. This information, based on monitoring the expression of selected genes, led to the conclusion that disease status was an independent factor from cultivar and phytoplasma type, meaning that the effects of phytoplasma were great enough to be distinguished from the variability caused by cultivar, phytoplasma type, location and microclimate differences.

As expected, some variability in expression data was observed, since the plants were growing in uncontrolled conditions. Interestingly, many of the incorrectly classified samples after LDA analysis can be explained by taking some eco-physiological factors and knowledge on phytoplasma distribution in infected plants into account. For example, two Ic samples (36, 37) showed extremely high phytoplasma content measured by qPCR (Table 3). Such severe infection (both shoots were even found dry later in the season) was most probably the cause of a very high expression of *Adh1* and *SuSy*. Phytoplasmas are often unevenly distributed within infected plants (Berges *et al*., 2000) and can therefore be absent in the sampled tissue or can be present in a low number but the plant as a whole will still show symptoms and will behave as if infected both morphologically (symptoms) and physiologically (expression of *Adh1*, *SuSy*), as in samples 36-Ic, 63-Ib, 82-Ip and 83-Ip (Figs 2 and 3). Extreme environmental conditions as a result of a plant's position in the vineyard can also affect gene expression. This was observed in Hc samples 49, 52 and 58, which were all sampled from plants growing in the first row of the vineyard, with consequently stronger exposure to sunlight and temperature fluctuations than samples with a more central position. However, not all the observed variation could be explained by eco-physiological factors. The possibility of some biotic and microclimate differences exists, such as soil availability or composition and differences in agricultural practice applied to a specific plant.

The variability in gene expression within the groups of plants (healthy, recovered and infected) shows that each individual plant responds in a slightly different way. Despite this natural variation the extent of phytoplasma infection, or type of phytoplasma and diversity between clones within cultivar, the response of plants to phytoplasma infection at the level of *Adh1* and *SuSy* expression was so intense and uniform that it was possible to detect it in grapevine plants, regardless of all other biotic and abiotic variables. This work thus demonstrates that the response is general, and hence contributes to the understanding of the physiology of phytoplasma-plant interaction in natural conditions. An additional value of field conducted experiments, as a more robust approach to the study of plant-pathogen interaction, is the identification of strong signals that can be used as potential markers of plant disease status. However, before a certain gene can be used as a marker of a plant disease, additional validation studies including comparison with the conventional detection methods (PCR-RFLP, real-time PCR) on a large number of samples and perhaps also on other plant tissues (e.g. leaf laminas) should be carried out.

Acknowledgments

The authors gratefully acknowledge Fabio Quaglino (Istituto di Patologia Vegetale, Milano, Italy) and Irena Benedetič from the Counselling service of vine cellar Vinska klet 'GORIŠKA BRDA' for their contribution to the research work. We would also like to acknowledge Roger Pain for reviewing the manuscript. This project was supported by the Slovenian Research Agency (PhD grant of the first author), Ministry of Higher Education, Science and Technology of Slovenia (contracts J4-6459, J4-6459) and partially by Ministero Politiche Agricole e Forestali of Italy.

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