

RESEARCH PAPER

Proteomic analysis reveals differences between *Vitis vinifera* L. cv. Chardonnay and cv. Cabernet Sauvignon and their responses to water deficit and salinity

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Abstract

The impact of water deficit and salt stress on two important wine grape cultivars, Chardonnay and Cabernet Sauvignon, was investigated. Plants were exposed to increasing salinity and water deficit stress over a 16 d time period. Measurements of stem water potentials, and shoot and leaf lengths indicated that Chardonnay was more tolerant to these stresses than Cabernet Sauvignon. Shoot tips were harvested every 8 d for proteomic analysis using a trichloroacetic acid/acetone extraction protocol and two-dimensional gel electrophoresis. Proteins were stained with Coomassie Brilliant Blue, quantified, and then 191 unique proteins were identified using matrix-assisted laser desorption ionization time of flight/time of flight mass spectrometry. Peptide sequences were matched against both the NCBI nr and TIGR *Vitis* expressed sequence tag (EST) databases that had been implemented with all public *Vitis* sequences. Approximately 44% of the protein isoforms could be identified. Analysis of variance indicated that varietal difference was the main source of protein expression variation (40%). In stressed plants, reduction of the amount of proteins involved with photosynthesis, protein synthesis, and protein destination was correlated with the inhibition of shoot elongation. Many of the proteins up-regulated in Chardonnay were of unclassified or of unknown function, whereas proteins specifically up-

regulated in Cabernet Sauvignon were involved in protein metabolism.

Key words: 2-DE, grapevine, MALDI-TOF/TOF, salinity, shoot, water deficit.

Introduction

Grapevine (*Vitis vinifera* L.) is a perennial woody vine that produces the most economically important fruit crop in the world. Relative to other crop plants, grapevines are fairly tolerant to water deficit, but are susceptible to significant damage from long-term salinity. With the shortage of freshwater resources and the increase of saline soils in irrigated regions around the planet, it is important to develop crops that are able to sustain productivity under such challenging conditions.

Many recent proteomic studies have been performed on various species and under different abiotic stresses, such as water deficit (Salekdeh *et al.*, 2002; Hajheidari *et al.*, 2005; Vincent *et al.*, 2005; Ali and Komatsu, 2006; Jorge *et al.*, 2006), high salinity (Abbasi and Komatsu, 2004; Lee *et al.*, 2004; Kim *et al.*, 2005; Yan *et al.*, 2005; Askari *et al.*, 2006; Parker *et al.*, 2006), low temperature (Tafforeau *et al.*, 2002; Bae *et al.*, 2003; Imin *et al.*, 2004; Renaut *et al.*, 2004; Cui *et al.*, 2005; Amme *et al.*, 2006), heat (Majoul *et al.*, 2003, 2004), high light (Nam *et al.*, 2003; Phee *et al.*, 2004), ozone

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(Agrawal *et al.*, 2002), and metal toxicity (Requejo and Tena, 2005; Sarry *et al.*, 2006). To our knowledge, there are only five proteomic studies using grapevine (Tesnières and Robin, 1992; Sarry *et al.*, 2004; Carvalho *et al.*, 2005; Castro *et al.*, 2005; Vincent *et al.*, 2006). Although powerful mass spectrometry-based techniques have emerged recently, such as isotope-coded affinity tagging (ICAT; Gygi *et al.*, 1999), mass-coded abundance tagging strategies (MCAT; Cagney and Emili, 2002), stable isotope labelling by amino acids in cell culture (SILAC; Ong *et al.*, 2002), or isobaric tags for relative and absolute quantitation (iTRAQ; Ross *et al.*, 2004), as well as direct isotopic labelling methods using ^{18}O (Rao *et al.*, 2005) or D_2O (Che and Fricker, 2005), two-dimensional electrophoresis (2-DE) remains one of the most efficient strategies to isolate proteins showing quantitative variation in response to a treatment (Rabilloud, 2002; Görg *et al.*, 2004). The reproducibility of 2-DE and the accuracy of sample comparison were recently improved with the introduction of the difference gel electrophoresis (DIGE; Unlu *et al.*, 1997) technique.

Varietal differences have been exploited for centuries in agriculture. Today, many breeding strategies use genetic variations between cultivars to improve the performance of an elite variety. In grapevine in particular, and in fruit crops in general, the choice of a variety is of critical importance because such differences often drive consumer preference for a particular table grape or wine. An intense artificial selection and a vegetative mode of reproduction appear to account for the genetic structure of cultivated *V. vinifera* varieties (Aradhya *et al.*, 2003; This *et al.*, 2006). Improved disease resistance, abiotic stress tolerance, or quality factors are important targets for genetic improvement of grapevine cultivars (Vivier and Pretorius, 2002; Sarry *et al.*, 2004). Several groups have investigated abiotic stress effects in grapevine (Benson and Roubelakis-Angelakis, 1994; Flexas *et al.*, 1999; Papadakis and Roubelakis-Angelakis, 1999; Gaudillère *et al.*, 2002; Lesniewska *et al.*, 2004) or have studied grapevine shoot development (Castelan-Estrada *et al.*, 2002; Lovisolo *et al.*, 2002; Lebon *et al.*, 2004); yet, none of them used any of the 'omics' technologies available today. Here, a proteomic study of the response to abiotic stresses of two of the most prominent *V. vinifera* cultivars, Chardonnay and Cabernet Sauvignon, is presented. Both varieties display physiological differences when exposed to water deficit and salinity, with Chardonnay appearing to be more tolerant to water deficit and salinity. Quantitative analysis of two-dimensional gels from shoots showed that varietal differences mainly account for changes in protein expression patterns. Some of the stress- and cultivar-responsive proteins are discussed in relation to the physiological adaptations of Chardonnay and Cabernet Sauvignon.

Materials and methods

Plant material and experimental conditions

Two-year-old rooted cuttings of dormant *V. vinifera* L. cv. Chardonnay (clone #6) and cv. Cabernet Sauvignon (clone #8) were obtained from Inland Desert Nursery (Benton City, WA, USA) and kept at 4 °C under low light in a cold room until potted in soil (Scotts MetroMix 200 medium). Cuttings were grown in a greenhouse in individual pots under controlled temperature and supplemental sodium halide light (day/night=25/17 °C; 16 h/8 h at a minimum PAR of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Plants were irrigated every third day with tap water. Every 30 d, the plants were irrigated with 0.5 l of a nutrient solution as described in Gibeaut *et al.* (1997). Upon producing an unbranched solitary shoot of 80–100 cm, the shoot was pruned to 60 cm as described in Cramer *et al.* (2005). Lateral shoots, buds, and young flowers were removed, leaving only winter buds and leaves. Plants were fertilized with 1.0 l of Gibeaut's solution every 2 d, a total of five times, to promote growth of a new shoot. The experiment began when new shoots grew and gave rise to a minimum of two new nodes (young leaves). Fertilization was not maintained over the course of the experiment.

The experiment consisted of a completely randomized factorial design consisting of two cultivars (Chardonnay and Cabernet Sauvignon) $\times$ 3 treatments (control, water deficit, and salinity) $\times$ 3 times (day 0, day 8, and day 16), with 3–6 biological replicates. Three biological replicates were used for the proteomic analyses. On day 0, only three control plants were measured (no stress treatments as they were not initiated yet), bringing the total number of plants to analyse to 42 (for each cultivar: three controls on day 0, three controls+three water-stressed+three salt-stressed on day 8, and three controls+three water-stressed+three salt-stressed on day 16). The subsequent effects of water deficit and salinity on the growth, water potentials, and proteomes of Chardonnay and Cabernet Sauvignon over the course of 16 d were studied (October 2003). For water deficit treatments, watering was completely stopped at the onset of the experiment (day 0). Control plants and salt-stressed plants were watered daily with 4.0 l of tap water or salt solution throughout the experiment. Stem water potentials of the plants were measured, and a calculated concentration of salt solution was added to the salinity-treated plants so that the water potentials of these plants matched those of the water deficit-treated plants. Consequently, salt concentration was increased over time as follows. On day 1, salt-treated vines were given 10 mM NaCl and 1 mM CaCl_2 . From day 2 to day 6, they received 20 mM NaCl and 2 mM CaCl_2 daily. During days 7 and 8, 55 mM NaCl and 5.5 mM CaCl_2 were supplied to the plants. From day 9 to day 12, 75 mM NaCl and 7.5 mM CaCl_2 were added daily to the watering solution. During days 13 and 14, the vines received 175 mM NaCl and 17.5 mM CaCl_2 , and 250 mM NaCl and 25 mM CaCl_2 on days 15 and 16.

Shoot tips (cut below the fourth leaf, thus including the apex, stem, four leaves, and tendrils) were harvested on days 0, 8, and 16. Two shoots were harvested per plant, pooled in a 50 ml Falcon tube, instantly frozen in liquid nitrogen, and stored at -80°C until further use.

Physiological measurements

Stem water potential: Fully mature leaves from between the second and sixth oldest nodes of the main shoot were selected for stem water potential measurement as described in McCutchan and Shackel (1992). Each measurement was performed using a pressure chamber (3005 Plant Water Status Console, Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Xylem sap was also collected to measure the solute potential of the sap with a vapour pressure osmometer (Model 5500, Wescor Inc., Logan, UT, USA). Solute potential was calculated as in Cramer and Bowman (1991),

and the water potential of the xylem was corrected for variation in solute accumulation in the salt-treated plants (data not shown). The water potential was recorded every 4 d, using different plants in each measure.

Growth measures: Shoot length (mm) and leaf length (mm) were measured every 4 d. A leaf was not considered apparent until it had reached at least 10 mm in length; otherwise, it was assumed to be indistinguishable from the shoot apex. Reference starting points along the shoots for growth measurements were marked with tape, resulting in all shoot tips bearing exactly two leaves from the reference point on day 0.

Protein analysis

Protein extraction: Grapevine shoots were finely ground in liquid nitrogen, then the proteins were extracted following the procedure described by Damerval *et al.* (1986). Proteins were resuspended in R2D2 buffer developed by Méchin *et al.* (2003). For each sample, 90 µl of resolubilization solution was used to resuspend 1 mg of pellet. Once resolubilized, the protein extract was stored at -80°C until use. Protein concentration was determined using an EZQ Protein Quantitation Kit (Invitrogen, Carlsbad, CA, USA) with ovalbumin as standard, according to the manufacturer's instructions.

Two-dimensional PAGE: Before use, the protein extracts were allowed to thaw at room temperature; they were then centrifuged at 14 000 g for 15 min at 24°C . A volume of 185 µl (~ 0.2 mg of protein) of supernatant was loaded onto immobilized pH gradient (IPG) strips (ReadyStrip 110 mm, pH 4–7, Bio-Rad, Hercules, CA, USA). Isoelectric focusing (IEF) was performed using a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) system. Strips were passively rehydrated with the protein extracts for 1 h. Wicks were then added to the electrodes and strips were covered with mineral oil and further rehydrated for 12 h at 50 V. IEF conditions were: 50–250 V linear gradient for 20 min, 250–4000 V linear gradient for 2 h, and 4000–8000 V rapid gradient until a total of 18 000 Vh was reached. The strips were then equilibrated twice for 15 min each as described in Görg *et al.* (1987). The second dimension was run on 12.5% Criterion TRIS-HCl resolving gels (Bio-Rad, Hercules, CA, USA) for 1 h at 200 V in a Criterion Dodeca cell. Two-dimensional gels were stained using a colloidal Coomassie brilliant blue (CBB) G-250 procedure, according to Neuhoff *et al.* (1988).

Two-dimensional image processing and statistical analyses: Two-dimensional gels were digitized with a Versadoc Imaging system and processed using PDQuest software V.7.3 (Bio-Rad, Hercules, CA, USA). Spot quantity normalization occurred throughout the whole matchset, which included all 42 two-dimensional gels. The gel used as reference in the PDQuest matchset corresponded to a co-migration of protein extracts from both Chardonnay and Cabernet Sauvignon control plants, and was subsequently used to excise spots for protein identification (see below).

This study was based on an unbalanced experimental design in which the first time point (day 0) was represented only by control plants. All three treatments (control, water deficit, and salinity) were measured during the successive temporal stages (days 8 and 16). A balanced experiment was obtained by converting protein levels of days 8 and 16 into percentages with respect to the protein levels of the controls measured on day 0 for both varieties (Chardonnay and Cabernet Sauvignon). Controls on day 0 were then removed from the data set. Therefore, spot quantities for each stress treatment on days 8 and 16 were expressed relative to their controls on day 0. It was verified that the variation due to the technique (using three

technical replicates, $\text{SE}=0.05$) was insignificant compared with the variation resulting from the experimental conditions (three biological replicates; $\text{SE}=0.18$). Spots were considered reproducible when present in at least two of the three biological replicates. Statistical analyses were performed on those 758 spots deemed reproducible.

A full factorial analysis of variance (ANOVA) was performed on each protein to determine whether varietal effects across time were of significance, using the statistical programming language R. The following linear model was used for the ANOVA:

$$y_{ijk} = V_i + D_j + T_k + (VD)_{ij} + (VT)_{ik} + (DT)_{kj} + (VDT)_{ijk} + e_{ijk} \quad (1)$$

where y_{ijk} denotes the protein level measured for variety i , day j , and treatment k , with $1 \leq i \leq 2$, $1 \leq j \leq 2$, and $1 \leq k \leq 3$. The terms V_i , D_j , and T_k measure the effect of the variety, day, and treatment, respectively. The interaction terms $(VD)_{ij}$, $(VT)_{ik}$, and $(DT)_{kj}$ account for the interaction between variety and day, variety and treatment, and day and treatment, respectively. Similarly, the term $(VDT)_{ijk}$ represents the interaction of variety, day, and treatment. An ANOVA was performed on each protein using the linear model above.

An adjustment for the false discovery rate (Benjamini and Hochberg, 1995) was made on the P -values of the individual effects. Any protein with an adjusted P -value of <0.05 associated with a specific effect was deemed to have a significant change attributed to the specified effect. Additionally, a forward stepwise regression was performed to determine which proteins could be used as predictors of shoot length and water potential, using JMP software (SAS Institute, Cary, NC, USA).

Protein identification: To the best of our knowledge, no proteomic map of grapevine shoot exists. It was decided to establish a working map by identifying the proteins which were expressed abundantly at the onset of treatment and then to follow their expression over time in relation to water or salt stresses in both cultivars. A total of 202 spots were excised from a gel in which two samples had co-migrated (equal proportions of protein samples obtained from Chardonnay and Cabernet Sauvignon both grown under control conditions and collected on day 0) under the electrophoretic conditions described above. To increase the chance of protein identification, the 186 most abundant spots were excised from the reference gel. An additional 16 spots proven significant in the two stepwise regression analyses were subsequently excised. Excised spots were trypsin digested and analysed using matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry (MS) as described in Vincent *et al.* (2006). The peptide lists generated by the MASCOTTM software were searched against both the non-redundant (nr) protein database of NCBI and the TIGR *Vitis* expressed sequence tag (EST) database.

Results

Physiological responses of grapevines subjected to water and salt stresses

Stem water potential: The stem water potential of control plants ranged between -0.16 MPa and -0.37 MPa over the course of the experiment (Fig. 1). No significant differences were detected between cultivars at any time or between control and stressed grapevines until after day 4. On day 8, both water deficit-treated and salt-stressed

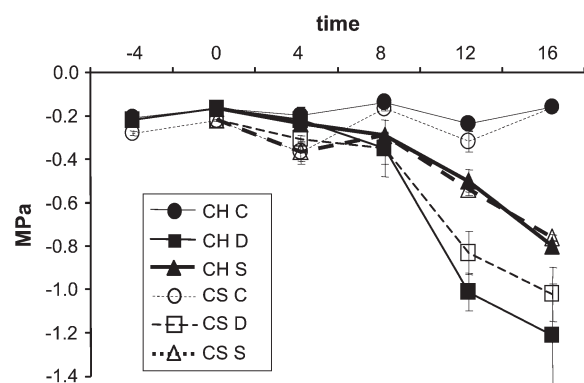


Fig. 1. Stem water potential over time (from day -4 to day 16). Vertical bars show the SE ($n=6$). CH, Chardonnay; CS, Cabernet Sauvignon; C, control; D, water deficit; S, salinity.

plants had lower stem water potentials than the controls. On days 12 and 16, the stem water potential of the water deficit-treated grapevines decreased more rapidly than that of NaCl-stressed vines and reached a maximum of -1.0 MPa to -1.2 MPa depending on the cultivar. The stem water potential of the salt-treated plants did not decrease to the same extent and reached a maximum of -0.85 MPa in both cultivars. The general trend after day 8 was an intermediate water status of the salt-stressed plants between that of the controls and water deficit-treated plants.

Shoot and leaf length: The shoot length response to water deficit and salinity treatment differed between the two cultivars (Fig. 2A). Under optimal conditions, Cabernet Sauvignon shoots grew slightly faster than Chardonnay, but this difference was not statistically significant. With the salt treatment, the shoot growth of Chardonnay was not affected relative to controls, whereas Cabernet Sauvignon shoot length was significantly reduced by day 12. With water deficit, Chardonnay shoot length was affected by day 8 relative to its control, whereas Cabernet Sauvignon shoot elongation decreased significantly as early as day 4.

When individual leaf lengths were measured, the responses to the treatments differed from one leaf to another, as well as between cultivars. Leaves were observed to grow faster in well-watered Cabernet Sauvignon grapevines than in Chardonnay, particularly at later developmental stages. Leaf 1 length appeared to be reduced only by water deficit on days 12 and 16 (Fig. 2B). The same observation held true for leaves 2 and 3, which showed a more acute reduction under water stress (Fig. 2C, D). Then the pattern changed for leaf 4 and the younger leaves. Salt stress reduced the length of leaf 4 in both cultivars (Fig. 2E), although to a lesser extent than water deficit. As previously observed in shoots, Cabernet Sauvignon seemed to be more affected by salt and water stresses than Chardonnay when the growth of leaves 4–8 was considered (Fig. 2F–I). In Cabernet Sauvignon plants,

elongation of leaves 6 and 7 was stopped by water deficit and no new leaves were produced after leaf 7. The inhibition in Chardonnay occurred at later developmental stages than that in Cabernet Sauvignon; water deficit stopped the elongation of leaves 7 and 8 and no new leaves were produced after leaf 8.

Protein identified in grapevine shoots

Database search and cross-contamination: A consensus map of both varieties was generated from a reference gel (Fig. 3A). Out of 202 excised spots, no MS data were obtained for three spots (spot #47, spot #63, and spot #91). The remaining 199 spots were examined for spot cross-contamination (several proteins identified in a single spot) by observing the MS spectra. If cross-contamination was found, the quantitative data associated with the spot was discarded. A total of 191 proteins led to a unique hit (including proteins of unknown function). Protein identities are indicated in Table 1.

A number of protein identities had low protein scores, as expected, due to the incomplete sequencing effort of the grapevine genome (http://www.evry.inra.fr/public/projects/genome/grape_fr.html), and nucleotide sequence data are available only from a limited number of EST sequences in *Vitis*. The EST libraries do not represent all genes of a given organism, and the gene sequences, whose quality varies, are only partial.

On occasion, the MS analysis of one particular spot led to multiple protein identifications (eight spots out of 199, 4.02%), indicating that several proteins had co-migrated (otherwise known as cross-contamination). Spot density was high because of the large amount of proteins loaded on small gels (11 cm), although the 2-D gel resolution was improved by using a narrow pH gradient (4–7). In general, spot resolution was of high quality on the 2-D gels; however, some overlaps were detectable. The phenomenon was more dramatic when different proteins co-migrated at precisely the same location on the 2-D gel. Technical improvements in the sensitivity and accuracy of MS analyses allowed the detection and identification of proteins co-localizing in the same spot on 2-D gels. This phenomenon is not frequently discussed in the literature. Méchin *et al.* (2004) reported that almost 10% (60 spots out of 632 excised spots) of the spots contained cross-contamination in maize seeds. Parker *et al.* (1998) proposed a method to detect cross-contaminations. Cross-contamination can be partially overcome by reducing the complexity of the protein extract by performing some pre-fractionation methods, or increasing the 2-D gel resolution by using both a narrower pH gradient in the first dimension and a wider percentage polyacrylamide gradient of the SDS–polyacrylamide gel in the second dimension. In this study, analysis of MS/MS spectra revealed such co-migration, and the protein quantity for

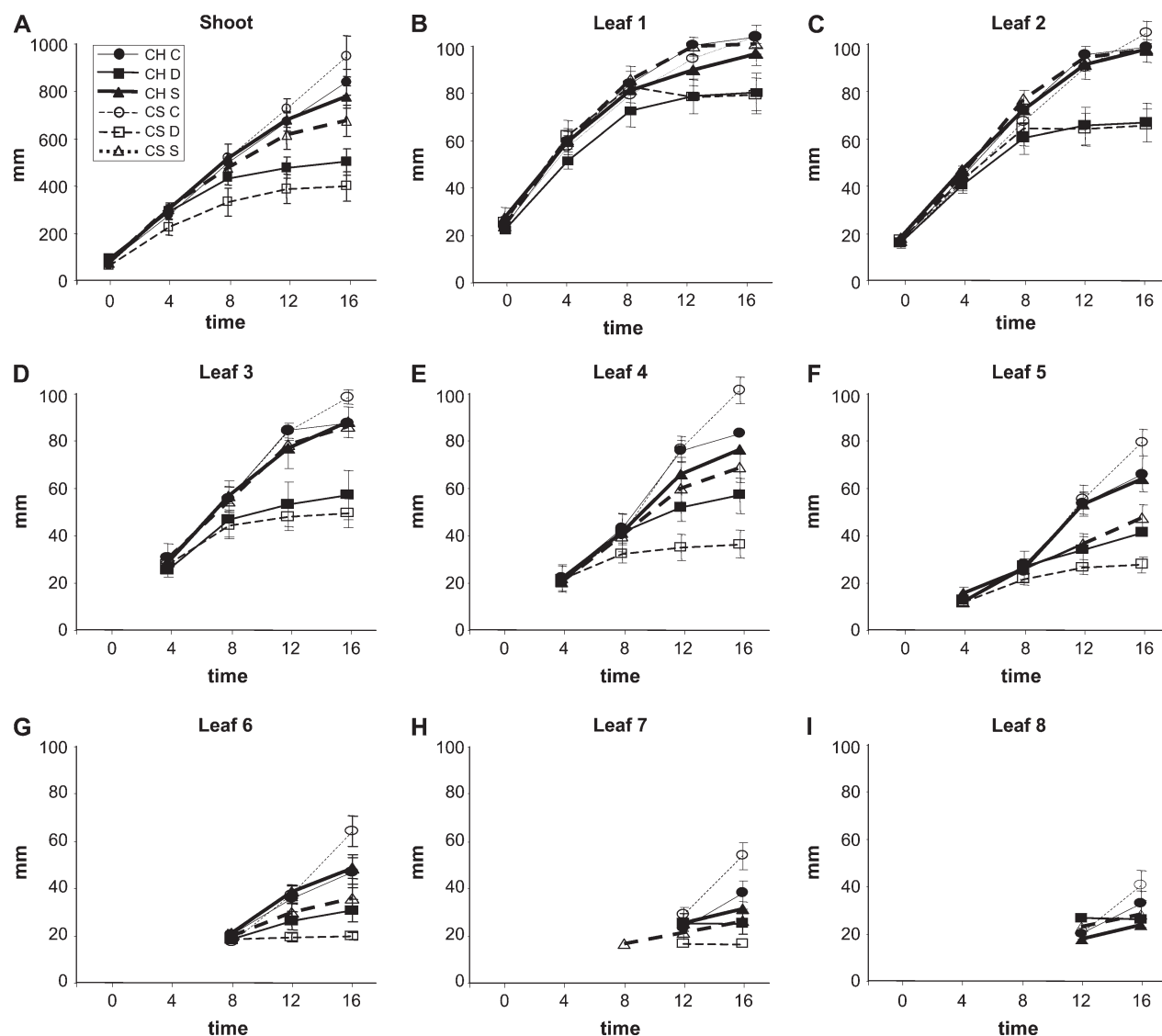


Fig. 2. Length measurement over time (from day 0 to day 16) of the shoot and the leaves along it, leaf 1 being the oldest and leaf 8 the youngest. (A) Shoot length. (B) Leaf 1 length. (C) Leaf 2 length. (D) Leaf 3 length. (E) Leaf 4 length. (F) Leaf 5 length. (G) Leaf 6 length. (H) Leaf 7 length. (I) Leaf 8 length. Vertical bars show the SE ($n=6$). CH, Chardonnay; CS, Cabernet Sauvignon; C, control; D, water deficit; S, salinity.

these particular spots was considered inaccurate, and therefore disregarded (eight spots). Of the 191 remaining spots, 142 (74%) led to a known protein annotation.

Protein isoforms: Protein identification revealed several protein isoforms (Table 1). Out of 191 identified spots, 83 bore the same annotation as another spot (43.5%, see inset in Fig. 3B). In some cases, such as for ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) large subunit (hereafter referred to as RBCL; spot #11, spot #65, spot #88, spot #90, and spot #173), up to five isoforms of the same protein were isolated. One of the isoforms (spot #11) exhibited a molecular mass (M_r) of 20 000 and an isoelectric point (pI) of 5.8, whereas the four others presented a lower M_r (15 000) and were

distributed along a pI from 4.6 to 5.4. According to the theoretical M_r and pI provided by the ExPASy website (http://www.expasy.org/tools/pi_tool.html), RBCL should bear an M_r of 51 000 and a pI of 7.5, which is beyond the pI range of the gels used in this study. Therefore, those spots probably corresponded to degraded forms of RBCL. Among the 202 excised spots, none corresponded to undegraded forms of RBCL. Because the protein map did not cover all the spots resolved under the electrophoretic conditions (Fig. 3A), it is possible that they were missed. Alternatively, the undegraded form of RBCL could have been expressed in such low levels in grape shoots that its corresponding spot on 2-D patterns was neither excised nor stained. Perhaps the totality of RBCL molecules present in the shoot samples were completely degraded

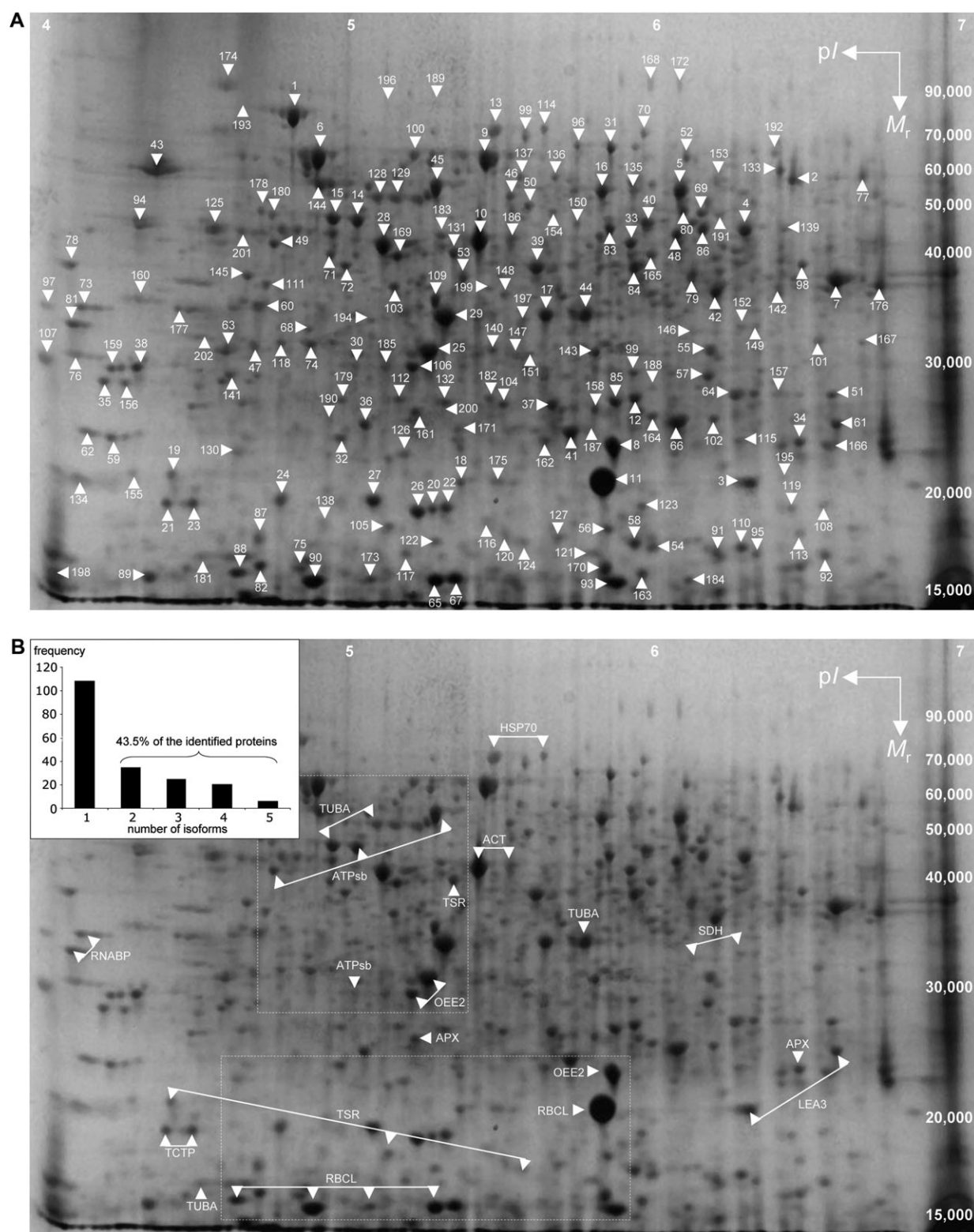


Fig. 3. Reference 2-D map with two protein extracts obtained from Chardonnay and Cabernet Sauvignon shoots grown under control conditions and collected on day 0. The isoelectric point (pI) extends from pH 4 to 7 and the molecular mass (M_r), from 15 000 to 90 000. (A) 2-D map with the 199 excised spots. (B) Some protein isoforms. ACT, actin; APX, ascorbate peroxidase; ATPsb, ATP synthase beta-subunit; HSP70, heat shock protein 70 kDa; LEA3, late embryogenesis abundant protein, group 3; OEE2, oxygen-evolving enhancer protein 2; RBCL, ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit; RNABP, RNA-binding protein; SDH, NAD-dependent sorbitol dehydrogenase; TCTP, translationally controlled tumour protein homologue; TSR, transformer serine/arginine-rich ribonucleoprotein; TUBA, tubulin alpha-chain. The insert shows the histogram of the frequency of isoforms. Dashed rectangles indicate areas of isoforms illustrated in Supplementary Figs S1 and S6 at JXB online.

Table 1. Protein identities of the 202 excised spots

Excised spot no. ^a	Protein code ^b	Accession no. ^c	Function annotation	M_r (10^3) ^d	pI ^e	Protein score ^f	MATdb functional classification (FunCat)
1	CCA	gil2129851	tRNA adenylyltransferase	68.9	6.2	49	Protein synthesis
2	ATPsf1	gil3410894	F1 ATPase α subunit	55.0	6.0	530	Electron transport/membrane-associated energy conservation
3	LEA3	gil7387828	Late embryogenesis abundant protein, group 3	23.2	8.8	52	Stress
4	EPID	gil15241945	NAD-dependent epimerase/dehydratase family	15.5	6.2	390	C-compound and carbohydrate metabolism
5	ENO1	TC45145	Enolase 1	47.2	5.5	343	Glycolysis and gluconeogenesis
6	CPN60a	gil1351030	60 kDa chaperonin α subunit	62.1	5.1	157	Photosynthesis
7	bHLH	gil18397368	bHLH protein			50	Transcription
8	OEE2	gil131390	Oxygen-evolving enhancer protein 2, chloroplastic	28.5	8.9	230	Photosynthesis
9	CPN	gil15231255	Chaperonin, putative			291	Protein destination
10	ACT	gil11276970	Actin	41.8	5.4	690	Cellular biogenesis
11	RBCL	gil16973406	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit	52.6	6.2	314	Photosynthesis
12		gil14719331	3- β Hydroxysteroid dehydrogenase/isomerase protein putative	31.3	9.1	181	
12		TC40769	Protein At2g37660, chloroplast			124	
13	HSP70	gil399940	Heat shock 70 kDa protein mitochondrial	73.1	6.4	142	Protein destination
14	ATPsb	gil14718068	ATP synthase β subunit	59.7	6.2	703	Electron transport/membrane-associated energy conservation
15	TUBa3	gil8928432	Tubulin α -3 chain	49.6	5.1	713	Cellular biogenesis
16	RCA1	TC38352	Rubisco activase α form precursor	52.1	6.4	509	Photosynthesis
17	RCA1	TC38353	Rubisco activase α form precursor	52.1	6.4	249	Photosynthesis
18	na	gil8885565	emblCAB67639.1 ~ gene_id:F15L12.3			53	Unknown protein
19	TSR	gil18390758	Transformer serine/arginine-rich ribonucleoprotein, putative	38.1	10.6	51	Protein synthesis
20	ALL	TC45421	Pollen allergen-like protein	13.3	9.3	401	Unknown protein
21	TCTP	gil549063	Translationally controlled tumour protein homologue	18.9	4.5	193	Unclassified protein
22	PPIase	TC40313	Peptidyl-prolyl <i>cis-trans</i> isomerase, chloroplastic	28.2	8.8	245	Protein destination
23	TCTP	gil549063	Translationally controlled tumour protein homologue	18.9	4.5	161	Unclassified protein
24	ETA1	TC45771	Early tobacco anther 1	17.4	4.9	266	Unknown protein
25	OEE2	gil15408655	Putative 33 kDa oxygen-evolving protein of photosystem II	28.5	8.9	230	Photosynthesis
26	PDX	TC45159	Peroxioredoxin			169	Stress
27	na	gil15240532	Hypothetical protein			51	Unknown protein
28	RCA1	TC38353	Rubisco activase α form precursor	52.1	6.4	251	Photosynthesis
29	MHYD	TC45122	Metal-dependent hydrolase-like protein	29.2	6.2	648	Unclassified protein
30	ATPsb	gil231586	ATP synthase β chain, mitochondrial	59.7	6.2	210	Electron transport/membrane-associated energy conservation
31	na	gil15241041	Expressed protein			42	Unknown protein
32	na	gil15240032	Hypothetical protein			54	Unknown protein
33	na	gil15217625	Hypothetical protein			34	Unknown protein
34	APX	TC45171	Ascorbate peroxidase cytosolic	27.4	5.7	597	Stress
35	na	TC45500	At1g79210/YUP8H12R_1			221	Unknown protein
36	SOD-Mn	gil7484734	Superoxide dismutase (Mn)	23.4	5.6	228	Stress
37	NPAC	TC45057	Nascent polypeptide-associated complex α chain	12.4	4.4	311	Unclassified protein
38	NAC	gil15229149	α NAC-related protein			57	Unknown protein
39	FH3	gil729508	Flavonone-3-hydroxylase	40.3	5.3	521	Secondary metabolism
40	SAMS	gil3024126	S-Adenosylmethionine synthetase 1	42.8	5.3	473	Unclassified protein
41	CAB8	gil115813	Chlorophyll A-B-binding protein 8, chloroplastic	28.5	5.2	106	Photosynthesis
42	ALDL	TC38366	Latex plastidic aldolase-like protein	42.7	8.4	316	Glycolysis and gluconeogenesis
43	CRT	TC38845	Calreticulin precursor	48.5	4.5	414	Cellular communication/signal transduction
44	TUBa	gil9965991	Tubulin α chain			560	Cellular biogenesis
45	ATPsb	gil231586	ATP synthase β chain, mitochondrial	59.7	6.2	650	Electron transport/membrane-associated energy conservation
46	TFB3	gil15226706	Transcriptional factor B3 family	27.6	9.1	76	Transcription

Table 1. (Continued)

Excised spot no. ^a	Protein code ^b	Accession no. ^c	Function annotation	M_r (10^3) ^d	pI ^e	Protein score ^f	MATdb functional classification (FunCat)
47		Lost					
48	LDOX	gil1730109	Leucoanthocyanidin dioxygenase	40.4	5.2	193	Secondary metabolism
49	ATPsb	gil5763976	ATP synthase β chain	59.7	6.2	38	Electron transport/membrane-associated energy conservation
50	RPT3	gil15237159	Proteasome 26S AAA-ATPase subunit RPT3	45.7	5.4	300	Protein degradation
51	PSA4	gil1709761	Proteasome subunit α type 4	27.5	6.6	120	Protein degradation
52	KARI	gil6225542	Ketol-acid reductoisomerase, chloroplastic	63.8	6.4	152	Amino acid metabolism
53	ADK	gil4582787	Adenosine kinase	37.8	5.3	61	Electron transport/membrane-associated energy conservation
54	RISP	TC45147	Cytochrome B6-F complex iron-sulphur subunit, chloroplastic	24.1	7.6	307	Electron transport/membrane-associated energy conservation
55	na	TC38739	F18O14.34			328	Unknown protein
56	RPS12	TC46162	Ribosomal 40S protein S12	15.3	5.3	392	Protein synthesis
57	ALD	TC38325	Fructose 1,6-bisphosphate aldolase	41.9	9.0	335	Glycolysis and gluconeogenesis
58	na	TC38872	GblAAF26101.1			315	Unknown protein
59	HMMR	gil15242952	Hyaluronan-mediated motility receptor-related protein	98.5	5.1	44	Unclassified protein
60	SAMS	gil7340731	S-Adenosylmethionine synthetase	42.8	5.3	106	Unclassified protein
61	LEA3	gil7387828	Late embryogenesis abundant protein, group 3	23.2	8.8	40	Stress
62	na	gil15290078	P0004A09.6			28	Unknown protein
63		lost					
64	PSA6	gil12229897	Proteasome subunit α type 6	27.6	6.2	180	Protein degradation
65	RBCL	gil14599586	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit	52.6	6.2	399	Photosynthesis
66	CPN21	TC38847	Chaperonin 21 chloroplastic	26.8	8.9	262	Protein destination
67	GRP	TC38485	Glycine-rich RNA-binding protein	15.7	5.3	561	Protein synthesis
68	ACT2/7	gil15242516	Actin 2/7			72	Cellular biogenesis
69	na	gil15983513	At3g60750/T4C21_160			85	Unknown protein
70	SDH	gil15224174	Succinate dehydrogenase, putative			119	Tricarboxylic acid pathway
71	CHL1	gil3334150	Magnesium chelatase subunit chlI, chloroplastic	46.3	6.1	113	Photosynthesis
72	SBPase	gil3914940	Sedoheptulose-1,7-bisphosphatase, chloroplastic	42.4	6.2	196	Photosynthesis
73	RNABP	Singleton	RNA-binding protein precursor			212	Protein synthesis
74	na	gil18421713	Expressed protein			47	Unknown protein
75	UGDH	gil15228687	UDP-glucose dehydrogenase-related	53.1	5.6	177	Electron transport/membrane-associated energy conservation
76	eEF1b	TC39070	Elongation factor 1B α -subunit	3.2	6.0	117	Protein synthesis
77	na	gil18057121	Hypothetical protein			37	Unknown protein
78	DEL	gil13346180	GHDEL61	69.8	5.5	31	Transcription
79	na	gil15235233	Hypothetical protein			48	Unknown protein
80	KAS1	gil7385201	Beta-ketoacyl-ACP synthetase I	32.1	9.5	80	Lipid, fatty acid and isoprenoid metabolism
81	RNABP	gil1076251	RNA-binding protein			67	Protein synthesis
82	NMCP1	gil15239130	Nuclear matrix constituent protein 1 (NMCP1)-related	121.2	5.3	39	Unclassified protein
83	LEA	gil7489493	LEA protein			49	Stress
84	PDI	gil15623934	Protein disulphide isomerase putative	39.7	6.0	173	Protein destination
85	TP1c	gil13431949	Triosephosphate isomerase, chloroplastic	33.3	7.7	174	Photosynthesis
86	GAPb	gil256965	Glyceraldehyde-3-phosphate-dehydrogenase subunit GapB			36	Photosynthesis
87	TRANSF	gil18419528	Transposase	92.6	7.8	63	Unclassified protein
88	RBCL	gil7008043	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit	52.6	6.2	172	Photosynthesis
89	TF	gil15220876	Transcription factor-related			133	Transcription
90	RBCL	gil2342928	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit	52.6	6.2	393	Photosynthesis
91		lost					
92	CE	gil18409633	Ubiquitin-conjugating enzyme family	39.5	9.0	96	Protein degradation
93	PPR	gil15240704	Pentatricopeptide repeat (PPR)-containing protein	59.7	6.2	50	Unclassified protein

Table 1. (Continued)

Excised spot no. ^a	Protein code ^b	Accession no. ^c	Function annotation	M_r (10 ³) ^d	pI ^e	Protein score ^f	MATdb functional classification (FunCat)
94	na	TC38641	At3g23600/MDB19_9			141	Unknown protein
95	RT	gil22327496	Non-LTR retroelement reverse transcriptase putative	158.4	9.2	27	Unclassified protein
96	na	gil11994791	Gene_id:T19N8.13			66	Unknown protein
97	GRIP	gil7406665	Ripening-related protein putative	18.0	4.0	43	Unclassified protein
98	RGP	TC45606	Reversibly glycosylated polypeptide	41.3	5.8	299	Cellular transport and transport mechanisms
99	SPK1	gil15232969	Guanine nucleotide exchange factor	206.8	5.8	91	Protein destination
100	na	gil15239036	Putative protein: At5g05210.1			81	Unknown protein
101	XET	TC38660	Xyloglucan endotransglycosylase	34.3	8.6	399	Cellular biogenesis
102	na	gil15241964	Putative protein: At5g56850.1			49	Unknown protein
103	na	gil15225090	Hypothetical protein: At2g31480.1			46	Unknown protein
104	na	gil25370530	Hypothetical protein T32G9.36			46	Unknown protein
105	TSR	gil18390758	Transformer serine/arginine-rich ribonucleoprotein, putative	38.0	10.6	57	Protein synthesis
106	OEE2	gil15408655	Oxygen-evolving 33 kDa protein of photosystem II putative	28.5	8.9	239	Photosynthesis
107	na	gil11994791	Gene_id:T19N8.13			73	Unknown protein
108	na	gil22326478	Hypothetical protein: At2g46180.1			91	Unknown protein
109	FK	gil21592495	Fructokinase-like protein	34.7	6.9	101	C-compound and carbohydrate metabolism
110	na	gil25411592	Hypothetical protein: At2g15000			43	Unknown protein
111	HSP	gil6969976	Heat shock protein high molecular weight			165	Protein destination
112	AK	gil5305496	Adenylate kinase	24.5	6.5	62	Electron transport/membrane-associated energy conservation
113	PR10	gil11182124	Pathogenesis-related protein 10	17.2	5.7	300	Stress
114	HSP70	gil27476086	Heat shock 70 kDa protein putative, mitochondrial	73.1	6.4	152	Protein destination
115	BQR	TC44925	1,4-Benzoquinone reductase-like protein	21.7	6.3	120	Electron transport/membrane-associated energy conservation
116		gil27227801	Structural maintenance of chromosomes 1 (SMC1) protein	140.9	7.8	78	
116		TC47349	Plastid protein At2g33430/F4P9.20			54	
117	RPS14	gil14916695	Ribosomal 30S protein S14 chloroplastic	11.7	11.2	54	Protein synthesis
118		gil15228575	Hypothetical protein: At3g03510.1			63	
118		TC39070	Elongation factor 1B α -subunit			47	
119	PRX	TC46190	Peroxisome protein mitochondrial	21.4	9.0	97	Stress
120	NACK2	gil19570249	Kinesin-like protein NACK2	107.2	8.5	60	Cellular biogenesis
121	SCL30a	gil15231285	Arginine/serine-rich splicing factor SCL30a	30.1	11.6	92	Protein synthesis
122	DMQ	TC38745	S-Adenosylmethionine:2-demethylmenaquinone methyltransferase	17.8	6.1	280	Unclassified protein
123	na	gil15227974	Hypothetical protein: At2g42650.1			63	Unknown protein
124	TSR	gil28973767	Transformer serine/arginine-rich ribonucleoprotein, putative	38.0	10.6	56	Protein synthesis
125	na	gil21952850	Hypothetical protein			74	Unknown protein
126	na	gil15236723	Putative protein: At4g17990.1			63	Unknown protein
127	na	gil29027824	OSJNBb0055I24.7			54	Unknown protein
128	TUBa	gil6723478	Tubulin α chain			220	Cellular biogenesis
129	na	gil18421036	Expressed protein: At5g26731.1			36	Unknown protein
130	na	gil15227974	Hypothetical protein: At2g42650.1			56	Unknown protein
131	TSR	gil18390758	Transformer serine/arginine-rich ribonucleoprotein, putative	38.0	10.6	56	Protein synthesis
132	na	gil22329097	Putative protein: At4g32420.1			86	Unknown protein
133	PPB	gil12802327	Processing peptidase β subunit mitochondrial	58.9	6.6	97	Protein destination
134	SAMS	gil7340731	S-Adenosylmethionine synthetase	42.8	5.3	129	Unclassified protein
135	na	gil18413957	Expressed protein: At4g13230.1			70	Unknown protein
136	IPS	TC45187	Inositol-3-phosphate synthase	56.4	5.4	132	C-compound and carbohydrate metabolism
137	SRP	gil26452521	Serine/arginine-rich protein putative	28.0	11.5	76	Protein synthesis
138	na	gil25370530	Hypothetical protein T32G9.36			50	Unknown protein

Table 1. (Continued)

Excised spot no. ^a	Protein code ^b	Accession no. ^c	Function annotation	M_r (10^3) ^d	pI ^e	Protein score ^f	MATdb functional classification (FunCat)
139	GLOB	gil13507023	Globulin 7S			44	Storage protein
140	H1	gil82088	Histone H1-like protein	24.9	10.7	82	Cellular biogenesis
141	RBP29	TC45487	29 kDa ribonucleoprotein A, chloroplastic	36.0	5.2	232	Protein synthesis
142	na	gil11994791	Gene_id:T19N8.13			62	Unknown protein
143	KIN	gil13161377	Kinesin putative	93.1	5.8	70	Cellular biogenesis
144	na	gil25402998	Protein T23E23.14			78	Unknown protein
145	PSII	gil21537121	Photosystem II			48	Photosynthesis
146		TC45858	NAD-dependent sorbitol dehydrogenase	40.2	6.7	73	
146		gil629469	Ribosomal protein S3			76	
147	CapLEA-1	TC24418488	Late embryogenesis abundant protein 1	19.1	8.6	71	Stress
148	CSase	TC38757	Cysteine synthase	33.8	5.9	373	Amino acid metabolism
149	RE	gil15217255	Retroelement putative			66	Unclassified protein
150		gil11994791	Gene_id:T19N8.13			81	
150		TC38332	S-Adenosylmethionine synthetase 2			50	
151	RLK	gil15220362	Receptor-like kinase, putative	75.3	6.0	55	Protein destination
152	SDH	TC45858	NAD-dependent sorbitol dehydrogenase	40.2	6.7	182	C-compound and carbohydrate metabolism
153	RNP	gil16905200	Small ribonucleoprotein putative			89	Protein synthesis
154	POLX	gil130582	Retrovirus-related Pol polyprotein from transposon TNT 1–94	151.1	8.8	59	Unclassified protein
155	MAT	gil6683296	Maturase	61.1	9.6	72	Protein destination
156	na	gil15240846	Putative protein: At5g38150.1			81	Unknown protein
157	na	gil15228104	Unknown protein: At2g37230.1			82	Unknown protein
158	MYO	gil15238181	Myosin heavy chain-like protein	60.5	5.1	77	Cellular biogenesis
159	RPS14	gil6066165	Ribosomal protein S14	11.7	11.2	51	Protein synthesis
160	na	gil15241964	Putative protein: At5g56850.1			92	Unknown protein
161	APX	gil555576	Ascorbate peroxidase	27.4	5.7	84	Stress
162	PSB1	gil8671502	Proteasome 20S β 1 subunit	24.3	6.4	58	Protein degradation
163	na	gil15227974	Hypothetical protein: At2g42650.1			78	Unknown protein
164	MAT	gil6683296	Maturase	61.1	9.6	70	Protein destination
165	na	gil11994791	Gene_id:T19N8.13			80	Unknown protein
166	na	gil25407040	Protein T23G18.18			65	Unknown protein
167	na	gil15240846	Putative protein: At5g38150.1			64	Unknown protein
168	na	gil26983792	Unknown protein			50	Unknown protein
169		TC38505	Phosphoribulokinase, chloroplast precursor	44.5	5.7	60	
169		gil4240041	ZmGR2c			71	
170	SR1	gil22329298	Pre-mRNA splicing factor SF2	33.7	11.1	60	Protein synthesis
171		TC46643	Multicatalytic endopeptidase complex, 20S proteasome, β subunit	25.1	5.3	96	
171		gil27754263	Unknown protein			100	
172	na	gil18421036	Expressed protein: At5g26731.1			67	Unknown protein
173	RBCL	gil2342976	Ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit	52.6	6.2	126	Photosynthesis
174	na	gil22831335	Hypothetical protein			53	Unknown protein
175	RPL39	gil18396099	Ribosomal protein L39 putative	6.4	12.3	41	Protein synthesis
176	SRP	gil15219287	Arginine/serine-rich protein, putative	45.3	12.4	160	Protein synthesis
177	na	gil15225090	Hypothetical protein: At2g31480.1			94	Unknown protein
178		TC40636	RAD23 protein	41.5	4.7	70	
178		gil25404204	Hypothetical protein F16M22.3			52	
179	DLH	gil15225693	Dienelactone hydrolase	25.9	5.3	93	Unclassified protein
180	na	gil15227736	Unknown protein: At2g30330.1			27	Unknown protein
181	TUBa	gil5757890	Tubulin α chain			140	Cellular biogenesis
182	CFI	gil1705761	Chalcone-flavonone isomerase	22.9	4.8	120	Secondary metabolism
183	DRP	gil118927	Desiccation-related protein	16.3	5.9	80	Stress
84	na	gil15225090	Hypothetical protein: At2g31480.1			101	Unknown protein
185	MAT	gil20270786	Maturase	61.1	9.6	55	Protein destination
186	ACT	gil9082317	Actin	41.8	5.4	154	Cellular biogenesis
187	PRp27	gil5360263	NtPRp27	27.4	9.3	116	Stress
188	TPic	TC38767	Triosephosphate isomerase, chloroplastic	33.3	7.7	312	Photosynthesis
189	RPS7	gil44388	Ribosomal protein S7			40	Protein synthesis
190	XET	TC38660	Xyloglucan endotransglycosylase	34.3	8.6	62	Cellular biogenesis
191	na	gil21436019	AT5g28840/F7P1_20			509	Unknown protein

Table 1. (Continued)

Excised spot no. ^a	Protein code ^b	Accession no. ^c	Function annotation	M_r (10 ³) ^d	pI ^e	Protein score ^f	MATdb functional classification (FunCat)
192	RPL36	gil54022800	Ribosomal protein L36, putative			50	Protein synthesis
193	na	gil34911446	P0431H09.23			44	Unknown protein
194	SPDS	TC39507	Spermidine synthase			366	Spermidine metabolism
195	RBC	gil11967693	Ribulose 1,5-bisphosphate carboxylase-oxygenase			190	Photosynthesis
196	HSP70	gil6911549	Heat shock protein 70 kDa	73.1	6.4	127	Protein destination
197	APX	gil21553684	Ascorbate peroxidase, thylakoid-bound	27.4	5.7	83	Stress
198	RP1	TC46970	60S acidic ribosomal protein P1			105	Protein synthesis
199	RP0	TC45436	60S acidic ribosomal protein P0			93	Protein synthesis
200	na	TC38641	Unknown protein			261	Unknown protein
201	HSP70	gil48716124	Heat shock protein 70 kDa	73.1	6.4	130	Protein destination
202	CPN60	gil765192	Chaperonin-60 LS2	4.5	6.4	96	Protein destination

^a An excised spot no. highlighted in bold represents cross-contamination.

^b A protein code bearing 'na' annotation stands for 'not available'.

^c Accession numbers prefixed with either 'gil' or 'TC' are from NCBI Entrez and TIGR Grape Gene Index databases, respectively.

^d Theoretical molecular weight computed by the ExPASy website.

^e Theoretical isoelectric point computed by the ExPASy website.

^f Mascot protein score from MALDI-TOF/TOF MS analysis. A Mascot score below 77 indicates that the identification given is only tentative ($P > 0.05$).

due to the growing conditions (artificial light of the greenhouse). Because most of the identified proteins resolved at their expected pI and M_r , protein degradation related to technical handling tended to be ruled out. A more likely explanation is to be found in the nature of the plant material being dealt with. Grapevine shoots are mainly composed of stem tissues, whose main function is not photosynthesis, hence a low RuBisCo content. The expression profile varied from one RBCL isoform to another, with differences due to the cultivars, treatments, or developmental stages (see Supplementary Fig. S4A–E at JXB online). Houtza and Portis (2003) have reported that a number of co- and post-translational modifications can affect RBCL. RBCL can also be subject to proteolysis (Albuquerque *et al.*, 2001), which is enhanced under oxidative conditions (Penarrubia and Moreno, 1990). Degradation products of RBCL, though of greater M_r than in the present study, were also found in leaves of micropropagated grapevine plantlets (Hajdich *et al.*, 2001; Carvalho *et al.*, 2005).

Other proteins such as heat shock protein 70 kDa (HSP70, M_r 70 000, spot #13 and spot #114), chaperonin 60 kDa (CPN60a, M_r 60 000, spot #6), translationally controlled tumour protein homologue (TCTP, M_r 19 000, spot #21 and spot #23), actin (ACT, M_r 42 000, spot #10 and spot #186), and ascorbate peroxidase (APX, M_r 28 000, spot #34 and spot #161) were located at the correct M_r . Shifts of protein pI and/or M_r of some isoforms are indicated in Fig. 3B. A tool calculating those shifts on 2-D gels has been devised (Halligan *et al.*, 2004). Examples of protein isoform expression patterns are presented in Supplementary Figs S2–S6 at JXB online.

Functional classification: Figure 4 presents the functional classification of the proteins according to the annotation in

the MIPS *Arabidopsis thaliana* database (MAtdB <http://mips.gsf.de/proj/thal/db>; Schoof *et al.*, 2002). The highest percentage (27%) corresponded to proteins of unknown function. Up to 10% of the identified proteins were involved in photosynthesis; this was expected, as the shoot tip, a photosynthetically active part of the grapevine, was collected. Unclassified proteins (9%) correspond to gene products of known function, but acting in various pathways, hence belonging to multiple categories. Protein metabolism (2% transcription, 12% protein synthesis, 8% protein destination, and 3% protein degradation) represented 25% of the identified proteins, which is consistent with the fact that the shoot tip is an actively growing organ where both cell production and elongation take place. Interestingly, in the control samples, 7% of the proteins identified have been previously reported to be involved in stress, indicating possible roles for these proteins in general metabolism in addition to their role in plant adaptation to severe conditions.

Statistical analyses using reproducible spots

Stepwise regression and correlation analyses: The present study demonstrated that abiotic stresses affected Chardonnay and Cabernet Sauvignon at both physiological and protein levels over time. It was hypothesized that some of the changes in protein expression levels could be due to the physiological adjustments occurring in response to the abiotic stresses. A forward stepwise regression model was used to identify which, if any, protein expression levels (independent variable) were best correlated with each of the physiological parameters (dependent variables) of water potential or shoot length. This analysis was performed with all 758 quantified proteins. Shoot length

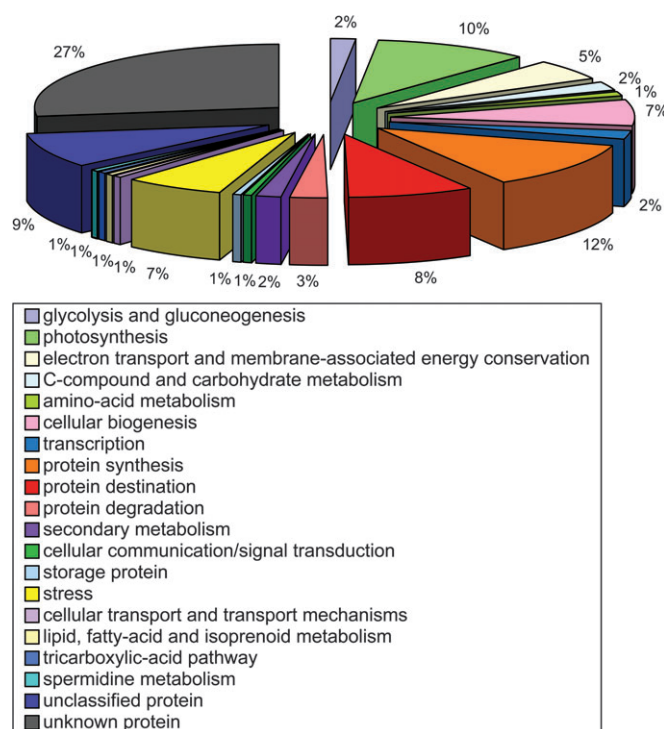


Fig. 4. Functional classification according to the annotation in the MIPS *Arabidopsis thaliana* database (MATDB Funcat) of the 191 identified proteins (Table 1).

was completely predicted ($R^2=0.998$) by eight proteins (Table 2A), and water potential was completely predicted ($R^2=1.0$) by eight proteins (Table 2B).

Pairwise correlations were computed across the expression profiles of these eight proteins, and none was found to be above 0.5, with P -values below 0.05 (data not shown). The eight proteins retained by each model were excised, and identified using MS analysis. No obvious molecular pattern could be found in the model describing water potential (Table 2A), whereas most proteins were involved in protein metabolism (either synthesis or destination) in the model fitting shoot length measurements (Table 2B).

ANOVA: Table 3 shows the number of spots with statistically significant main effects and/or interaction effects associated with the model in equation (1). Protein expression was mainly affected by the varietal (V) effect (305 spots out of 758, 40%); among those, 164 spots were up-regulated in Chardonnay, 137 spots were up-regulated in Cabernet Sauvignon, and four spots showed no variation between both cultivars. The day (D) and treatment (T) effects affected protein expression levels to a lesser extent (199 and 115 spots, respectively). Supplementary Tables S1 and S2 (available at *JXB* online) list the identified proteins showing significant variation for the T and V effects, respectively. Interest-

ingly, 120 spots showed significant variation when the day×treatment (DT) interaction was considered. This suggested that the grapevine response to the treatment also depended on the developmental stage of the plant or the duration of the stress. The variety×day (VD) interaction was shown to be a significant effect for fewer proteins (89 spots), indicating that the cultivars were not subjected to as much variation as the other factors over time at the protein level. Similarly, 79 proteins showed a significant variety×treatment (VT) interaction, thus indicating that a number of proteins were cultivar specific in their response to abiotic stresses. Differences in salt and water deficit tolerance might reside within this set of proteins.

Most of the proteins showed significant effects for several main factors and/or interactions, potentially highlighting the complex regulatory network of grapevine adaptation to the abiotic stresses at the protein level. For instance, more than a third of the spots with a significant V effect (305 spots) showed evidence of a significant D effect (106 spots). Similarly, a quarter of the proteins (81 spots) had a significant T effect (data not shown). For those spots with significant effect(s), the functional classification of the identified proteins has been represented according to the MATDB molecular function annotation (see Supplementary Fig. S7 at *JXB* online).

Discussion

Chardonnay appears to be more tolerant than Cabernet Sauvignon to water deficit and salinity at the physiological level

In this study, grapevine growth was significantly affected by the abiotic stresses. The stem water potential of control vines remained steady over time, whereas water deficit-treated and salt-stressed plants had significantly lower stem water potentials on day 8. The decrease of stem water potential in water deficit-treated plants was approximately twice that of salt-stressed plants on days 12 and 16.

At the molecular level, a pathogenesis-related protein characterized in tobacco, NtPRp27 (Okushima *et al.*, 2000), accounted for 82% of the stepwise regression model for water potential. This protein was up-regulated at the last developmental stage under the stress conditions. In addition to the pathogen and wound responses in tobacco, NtPRp27 transcripts were also observed to accumulate following water deficit stress or abscisic acid (ABA) treatment, but not under salinity conditions (Okushima *et al.*, 2000). The greater effect of water stress on NtPRp27 abundance compared with salt treatment reflects what was observed in grapevine shoots.

While stress levels were not different between cultivars, as indicated by the equal water potentials for the different stress treatments, shoot length measurements showed a clear distinction between cultivars. Chardonnay shoot

Table 2. Forward stepwise regression using all the 758 reproducible proteins in the model and (A) water potential or (B) shoot length

Step	Parameter	R^2	Protein identity	MatDB functional classification
(A)				
1	187_PRp27	0.8175	NtPRp27	Stress
2	188_TPIc	0.9438	Triosephosphate isomerase, chloroplastic	Photosynthesis
3	189_RPS7	0.9754	Ribosomal protein S7	Protein synthesis
4	190_XET	0.9910	Xyloglucan endotransglycosylase	Cellular biogenesis
5	191_na	0.9973	AT5g28840/F7P1_20	Unknown protein
6	192_RPL36	0.9992	Ribosomal protein L36, putative	Protein synthesis
7	193_na	0.9998	P0431H09.23	Unknown protein
8	194_SPDS	1.0000	Spermidine synthase	Spermidine metabolism
(B)				
1	195_RBC	0.7705	Ribulose 1,5-bisphosphate carboxylase-oxygenase	Photosynthesis
2	196_HSP70	0.9310	Heat shock protein 70 kD	Protein destination
3	197_APX	0.9777	Ascorbate peroxidase, thylakoid-bound	Stress
4	198_RP1	0.9922	60S acidic ribosomal protein P1	Protein synthesis
5	199_RP0	0.9988	60S acidic ribosomal protein P0	Protein synthesis
6	200_na	0.9995	Unknown protein	Unknown protein
7	201_HSP70	0.9998	Heat shock protein 70 kDa	Protein destination
8	202_CPN60	0.9998	Chaperonin-60 LS2	Protein destination

elongation was slowed down by water deficit, but not by salinity, whereas Cabernet Sauvignon shoot length was significantly reduced by both salinity and water deficit, as compared with control. When the leaves along the shoot were considered, leaves that were developed before the treatment onset (leaves 1–3) seemed to cope better with the stresses than the younger ones, indicating that cell production and elongation may have been affected in the young leaves (leaves 4–8). As observed for shoot growth, salinity had almost no effect on leaf growth of Chardonnay. With water deficit, leaf length was altered to a lesser extent in Chardonnay than in Cabernet Sauvignon, even though water potentials were lower in Chardonnay than in Cabernet Sauvignon. Therefore, Chardonnay appears to be more resistant to both water deficit and salinity than Cabernet Sauvignon.

Stepwise regression indicated that the inhibition of shoot growth was best correlated with several proteins involved either in protein synthesis, 60S acidic ribosomal P0 and P1 proteins, or in protein destination, two 70 kDa heat shock proteins (HSP70) and a 60 kDa LS2 chaperonin (CPN60). Because of their well-known role as molecular chaperones, HSP70 and CPN60 were deliberately classified in the protein destination category. However, many studies reported their involvement in heat stress initially, and various biotic or abiotic stresses later on (Wang *et al.*, 2004). Because the chaperones and ribosomal proteins included in the stepwise regression model accumulated under the present stress conditions, it can be hypothesized that they played an active role in maintaining protein metabolism in grapevine shoots.

It is a common observation that wine quality benefits from mild drought periods, thus improving berry composition without any significant yield loss. Therefore,

Table 3. Among the 758 reproducible proteins, number of significant proteins (adjusted P-value $P < 0.05$) for each effect of the ANOVA model (equation 1)

Factors	$P < 0.05$
V	305
D	199
T	115
V×D	89
V×T	79
D×T	120
V×D×T	111

monitoring the water status of grapevine plants, by following the carbon isotope composition (Gaudillère *et al.*, 2002) or by using thermal and visible imagery (Jones *et al.*, 2002; Grant *et al.*, 2006; Möller *et al.*, 2007), should favour grape cultivation for wine production. Drought effects are not limited to berry; vegetative organs are affected as well. Stoll *et al.* (2000) showed that drying roots of grapevine plants produced chemical signals, such as ABA concentration in xylem sap (Correia *et al.*, 1995), which were transported to the leaves, thus decreasing stomatal conductance. Drought caused the reduction of CO₂ assimilation and the enhancement of O₂ uptake, due to stomatal closure, along with a decrease in the activity of various enzymes of the Calvin cycle (Flexas *et al.*, 1999; Maroco *et al.*, 2002). Grapevine shoot organogenesis was shown to be under the influence of soil water deficit, and not related to competition for assimilates between axes of various sink strength (Lebon *et al.*, 2006); however, unlike what is presented in the study reported here, no varietal difference was reported in their experiments.

Protein identification of grapevine shoots reveals many proteins of unknown function, and a significant proportion of isoforms

In the present study, the largest category (27%) of the identified proteins corresponded to genes of unknown function, with a rate comparable with the one reported for genome annotation. In the fully sequenced genome of *A. thaliana* (Arabidopsis Genome Initiative, 2000) and draft sequence of the rice genome (Goff *et al.*, 2002), 30% of the genes were of unknown function. Koller *et al.* (2002) found that 30% of proteins had no known function or poor homology in rice seeds. Among grapevine ESTs, 19% of the genes did not match any annotation (Goes da Silva *et al.*, 2005), which was lower than the proportion of unknown proteins. The functional categorization drawn from a grape berry 2-D map (Sarry *et al.*, 2004) differed significantly from the present classification in grape shoots. Among other things, the proportion of proteins participating in protein metabolism was small in grape berries (13%) compared with grape shoot (25%), whereas the proportion of proteins involved in stress was greater in berries (19%) than in shoots (7%). These differences may be accounted for by the different organ sources (berry mesocarp versus shoot) of the tissue analysed as well as by grapevine cultivar difference (Gamay Noir versus Chardonnay/Cabernet Sauvignon).

Up to 43.5% of the excised proteins corresponded to isoforms in this study. This is a common observation reported in proteomic studies on different species such as alfalfa (Valot *et al.*, 2004; Watson *et al.*, 2004), ginseng (SI Kim *et al.*, 2003; Nam *et al.*, 2003), barley (Finnie *et al.*, 2004; Ostergaard *et al.*, 2004), and rice (ST Kim *et al.*, 2003). In maize seeds, isoforms represented 71% of the identified proteins, with one protein being present as 26 isoforms (Méchin *et al.*, 2004). In the present study, it was possible to identify several isoforms without trying to characterize them further. Expression profiles differed from one isoform to another, potentially giving some hints about their function (see Supplementary Figs S1–S6 at JXB online). The presence of isoforms raises the important question of protein redundancy and activity. Those diverse forms of protein could have different origins. They could be various gene products encoded by different genes but having the same function. They could be expressed in various organs or tissues, or act in different cell compartments. For instance, three ascorbate peroxidases were identified; two of them were cytosolic (spot #34 and spot #161) whereas the other one was chloroplastic (spot #197). Isoforms could also arise from alternative splicing, encoded by the same gene but yielding different mRNAs, hence different proteins. Alternatively, they could be explained by post-translational modification (PTM) wherein the same protein adopts several forms by adding non-protein components. PTMs affect many proteins and

can result from phosphorylation, glycosylation, sulphation, etc., and are preferentially associated with certain amino acids (Huber and Hardin, 2004). Though 2-DE cannot indicate whether those isoforms are encoded by different copies of the same or related genes or whether they correspond to different forms of the same gene product, such a technique gives the opportunity to isolate post-translationally modified proteins. Other tools, such as affinity chromatography, are needed to characterize them further.

Few proteins respond to abiotic stresses

ANOVAs indicated that only 15% (115 out of 758) of the spots showed significant variation in response to the water deficit and salinity stress treatments; among them, 36 spots were identified and their annotations suggest they belong to various functional categories (see Supplementary Table S1 at JXB online). A few identified proteins, displaying apparent overexpression under the present stress conditions, were of particular interest. A nuclear matrix constituent protein 1-related (NMCP1, spot #82) was significantly up-regulated by both water deficit and salinity stresses at the oldest developmental stage (day 16), regardless of the variety; however, protein levels reached in salt-stressed Cabernet Sauvignon vines on day 16 were equivalent to those expressed on day 0 (see Supplementary Fig. S8A at JXB online). This protein was found to be exclusively localized at the nucleus periphery of carrot cells (Masuda *et al.*, 1997) and seemed to be involved in restructuring of the nuclear envelope during mitosis (Masuda *et al.*, 1999). To our knowledge, no study has mentioned a potential role of NMCP1 in abiotic stress. Another novel result was the overexpression of a ribosomal protein L39 (RPL39; spot #175), under both stresses, but particularly under water deficit, on the last developmental stage analysed (see Supplementary Fig. S8B at JXB online). As far as is known, this is the first time that an increase in the amount of RPL39 under abiotic stress has been reported in plants. In yeast, this protein is a 60S ribosomal subunit implicated in translational accuracy (Dresios *et al.*, 2000). Both NMCP1 and RPL39 bore fairly low MASCOT protein scores (39 and 41, respectively; Table 1), therefore one must remain cautious about the tentative annotations, and the involvement of such proteins in these processes remains to be evidenced.

Three spots identified as photosynthetic enzymes, two RBCL isoforms and a chloroplastic sedoheptulose-1,5-bisphosphatase (SBPase), were up-regulated under both abiotic stress conditions, water deficit in particular (spot #88 Supplementary Fig. S1A, spot #65 Supplementary Fig. S1D, and spot #72 Supplementary Fig. S8C at JXB online). Interestingly, a spot loosely annotated photosystem II (PSII, spot#145, Supplementary Fig. S8D at JXB

online) was down-regulated by both water and salt stresses. However, it should be noted that the protein score attached to the MS identification of this spot was poor (protein score=45, Table 1); thus, its implication in photosynthesis needs to be confirmed. Other RBCL isoforms (Supplementary Fig. S1B, C, E at *JXB* online) or proteins involved in photosynthesis, such as RuBisCO activase, Mg-chelatase, chloroplastic phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase, chloroplastic triose phosphate isomerase, and oxygen-evolving protein of photosystem II, were not affected by abiotic stresses, highlighting the diversity of response to stress within a gene family or a particular biochemical pathway. An increased expression of four isoforms of RBCL was observed under drought stress in sugar beet leaves (Hajheidari *et al.*, 2005). Both small and large subunits of RuBisCO were down-regulated under ozone stress in rice leaf (Agrawal *et al.*, 2002). Maroco *et al.* (2002) reported a reduced activity of several enzymes of the Calvin cycle in drought-stressed grapevine. Photosynthesis dysfunction caused by stress can be associated with oxidative stress (Mittler *et al.*, 2004). While it appears that this is not the case in the present study because none of the antioxidant enzymes identified (ascorbate peroxidase, superoxide dismutase, or peroxiredoxin) appeared to be up-regulated by stress, it is possible that other isozymes, which were not detected here, were affected or that substrate levels rather than protein amounts were responsible for oxidative stress. Interestingly, protein response to abiotic stress is generally greater on the last developmental stage of grapevine shoots, reflecting what was observed at the physiological level.

Protein expression is mainly affected by cultivar differences

ANOVA indicated that protein quantity was most strongly affected by cultivar, then time, and finally treatment. Potentially, proteins being expressed at higher levels in one cultivar compared with the other could account for the differences observed at the morphological and physiological levels. Some functional categories were specific to a particular variety. Proteins involved in protein destination and degradation are specifically expressed in greater amounts in Cabernet Sauvignon (see Supplementary Table S2, as well as inset in Supplementary Fig. S7 at *JXB* online). A chloroplastic peptidyl-prolyl *cis-trans* isomerase (PPIase), involved in protein folding, was clearly more abundant in Cabernet Sauvignon, relative to Chardonnay (spot #101, Supplementary Fig. S9A at *JXB* online). Two other proteins also participating in protein degradation, a proteasome subunit alpha type 4 (PSA4, spot #51, Supplementary Fig. S9B at *JXB* online) and a ubiquitin-conjugating enzyme family member (CE, spot #92, Supplementary Fig. S9C at *JXB* online), further

exemplified this phenomenon. The apparent activation of the proteolysis machinery of Cabernet Sauvignon is consistent with the observation that this grapevine cultivar was less able to cope physiologically with water and salt stresses. A xyloglucan endotransglycosylase (XET), part of the XTH family of enzymes (Rose *et al.*, 2002), a cell wall-modifying enzyme associated with cell elongation (Wu *et al.*, 2005), was expressed at higher abundance during the initial developmental stage day 0 in Chardonnay and decreased over time regardless of the treatment, compared with Cabernet Sauvignon wherein XET amounts remained low except on day 8 (spot #101; see Supplementary Fig. S9D at *JXB* online). At the physiological level, shoot growth was better sustained under stress conditions in Chardonnay, compared with Cabernet Sauvignon. It can be hypothesized that the elevated amounts of XET observed in Chardonnay may participate in maintenance of shoot elongation in this species, relative to Cabernet Sauvignon.

In this study, 45% (15 out of 33 spots) of the excised proteins observed to be more abundant in Chardonnay shoots did not yield any annotation. One explanation for this low rate of identification is that these gene products were specific to Chardonnay and account, in part, for its enhanced physiological tolerance to abiotic stress. Additionally, some functional categories were common to both cultivars, but were preferentially expressed in one variety and not the other. Although we demonstrated that a significant proportion of protein isoforms were identified, no isoforms were shared between the two cultivars (Supplementary Table S2 at *JXB* online). For instance, two ATP synthase beta chain isoforms (ATPsb) were more abundant in Chardonnay whereas three RBCL isoforms were up-regulated in Cabernet Sauvignon.

Among the proteins displaying a significant effect for variety factor, 10% (79 out of 758) presented significant effects for variety×treatment (VT) interaction as well, of which nine were identified by MS (see Supplementary Table S3 and VT pie chart in Supplementary Fig. S7 at *JXB* online). These proteins responded to abiotic stresses, albeit displaying varietal differences (see Supplementary Fig. S10 at *JXB* online). Most of these proteins showed significant effects for treatment (*T*) factor as well (see Supplementary Table S3 at *JXB* online, spots #113, 78, 7, 149, and 38). They might explain the cultivar differences in stress adaptation. For instance, an NAC-related protein (NAC, spot #38) was significantly down-regulated under water stress and, to a greater extent, under salinity conditions in Cabernet Sauvignon, but showed no distinct responses to treatments in Chardonnay (see Supplementary Fig. S10H at *JXB* online). No function was reported for this NAC protein. Likewise, a mitochondrial peroxiredoxin (PRX, spot #119) was strongly induced by salt stress on day 8 in Cabernet Sauvignon, whereas it was almost not affected by any treatment in Chardonnay over

time (see Supplementary Fig S10C at *JXB* online). PRXs belong to the antioxidant system, thus scavenging reactive oxygen species (Dietz *et al.*, 2006). It can be hypothesized that the salt-treated shoots from Cabernet Sauvignon suffered from oxidative stress on day 8, hence presenting more damage at the physiological level; however, it must be emphasized that none of the other antioxidant enzymes were significantly up-regulated under the present stress conditions. Among these proteins showing a significant effect for VT interaction, proteins beside PRX have been reported to be involved in response to abiotic or biotic stresses. Pathogenesis-related proteins 10 (PR-10) have been associated with a variety of stress responses in plants; in ginseng, they displayed an RNase activity (Hoffmann-Sommergruber, 2002). In grapevine, Castro *et al.* (2005) observed the induction of several PR-10 isoforms upon herbicide applications. Under the present conditions, PR-10 (spot #113) was markedly up-regulated by salt treatment over time and water deficit stress on day 16 in Cabernet Sauvignon shoots, but not in Chardonnay (see Supplementary Fig. S10B at *JXB* online). Thus, this protein did not seem to participate in the relative stress tolerance displayed by Chardonnay. bHLH proteins correspond to a large family of basic helix-loop-helix transcription factor members which have been involved in plant adaptation to abiotic stresses, such as drought and wounding (Kim and Kim, 2006), salinity (Kiribuchi *et al.*, 2005), and iron deficiency (Yuan *et al.*, 2005; Ogo *et al.*, 2006). In the present experiment, the up-regulation of bHLH protein (spot #7) under stress conditions on day 8 was followed by almost a repression on day 16 in Cabernet Sauvignon, while its abundance was increased under salt conditions in Chardonnay (see Supplementary Fig. S10E at *JXB* online), potentially explaining the sustained shoot elongation observed in this variety. Interestingly, another protein containing a bHLH domain, GHDEL61 protein (DEL, spot #78), also presented an increased abundance in salt-stressed shoots from Cabernet Sauvignon on day 8 (see Supplementary Fig. S10D at *JXB* online). The relevance of such an observation in grapevine adaptation to abiotic stress is yet to be elucidated. Another potential candidate in cultivar-dependent responses to abiotic stress might be found in a pentatricopeptide repeat-containing protein (PPR, spot #93), which displayed an up-regulation under salinity on days 8 and 16, as well as under water deficit on day 16 in Chardonnay relative to the controls; almost the opposite pattern could be noted in Cabernet Sauvignon (see Supplementary Fig. S10F at *JXB* online). PPR proteins contain a degenerate 35 amino acid repeat motif; they bind to RNA and affect the post-transcriptional regulation of chloroplastic or mitochondrial genes (Okuda *et al.*, 2006; Schimtz-Linneweber *et al.*, 2006). A total of 466 PPR proteins were described in *Arabidopsis thaliana* (Rivals *et al.*, 2006). To our knowledge, this protein has never been

reported to participate in the stress response in plants. It can be hypothesized that, when subjected to environmental constraints such as drought or salinity, *V. vinifera* cv. Chardonnay copes by enhancing RNA editing of the major gene products involved in growth processes. Again, care should be taken when drawing such conclusions, as the protein score at which PPR was identified was low.

Another proteomic study also highlighted the variation in protein amount, albeit to a lesser extent, between various grapevine cultivars (Sarry *et al.*, 2004). None of the aforementioned proteins, however, was identified in berry mesocarp. Major changes in protein pattern from one cultivar to another were also reported in rice during drought (Salekdeh *et al.*, 2002) or salt stress (Abbasi and Komatsu, 2004), as well as in wheat species differing in salt sensitivity (Majoul *et al.*, 2000; Ouerghi *et al.*, 2000). There are many phenotypical differences between Chardonnay and Cabernet Sauvignon, the most obvious being the colour of the berries. Shoot tips and young leaves also differ (Bettiga, 2003; Wolpert, 2003). Genetic variations have also been reported between grapevine cultivars (Aradhya *et al.*, 2003; Adam-Blondon *et al.*, 2004; Riaz *et al.*, 2004; This *et al.*, 2006); Cabernet Sauvignon resulted from a cross between Cabernet Franc and Sauvignon Blanc, while Chardonnay was a cross between Pinot Noir and Gouais Blanc (Bowers *et al.*, 1999). In this study, cultivar differences have been observed at both physiological and protein levels in relation to abiotic stress adaptation over time. These observed variations probably arose from the genetic differences between these two cultivars.

Conclusions

Here, the first proteomic study on grapevine shoots of two of the most important cultivars worldwide, Chardonnay and Cabernet Sauvignon, exposed to water or salt stress for either 8 d or 16 d, is presented. The adverse effects of both treatments were assessed at a physiological level by measuring the stem water potential, and shoot and leaf elongation. Significant differences in leaf elongation were found between cultivars in response to salinity and water deficit, with Chardonnay apparently more stress tolerant than Cabernet Sauvignon. Statistical analyses showed that protein quantities varied mainly in response to the cultivars, then with time, and finally with the stress. Many of the proteins up-regulated in Chardonnay were of unclassified or of unknown function, whereas proteins specifically up-regulated in Cabernet Sauvignon were involved in protein metabolism. Among the proteins responding to stress treatments, some have previously been involved in stress adaptation, such as PR-10, PRX, and bHLH, while other proteins, such as NMCP1, RPL39, DEL, and PPR, were described as being affected by abiotic stress in plants for the first time. Further analyses are needed to determine the functional role of many of these

proteins in *V. vinifera*. Proteins specific to each cultivar are being investigated. By combining physiology and protein analyses, better characterization of the grape shoot response to two major abiotic stresses could be obtained.

Supplementary data

The Supplementary data mentioned herein can be found at *JXB* online.

Supplementary Tables S1, S2, and S3 list the identified proteins with significant effect ($P < 0.10$) for, respectively, the treatment (*T*) factor, the variety (*V*) factor, and the variety $\times$ treatment (*VT*) interaction in the ANOVA model.

Supplementary Figs S1–S6 illustrate the expression patterns of the isoforms identified for the following proteins: RuBisCO large subunit, actin, cytosolic ascorbate peroxidase, RNA-binding protein, heat shock protein 70 kDa, and ATP synthase beta subunit.

Supplementary Fig. S7 presents the functional classification according to MAtDB of the identified proteins significant for each individual effect of the ANOVA model. Supplementary Figs S8, S9, and S10 show examples of proteins responding to treatment, variety, and their interaction, respectively.

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