JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Proteome Changes in Leaves from Grapevine (*Vitis vinifera* L.) Transformed for Alcohol Dehydrogenase Activity

FRANÇOIS-XAVIER SAUVAGE,† MARTINE PRADAL,† PHILIPPE CHATELET,§ AND CATHERINE TESNIERE*,†

UMR1083, Sciences Pour l'Oenologie, and UMR1098, Biologie des Espèces Pérennes Cultivées, INRA, F-34060 Montpellier, France

A proteomic approach has been used to study changes in leaf protein content from plants transformed for alcohol dehydrogenase (ADH) activity. Individual quantitative analysis of 190−436 spots separated by two-dimensional electrophoresis was performed, and spots displaying significant quantitative changes between control (C), sense (S), and antisense (R) transformants were selected using Student's *t* test. Of the 14 spots selected and further analyzed after trypsic digestion, 9 could be identified by MS analysis and 5 by LC-MS/MS. Identified proteins had mainly a chloroplastic origin: four rubisco large subunits, one rubisco binding protein, two glutamine synthetases, one elongation factor Tu, one ATP synthase $\beta$ subunit, and one plastidic aldolase. Proteins with other localization were also identified, such as a UDP-glucose pyrophosphorylase, a mitochondrial aminomethyltransferase, a linalool synthase, which comigrated with the protein identified as elongation factor Tu, an enolase comigrating with a glyceraldehyde 3-phosphate dehydrogenase, and a mixture of eight proteins among which were a dehydroascorbate reductase, a chalcone isomerase, and a rubisco activase. The results emphasize the changes in carbon metabolism-associated proteins linked to the alteration in ADH activity of grapevine transformant leaves.

**KEYWORDS: Alcohol dehydrogenase; grapevine; leaves; proteins; transformants; *Vitis vinifera* L.**

## INTRODUCTION

Several approaches have been undertaken to assess the functional role of alcohol dehydrogenase (ADH) through genetic transformation. Resulting studies have shown a role of the ADH function in development, stress responses, and metabolite synthesis (*1*−*4*). More recently were produced a number of transgenic transformants with modified levels of ADH activity to study the role of ADH in grapevine (*5*). Initial analysis of these transformants indicated changes in sucrose content, in some phenolic compounds, and in volatile secondary metabolites, especially those from the glycosydically bound fraction.

In recent years, proteomics has been successfully applied for a systematic analysis of gene product modifications to a number of plant species submitted to a wide range of conditions (*6*−*10*). The technological progress made in the separation of proteins in two-dimensional electrophoresis (2-DE) gel coupled with the development of mass spectrometric techniques has allowed powerful analysis of proteome changes.

In the present study, proteomics has been used to increase our knowledge of the involvement of the ADH function in the differential leaf protein expression from transformed grapevine plants. We report a comparative proteome approach, based on the separation of protein extracts by 2-DE and subsequent identification of the relevant spots using sensitive mass spectrometry techniques. Among the most affected spots, the majority of proteins were identified as being related to chloroplasts or to primary metabolism.

## MATERIALS AND METHODS

**Plant Material.** The transformed grapevine plants (*Vitis vinifera* L.) were obtained in a previous experiment (*5*). The transgenic plants were grown in greenhouse conditions, and for each control, sense, and antisense line, samples from young, fully expanded leaves were recovered. To randomize biological variations, 20 leaves were collected on 4−5 plants of the same line, pooled, washed, and quickly dried with tissue paper. Leaves were then frozen in liquid nitrogen and stored at −80 °C prior to protein extraction.

**Protein Extraction and Determination.** Plant material was ground under liquid nitrogen, and 400 mg of powder was suspended in 1 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, containing 10 M urea). After homogenization and sonication for 15 min, the mixture was centrifuged (13000*g* at 4 °C for 15 min). One volume of TCA (30% w/v) was then added to 2 volumes of supernatant. After homogenization, the mixture was transferred at −20 °C for 1 h. Precipitated material was then collected by centrifugation (13000*g* for 5 min) and the pellet washed three times with acetone and once with ethanol. Precipitated proteins were then dissolved using 500 $\mu$L of lysis buffer [7 M urea,

* Address correspondence to this author at UMR 1083, Sciences Pour l'Oenologie, INRA, 2 Place Viala, 30460 Montpellier cedex 01, France (Telephone: +33 4 99 61 25 31. Fax: +33 4 99 61 28 57. E-mail: tesniere@supagro.inra.fr).

† UMR1083, Sciences Pour l'Oenologie, INRA.

§ UMR1098, Biologie des Espèces Pérennes Cultivées, INRA.

10.1021/jf063723w CCC: $37.00 
Published on Web 03/10/2007

2% CHAPS (w/v), 2 M thiourea, 0.2% DTT (w/v)], and insoluble material was eliminated by centrifugation. One aliquot of the supernatants was used for the determination of protein concentration, using the Bradford method (*11*), and the remaining part was stored at −80 °C until assayed.

**2-DE and Gel Image Analysis.** Proteins were applied by rehydration onto immobilized nonlinear pH gradient 3−10 Immobiline Dry-strips (18 cm) for 10 h at 20 °C. Isoelectric focusing (IEF) was performed on an IPGphor system (GE Healthcare) by increasing the voltage in the different following steps: 9 h at 50 V, 1 min gradient to 300 V, 30 min at 300 V, 3 h gradient to 8000 V, 11 h at 8000 V, and 3 h gradient to 300 V, for a total of 102 kV·h. After IEF, strips were equilibrated for 15 min in equilibration buffer [50 mM Tris-HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2 M thiourea; 2% (w/v) SDS] with 2% (w/v) DTT, then replacing DTT by 2.5% iodoacetamide (w/v). Second-dimension SDS gels were run in 11% (w/v) acrylamide using an ISODALT apparatus (GE Healthcare) at 15 °C in a Tris−Gly buffer [25 mM Tris-HCl; 192 mM glycine; 0.1% (w/v) SDS]. Running conditions were 2 h at 20 mA/gel, 2 h at 30 mA/gel and 40 mA/gel until the forehead blue reached the bottom of the gel. These conditions allowed the resolution of polypeptides with a molecular mass ranging from 20 to 200 kDa. Three gel replicates were produced for each sample.

The 2-DE gels were stained with colloidal Coomassie Brillant Blue (CBB) G-250 (Bio-Rad) according to the method of ref *12*. Gel images were obtained with an Image Scanner (GE Healthcare). As usual in gel analysis, to reduce background noise and eliminate unexploitable spots, a maximal area common to all nine gels was defined using selected major proteins bordering each side of the gels. In all cases, this area corresponded to at least 95% of the total gel. Noise reduction, background subtraction, spot detection, quantification, gel-to-gel matching, and differential protein display analysis were carried out using the TotalLab ImageMaster 2-D Platinum version 5 software (GE Healthcare). As three conditions were analyzed [control (C), sense-transformed (S), and antisense-transformed (R) plants] and three independent replicates were performed per condition, image analysis was carried out considering all nine gels. After normalization on each gel, the spot intensity for each protein was quantified using three gels from the same extraction and used to compare the protein level among the samples. Proteins exhibiting variation among the samples were selected according to Student's *t* test. All proteins exhibiting at least 2-fold changes in all replicates were considered to be differentially regulated proteins, and the corresponding spots were excised.

**Protein Identification by Mass Spectrometry Analysis (MS).** Protein spots excised from the CBB gels were destained, then digested by sequence-grade trypsin (GOLG, Promega), and treated as previously described (*13*). The final extract was introduced directly into an UltraFlex MALDI-TOF mass spectrometer (Brucker-Franzen Analytik, Bremen, Germany) in a reflectron mode with an accelerating voltage of 25 kV and a delayed extraction of 50 ns. Peptide mass fingerprint spectra were calibrated using trypsin autolysis products (*m*/*z* 842.51, 1045.56, and 2211.10) as internal standards and were acquired in an automatic mode using the AutoXecuteTM module of FlexcontrolTM (Brucker-Franzen Analytik). Protein identification was performed by searching for *Viridiplantae* proteins in the Swiss-Prot and nonredundant Trembl databases, using the Mascot search engine, by peptide mass fingerprint analysis. The following parameters were applied: monoisotopic mass accuracy, 50 ppm; missed cleavages, 1; allowed fixed modifications, carbamidomethyl (C); and variable modifications, oxidation (M).

**Protein Identification by LC-MS/MS Analysis.** Samples (1 μL) were analyzed online using nanoflow HPLC nanoelectrospray ionization on a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR Pulsar-*i*, Applied Biosystems, Foster City, CA) coupled with an Ultimate 3000 HPLC (Dionex, Amsterdam, The Netherlands). Desalting and preconcentration of samples were carried out online on a Pepmap precolumn (0.3 mm × 10 mm). An AB gradient (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) consisting of 0−40% B in 30 min and 40−80% B in 5 min at 300 nL/min was used to elute peptide from the capillary (0.075 mm × 150 mm) reverse-phase column (Pepmap, Dionex). Nanoelectrospray mass spectrometry was performed online on a Q-TOF fitted with an uncoated silica PicoTip Emitter (NewOjective, Woburn, MA) with an outlet diameter of 8 μm. Spectra were recorded using Analyst QS 1.1 software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage (IS), 1800 V; curtain gas (CUR), 25; declustering potential (DP), 75 V; focusing potential (FP), 265 V; declustering potential 2 (DP2), 15 V. Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient. Every 7 s, the instrument cycled through acquisition of a full-scan spectrum (1 s) and two MS/MS spectra (3 s each). Peptide fragmentation was performed using nitrogen gas on the most abundant doubly or triply charged ions detected in the initial MS scan, with a collision energy profile optimized according to peptide mass (using manufacturer parameters) and an active exclusion time of 0.60 min. All MS/MS spectra were searched against the *Vitis vinifera* entries of either the Swiss-Prot or Trembl database (http://www.expasy.ch) or ESTs in Genbank databases (http://www.ncbi.nlm.nih.gov/Genbank/index.html) by using the Mascot v 2.1 algorithm (http://www.matrixscience.com), by sequence query analysis. All significant hits were manually inspected.

## RESULTS

**Proteome Changes in Grapevine Leaves from ADH Transformants.** The total proteins from grapevine leaves were resolved in 190−436 spots in highly reproducible SDS−polyacrylamide gels (isoelectric focusing pH range, 3−10; size, 18 cm; SDS-PAGE gel size, 23 cm × 20 cm). **Figure 1** shows a selection of the entire image of the CBB-stained 2-D gels of total extracted proteins from leaves of control (C), sense-transformed (S), and antisense-transformed (R) plants. A Student's *t* test was performed to select proteins displaying significant changes. Overall, the protein levels of 21, 57, and 35 spots were found to be altered by transformation between, respectively, R and S, C and S, and R and C (**Table 1**). Compared to spots from C, the majority of the spots from R and S were down-regulated with, respectively, 80 and 82% of the total altered proteins.

From **Table 1**, only proteins showing over 2-fold changes in all three replicate gels from the same protein extraction and with a minimal volume of 0.1% were considered to be differentially regulated proteins and in sufficient quantity to be analyzed. These spots (*14*) were excised from the gels, digested with trypsin, and analyzed with a MALDI-TOF mass spectrometer. For five spots for which identification by MALDI-TOF analysis could not be obtained, a MS/MS analysis was performed.

**Identification of Proteins by MS Analysis.** This technique allowed the identification, from 9 of the excised spots, of a total of 10 proteins, with spot 692 corresponding to a mixture of 2 proteins (**Table 2**). Low scores after MS analysis did not allow spots 484, 485, 527, 583, and 642 to be identified. The identified proteins were mainly of chloroplastic origin. These included three rubisco large subunits for spots 561, 597, and 619 (with scores of, respectively, 130, 68, and 74), a rubisco binding protein for spot 427 (with a score of 69), a glutamine synthetase for spot 684 (with a score of 75), an elongation factor Tu for spot 692 (with a score of 69), and an ATP synthase β subunit for spot 444 (with a score of 71). Proteins with other localizations were also identified, such as a UDP-glucose pyrophosphorylase for spot 453 (with a score of 60), a mitochondrial aminomethyltransferase for spot 520 (with a score of 72), and a linalool synthase for spot 692 (with a score of 68).

**Identification of Proteins by MS/MS Analysis.** The five unidentified spots (484, 485, 527, 583, and 642) were analyzed using MS/MS. The identified proteins (**Table 3**) included an additional glutamine synthetase for spot 484, a mixture of enolase and glyceraldehyde-3-phosphate dehydrogenase for spot

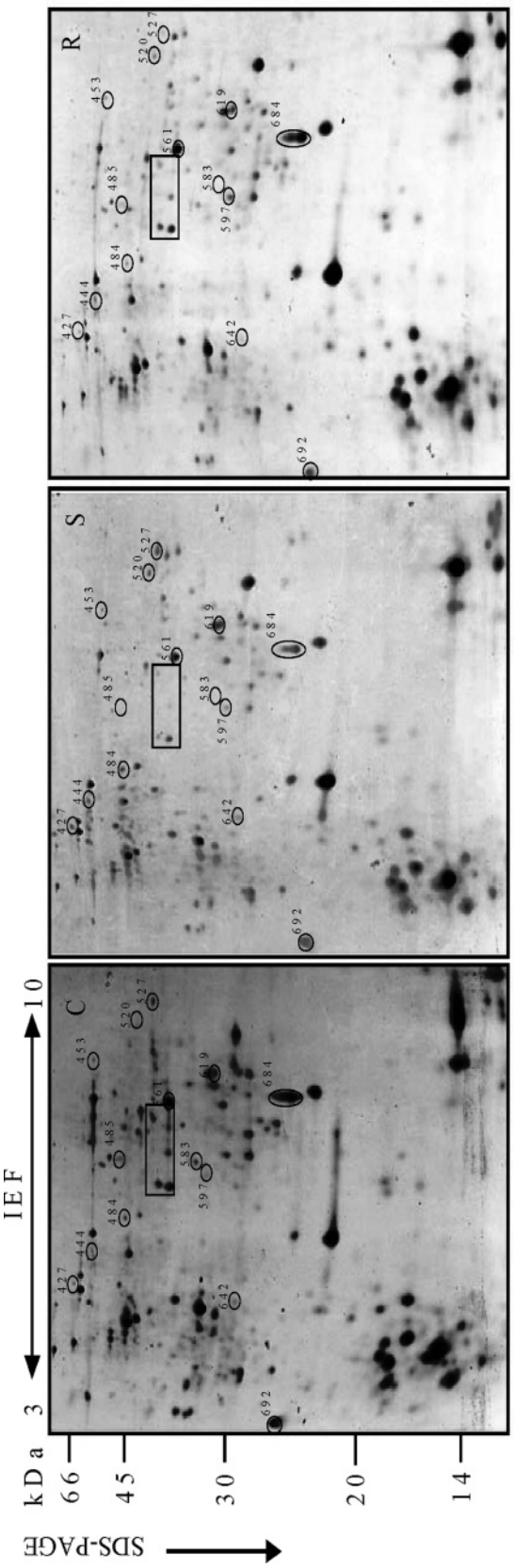


**Figure 1.** 2-DE of proteins from grapevine leaves of alcohol dehydrogenase transformants. Proteins isolated from the control (C), sense-transformed (S), and antisense-transformed (R) plants are shown. Gels were stained with Coomassie Brillant Blue G-250. Positions of proteins with significant changes between transformants are indicated by circles. The putative position of ADH is indicated by a rectangle.

**Table 1.** Two-Sample Student's *t* Test Analysis To Compare the Relative Volume of Each Individual Spot[a]

| C/S | | C/R | | S/R | |
|---|---|---|---|---|---|
| spot | calcd Student *t* | spot | calcd Student *t* | spot | calcd Student *t* |
| 853 | 51.89 | 607 | 21.42 | 427 | 10.92 |
| 770 | 37.16 | 435 | 19.90 | 485 | 9.46 |
| 607 | 21.42 | 464 | 14.58 | 563 | 9.44 |
| 435 | 19.90 | 792 | 10.73 | 575 | 7.80 |
| 676 | 15.07 | 718 | 9.87 | 629 | 7.27 |
| 464 | 14.58 | 694 | 7.74 | 597 | 5.30 |
| 575 | 13.78 | 839 | 6.79 | 538 | 4.49 |
| 519 | 13.56 | 578 | 5.64 | 444 | 4.16 |
| 427 | 13.25 | 745 | 5.13 | 642 | 4.12 |
| 454 | 12.45 | 512 | 5.06 | 684 | 4.10 |
| 572 | 10.81 | 577 | 5.03 | 572 | 3.85 |
| 631 | 10.50 | 747 | 4.89 | 527 | 3.39 |
| 718 | 9.87 | 570 | 4.80 | 692 | 3.39 |
| 477 | 9.29 | 729 | 4.66 | 637 | 3.21 |
| 755 | 8.67 | 763 | 4.64 | 540 | 3.20 |
| 637 | 7.86 | 625 | 4.63 | 466 | 3.11 |
| 529 | 7.57 | 468 | 4.57 | 714 | 3.09 |
| 757 | 7.33 | 801 | 4.40 | 561 | 3.06 |
| 625 | 6.74 | 675 | 4.38 | 484 | 2.86 |
| 476 | 6.50 | 496 | 4.19 | 520 | 2.84 |
| 445 | 6.42 | 480 | 4.06 | 786 | 2.82 |
| 792 | 6.17 | 699 | 3.93 | | |
| 618 | 5.99 | 692 | 3.84 | | |
| 714 | 5.35 | 723 | 3.64 | | |
| 558 | 5.09 | 485 | 3.53 | | |
| 595 | 4.73 | 757 | 3.49 | | |
| 729 | 4.66 | 672 | 3.47 | | |
| 444 | 4.44 | 561 | 3.34 | | |
| 583 | 4.44 | 627 | 3.24 | | |
| 801 | 4.40 | 489 | 3.13 | | |
| 557 | 4.40 | 849 | 3.08 | | |
| 675 | 4.38 | 678 | 3.06 | | |
| 670 | 4.37 | 582 | 3.01 | | |
| 642 | 4.28 | 610 | 2.97 | | |
| 496 | 4.19 | 651 | 2.86 | | |
| 619 | 4.15 | | | | |
| 559 | 4.10 | | | | |
| 597 | 4.09 | | | | |
| 480 | 4.06 | | | | |
| 680 | 4.02 | | | | |
| 699 | 3.93 | | | | |
| 488 | 3.90 | | | | |
| 577 | 3.89 | | | | |
| 661 | 3.65 | | | | |
| 723 | 3.64 | | | | |
| 739 | 3.59 | | | | |
| 627 | 3.32 | | | | |
| 647 | 3.18 | | | | |
| 629 | 3.17 | | | | |
| 489 | 3.14 | | | | |
| 520 | 3.13 | | | | |
| 574 | 3.12 | | | | |
| 849 | 3.08 | | | | |
| 678 | 3.06 | | | | |
| 413 | 2.92 | | | | |
| 602 | 2.91 | | | | |
| 453 | 2.86 | | | | |

[a] When comparing two means (C/S, C/R, or S/R), three treatments (C, S, and R), and three replicates per treatment, the number of degrees of freedom is 4 [(3 + 3) − 2]. The statistical probability at 95% of two means being different is significant for a calculated (calcd) value of $t > 2.78$.

485, an additional rubisco large subunit for spot 527, a plastidic aldolase for spot 583, and a mixture of eight proteins, among which were a dehydroascorbate reductase, a plastidic aldolase, a rubisco activase, and a chalcone isomerase, for spot 642.

**Effect of Transformation on Protein Content.** Compared to the control line, sense and antisense lines behave significantly differently. S transformant leaves (**Figure 2A−G**) displayed an

**Table 2.** Transformation Responsive Proteins Identified by MS Analysis after IEF/SDS-PAGE

| spot[a] | masses[b] | p*I*[c] | identified protein (species) | accession no.[d] | matching peptides[e] | score | %[f] |
|---|---|---|---|---|---|---|---|
| 427 | 56900/63287 | 5.50/5.85 | rubisco binding protein (*Pisum sativum*) | P08927 | 6 | 69 | 17 |
| 444 | 52800/52738 | 5.70/5.28 | ATP synthase $\beta$ subunit (*Vitis vinifera*) | Q3ZU94 | 11 | 71 | 23 |
| 453 | 49300/51640 | 8.10/6.36 | UDP-glucose pyrophosphorylase like protein (*Solanum tuberosum*) | Q3HVN2 | 6 | 60 | 18 |
| 520 | 41400/44759 | 8.60/8.55 | aminomethyltransferase mitochondrial precursor (*Arabidopsis thaliana*) | O65396 | 6 | 72 | 18 |
| 561 | 37300/52310 | 6.90/6.22 | rubisco large subunit (*Uncaria* sp.) | Q8HT55 | 14 | 130 | 31 |
| 597 | 34700/49894 | 5.90/6.18 | rubisco large subunit fragment (*Capparis spinosa*) | Q7YNU8 | 7 | 68 | 17 |
| 619 | 33400/49434 | 8.00/6.75 | rubisco large subunit fragment (*Reaumuria cistoides*) | Q8MFQ5 | 9 | 74 | 20 |
| 684 | 29500/47502 | 7.00/6.77 | glutamine synthetase leaf isozyme chloroplast precursor (*Phaseolus vulgaris*) | P15102 | 7 | 75 | 29 |
| 692 | 29700/32014 | 4.60/4.54 | elongation factor Tu (*Halochlorococcum moorei*) | Q69B00 | 6 | 69 | 39 |
| | 29700/65888 | 4.60/5.85 | linalool synthase (*Artemisia annua*) | Q9SPN0 | 9 | 68 | 22 |

[a] Spots are numbered accordingly to **Figure 1**. [b] Observed molecular mass determined on the gel (Da)/theoretical molecular mass (Da). [c] Observed p*I* determined on the gel/theoretical p*I*. [d] Accession number in Swiss-Prot and TrEMBL databases and organism assignment after BLAST homology searches. [e] Number of peptide mass fingerprinting matching with the reference protein. [f] Percentage of amino acids in reference proteins covered by matching peptides.

**Table 3.** Transformation Responsive Proteins Identified by LC-MS/MS Analysis after IEF/SDS-PAGE

| spot[a] | mass[b] | p*I*[c] | identified protein | accession no.[d] | no. of peptide sequences[e] | score | sequence |
|---|---|---|---|---|---|---|---|
| 484 | 42500 | 5.80 | glutamine synthetase | CD711006 | 2 | 45 | EDGGYELIK |
| | | | | | | 31 | AILNLSLR |
| | | | | CF608137 | 1 | 28 | LDDLLNMDIRPYTDK |
| 485 | 42200 | 6.00 | enolase | BM436891 | 4 | 40 | EGLELLK |
| | | | | | | 44 | ACNALLLK |
| | | | | | | 54 | VQIVGDDLLVTNPK |
| | | | | | | 80 | NQIGTVTESIEAVK |
| | 42200 | 6.00 | glyceraldehyde 3-phosphate dehydrogenase | CB975696 | 1 | 53 | VSAAVPSGASTGIYEALELR |
| | | | | CB971973 | 1 | 52 | IVDNETISVDGKPIK |
| | | | | CD711955 | 1 | 56 | VVDLAHLVAAK |
| 527 | 39800 | 8.80 | rubisco large subunit | CD007779 | 3 | 63 | GGLDFTK |
| | | | | | | 43 | AVYECLR |
| | | | | | | 61 | DDENVNSQPFMR |
| 583 | 35300 | 6.20 | plastidic aldolase | CB975326 | 7 | 39 | EAAWGLAR |
| | | | | | | 64 | ANSLAQLGK |
| | | | | | | 60 | ALQNTCLK |
| | | | | | | 71 | EAQEALLIR |
| | | | | | | 73 | MVDVLVEQK |
| | | | | | | 71 | ATPEQVADYTLK |
| | | | | | | 53 | GLVPLVGSNDESWCQGLDGLASR |
| | | | | CA808522 | 1 | 77 | LASIGLENTEANR |
| 642 | 32200 | 5.50 | dehydroascorbate reductase | CA815817 | 2 | 58 | ISPGGTVPVMK |
| | | | | | | 57 | DISAVDLSLGPK |
| | | | plastidic aldolase | CB974958 | 2 | 43 | MVDVLVEQK |
| | | | | | | 47 | ATPEQVAQYTLK |
| | | | rubisco activase | BM436326 | 3 | 40 | EAADIIR |
| | | | | | | 43 | LVVHITK |
| | | | | | | 73 | VPIIVTGNFSTLYAPLIR |
| | | | chalcone isomerase | BQ798064 | 2 | 42 | VSENCVAFWK |
| | | | | | | 72 | LLTEAVLESIIGK |

[a] Spots are numbered accordingly to **Figure 1**. [b] Observed molecular mass determined on the gel (Da). [c] Observed p*I* determined on the gel. [d] Accession number in GenBank databases, assignment after BLAST homology searches in EST *Vitis vinifera* database. [e] Number of peptide mass fingerprinting matching with the reference protein.

increased content in a first group of proteins comprising a rubisco-binding protein (spot 427), an ATP synthase $\beta$ subunit (spot 444), a UDP-glucose pyrophosphorylase (spot 453), a glutamine synthetase (spots 484), an aminomethyltransferase (spot 520), and two rubisco large subunits (spots 527 and 619). In contrast, these leaves (**Figure 2H**−**K**) displayed a decreased content in a second group of proteins comprising a mixture of enolase and glyceraldehyde-3-phosphate dehydrogenase (spot 485), a plastidic aldolase (spot 583), a rubisco large subunit (spot 597), and a glutamine synthetase (spot 684). R transformants (**Figure 2L**,**M**) displayed only a third protein group with decreasing content and composed of a rubisco large subunit (spot 561) and a mixture of elongation factor Tu and linalool synthase (spot 692).

## DISCUSSION

It has been previously shown that variation in ADH expression obtained by transformation of grapevine could modify some aspects of leaf primary and secondary metabolism (*5*). Here, we evaluated whether such changes could result in significant alterations at the proteomic level.

We found 72% matching spots between the three repetitions obtained with the C sample, 65% with the R sample, and 53% with the S sample, but when only spots with a relative volume above 0.1% were considered, matching percentages rose, respectively, to 74, 81, and 80% (data not shown). At this stage, protease activity could be considered at most very limited, because it would have led to lower, random matching and also because of the presence of polypeptides with a high molecular

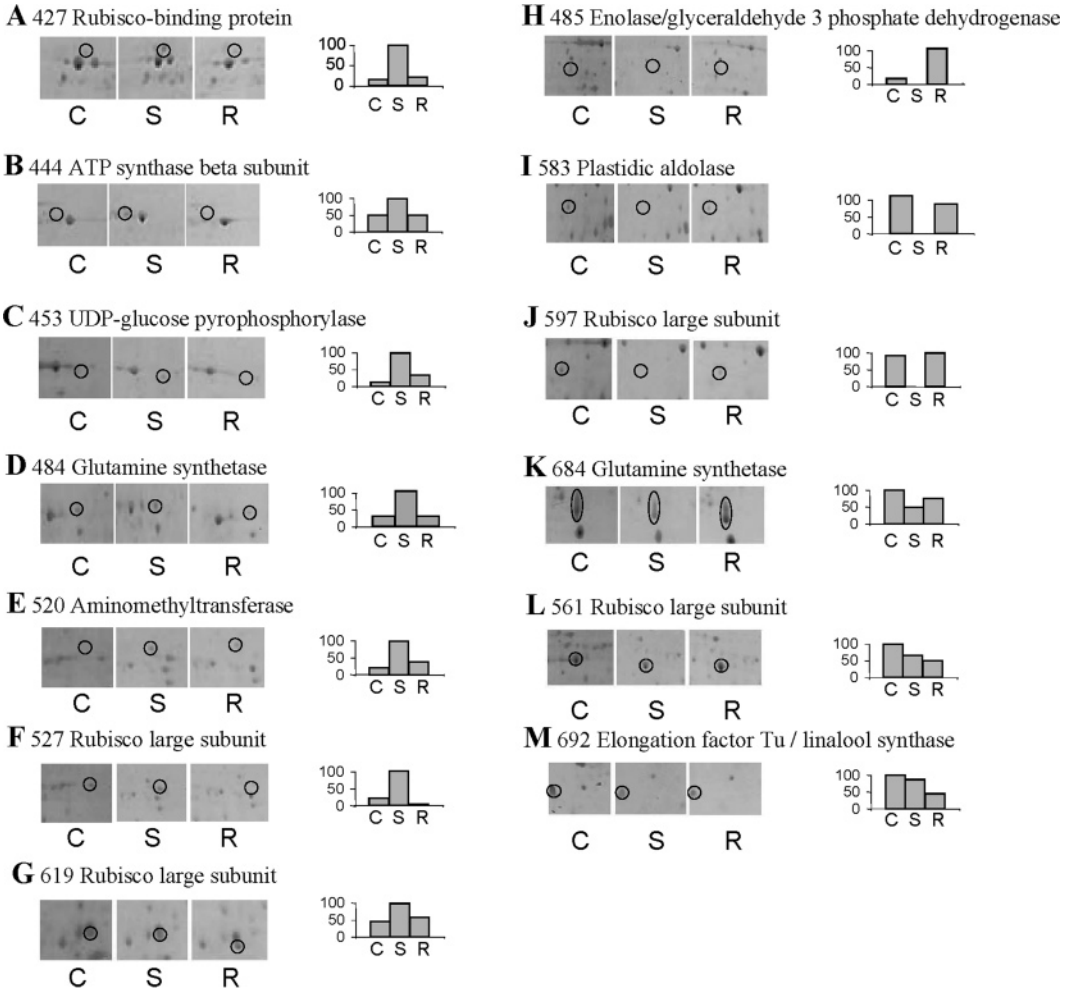


**Figure 2.** Grapevine protein levels changed by the transformation and identified by MALDI-TOF: **A**–**G**, protein levels increased in leaves from S transformants (**A**, rubisco-binding protein; **B**, ATP synthase $\beta$ subunit; **C**, UDP-glucose pyrophosphorylase; **D**, glutamine synthetase; **E**, aminomethyltransferase; **F** and **G**, rubisco large subunits); **H**–**K**, protein levels decreased in leaves from S transformants (**H**, enolase and glyceraldehyde-3-phosphate dehydrogenase; **I**, plastidic aldolase; **J**, rubisco large subunit; **K**, glutamine synthetase); **L** and **M**, protein levels decreased in leaves from R transformants (**L**, rubisco large subunit; **M**, elongation factor Tu and linalool synthase). C, control; S, sense-transformed; R, antisense-transformed. Detailed information for the proteins is provided in **Tables 2** and **3**. The level of change found within each spot is shown in bars on the right. The highest level found among transformants is shown as 100.

weight. Thus, results indicated that only a few proteins were found to be strongly altered between the three conditions of transformation, C, S, and R. As ADH variations between C and S lines had already been reported at both transcript and enzyme activity levels (*5*), we first checked whether noticeable differences in ADH protein content could be observed (in a 5.5−5.8 pH range with molecular mass ranging from 42 to 44 kDa). However, no such related spot could be detected (**Figure 1**).

Only spots that showed over 2-fold changes were analyzed by two complementary techniques including MS and MS/MS analyses. The results obtained with both methods were coherent, with most of the identified proteins being chloroplast-related. Fragments of large rubisco subunits, ATP synthase $\beta$ subunit, glutamine synthetase, aminomethyltransferase, and UDP-glucose pyrophosphorylase have already been found in proteome analysis from leaves of various origins (*6*, *9*, *10*). For rubisco, some large subunits exhibited various variations, increasing or decreasing in S transformants and decreasing in R transformants. Given the presence of different isogenes, the possibility cannot be excluded that the apparently conflicting results may in fact reflect differential expression of the large rubisco subunits in grapevine transformants. Rubisco-binding protein participates in the formation of the rubisco complex. In fact, this chaperone protein is supposed to ensure the correct assembly of the complex (*14*), by assembling the mature large and small rubisco subunits into competent units. The level in rubisco-binding protein is increased >5-fold in S transformants, suggesting a change in the rubisco complex formation in these leaves. These changes are to be related to the drastic decrease in the content of a rubisco large subunit (spot 597), also observed in S leaves, suggesting that this subunit participates to the complex formation.

ATP synthase $\beta$ subunit participates in the structure of the $F_1$−ATPase complex, which is involved in the ATP synthesis and hydrolysis coupled to proton translocation across the thylakoid membrane. The content of ATP synthase $\beta$ subunit also increased in S transformants, suggesting some changes in the $F_1$−ATPase complex formation. Aminomethyltransferase, the content of which is increased in S transformants, has a subcellular, mitochondrial location and is involved in the glycine cleavage system. As glycine is synthesized in illuminated leaves during photorespiration, it can be suggested that ADH is involved in light-dependent processes. Increase and decrease of another light-modulated chloroplast enzyme, glutamine synthetase, were both observed in S transformants. Chloroplast glutamine synthase, known to be constitutively expressed in

mature leaves, contributes to the assimilation of ammonium produced by photorespiration (*15*, *16*). Finally, the content of other proteins related to chloroplast such as glyceraldehyde 3-phosphate dehydrogenase and plastidic aldolase was reduced in S transformants.

Most of the identified proteins displaying an increasing or decreasing content in ADH overexpressing S transformants are directly or indirectly related to chloroplasts. This could suggest that photosynthetic metabolism has been changed by modifying the level of ADH activity in grapevine leaves. This is to be compared to the higher level in chlorophyll contents observed in S transformants (*5*).

Some other proteins, such as UDP-glucose pyrophosphorylase, the level of which increases in S transformants, are also related to energy metabolism. In fact, UDP-glucose pyrophosphorylase is positioned at the crossroads of sucrose synthesis and breakdown. In *Arabidopsis* leaves, UDP-glucose pyrophosphorylase is strongly up-regulated by sucrose, this effect being independent of the hexokinase status (*17*). In some cases, however, UDP-glucose pyrophosphorylase activity is correlated to sucrose breakdown (*18*). The substrate/product of the enzyme is a key metabolite for carbohydrate metabolism in photosynthetic tissues. Another supposed role of UDP-glucose pyrophosphorylase is to provide UDP-glucose for the synthesis of the carbohydrate moiety of glycoproteins and for other glycosylation reactions (*19*). In fact, this substrate is used by the majority of the glycosyltransferases as a sugar nucleotide donor in the synthesis of secondary metabolites. In the present study, a significant increase in the UDP-glucose pyrophosphorylase content was observed in leaves from S transformants, compared to the controls. This is to be related with the drastic decrease in sucrose content observed in these transformants (*5*). This decrease, together with the higher UDP-glucose pyrophosphorylase protein content, could suggest that UDP-glucose is also affected by these changes. In addition, the higher content in glycosylated volatile compounds observed in the S transformants (*5*) is coherent with a participation of UDP-glucose to the glycosylation of these molecules. Elongation factor Tu is involved in protein biosynthesis and a decrease in its content was observed in R transformants. Such an observation suggests that the pathways of protein biosynthesis could be altered by antisense transformation. This spot contained also an additional protein identified as linalool synthase but, although this protein is involved in the terpene biosynthetic pathway, no changes in related volatile compounds could be observed in these transformants (*5*).

In a previous study, we found a large increase in the glycosidically bound fraction of the volatile compounds of S transformants (*5*). One would have therefore expected to identify some of the related proteins in the present study. Identification of proteins related to the secondary metabolism was probably limited by the paucity of sequence data available. Alternatively, content variations could possibly be too small, or below the stringent 2-fold selection threshold. In all cases, these proteins are probably in too low amount to be detected in whole leaf extracts. Cellular fractionation, as well as pH scale reduction in the first electrophoretic separation, could be an alternative to improve identification possibilities. In the present study, differentially expressed proteins in grapevine leaves have been identified and discussed in relation to plant biochemical processes. In leaves from transformants overexpressing ADH, no variation in the content of proteins related to the secondary metabolism was revealed, whereas biochemical data previously showed that this metabolism was affected (*5*). In the results reported here, variations in these transformants concern mainly proteins related to the primary metabolism. In leaves from ADH antisense transformants, the variations concern a more limited number of protein than expected, which could likely be due to some compensatory mechanisms as already observed (*5*). It is also noticeable that transgenic plants transformed for ADH expression displayed large content variation for several proteins, even though actual variations of ADH content were not detectable; this suggests the unexpected involvement of ADH, in some way unidentified as yet, in several basic metabolism pathways.

In conclusion, the present study emphasizes the importance of changes in the abundance of chloroplastic proteins and in sugar−phosphate substrates in relation to increased ADH activity. The changes in the carbon metabolism-associated proteins presumably reflect altered patterns of carbon flux in response to changes in ADH activity in transformed plant leaves.

## ACKNOWLEDGMENT

We acknowledge the technical assistance of G. Lopez (UMR BEPC, Montpellier) for his care of the plants. We also thank Dr. G. Albagnac (CEPIA Department of INRA) for supporting our research effort. We give many thanks to J. Poncet and P. Jouin for mass spectrometry analysis and help in database searches.

## LITERATURE CITED

(1) Conley, T. R.; Peng, H. P.; Shih, M. C. Mutations affecting induction of glycolytic and fermentative genes during germination and environmental stresses in *Arabidopsis*. *Plant Physiol.* **1999**, *119*, 599−608.

(2) Rousselin, P.; Toro Perea, N.; Dolferus, R.; Tahar, B.; Caboche, M.; Jacobs, M. Complementation of an alcohol dehydrogenase-deficient *Nicotiana plumbaginifolia* mutant by transformation with the *Arabidopsis thaliana* Adh gene. *Trans. Res.* **1994**, *3*, 376−387.

(3) Shiao, T.; Ellis, M. H.; Dolferus, R.; Dennis, E. S.; Doran, P. M. Overexpression of alcohol dehydrogenase or pyruvate decarboxylase improves the growth of hairy roots under hypoxia. *Biotechnol. Bioeng.* **2002**, *77*, 455−461.

(4) Speirs, J.; Lee, E.; Holt, K.; Yong-Duk, K.; Steele Scott, N.; Loveys, B.; Schuch, W. Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit affects the balance of some flavor aldehydes and alcohols. *Plant Physiol.* **1998**, *117*, 1047−1058.

(5) Tesniere, C.; Torregrosa, L.; Pradal, M.; Souquet, J. M.; Gilles, C.; Dos Santos, K.; Chatelet, P.; Gunata, Z. Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. *J. Exp. Bot.* **2006**, *57*, 91−99.

(6) Castro, A. J.; Carapito, C.; Zorn, N.; Magné, C.; Leize, E.; Van Dorsselaer, A.; Clément, C. Proteomic analysis of grapevine (*Vitis vinifera* L.) tissues subjected to herbicide stress. *J. Exp. Bot.* **2005**, *56*, 2783−2795.

(7) Jones, A. M. E.; Thomas, V.; Truman, W.; Lilley, K.; Mansfield, J.; Grant, M. Specific changes in the *Arabidopsis* proteome in response to bacterial challenge: differentiating basal and R-gene mediated resistance. *Phytochemistry* **2004**, *65*, 1805−1816.

(8) Rakwal, R.; Komatsu, S. Role of jasmonate in the rice (*Oryza sativa* L.) self-defense mechanism using proteome analysis. *Electrophoresis* **2000**, *21*, 2492−2500.

(9) Schlesier, B.; Berna, A.; Bernier, F.; Mock, H. P. Proteome analysis differentiates between two highly homologues germin-like proteins in *Arabidopsis thaliana* ecotypes Col-0 and Ws-2. *Phytochemistry* **2004**, *65*, 1565−1574.

(10) Wilson, K. A.; McManus, M. T.; Gordon, M. E.; Jordan, T. W. The proteomics of senescence in white clover *Trifolium repens* leaves. *Proteomics* **2002**, *2*, 1114−1122.
(11) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(12) Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255−262.
(13) Giribaldi, M.; Perugini, L.; Sauvage, F. X.; Schubert, A. Analysis of protein changes during grape berry ripening by 2D-electrophoresis. *Proteomics* **2007**, in press.
(14) Demirevska-Kepova, K.; Simova-Stoilova, L.; Kjurkchiev, S. Barley leaf rubisco, rubisco binding protein and rubisco activase and their protein/protein interactions. *Bulg. J. Plant Physiol.* **1999**, *2*, 31−44.
(15) Pérez-Rodríguez, J.; Valpuesta, V. Expression of glutamine synthetase genes during natural senescence of tomato leaves. *Physiol. Plant.* **1996**, *97*, 576−582.
(16) Feller, U.; Fischer, A. Nitrogen metabolism in senescing leaves. *Crit. Rev. Plant Sci.* **1994**, *13*, 241−273.
(17) Ciereszko, I.; Johansson, H.; Kleczkowski, L. A. Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in *Arabidopsis*. *Biochem. J.* **2001**, *354*, 67−72.
(18) Magel, E.; Abdel-Latif, A.; Hampp, R. Non-structural carbohydrates and catalytic activities of sucrose metabolizing enzymes in trunks of two *Juglans* species and their role in heartwood formation. *Holzforschung* **2001**, *55*, 135−145.
(19) Kleczkowski, L. A.; Geisler, M.; Ciereszko, I.; Johansson, H. UDP-Glucose pyrophosphorylase. An old protein with new tricks. *Plant Physiol.* **2004**, *134*, 912−918.

**Received for review December 22, 2006. Revised manuscript received January 25, 2007. Accepted January 30, 2007.**

JF063723W