

Identification of Grapevine Cultivar Biomarkers Using Surface-Enhanced Laser Desorption and Ionization (SELDI-TOF-MS)

Giovanni Povero,¹ Massimo Papale,² Loreto Gesualdo,³ Amedeo Alpi,⁴ Pierdomenico Perata,¹ and Elena Loreti^{5*}

Abstract: Protein-based tools for identifying plant varieties have developed rapidly, parallel to the evolution of gene-based technologies. In this study, we used ProteinChip technology to analyze the protein profiles of four different varieties of grapevines. This technique incorporates surface-enhanced laser desorption/ionization with mass spectrometry for rapid profiling and a comparison of protein profiles. Results revealed that several protein signals were differentially expressed in the varieties used, suggesting that this technology could potentially be used to identify cultivar-specific biomarkers in grapevines.

Key words: biological markers, grape, mass spectrometry, ProteinChip, *Vitis vinifera* L.

The genus *Vitis* comprises three different natural groups depending on geographical location: Asia (40 to 60 species), North America (25 species), and a single European species, *Vitis vinifera* L. The domesticated grapevine (*V. vinifera*) is of great economic importance as a table grape, for raisins and juices, and for production of wine and its derivatives (liquors and vinegars). In *V. vinifera* L., ~6,000 cultivars are classified (Alleweldt and Dettweiler 1994), of which fewer than 400 are commercially important.

In the last century, the classification of grapevine cultivars was based on ampelography, from the Greek *ampe-los* (vine) and *graphos* (writing), which is the discipline that studies, identifies, and classifies different grapevine varieties. Traditionally, ampelography was performed by comparing the shape and color of the various organs of the plant during the different stages of growth (Galet 1991, 2000). Genomic and proteomic techniques have enhanced

the robustness and scientific rigor of variety classification that was formerly conducted using a narrower group of techniques, based primarily on phenotypic characterization. Grapevine genetics and grapevine genomic resources have developed greatly in the 21st century, including the complete sequencing of the grapevine genome (Jaillon et al. 2007, Velasco et al. 2007). Molecular marker techniques have been developed to identify and genotype different cultivars (for reviews see Agarwal et al. 2008, Kumar et al. 2009), such as restriction fragment length polymorphism (RFLP; Bowers and Meredith 1996), random amplification of polymorphic DNA (RAPD; Grando et al. 1995, Tessier et al. 1999), amplified fragment length polymorphism (AFLP; Cervera et al. 1998, 2000), and polymorphic microsatellite length determinations (Botta et al. 1995, This et al. 2004).

Although DNA-based technologies are extremely powerful, a complementary approach is to use proteomics to distinguish among varieties. An array of new protocols, platforms, and workflows has been proposed for proteomics, requiring sophisticated equipment and expertise for their complexity (Jorrin-Novo et al. 2009). Little is known about the proteins present in grape berries (Deytieux et al. 2007). Peptides and proteins contribute considerably to the quality of the product, as they affect the taste, clarity, and stability of wine. The molecular weight of the majority of grapevine proteins ranges from 20 to 30 kDa (Ferreira et al. 2001). An analysis of proteins is based on either electrophoresis or chromatographic separation, among which one- or two-dimensional (2-D) electrophoresis, capillary electrophoresis, isoelectric focusing, affinity chromatography, immunodetection, and high-performance liquid chromatography (HPLC) are commonly used. In the case of 2-D electrophoresis, proteins are first separated based on their isoelectric point (pI) and then by their molecular mass. Some of these techniques have a high resolving power and can reveal thousand of proteins, but require a large sample and are time-consuming and expensive. Two-dimensional electrophoresis, in particu-

¹Plant Lab, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, 56127 Pisa, Italy; ²Department of BioAgroMed, Core Facility of Proteomics and Mass Spectrometry, University of Foggia, Via Napoli 52, 71100 Foggia, Italy; ³Department of Biomedical Sciences and BioAgroMed, University of Foggia, Via L. Pinto 1, 71100 Foggia, Italy; ⁴Department of Crop Plant Biology, University of Pisa, Via Mariscoglio 34, 56124 Pisa, Italy; and ⁵National Research Council-IBBA, Via G. Moruzzi 1, 56100 Pisa, Italy.

*Corresponding author (email: elena.loreti@ibba.cnr.it; tel: +39 050 2216541; fax: +39 050 2216532)

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lar, requires technical expertise, especially for the production of reproducible gels. In addition, low-molecular-mass proteins (<20 kDa) are not well represented by 2-D electrophoresis, which is crucial because of the size of most grapevine proteins. If sufficient sample is present on the gel, then proteins can be excised from the gel, subjected to in-gel proteolysis, and analyzed by mass spectrometry (MS). Mass spectrometry, which separates ionized molecules (gaseous ions) according to their specific charge/mass ratio, is not quantitative per se, but the combination of stable isotope labeling, advanced multidimensional chromatography, and MS enables the identification and quantitation of many hundreds of proteins in a single comparative experiment. (Gruhler et al. 2005). Most quantitative proteomic strategies rely on the incorporation of stable isotopes into proteins or peptides, which are then compared with an unlabeled control sample by mass.

ProteinChip array is an innovative proteomic technology that uses MS (Hutchens and Yip 1993). It is based on surface-enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS) and has overcome many of the limitations of 2-D electrophoresis and matrix-assisted laser desorption/ionization (MALDI)-TOF-MS. The SELDI technique allows simplified sample extraction followed by an investigation of proteins with on-chip binding and detection. The ProteinChip arrays consist of different chromatography surfaces capable of retaining proteins depending on their physical and chemical properties (Merchant and Weinberger 2000). The surface of the array mirrors the properties of conventional chromatography media such as reverse phases, anion and cation exchange, metal affinity, and normal phases. Because proteins are fixed to a specific surface, the unbound protein can easily be removed by washing before MS. This is a high-throughput technique for analysis of complex biological samples. It can detect multiple protein changes simultaneously with a high sensitivity and specificity and can be used for biomarker discovery (Chapman 2002). This approach has been particularly used in predictive medicine for the implementation of novel biomarkers for detecting disease (Reddy and Dalmaso 2003, Xiao et al. 2005, Droin et al. 2009, Wiesner 2004, Poon 2007). SELDI techniques have also been applied in plant sciences (Ebert et al. 2008, Badri et al. 2009) and to characterize wine proteins (Weiss et al. 1998).

Correct varietal identification is of scientific and practical importance in all arboriculture fields, but particularly with grapevines because of the number of cultivars (Alleweldt and Dettweiler 1994). It is also difficult to identify seed-propagated juvenile grapevines because they do not have the typical morphological traits of adult plants until they are approximately five years old. Protein-based studies could provide an innovative approach in identifying a specific cultivar. The SELDI approach has not previously been used to detect markers for the identification of different grapevine cultivars.

The aim of this study was to evaluate the ability of SELDI-TOF-based ProteinChips to discriminate among

grapevine varieties and to determine whether this technique can identify protein markers specific to a grapevine variety and to a group of grapevine varieties. Four different Italian grapevine varieties were selected, two white (cvs. Italia and Regina) and two black (cvs. Abrusco and Sangiovese). Italia is the main table grape in Italy and Regina is another important variety. Sangiovese originates in Tuscany, is the most widespread black grape cultivar for wine production in Italy, and produces wines that are spicy, with good acid levels, smooth texture, and medium body. Abrusco also originates in Tuscany and is largely used to intensify wine color because of its high anthocyanin concentration. Regina and Italia have slightly different berry shapes, whereas Sangiovese and Abrusco have similar berries. Results showed that the ProteinChip was useful not only to distinguish among different varieties but also to recognize protein markers that are specific for each variety among those tested. This approach could thus be a valuable tool for the identification of grape varieties using their protein profiles.

Materials and Methods

Protein extraction. Grape berries from *V. vinifera* cvs. Regina, Italia, Abrusco, and Sangiovese were harvested from a vineyard in Tuscany at full ripeness: 18.1, 21.7, 22.8, and 24.3 Brix, respectively. Italia derives from a breeding program carried out in Italy in 1911 by geneticist Alberto Pirovano, who obtained Italia by crossing Bicane with Muscat Hamburg (a cross between Schiava Grossa and Muscat of Alexandria). Regina, also known as Afus Ali, is an old variety from Lebanon; Abrusco and Sangiovese are Italian varieties (<http://www.vivc.bafz.de>). Proteins were extracted by homogenizing frozen berry skin tissue. Each biological replicate derived from a pool of 20 berry skins. The extraction protocol used was phenol-based (Zheng et al. 2007). Two denaturing buffers (DB) were used to resuspend the pellet in the final steps of the extraction procedure. A DB1 buffer (7 M urea, 2.0 M thiourea, 4% [w/v] CHAPS, 10 mM DTE, 1% [v/v]) was used for Q10, H50, and CM10 ProteinChip arrays, while a DB2 buffer (7 M urea, 2.0 M thiourea, 4% [w/v] CHAPS) was used for IMAC-30 arrays, as DTE interferes with protein binding with this ProteinChip surface. Protein concentration was measured by Bradford's method (Bradford 1976), using a protein-dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as a standard. Four biological replicates were analyzed.

ProteinChip arrays, preparation, and analysis. Four ProteinChip (Bio-Rad) types were used to determine the protein profiles of grape varieties: (1) H50, which binds proteins by hydrophobic interaction; (2) CM10, which binds proteins by cationic exchange; (3) copper-coated IMAC-30, which binds metal-binding proteins, and (4) Q10, which binds proteins through strong anionic exchanges. The ProteinChips were prepared according to the manufacturer's instructions. They were first washed twice, each type with its specific binding/washing buffer: 10% acetonitrile and

0.1% trifluoroacetic acid (TFA) for H50; 100 mM sodium acetate, pH 4, for CM10; 100mM Tris-HCl, pH 8.8, for Q10; and 0.1M Tris-HCl, pH 7.4, for IMAC-30, which required preliminary loading with Cu^{++} and neutralization by sodium acetate (pH 4), before exposure to its specific binding buffer. ProteinChips were loaded with 30 μg berry skin proteins from each sample in duplicate for each biological replicate ($n = 4$). After a 30-min incubation with continuous shaking, the ProteinChip arrays were washed twice with 150 μL buffer, and finally with 200 μL deionized Millipore water followed by air drying for 20 min. A saturated solution of sinapinic acid (Ciphergen, Freemont, CA) was then prepared in 50% acetonitrile, 0.1% TFA. Sinapinic acid was diluted by 50% in 50% acetonitrile and 1% TFA solution, and 2 μL was applied to each spot. All ProteinChips were read by adopting the same protocol (laser energy 6,000 nanojoule; matrix attenuation 2,500; focus mass 10,000; sample rate 800; partition 1 of 4; acquired mass range from 3,000 to 30,000). The software was externally calibrated using the All-in-One Protein Standard II kit (Bio-Rad), and all spectra were normalized using a total ion current.

The analysis was performed in a mass-to-charge ratio (m/z) range from 3000 to 30,000 with ProteinChip Data Manager 3.5 (Bio-Rad), considering those detected in ~20% of all spectra only as real peaks (mean peak threshold = 20%) and with a signal/noise and valley depth ratio greater than 5. After acquisition, SELDI spectra were grouped according to grapevine varieties, and a list of differently expressed ($p < 0.05$) mass peaks was compiled by applying the Mann–Whitney non-parametric test. A receiver operating characteristics (ROC) curve was also determined for each differently expressed mass peak and the optimal area under the curve (AUC) was defined as the one that best segregated two grape varieties. The definition of the best surface (i.e., ProteinChip type) for protein profiling studies often relies on some technical considerations such as the number of mass peaks that can be recognized in the defined mass range or the overall quality of the mass spectra defined on the basis of both intensity and resolution of each peak. Obviously, the higher the number of signals in a certain mass spectra, the greater the possibility of identifying a set of mass peaks specifically associated to each cultivar. The results obtained indicated that Q10 could be considered overall the best ProteinChip for cultivar profiling. However, cultivar-specific markers were identified by using the whole set of arrays because, even in a spectrum showing a lower number of mass peaks, it would be possible to recognize one or few peaks closely associated with each cultivar.

Results and Discussion

Comparison of SELDI protein profiles. Each cultivar expressed a particular and conserved protein pattern and the protein profile on each chip was highly replicable using four biological replicates (Supplemental Figure 1). We initially compared the protein profiles of black grape and white grape varieties. Analyses of SELDI spectra identi-

fied approximately 70 differently expressed ($p < 0.05$) mass peaks in the chosen mass range (3 to 30 kDa). Of these, some peaks were higher in Abrusco and Sangiovese cultivars, while others were higher in both the Regina and Italia cultivars.

The distribution of selected peak intensities in the comparison between white grapes (Italia, Regina) and black grapes (Abrusco, Sangiovese) in all biological replicates was determined. We identified the 15.671 kDa peak on a Q10 matrix and the corresponding box-plot indicated that this peak is unique to the Italia and Regina cultivars (Figure 1A). Moreover, the distribution of intensities of the 11.093 kDa peak on CM10 matrix suggested that this mass peak was characteristic for Abrusco and Sangiovese (Figure 1B). Results suggest that these proteins could be good markers for white grapes and black grapes, respectively.

Identification of specific varietal biomarkers. The SELDI data allowed identification of specific proteins present in the two different groups. The next step was to verify whether the ProteinChip technology enabled identification of protein markers specific to a single grapevine variety, which was achieved by analyzing a comparison of the intravarietal protein profiles. To determine the most suitable marker for Regina, the protein profiles of Regina and Italia were compared and over 100 protein peaks in common to both varieties were found. On the basis of the p value calculation, a panel of differently expressed mass peaks were identified in Italia and Regina, and therefore specific to each variety (Supplemental Table 1). Nine significant mass peaks ($p < 0.05$) were identified with the ProteinChip CM10, seven with ProteinChips H50 and Q10, and nine with IMAC-30.

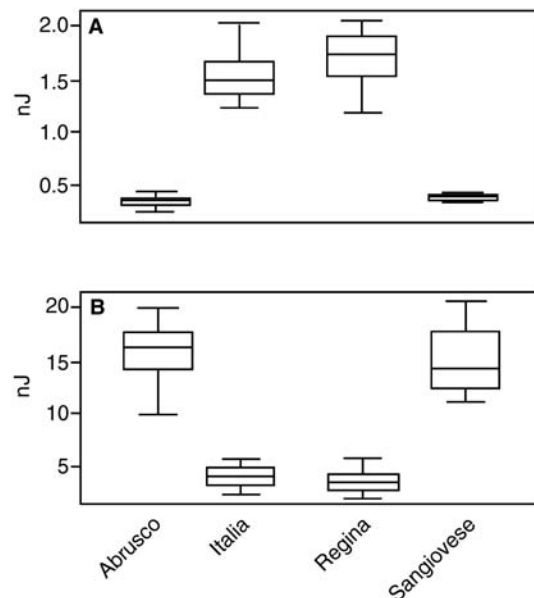


Figure 1 Distribution of peak intensities in a comparison between white grapes (Italia, Regina) and black grapes (Abrusco, Sangiovese). (A) Distribution of intensities, expressed as nanojoule (nJ) of the 15.671 kDa peak on Q10 matrix. (B) Distribution of intensities of the 11.093 kDa peak on CM10 matrix.

Table 1 Cultivar-specific protein mass peaks. Statistically significant peaks ($p < 0.05$) are reported. For each mass peak, the chip used is indicated. The estimated area under curve (AUC) for each differently expressed mass peak was also measured to define sensitivity and specificity. All mass peaks had AUC values equal to 1, indicating 100% sensitivity and specificity.

Marker	Mass (kDa)	p value	AUC	Chip
Italia	3.977	0.0076	0.9375	H50
Regina	6.858	0.00360935	1	IMAC30
Regina	9.202	0.00360935	1	IMAC30
Regina	3.053	0.00360935	1	H50
Sangiovese	7.284	0.00360935	1	CM10
Sangiovese	6.436	0.00360935	1	CM10
Sangiovese	3.506	0.00360935	1	H50
Abrusco	8.413	0.00360935	1	CM10
Abrusco	3.408	0.00360935	0.9791	H50
Abrusco	13.019	0.00360935	0.9791	Q10

The next step was to evaluate whether these mass peaks could differentiate Regina or Italia from Abrusco and Sangiovese grape samples. Each cultivar was compared with all the others and protein peaks specific for Italia or Regina were identified (Table 1). A graphical representation of SELDI spectra and the corresponding box-plot for a representative protein peak was developed. The mass spectra highlighted the presence of the 3.053 kDa peak only in the Regina grape samples, using an H50 array (Figure 2A), and virtual gel-view fingerprints were constructed (Figure 2B). Furthermore, the box-plot indicating the distribution of the intensities confirmed that this protein was highly expressed in the Regina grape compared with the other varieties (Figure 2C). Overall results suggested that this protein could be a selective marker for this variety. The same criteria were applied to find a specific mass peak corresponding to a specific protein and representing a marker specific to Italia (H50 ProteinChip; Figure 2D).

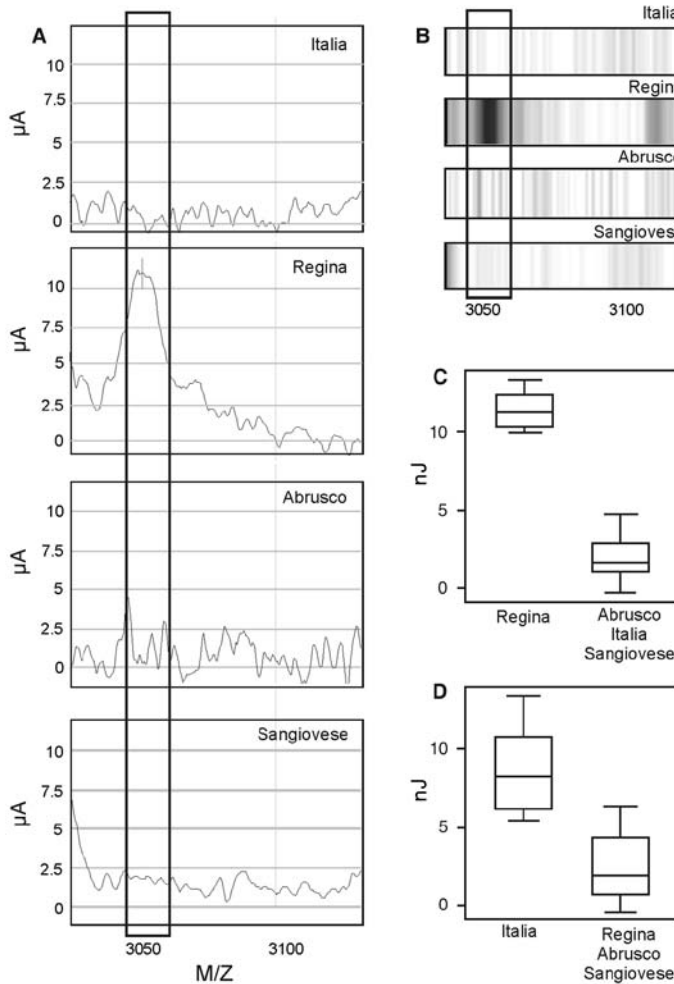


Figure 2 Identification of cultivar-specific protein peaks for table grape cultivars. (A) SELDI-MS spectra of protein samples extracted from grape berries on H50 ProteinChips. (B) Virtual gel-view fingerprints of protein samples extracted from berries on H50 ProteinChips. (C) Distribution of intensities, expressed as nanojoule (nJ) of the 3.053 kDa peak on H50 matrix. (D) Distribution of intensities (box-plot) of the 3.977 kDa peak on H50 matrix.

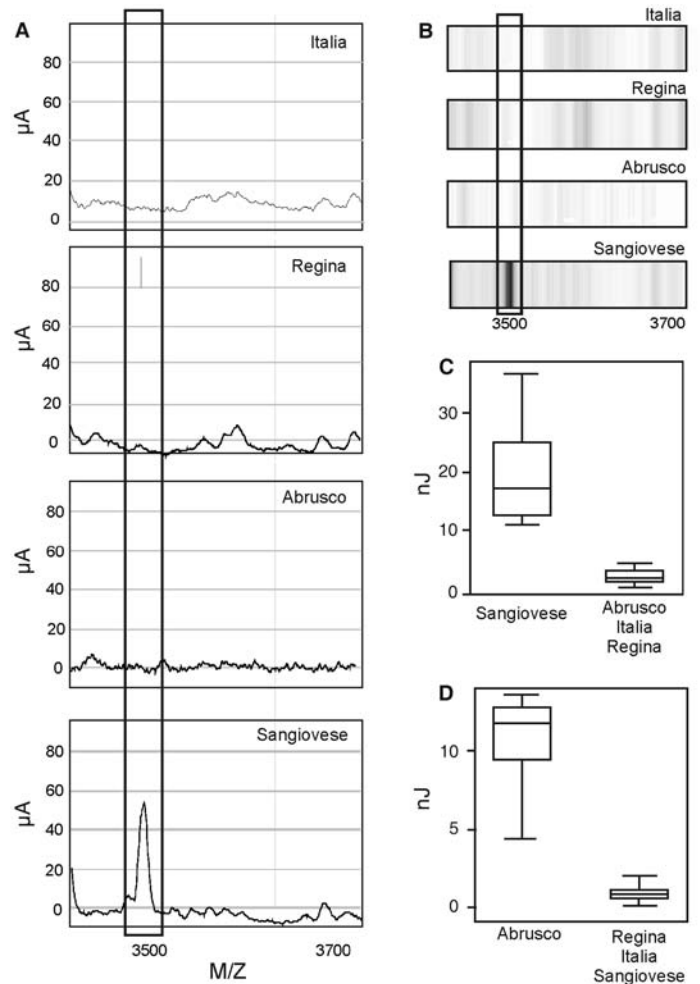


Figure 3 Identification of cultivar-specific protein peaks for winegrape cultivars. (A) SELDI-MS spectra of protein samples extracted from grape berries on H50 ProteinChips. (B) Virtual gel-view fingerprints of protein samples extracted from berries on H50 ProteinChips. (C) Distribution of intensities, expressed as nanojoule (nJ) of the 3.507 kDa peak on H50 matrix. (D) Distribution of intensities (box-plot) of the 8.413 kDa peak on CM10 matrix.

Cultivar-specific markers were also selected for Abrusco and Sangiovese grape samples (Supplemental Table 1), with the same approach used to select the specific varietal markers of the Regina and Italia samples. We initially identified differentially expressed protein peaks in the two black grapes. These peaks were then validated as Abrusco- or Sangiovese-specific markers in the four varieties. The mass spectra highlighted the presence of a 3.507 kDa peak only in the Sangiovese grape samples, using an H50 array (Figure 3A, B). The box-plot indicating the distribution of the intensities confirmed that this protein is only highly expressed in Sangiovese (Figure 3C). Mass peaks specific to Abrusco were also identified using the CM10 ProteinChip (Figure 3D).

Conclusions

This study used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) ProteinChip array technology on grape berry skin samples. This technique offers enhanced sensitivity that is ideal for the analysis of small sample volumes and allows for the screening of low-molecular-weight proteins. The SELDI-TOF-MS approach has several advantages for biomarker analysis: it overcomes the problem of the quantity of the starting material, has a high-throughput capacity, and is reproducible. Our results suggest that SELDI-TOF-MS would also be a valuable tool for biomarker identification in plant and crop sciences. Each grapevine variety analyzed displayed a protein pattern that distinguished it from the others: there was a protein pattern common to white grapes that distinguished them from black grapes and vice versa among the four varieties tested and a series of SELDI mass clusters associated with each variety could be identified.

The similar pattern observed in Regina and Italia and in Abrusco and Sangiovese is unlikely to arise from common ancestors and instead suggests common markers in black/white grape groups. The environment is likely to play an important role in the pattern of biomarkers and is a topic that deserves a specific study with grapes of the same variety collected from different environments. Although the purification and sequencing of the SELDI biomarkers is necessary to further clarify their biological role, this study appears to be the first to highlight the feasibility of SELDI protein profiling for identifying potential biomarkers for grape varieties. This methodology should be considered complementary to DNA-based techniques for variety genotyping. The SELDI-based proteomic approach could also be a rapid way to screen and characterize wine proteins. In recent years, identifying the different varieties in wine has been problematic. Exploiting protein differences among grapes to identify cultivars could be the first important step in identifying the different cultivars used to produce specific wines.

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