Modulation of Protein Phosphorylation, N-Glycosylation and Lys-Acetylation in Grape (Vitis vinifera) Mesocarp and Exocarp Owing to Lobesia botrana Infection*

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Grapevine (Vitis vinifera) is an economically important fruit crop that is subject to many types of insect and pathogen attack. To better elucidate the plant response to Lobesia botrana pathogen infection, we initiated a global comparative proteomic study monitoring steady-state protein expression as well as changes in N-glycosylation, phosphorylation, and Lys-acetylation in control and infected mesocarp and exocarp from V. vinifera cv Italia. A multi-parallel, large-scale proteomic approach employing iTRAQ labeling prior to three peptide enrichment techniques followed by tandem mass spectrometry led to the identification of a total of 3059 proteins, 1135 phosphorylation sites, 323 N-linked glycosylation sites and 138 Lys-acetylation sites. Of these, we could identify changes in abundance of 899 proteins. The occupancy of 110 phosphorylation sites, 10 N-glycosylation sites and 20 Lys-acetylation sites differentially changed during L. botrana infection. Sequence consensus analysis for phosphorylation sites showed eight significant motifs, two of which containing up-regulated phosphopeptides (X-G-S-X and S-X-X-D) and two containing down-regulated phosphopeptides (R-X-X-S and S-D-X-E) in response to pathogen infection. Topographical distribution of phosphorylation sites within primary sequences reveal preferential phosphorylation at both the N- and C termini, and a clear preference for C-terminal phosphorylation in response to pathogen infection suggesting induction of region-specific kinase(s). Lys-acetylation analysis confirmed the consensus X-K-Y-X motif previously detected in mammals and revealed the importance of this modification in plant defense. The importance of N-linked protein glycosylation in plant response to biotic stimulus was evident by an up-regulated glycopeptide belonging to the disease resistance response protein 206. This study represents a substantial step toward the understanding of protein and PTMs-mediated plant-pathogen interaction shedding light on the mechanisms underlying the grape infection. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.020214, 945–956, 2012.

Grapevine (Vitis vinifera) is considered one of the most commercially important fruit crops because of the variety of derived food products including wine, table grape, and raisins. Despite its agronomic importance it has only recently emerged as a subject of systems biological investigations. The availability of high-throughput analysis methods and a high-quality draft of the grapevine genome sequence (1) have led to the recent application of next-generation sequencing to investigate grape berry development and pathogen infection, although few proteomic studies are available. Upon infection, plants dramatically change at the molecular and biochemical levels as reflected by global transcriptome (2–4), proteome (5, 6), and metabolome (7) system analyses. These studies demonstrate the importance of the complex modulation of plant metabolism and defense responses during pathogen infection. These changes activate a series of biological signaling mechanisms such as Ca^{2+} influx (8, 9), kinase cascades (10, 11), reactive oxygen species (12, 13), and phytohormone signaling pathways (14). However, so far none of these studies has addressed quantitative changes of grapevine protein post-translational modifications (PTMs) upon pathogen infection, which we believe would provide novel insights into the
underlying molecular processes that may eventually yield novel strategies for pathogen control in the field.

Grapevine moth Lobesia botrana (Lepidoptera: Tortricidae) is one of the major pests on grapes because of its wide geographical distribution and the heavy damage it can inflict on vineyards, affecting plant health, yield, and quality of the fruit. Originally described as European grape moth it has also been found in vineyards in United States, South America, and Japan. L. botrana is a key pest of table and wine grape (V. vinifera), reducing yield and increasing susceptibility to fungal infections (15). Adult L. botrana oviposits on the grape berries and it causes direct damage to clusters when larvae feed within the berries, and indirect damage by fungal infestation of injured berries by Botrytis and other secondary fungi such as Aspergillus, Alternaria, Rhizopus, Cladosporium, and Penicillium (16). Even though improvements in the management of this pest have been reported using insecticide (17) and pheromone-based strategies (18), little is known about the signaling mechanisms in grapes in response to L. botrana. Therefore, a comprehensive qualitative description of the molecular events that change is important to obtain biological insight into the infection mechanism.

In this work, we studied quantitative changes of the proteomes in the exocarp and mesocarp of V. vinifera “Italia cv” infected by L. botrana. Our aims were to gain insight into the sequence of events that occur in the V. vinifera “Italia cv” proteome and PTMs during infection, and to identify proteins that contribute to the susceptibility and response of this grapevine to L. botrana. We identified a total of 3059 proteins in grape berries in which 899 proteins were found to be significantly changed in abundance upon L. botrana infection. The number of differentially expressed proteins during the infection included proteins involved in photosynthesis, metabolism, disease/defense, protein destination, and protein synthesis. Using a large scale multiplex PTM analysis we have mapped the plant-pathogen signaling pathway showing among other regulations, the PTM regulation of the Ca²⁺ calmodulin-like protein (CaMCM1) and the disease resistance response protein 206 (DRR206). In addition, a new Lys-acetylation motif in plants has been identified (X-K-Y-X). Moreover, we have identified eight phosphorylation motifs with a remarkable phosphorylation preference for both proteins N- and C termini. To our knowledge, this work represents the first comprehensive study of protein phosphorylation, N-glycosylation, and Lys-acetylation on a plant system after infection with a pathogen.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**Poros R2 and R3 reversed-phase material was from PerSeptive Biosystems (Framingham, MA). TiO₂ beads were obtained from GL Science (Tokyo, Japan). Empore™ C8 extraction disk was from 3 M Bioanalytical Technologies (St Paul, MN). Ammonia solution (25%) was from Merck (Darmstadt, Germany). RapiGest™ SF Surfactant was from Waters (Milford, MA). The iTRAQ® reagents were from Applied Biosystems (Foster City, CA). All other chemicals were obtained from Sigma (Steinheim, Germany).

**Plant Material—**Grape clusters of V. vinifera Italia table grape variety were collected in September 2010 from a vineyard located at the CRA-UTV (Turi, Apulian region, Southern Italy, long. 40.56° E, lat. 17.12° N) planted in 1999. The vines were planted on 140-RU Ruggeri (Vitis berlandieri × Vitis rupestris) rootstocks, with a spacing of 2.5 m between rows and 2.5 m within a row and trained to a trellis system named “double tendone.” This vineyard was surveyed for pests for all the previous two years and the only grape moths that were found on it was L. botrana. The grape clusters, healthy and attacked by L. botrana were randomly collected from the same vineyard at the ripening phenological stage (19).

**Tissue Preparation—**Intact and attacked grape berries were carefully peeled on ice, the seeds were removed, and the exocarp and the mesocarp were immediately frozen in liquid nitrogen. Samples were transferred to a mortar chilled with liquid nitrogen and ground to powder using a pestle.

**Phenol Protein Extraction—**Exocarp (1.5 g) and mesocarp (3 g) were resuspended with 15 ml of 0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl; 50 mM EDTA; 10 mM diithiothreitol, pH 7.5 with protease and phosphatase inhibitor cocktails (Roche - Mannheim, Germany) and 15 ml of phenol saturated with Tris-HCl, pH 7.5 (20). The extraction was performed under shaking and at 4 °C for 2 h. Following centrifugation (5000 × g for 30 min at 4 °C), the phenol phase containing proteins was collected and transferred to new tubes. Proteins were precipitated with five volumes of cold 0.1 M ammonium acetate in methanol overnight at −20 °C. Protein pellet was resuspended in a solution of 6 M urea; 2 M thiourea; RapiGest™ 0.1% (v/v); and 200 mM triethylammonium bicarbonate (TEAB).

**In-solution Protein Digestion and Peptide Labeling—**Proteins were reduced with 10 mM dithiothreitol for 1 h at room temperature and alkylated with 40 mM iodoacetamide at room temperature in the dark for 40 min. Samples were diluted seven volumes with 200 mM TEAB and digested with 1:50 (w/w) trypsin (Promega, Madison, WI) overnight at 37 °C. Enzymatic reaction was quenched adding formic acid (FA) to 1% final concentration. Sample was centrifuged at 15000 × g for 10 min at 4 °C to collect the supernatant and to remove the acid labile surfactant RapiGest™. Protein quantitation was performed by amino acid analysis. Peptides were labeled with iTRAQ® reagent according to manufacturer’s specifications and lyophilized.

**Phosphopeptide Enrichment—**TiO₂ chromatography was used to enrich samples for phosphopeptides as described previously (21–24) with minor modifications. Sample (150 μg) was resuspended in 80% acetonitrile (ACN) (v/v), 5% (v/v) trifluoroacetic acid (TFA), 1 M glycine acid in a polypropylene tube containing 1 mg TiO₂ beads and incubated with constant shaking for 30 min. The TiO₂ beads were washed with 80% ACN (v/v), 2% (v/v) TFA and then 20% ACN (v/v), 0.1% (v/v) TFA, and phosphopeptides were eluted with 70 μl 1.5% NH₄ for 15 min with constant shaking. Sample containing phosphopeptides was desalted on a Poros R3 micro-column as described below and then lyophilized.

**Glycopeptide Enrichment and Enzymatic Deglycosylation—**Zwitterionic-hydrophilic interaction liquid chromatography (ZIC-HILIC) was performed to enrich glycosylated peptides as previously described (25). Briefly, 130 μg of labeled peptides were resuspended in 80% (v/v) ACN; 1% (v/v) TFA and loaded onto a HILIC GEOFader tip (Eppendorf, Hamburg, Germany) microcolumn packed with ZIC-HILIC resin (Sequant, Umeå, Sweden; particle size 10 μm). Following sample loading, micro-columns were washed with 80% ACN; 1% TFA, and glycopeptides were then eluted with 5% (v/v) FA and lyophilized.

Glycopeptides were resuspended in 50 mM sodium acetate pH 5.5, and deglycosylated with 0.5 U of N-glycosidase A (PNGase A), over-
night under shaking at 37 °C. The reaction was quenched with 1% (v/v) FA and the deglycosylated peptides were desalted on a Poros R2 microcolumn and lyophilized. In order to check for spontaneously chemical deamidation, an aliquot of the enriched glycopeptides was analyzed by LC-MS/MS without PNGase treatment, as previously suggested (26).

**Lys-Acetylated Peptide Enrichment**—Labeled peptides (130 μg) were resuspended in 50 mM MOPS-NaOH pH 7.2 (10 mM sodium phosphate, 50 mM sodium chloride), and incubated overnight at 4 °C with agaroase conjugated anti-acetyllysine antibody (ImmuneChem Pharmaceuticals Inc., Burnaby, Canada). The sample was then washed four times with 50 mM MOPS-NaOH pH 7.2, following by two times washing with UHQ water. Lys-acetylated peptides were eluted from beads twice with 1% TFA (in water) and desalted on a Poros R2 micro-column and lyophilized.

**Sample Desalting**—Samples were desalted using self-made micro-columns packed either with Poros R2 or R3 reversed-phase resin. Micro-columns were prepared by stamping out a small plug of C₈ material from a 3 m Empore™ C₈ extraction disk and placing the plug in the constricted end of a P10 or P200 tips. The reverse-phase resin (suspended in 100% ACN) was packed in the tip where the C₈ material prevented the beads from leaking. The micro-column was packed by the application of air pressure. Each acidified sample was loaded onto the micro-column and washed three times with 0.1% TFA. Peptides were eluted with 50% ACN, 0.1% TFA and 70% ACN, 0.1% TFA, respectively.

**HLIC Fractionation**—Prior to LC-MS/MS analysis, samples were pre-fractionated on a TSKgel Amide 80 HLIC HPLC column (length: 15 cm, diameter: 2 mm, particle size: 3 μm) as previously described (27, 28). Samples were resuspended in solvent B (90% ACN, 0.1% TFA). Peptides were eluted at 6 μL/min by decreasing solvent B in a linear manner (100–60%) in 26 min.

**Reversed Phase LC**—Sample was resuspended in 0.1% FA and loaded onto a reversed-phase in-house packed Reprosil-Pur C18-AQ column (3 μm; Dr. Maisch GmbH, Ammerbuch, Germany) (length: 17 cm; inner diameter: 100 μm; outer diameter: 360 μm) using an Easy-LC nanoHPLC system (Thermo Fisher Scientific, Proxeon, Odense, Denmark). The peptides were eluted from the column with a chromatography gradient from 0–34% solvent B (90% ACN, 0.1% FA) for 115 min at a flow rate of 300 nL/min directly into an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

The LTQ-Orbitrap XL instrument was operated in a data-dependent MS/MS scan mode. After a survey scan (400–1500 m/z; 30,000 resolution at 400 m/z) the top three most intense ions were selected for both low-resolution CID-MS/MS using MSA (normalized collision energy = 35) and high-resolution HCD-MS/MS (normalized collision energy = 55; 7500 resolution at 400 m/z) (29, 30).

To analyze phosphopeptides, we used a LTQ-Orbitrap XL and a LTQ-Orbitrap Velos. In the LTQ-Orbitrap XL, after a survey scan (30,000 resolution at 400 m/z) the top three most intense ions were selected for both low-resolution CID-MS/MS using MSA (normalized collision energy = 35) and high-resolution HCD-MS/MS (normalized collision energy = 55; 7500 resolution at 400 m/z) (29). For the phosphopeptides analyzed in the LTQ-Orbitrap Velos, after a survey scan (30,000 resolution at 400 m/z) the top seven most intense ions were selected for high-resolution HCD-MS/MS (normalized collision energy = 48; 7500 resolution at 400 m/z). Raw data were viewed in Xcalibur v2.0.7 (Thermo Fisher).

**Data Process and Database Search**—The MS/MS spectra were processed (smoothing, background subtraction, and centroiding) using Proteome Discoverer (Version 1.2, Thermo Fisher) and database searches were performed against a target and decoy (reverse) separate Vitis sp. database downloaded from NCBI 25th of July 2011 (78017 sequences) using an in-house Mascot server (v2.2, Matrix Science Ltd., London, UK). Trypsin was chosen as the enzyme allowing up to two missed cleavages. S-carbamidomethyl-cysteine was defined as a fixed modification. Oxidation (M), N-acetylation (protein N terminus), phosphorylation (S, T, and Y), deamidation (N and Q), Lys-acetylation, and iTRAQ® reagents (protein N terminus and Lys side-chain) were defined as dynamic modifications for the individual searches. The MS and MS/MS data were searched with a peptide ion mass tolerance of 10 ppm and a fragment ion mass tolerance of ± 0.6 Da (± 0.05 Da for high-resolution HCD spectra). Only high-confidence peptides with Mascot ion score ≥ 23, phosphopeptides with MD-score ≥ 9 (CID-MSA activation) or MD-score ≥ 13 (HCD activation) (31), and N-glycopeptides with deamidation sites within the PNGase consensus sequence (N-X-S/T/C, X ≠ F) were considered for further analysis. Lys-acetylated peptides were confirmed by manual inspection of the MS/MS spectra based on the presence of the immonium ion of the acetylated lysine (m/z 143.1179) and/or its derivative with an ammonia loss (m/z 126.0813). The site localization was confirmed by the presence of a 170 Da gap (acetylylsine residue) between consecutive b and y ions (32). Protein annotation was obtained using ProteinCenter (Thermo Fisher Scientific) and Blast2GO (33) (http://www.blast2go.com/b2ghome).

We used two biological replicates for the Proteome, N-Glycoproteome and Lys-Acetylproteome studies. In the phosphoproteome study, each of the two biological replicates was analyzed twice in the LC-MS/MS using CID-MSA/HCD, or only HCD, as described above in details. To control label efficiency and technical variability in the PTMs studies, two iTRAQ® labeling strategies (forward and reverse) were used; the uninfected samples were labeled with 114 and 116 and the infected samples were labeled with 115 and 117.

Data were normalized using log2-transformed median and the cutoff for significant regulated peptides between experimental data was set at two times the ratio for the standard deviation. It should be noted that only high-confidence peptides that were found regulated in all biological replicates were considered further in this study.

**Data Availability**—Raw mass spectrometer output files, annotated MS/MS spectra and excel tables containing all the identified peptides and PTMs are available for download at the Proteome Commons Tranche data repository [https://proteomecommons.org/groups-search.jsp] under the project name “Grape proteome and PTMome during parasite infection.” Assigned phosphopeptides and annotated spectra were deposited into The Plant Protein Phosphorylation Database [P3DB, http://p3db.org, (34)]. To visualize the download annotated MS/MS spectra, click on the Index.html file to open a table containing the peptide sequence and other useful information as activation type (CID or HCD), modification(s) site(s), and ion score. By clicking in the “Image” button, the related annotated spectra will be displayed.

**RESULTS**

**Experimental Setup**—Seeking to better understand the molecular events following grape infection by L. botrana, we developed a comprehensive large scale MS-based proteomics and PTMomics strategy as illustrated in Fig. 1. Uninfected and infected berries were peeled on ice and their exocarp and mesocarp were frozen in liquid nitrogen and pulverized with a pestle. Phenol extraction was performed as previously described (20). Protein pellets were solubilized, digested with trypsin, quantified by amino acid analysis, labeled with iTRAQ® reagents and combined in a 1:1 ratio (uninfected/infected tissues). We performed enrichment steps for glycosylated, phosphorylated, and Lys-acetylated peptides to min-
imize signal suppression caused by unmodified peptides during MS analysis. We used zwitterionic-hydrophilic interaction liquid chromatography (ZIC-HILIC) to enrich for glycopeptides (25) and the glycan moiety was then enzymatically released by PNGaseA. This enzyme was used because it can also cleave the oligosaccharides containing a fucose alpha(1–3)-linked to the asparagine-linked N-acetylglucosamine, commonly found in plants N-glycoproteins (35). To minimize false positives because of spontaneous chemical deamidation that may occur during sample preparation, we analyzed a small portion of the ZIC-HILIC-enriched glycopeptides without PNGase A treatment, as suggested by Palmisano et al. (26). Spontaneous chemical deamidation occurred at 14 Asn and 5 Gln on 17 N-glycopeptides (supplemental Table S1). Even though none of these peptides were found in our enzymatically deamidated dataset, it was possible to identify three spontaneous chemical deamidated peptides containing the PNGase A recognition motif (N-X-S/T/C; where X ≠ P), which could lead to data misinterpretation. Phosphorylated and Lys-acetylated peptides were enriched using TiO2 chromatography.

**Fig. 1. Experimental setup.** Uninfected and infected berries with the grapevine moth *L. botrana* were peeled on ice. Exocarp and mesocarp were frozen in liquid nitrogen and powdered using mortar and pestle. Following protein digestion, peptides were labeled with iTRAQ® and combined in 1:1 ratio. Phospho-, glyco- and Lys-acetyl-peptides were enriched by using TiO2 chromatography, ZIC-HILIC purification followed by enzymatic deamidation, and immunoprecipitation, respectively. Enriched peptides were prefractionated offline on a TSKGel Amide 80 HILIC HPLC column and subjected to LC-MS/MS analysis. *E(−)*, exocarp uninfected; *E(+)*, exocarp infected; *M(−)*, mesocarp uninfected; *M(+)*, mesocarp infected.
phy (21–24) and immunoprecipitation (36), respectively. Prior to LC-MS/MS analysis, all iTRAQ® labeled samples were prefractionated offline on a TSKGel Amide 80 HILIC HPLC column.

Proteome and PTM Coverage—By using combined proteomics and PTMomics approaches we were able to identify and quantify a total of 3059 proteins; 609 proteins were exclusively detected in the exocarp, 858 proteins were exclusive to the mesocarp, and 1592 proteins were identified in both tissues (Fig. 2A). Around 80% of these were unknown, unnamed or uncharacterized proteins. Therefore, substantial homology searching had to be performed using the Blast2Go software (33) to obtain putative functions for these proteins.

In this manuscript, the N-glycosylated, phosphorylated and Lys-acetylated sites were defined as “pN”, “pS/T/Y,” and “acK”, respectively. We used the MD-Score (31) to assign phosphosites, and the PNGase recognition motif (N-X-S/T/C; where X ≠ P) to assign correct N-glycosylation sites. Furthermore, only high-confidence (Mascot ion score ≥23) and rank 1 peptides were taken into consideration in this study. After applying these filters, we were able to identify 1135 phosphorylation sites, 323 N-glycosylation sites, and 138 Lys-acetylation sites (Fig. 2B). Even though we identified more Lys-acetylation sites than previous plant acetylproteome studies (37, 38), it is important to mention that iTRAQ can interfere with the Lys-acetylation immunoprecipitation efficiency, as previously reported (39) and this can reduce the number of identified Lys-acetylated peptides. The distribution of the 603 phosphorylation sites identified in the exocarp, and the 878 sites detected in the mesocarp was, respectively 89%/92% (pS); 10%/7% (pT); and 1%/1% (pY) (Fig. 2C). This distribution is similar to previous observations in Arabidopsis thaliana (40, 41) and rice (41). Fig. 2D represents the overlap between the proteins identified in the proteome and PTMome studies. The proteins identified in both approaches were used to determine whether a specific PTM was regulated due to protein differential expression or site occupancy. Among the 1736 proteins from the exocarp identified in the proteome study, 100 were also found in the phosphoproteome study, whereas 62 and 72 proteins were also identified in the N-glycoproteome and

![Fig. 2. Distribution of proteins and post-translational modification (PTM) sites. A. Total number of high-confidence proteins identified using the proteome and PTM approaches. B. Distribution of total number of identified PTMs sites in exocarp and mesocarp. C. Distribution of phosphosites identified in each grape tissue. D) Overlap of proteins identified in the proteome and PTMome studies. The numbers between brackets represent the total number of proteins identified on each study. E. Overlap of modified proteins.](image-url)
Lys-acetylproteome studies, respectively. In the same way, among the 1965 proteins identified in the mesocarp, 194/53/12 were also found in the phosphoproteome, N-glycoproteome, and Lys-acetylproteome, respectively. In addition, 13 proteins in total were both phosphorylated and Lys-acetylated, six proteins were phosphorylated and N-glycosylated, and six proteins were Lys-acetylated and N-glycosylated (Fig. 2E).

Fig. 3 depicts three representative MS/MS spectra of modified peptides regulated during the *Lobesia botrana* infection and mapped to proteins involved in the plant defense mechanisms. The deamidated N-linked glycopeptide YdNGSTLSVLGR (Fig. 3A) belongs to the “disease resistance response protein 206 (DRR206),” which plays an important role in the plant defense against fungi (42). The Lys-acetylated peptide VFDacKDQNGFISAELR (Fig. 3B) is a tryptic fragment of the “EF-hand calcium-binding protein,” which is orthologous to the “calcium-binding protein CML (CaMCML).” This protein seems to have a pivotal role in the plant innate immune system by inducing NO generation through the nitric oxide synthase (NOS) activation upon plant infection (43). Fig. 3C depicts the phosphopeptide EEYAsSDLDDLK that belongs to the protein “cyclin-dependent kinase F-1 (CDKF-1 or CAFK1),” which is involved in the control of cell cycle and differentiation during plant development (44) and abiotic or biotic stress (45). The N-glycosylated and Lys-acetylated peptides from DRR206 and CaMCML, respectively, were found up-regulated upon infection while the phosphorylated peptide from CDKF-1 was found down-regulated.

**Functional Distribution of PTMs—**Gene Ontology analysis revealed, as expected, that most of N-glycoproteins were localized in membrane, cell wall, extracellular space, endoplasmic reticulum, and vacuole in both exocarp (supplemental Fig. S1A) and mesocarp (Supplemental Fig. S1B). Moreover, analysis of mesocarp N-glycoproteins revealed a lysosomal and peroxisomal subcellular localization. Conversely, phosphorylated proteins were mainly localized to the nucleus and cytosol. Lys-acetylated proteins were localized in several cellular compartments in agreement with previous findings regarding a broad range of functions for this modification (37, 38).

Molecular function analysis indicates a major role for N-glycoproteins in catalytic activity whereas phosphoproteins show enrichment for DNA/nucleotide binding activity both in the exocarp and mesocarp, and protein binding activity in the exocarp (supplemental Fig. S2A and S2B). Biological process analysis also revealed an increased number of down-regulated proteins involved in transport dur-
ing infection. On the other hand, it is possible to notice an increased number of up-regulated molecules involved in protein biosynthesis. Cellular localization distribution revealed proteins located in ribosome, cytoskeleton, and cytosol over-represented in the up-regulated data set, whereas proteins localized in mitochondrion, vacuole, and thylakoid were over-represented in the down-regulated data set (supplemental Fig. S3). Taken together, these data suggest that during infection many metabolic processes shift to quiescence possibly to provide the energetic support for de novo synthesis of defense proteins.

**PTMs Motifs**—We used the motif-X algorithm (46) to identify sequence motifs from the phosphorylation and Lys-acetylation sites, using the *V. vinifera* database as background. We used a six amino acid residue sequence window surrounding the phosphorylated (S, T, or Y), and acetylated (K) residues. Only motifs with \( p < 10^{-6} \) were considered. Eight phosphorylation motifs were significantly enriched in our data set (Fig. 4A). The pS-P motif is a known target for the proline-directed kinase group, which includes mitogen-activated kinases and cyclin-dependent kinases [for review, see (47)]. In addition, casein kinase II (CKII) is known to phosphorylate Ser/Thr residues surrounded by acidic regions [pS-D-X-E; pS-X-X-D]. Furthermore, the recognition motif of 14-3-3 proteins [R-X-X-pS] was also found over-represented in our study. Strikingly,
we found two over-represented phosphorylation motifs in proteins N-, and C termini. Although these motifs have been already identified in other plants, the kinase involved in the phosphorylation of these sites is still unknown (48, 49). Using the recently developed ReportSites software (50) that maps the localization of phosphorylation sites within the protein, we confirmed an abundance of phosphosites localized in the distal portions of proteins, especially in their N-terminal portion (Fig. 4B).

Among the 1135 phosphorylation sites identified in our study, the site occupancy of 110 differentially changed during L. botrana infection (Fig. 4C). Analyzing the amino acid sequence of the phosphopeptides that contain these 110 sites, we observed a predominance of up-regulated phosphopeptides containing the protein C-terminal and [G-pS] motifs whereas the acidic motifs [R-X-X-pS] and [pS-D-X-E] were predominantly found in down-regulated phosphopeptides (Fig. 4D). These results suggest that during L. botrana infection kinases that preferentially phosphorylate the [G-pS] consensus sequence and phosphatases that remove phosphate groups from acidic motifs are activated whereas kinases and phosphatases that recognize the motifs [R-X-X-pS; pS-D-X-E] and [G-pS], respectively are inhibited.

The Lys-acetylation motif [acK-Y] was found over-represented in our data set (Fig. 4E). In a previous paper (37), only a very weak motif could be detected, with the acetylated lysines surrounded by Gly, Ala, Leu, and Arg amino acids. To our knowledge the acK-Y motif is the first report of the acetylation motif in plants, even though it has already been described for human (51).

### Table I

| Enzyme                                      | ID (NCBI)       | PTM site |
|---------------------------------------------|-----------------|----------|
| diacylglycerol kinase 5                     | gi225447673     | -        |
| glycerol kinase                             | gi22543625      | -        |
| pantothenate kinase 2                       | gi225457017     | -        |
| phosphoenolpyruvate carboxykinase           | gi29608358      | -        |
| protein kinase domain-containing protein     | gi297737665     | -        |
| xyulose kinase                              | gi147845091     | -        |
| inactive receptor kinase                    | gi225445372     | pS322    |
| pto kinase interactor                        | gi225432163     | pS80     |
| pfkb-like carbohydrate kinase family protein| gi225432552     | -        |
| galactose kinase                            | gi225432012     | -        |
| fggy family of carbohydrate kinase           | gi225446130     | -        |
| nak-type protein kinase                      | gi225427370     | -        |
| adenylate kinase b-like                     | gi225425838     | -        |
| cbl-interacting serine threonine-protein kinase 9 | gi229609825     | pS323    |
| cyclin-dependent kinase 1-1                 | gi147781538     | pS231    |
| protein kinase family protein               | gi225432364     | pS22     |
| ribose phosphate pyrophosphokinase          | gi296086310     | -        |
| serine threonine protein kinase              | gi297742124     | pS104    |
| adenylate kinase b-like                     | gi225466268     | -        |
| inactive receptor kinase                    | gi225445372     | -        |
| probable inactive receptor kinase at5g58300-like | gi225434317     | -        |
| nucleotide pyrophosphatase-like protein     | gi297738676     | -        |
| phosphoisoside phosphatase family protein   | gi225468413     | -        |
| serine threonine protein phosphatase 2a regulatory subunit | gi225455902 | -        |
| vacuolar h+-translocating inorganic pyrophosphatase | gi147785054 | pS699    |
| phosphoprotein phosphatase                  | gi225463785     | -        |
| soluble inorganic pyrophosphatase           | gi225426512     | -        |
| phosphoisoside phosphatase family protein   | gi225469602     | -        |
| probable inactive purple acid phosphatase 1-like | gi297730899    | -        |
| probable inactive purple acid phosphatase 29-like | gi296089042    | -        |
| serine threonine-protein phosphatase bs13 | gi225462440     | pS616    |
| type I inositol-trisphosphate 5-phosphatase 2-like | gi297742162    | -        |
| l-galactose-1-phosphate phosphatase         | gi225469270     | -        |
underlying the PTMomics tools to gain insights into the sequence of events underlying the V. vinifera infection, and to identify proteins that contribute to the susceptibility and response of this grapevine to L. botrana. Plants are constantly challenged by bacteria, fungi, and insects. Conserved antigenic molecules activate the plant defense system leading to expression of defense genes, cell wall thickness, and apoplastic acidification. Pathogenesis-related (PR) genes are a family of diverse proteins that are typically induced upon infection (52, 54). Indeed, five of six identified PR-proteins were found up-regulated in our study. Surprisingly, we found that the chitinase class III, known to be involved in plant defense responses, was down-regulated in the mesocarp during grape infection. In a study where a pine tree (Pseudotsuga menziesii) was infected with fungi and target genes expression were monitored over the time, the authors observed a transient decrease in the expression of chitinase genes (55). As we did not measure protein levels in a time-resolved manner, the down-regulation of chitinase class III could be explained by this transient expression profile. However, we cannot exclude the possibility that this isoform is in fact down-regulated during L. botrana infection.

Aquaporins are channel proteins specialized in the transport of water and/or small neutral solutes or gases through the membranes. These proteins can be modulated by differential protein expression as well as by PTMs (for review, see (56)). In a study where soybean gene expression was monitored upon fungal infection, a down-regulation of genes that encode aquaporins was observed. This is in agreement with our results as all detected aquaporins were down-regulated in infected berries. Moreover, the aquaporins isoforms PIP1;3 and PIP2;2 had their Lys-acetylation and phosphorylation sites positively regulated, respectively. However, it is still unclear whether these molecular events modulate the activity of PIP1;3 and PIP2;2.

In our study, we found that the occupancy of the acetylation site Lys95 on the calcium binding protein CML (CaMCML) increased during the L. botrana infection. Calcium (Ca<sup>2+</sup>) is an important secondary messenger involved in different defense

### DISCUSSION

In this study, we used state-of-the-art proteomics and PTMomics tools to gain insights into the sequence of events underlying the V. vinifera infection, and to identify proteins that contribute to the susceptibility and response of this grapevine to L. botrana. Plants are constantly challenged by bacteria, fungi, and insects. Conserved antigenic molecules activate the plant defense system leading to expression of defense genes, cell wall thickness, and apoplastic acidification. Pathogenesis-related (PR) genes are a family of diverse proteins that are typically induced upon infection (52, 54). Indeed, five of six identified PR-proteins were found up-regulated in our study. Surprisingly, we found that the chitinase class III, known to be involved in plant defense responses, was down-regulated in the mesocarp during grape infection. In a study where a pine tree (Pseudotsuga menziesii) was infected with fungi and target genes expression were monitored over the time, the authors observed a transient decrease in the expression of chitinase genes (55). As we did not measure protein levels in a time-resolved manner, the down-regulation of chitinase class III could be explained by this transient expression profile. However, we cannot exclude the possibility that this isoform is in fact down-regulated during L. botrana infection.

Aquaporins are channel proteins specialized in the transport of water and/or small neutral solutes or gases through the membranes. These proteins can be modulated by differential protein expression as well as by PTMs (for review, see (56)). In a study where soybean gene expression was monitored upon fungal infection, a down-regulation of genes that encode aquaporins was observed. This is in agreement with our results as all detected aquaporins were down-regulated in infected berries. Moreover, the aquaporins isoforms PIP1;3 and PIP2;2 had their Lys-acetylation and phosphorylation sites positively regulated, respectively. However, it is still unclear whether these molecular events modulate the activity of PIP1;3 and PIP2;2.

In our study, we found that the occupancy of the acetylation site Lys95 on the calcium binding protein CML (CaMCML) increased during the L. botrana infection. Calcium (Ca<sup>2+</sup>) is an important secondary messenger involved in different defense
mechanisms in plants. Changes in intracellular Ca\(^{2+}\) are translated into downstream signaling through various Ca\(^{2+}\)-sensor proteins (57, 58). These include calmodulins, calmodulin binding proteins, calcium-dependent protein kinases (CDPKs), and other EF-hand motif containing Ca\(^{2+}\)-binding proteins, and Ca\(^{2+}\)-binding proteins without EF-hands. CDPKs are plant-specific calcium sensors composed of many gene members (58) in *Arabidopsis*. A growing number of studies have revealed their roles in defense against biotic and abiotic stresses. Several CDPKs in *Arabidopsis* are involved in abscisic acid signaling and thus plant resistance to drought or salt stress (59, 60). CDPKs are activated by pathogen elicitors and are important for disease resistance (61). Even though these kinases seem to be crucial for the plant defense system, the CDPK identified in our study was not regulated upon infection. Furthermore, Ca\(^{2+}\) is associated with ROS and nitric oxide production in plants. A potato NADPH oxidase is phosphorylated by two CDPKs in a Ca\(^{2+}\)-dependent manner, which in turn elevates its ability to produce ROS (62). ROS are important secondary messengers responsible for the oxidative burst. Moreover, H\(_2\)O\(_2\) has a direct anti-pathogen effect, besides its role in cell signaling. Catalases and peroxidases are enzymes involved in H\(_2\)O\(_2\) removal and therefore have an important role as antioxidant agents. Following the grape infection, catalase was up-regulated, corroborating a previous observation (63). The authors observed an increased abundance of catalase after 72 h of fungal infection in *V. vinifera*. Surprising, peroxidase 4 was found down-regulated
in our study which suggested decreased expression of the gene upon moth infection.

In summary, we identified 899 proteins that were differentially regulated during grape infection by *L. botrana*. In this study, we dissected the grape immune system and revealed several unknown components. Furthermore, 21 kinases and 12 phosphatases were found differentially regulated following *L. botrana* infection, tightly regulating grape metabolism and signaling. To our knowledge, this is the first time that the acetylation motif X-K-Y-X is described in a plant system. These important findings need to be further explored to target specific protein modifications for better pest management but they represent a step forward toward a systems biology understanding of the grape-pathogen infection.

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