INTRODUCTION

Grapevines (*Vitis vinifera* L.) are an economically important fruit crop worldwide. They are used for the production of wine, table grapes, juice and raisins, and are worth millions of dollars every year for the US industry. Abiotic stresses affect both quality and quantity of grape production. Mild drought stress or the application of abscisic acid (ABA) increases the production of phenolic compounds such as anthocyanin, catechin and quercetin in the fruit and, in part because of their antioxidant activities, can benefit human health. Severe water deficit can reduce photosynthesis, inhibit vine growth, and decrease the quality of grapevines. Thus, there is an optimal level of drought stress that produces an optimal grape wine quality. A better understanding of grapevines responses to drought stress will allow one to minimize the loss of grapevine production and maximize grape quality.

ABA is a plant hormone that has important roles in developmental processes and adaptive stress responses in plants such as salt, cold and drought stress. ABA regulates plant responses by altering protein activities directly by post-translational modifications such as phosphorylation and nitrosylation, and indirectly by affecting the transcription of many genes. A model of ABA signaling has been constructed involving a central core pathway of PYR/PYL/RCAR receptors, 2C-type protein phosphatases (PP2C) and SNF1-related protein kinase 2 (SnRK2). Several transcription factors (AREB/ABFs) and ion channel proteins (SLAC1 and KAT1) are phosphorylated by SnRK2 kinases, but very likely there are many more proteins to be identified. Moreover, there may be other kinases in the ABA signaling pathway that have yet to be discovered.

Omic technologies have been used to gain better understanding of plant responses to stresses. Despite the great advances transcriptomic analyses have contributed to our understanding, there are far fewer proteomic and phosphoproteomic studies, which address a different level of plant regulation. Furthermore, recent studies from our lab indicate that the abundance of most proteins is not well correlated with transcript abundance.

In a previous study, the transcriptomic responses of grapevine to ABA were examined. Some of the results from that study indicated that the roots, which had been treated with 1 μM ABA for 2 h had 538 significantly differentially expressed genes (DEGs), whereas the leaf from the same plant had 69 significantly DEGs in response to the root treatment. Genes with significantly increased transcript abundance in leaves were involved in protein folding and the protein amino-acid phosphorylation process in roots. In this paper, we extend this study by examining the proteomic and phosphoproteomic responses of the grapevine leaves of vines whose roots were treated with ABA.

In this study, we identify proteins and phosphoproteins involved in the ABA signaling pathway in grapevine. A label-free approach was first used to identify and quantify changes in protein abundance. In addition, we utilized a second approach, using 6-plex isobaric mass tagging technology, labeling peptides with structurally identical tags but different reporter ions. Our data sets revealed motifs and phosphorylation sites that are consistent with other plant phosphoproteomes.
MATERIALS AND METHODS

Sample collection and ABA treatment

Rooted cuttings of Cabernet Sauvignon grapevines were grown in a growth chamber for 2 to 3 weeks before carefully transferring them to an aeroponic system located in a greenhouse under standard conditions (with supplemental sodium vapor lamp lighting (16 h light (minimum 400 μM m⁻² s⁻¹) at 28 °C and 8 h dark at 18 °C cycle). Each container (43.2 cm(L) × 27.9 cm(W) × 20.3 cm(H)) had a nebulizer with a fogger head size of 3.8 cm diameter × 4.4 cm height for each experimental replicate (three containers for control and three containers for ABA treated). The lid of each container had small holes large enough for several rooted plants to be passed through and into the container. Gibeaut's solution was used to provide the macronutrients and micronutrients to the vines. The pH of the solution was maintained at 6.0. Root and leaf samples were grown for 3 months before treatment.

ABA was applied to the roots by continually misting the roots with 10 μM ABA added to the Gibeaut's solution in the aeroponic system; leaves from the same vine (but not directly exposed to the misting solution) and exposed roots were collected after 2 h of root exposure with fresh control and ABA solutions. Root samples were quickly rinsed with tap water and collected leaf and root materials were rapidly frozen in liquid nitrogen before storage at −80 °C.

Label-free approach

Proteome sample preparation and LC–MS/MS analysis.

A phenol extraction protocol was used for Vitis vinifera leaves and is based on previous protocols (Vincent, Wheatley et al. 2006). Trypsin in-solution digestion, peptide extraction and fraction analysis by nanoflow liquid chromatography tandem mass spectrometry (LC–MS/MS) were conducted essentially as previously described. Briefly, three experimental replicates of ABA-treated leaves and untreated leaves were run separately on an LTQ Velos Pro mass spectrometer (Thermo, San Jose, CA, USA) for the sample-optimal gas phase fractionation. Chromatography was performed on an Easy-nLC II (Thermo) with magic C18 AQ column (3 μm bead size, 200 Å pore size, 0.1 mm inside diameter × 100 mm; Michrom Biosciences, Auburn, CA, USA). Each sample was analyzed in four 120-min LC–MS/MS runs at 0.5 μL min⁻¹, each using a different precursor mass range (400–506, 501–658, 653–913 and 908–1600). The m/z ranges for four gas phase fractionation per sample were optimized empirically by analyzing a mixture of pooled samples from m/z 400–1600, then creating gas phase fractionation fractions to approximately evenly distribute peptide observations among the four fractions.

Protein identification and quantification.

A protein database for V. vinifera was combined as in a previous report (Cramer et al. 2016). The X! Tandem and the GPM Cyclone (http://www.thegpm.org) in the automated mode using MudPIT filtering were used for peptide-spectrum matching. Protein and peptide false discovery rates (FDR) were calculated using reverse database searching.

Low-stringency protein identification data from individual replicate experiments were transformed into high-stringency data sets containing only reproducibly identified proteins using a suite of R modules known as the Spectral Counting Reporting Analysis Program (Scrappy). For a protein to be considered as reproducibly identified it needed to satisfy two criteria: it must be present with at least five peptides across all three replicates, an average of 1.7 peptides per experiment. The same program was used for calculation of protein abundance using normalized spectral abundance factors (NSAF), and determination of significantly differentially expressed proteins (P < 0.05) based on Student's t-test across three replicate NSAF values.

TMT labeling approach

Proteome sample preparation. The phenol protein extraction was used as mentioned above. The protein concentration was determined by using EZQ protein quantitation according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). Protein samples (10 mg) were digested with trypsin and desalted by Sep Pak Plus C18 cartridges (Waters Inc, Milford, MA, USA). Peptides (10 mg) were resuspended in 100 μL of 1% acetic acid.

Phosphopeptides enrichment and TMT labeling. The immobilized metal affinity chromatography (IMAC column; Qiagen Ni-NTA spin column (Cat No. 310144)) was loaded with 50 μL of peptides. The IMAC column was washed four times; twice with 1 bead volume of 0.1 M NaCl/25%AACN/0.1% acetic acid, once with 1 bead volume of 1% acetic acid, and once with half bead volume of ddH₂O. Peptides were eluted with 3 x bead volume using 6% NH₄OH (total 120 μL); the elution was collected in a silanized glass insert, and then dried with a speed-vac. The tandem mass tags (TMT) labeling method was followed according to the manufacturer’s instructions (TMT Fisher # 90061, Thermo Fisher Scientific, Ashville, NC, USA). The untreated leaf samples were labeled with TMT-126, TMT-127, and TMT-128, while ABA-treated leaf samples were labeled with TMT-129, TMT-130, and TMT-131. All TMT labeling samples were combined before sample fractionation using hydrophilic interaction liquid chromatography according to Pucci et al. (Pucci, Giuliano et al. 2009). A total of 40 fractions were evaporated to dryness in a vacuum centrifuge and resuspended in 100 μL 5% CAN and 0.1% formic acid for LC–MS/MS analysis.

LC–MS/MS analysis. Digested peptide samples were analyzed using LC–MS/MS at the Nevada Proteomics Center (University of Nevada, Reno, NV, USA). The peptides were separated and analyzed using a Michrom Paradigm Multi-Dimensional Liquid Chromatography instrument (Michrom Bioresearches Inc., Auburn, CA, USA) coupled with a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Peptide samples were dissolved in 100 μL of 0.1% formic acid and loaded onto a ZORBAX 300SB-C₁₈ 5-μm (5×0.3 mm) trap column (Agilent Technologies, Santa Clara, CA, USA), eluted from the trap, and then separated with a reverse phase Michrom Magic C₁₈AQ column (3 μm, 200 Å, 0.2×150 mm) by a gradient elution using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in ACN) at a flow rate of 2 μL min⁻¹. The gradient was set from 5 to 40% solvent B for 3 min, increased to 80% solvent B in 10 s and held at 80% solvent B for 1 min. MS spectra were recorded over the mass range of m/z 400–1600 with resolution of 60,000. The three most intense ions were isolated for fragmentation in the linear ion trap using CID with minimal signal of 500 and collision energy of 35.0 or using HCD with a minimal signal of 1000, collision energy of 55.0, and an activation time of 30 ms. Dynamic exclusion was implemented with two repeat counts, repeat duration of 15 s and exclusion duration of 90 s.

Protein identification and quantification.

All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific; version 1.0). Sequest was set up to search the uniprot_Vitis_20121010 database (54,242 entries). Search parameters included fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10.0 p.p.m., trypsin enzyme specificity, carbamidomethylation of cysteine as a fixed modification and oxidation of methionine, phosphorylation of serine, threonine and tyrosine and TMT6plex of lysine and the N-terminus as variable modifications. Scaffold (version Scaffold_4.4.1, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at > 95.0% probability and contained at least 1 identified peptide. Relative abundances of proteins were calculated based on Dayon et al. Briefly, a normalization of the reporter intensities by the sum of all the reporter intensities was made in order to determine the relative abundance of each reporter. Then the log₂ ratio of average TMTmasses (TMT-126, TMT-127, TMT-128) (ABA/Control) were obtained from the average values. Finally, an unpaired t-test was assessed to test the significance of the abundance difference (P < 0.05).

Motif analysis

In order to identify potential enzyme recognition sites, Scaffold PTM scans the data set for over-represented patterns in the amino acids surrounding modification sites. It uses the iterative statistical method described in a previous publication. Sequence logos were generated using Weblogo of all phosphorylation sites with Ascores ≥ 13 (P < 0.05). The frequency of each residue present in each data set is proportional to its height. The phosphorylation site is located at P on the residues flanking by 12 amino-acid residues (at position – 6 to +6).

RESULTS

Proteomic changes in leaves of grapevine in response to ABA

Leaves of Cabernet Sauvignon grapevines were collected after 2 h treatment with 10 μM ABA to the roots of the same vines. In this study, two approaches were employed to identify and quantify
proteomic changes (Figure 1). The first method was label-free quantitative shotgun proteomics using nanoflow liquid chromatography-tandem mass spectrometry (nanoLC–MS/MS). The second method was isobaric chemical labeling using TMT. The 6-plex TMTs were used to label leaf control (TMT129,130,131) and ABA-treated samples (TMT129,130,131) after phosphopeptide enrichment by IMAC. Protein identification and quantitation were analyzed after LC–MS/MS analysis.

Label-free quantitative proteomic analysis of grapevine leaves in response to ABA was quantified by normalized spectral abundance factors. Approximately 2533 non-redundant *Vitis vinifera* proteins in the UniProtKB database were identified at low stringency, with 363 and 310 proteins reproducibly identified in samples of leaf control and leaf ABA, respectively, at a FDR of 0.55% (Table 1; Supplementary File 1). Among the total of identified proteins, 20 upregulated and 13 downregulated proteins were significantly differentially expressed in the leaves in response to ABA treatment of the roots (*P* < 0.05) (Table 2). A functional analysis for Gene Ontology (GO) categories was analyzed with the Cytoscape (3.2.0, www.cytoscape.org) and the BinGO plugin (3.0.3, www.cytoscape.org) for the statistically significant proteins in response to ABA (Supplementary File 2), using a custom annotation derived from UniProt (uniprot.org), EnsemblPlants (plants.ensembl.org) and Gramene (gramene.org). 33,34 Photosynthesis, carbohydrate catabolic process and protein folding, carbohydrate catabolic process and response to abiotic stimulus were significantly over-represented proteins in response to ABA (Supplementary File 2), whereas protein folding was significantly over-represented only in upregulated proteins (F6HLR2, D7JSX8 and F6HDN4).

Some of the proteins decreased in abundance by ABA include photosynthetically related proteins such as a photosystem I complex protein (D7TAY3) and a oxygen-evolving enhancer 3 protein, PsbQ (F6HB84), part of photosystem II. A ribosomal protein (F6H5E3), involved in protein synthesis, was also decreased. Other proteins affected by ABA include proteins involved in amino acid, sugar and cell wall metabolism. A few proteins were increased in protein abundance by ABA including a voltage-dependent anion channel (A5AUG8), an NADP-dependent malic enzyme (P51615) and a putative oxygen-enhancer protein (Q6XQX7).

A total of 1011 proteins were identified by TMT labeling in the leaves of grapevine in response to 10 μM ABA for 2 h at an FDR of 0.62% at the protein level and 0.9% at the peptide level. (Supplementary File 3). A total of 787 proteins were tagged with TMT on the free amino terminus or lysine residues with a 0.61% protein FDR and 0.8% peptide FDR (Supplementary File 4). It should be noted that all proteins reported in this table with quantitation data have been observed in triplicates of both conditions, allowing for statistical evaluation of quantitation differences.

There were 20 proteins (11 phosphoproteins) that were significantly changed in protein abundance (*P* ≤ 0.05) in the leaves in response to the roots being treated with ABA (Table 3). All significantly differentially expressed proteins in response to ABA decreased in protein abundance. Biological process analysis (GO) of significant proteins changing in protein abundance in response to ABA revealed that several developmental processes were affected (*P* ≤ 0.05; Supplementary File 5). These proteins include the acetyltransferase component of the pyruvate dehydrogenase complex (D7T9L3), a Nck-associated protein 1 (D7T9L3) involved in actin remodeling, a component (F6HTWO) of the Cul4-RING E3 ubiquitin ligase complex, a DNA/RNA helicase (F6GT26), a protein involved in stability of Photosystem II (F6HVA4), a eukaryotic translation initiation factor (F6I2I6) and a ribosomal protein (A5AI30).

ABA affects phosphoproteins in grapevine

We utilized a new approach for the identification and quantification of phosphoproteins by enrichment of phosphopeptides prior to TMT labeling. This approach gives valuable data on phosphoprotein identification, phosphorylation sites and protein abundance changes. There were 219 phosphoproteins identified by TMT–LC–MS/MS (Supplementary File 6) and 116 of them were tagged with TMT. The phosphoproteins in this table are sorted by the number of discrete peptides identified; 145 proteins were identified from multiple peptides and 74 proteins were identified from single peptides. However, these proteins are not subject to the usual concerns associated with single peptide-based protein identifications because they were actually identified and quantified in triplicate experiments to allow for statistical evaluation.

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**Table 1.** Peptide/protein identification data of Cabernet Sauvignon leaves: control and ABA

| Condition | Low stringency redundant peptide count | Low stringency protein identifications | High stringency protein identifications | Protein FDR (%) |
|-----------|----------------------------------------|---------------------------------------|----------------------------------------|-----------------|
|           | R1          | R2          | R3          | R1          | R2          | R3          |                            |                                |
| Control   | 7238        | 6027        | 6099        | 888         | 840         | 821         | 363                   | 0.55                           |
| ABA       | 5269        | 5650        | 5715        | 767         | 1124        | 841         | 310                   | ND                             |

Abbreviations: FDR, false discovery rate; ND, not detected. R1, R2 and R3 denote replicate 1, replicate 2 and replicate 3. High-stringency protein indentifications were common to all three replicates.
The phosphoproteins tagged with TMT can be used for further quantification. The localized sites were identified and were reported with Ascor, which calculates the probability of correct phosphorylation site localization based on the presence and intensity of site-determining ions in MS/MS spectra. From the identified phosphoproteins, 192 non-redundant phosphorylation sites were found with Ascores \( \geq 10 \) (\( P \leq 0.05 \)) (Supplementary File 7). Identified phosphorylation sites were classified into 77% pSer, 20% pThr and 3% pTyr (Figure 2a). One percent of the proteins had three phosphorylation sites, 7% of the proteins had two phosphorylation sites and 92% of the proteins had one phosphorylation site, (Figure 2b). More than 50% of significant proteins in response to ABA (Table 3) were phosphoproteins. The novel phosphorylation sites with Ascores \( \geq 10 \) (\( P \leq 0.05 \)) were reported in Supplementary File 7. New phosphorylation sites for many proteins were identified; for example, pectinesterase (F6H777), a potassium efflux antipporter1 (F6H66) and an auxin efflux carrier (F6HFI3).

GO analysis for the phosphoproteins found in this study indicated that nucleotide binding is the most over-represented category of molecular function while membrane and transport is the most over-represented categories of the cellular component and biological process, respectively (Figure 3, Supplementary File 8). The plasma membrane was the highest membrane type for phosphoproteins in this study.

The putative motifs surrounding the phosphorylation sites of all phosphopeptides found in this experiment were analyzed with localization probability \( \geq 95\% \) and Ascores \( \geq 10 \) (\( P \leq 0.05 \)). Finding of motifs involving phosphorylation events provides valuable information about the specific binding of kinases to substrates. The amino acids around the phosphorylation sites from -6 to +6 were aligned to find the common motifs using WebLogo. The motifs found were [S–P], [S–D], [R–x–x–x–S] and [S–x–x–x–x–x–F] for phosphoserine, [T–P] for phosphothreonine and [Y–G] for phosphotyrosine (Figure 4; Supplementary File 9).

We also observed a similar response of specific phosphoproteins decreased in protein abundance after treatment with ABA to that which has been previously reported in phosphoproteins in Arabidopsis; for example, these included embryonic factor1 (AT2G38820; D7YS29), IQ-domain 32 (AT1G19870; F6H606), seed imbibition 1-like (AT5G40390; D7TWK5) and Tudor/PWWP/MBT protein (AT3G09670; F6HMK4).

Furthermore, the same leaf samples were used for all of the transcriptomic, proteomic and phosphoproteomic analysis. Transcriptomic data of ABA-treated leaves was obtained from microarrays, while proteomic profiles were obtained from TMT and label-free approaches. There were 508 identified proteins found in TMT tagged that were also found in the label-free method (Figure 5a). TMT quantified 787 proteins from 1011 identified proteins, while label-free quantified 360 proteins from 2533 identified proteins (Figure 5b). All three different approaches reported here can identify seven proteins that were found in common (Table 4). Of these seven proteins, just two showed significant effects by ABA, however, their abundance changes were not the same within all three measurement methods. One chlorophyll a/b binding protein (ASBA14) was increased in protein abundance by ABA based upon the TMT method, but with little or no effect for the label-free and microarray data in the leaves. Interestingly, this protein is phosphorylated. A heat-shock 70 protein, was increased in abundance by ABA in the label-free method, decreased in the TMT method, with little effect on the transcript levels.
Table 3. Annotation of proteins differentially expressed 2 h after 10 μM ABA treatment by TMT

| WT ID | Annotation | Log ratio: | P value | Phospho site | Peptide sequence |
|-------|------------|------------|---------|--------------|-----------------|
| VIT_13s0019g03620 | Tudor/PWP/MBT superfamily protein, group 2 | -1.84 | 1.07E-02 | | HSCHQVEEIKVPYK |
| VIT_13s0084g00160 | Proline-rich family protein | -0.94 | 1.60E-02 | | F6HVA4 |
| VIT_00s0361g00080 | Eukaryotic translation initiation factor-related protein | -1.10 | 1.97E-02 | | F6I216 |
| VIT_18s0001g01180 | Target of MYB protein 1 | -0.77 | 2.24E-02 | | F6H0F0 |
| VIT_01s0011g00820 | Remorin family protein | -1.04 | 2.38E-02 | | D7T9T5 |
| VIT_01s0011g01630 | Transcription activators | -1.30 | 2.44E-03 | | D7T9L3 |
| VIT_06s0009g02120 | Dr1-associated corepressor | -0.86 | 2.60E-03 | | D7T1D7 |
| VIT_09s0002g01800 | Dihydrolipoamide acetyltransferase | -1.08 | 2.66E-03 | | D7TZW9 |
| VIT_13s0019g00160 | Phosphorylation database (P3DB, http://p3db.org), listed 607 conserved plant phosphoproteins with 862 phosphosites identified in berries of grapevines (V. vinifera 'Italia cv') using iTRAQ labeling with TiO₂-phosphopeptide enrichment. In comparison, we found 219 phosphoproteins with 192 phosphosites in leaves of V. vinifera cv. Cabernet Sauvignon using an IMAC-phosphopeptide enrichment and TMT labeling method. In our study, many novel proteins were identified by differential expression analyses both at the RNA and protein levels. Moreover, the use of differentially expressed proteins to discern the influence of different cultivation conditions on the ABA signaling pathway. The results suggest that ABA treatment can affect the expression levels of various proteins involved in the ABA signaling pathway. The changes in protein abundance in response to ABA involve plant growth and photosynthesis. Overall, our results indicate that early changes of protein abundance in response to ABA involved plant growth and photosynthesis.

Proteins involved in photosynthesis are an important means to control plant growth and development. The inhibition of proteins involved in photosynthesis are correlated with the reduction of shoot elongation of grapevines under water deficit and salinity. In addition, studies of leaves and fruits treated with water deficit showed a decrease in gene expression involved in photosynthesis and a decrease in chlorophyll content. Our study found many significant phosphoproteins involved in cell cycle-related processes. There were significant changes of phosphoproteins in this study that were involved in growth and organ developmental processes. The changes of abundance of these proteins may result in the plant's acclimation to a drying environment.

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Phosphoproteomics of grapevine leaves to ABA
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in grapevine proteins. The phosphorylation of tyrosine (Tyr) in plants is less abundant due to the lack of receptor Tyr kinases.48 However, in silico analysis of the *Arabidopsis* genome indicated that ~4% of Arabidopsis kinases are tyrosine-specific kinases, which was close to the amount of pY found in our study. The proportions of phosphorylation sites on serine, threonine and tyrosine found in this study was consistent with the study on other plant species, such as 89.5% pS, 8.9% pT and 1.6% pY found in rice, and 87.7% pS, 9.9% pT and 2.4% pY found in Arabidopsis.46,49,50

We observed a decrease in protein abundance involved in serine family amino-acid metabolic process in leaves in response to ABA. Serines are commonly phosphorylated by kinases during cell signaling. In plants, the phosphorylated pathway of serine biosynthesis has had an important role in supplying serine to non-photosynthetic tissues under environmental stresses.51,52

The phosphorylation motifs are important to determine the binding of the kinase to its substrate.53 The identification of phosphorylation motifs and phosphorylation site localizations are important in understanding many signal transduction pathways. The motifs found in this study have been identified as the possible substrates of SnRK2s.10,36 The SnRK2s have been confirmed to phosphorylate [R–x–x–S] motifs in vitro.14,54 We found the phosphorylated [R–x–x–S] motif of the ABC transporter G family member 40 (ABCG40; F6HX69), which decreased in protein abundance in response to ABA. An ABC transporter was found to be able to transport ABA from the cytoplasm to the vacuole in order to control the level of ABA in the cytosol.55 ABCG40 is responsible for ABA transport into guard cells in *Arabidopsis*.56 The molecular mechanism of how ABA is transported has not yet been fully elucidated. It is possible that phosphorylation might be involved in this ABA transport mechanism.

Normally, membranes are the first sites of signaling to occur in response to stresses. In this context, many phosphoproteins found in this study are membrane proteins. It has been found that transport systems tend to be phosphorylated,57,58 which corresponds to our finding that transport is a major biological process of phosphoproteins. Altogether, these results indicate that ABA

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**Figure 2.** Proportional representation of phosphorylation sites on serine, threonine and tyrosine with Ascores ≥ 13 (P < 0.05) found in Cabernet Sauvignon leaf proteins whose roots had been treated with and without 10 μM ABA (a) and number of phosphosites, showing that most phosphopeptides were had one phosphosite (92%), followed by two phosphosites (7%) and three phosphosites (1%) (b). ABA, abscisic acid.

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**Figure 3.** Functional categories of 219 phosphoproteins identified by TMT–LC–MS/MS were analyzed with the Cytoscape (3.2.0) and BinGo plugin (3.0.3). LC–MS, liquid chromatography tandem mass spectrometry; TMT, tandem mass tags.
may be involved in the regulation of membrane transport systems in grapevine leaves via a protein phosphorylation process.\textsuperscript{58}

Analysis of omics in response to ABA

Label-free and labeling approaches have been found to be equally capable of reliably and accurately quantifying protein abundance levels.\textsuperscript{59,60} Our results confirmed the previous finding\textsuperscript{61} that compared label-free and an isobaric chemical labeling method. Combining label-free with the labeling approach provides a more complete picture for a proteomic study.

On the basis of our results of transcriptomic, proteomic and phosphoproteomic analyses, genes that significantly increased in transcript abundance in response to ABA in the roots, also had their protein abundance changed in the leaves. There might be a signal from the roots causing a change in protein abundance in the leaves. ABA has been proposed as a root-to-shoot signal during drought stress.\textsuperscript{62,63} Our work presented here demonstrated the changing of proteins in the leaves resulting from ABA-treated roots. The protein amino-acid phosphorylation process was a significantly overrepresented GO category of DEGs in response to ABA in roots, whereas the organ development process was a significantly overrepresented GO category for significant proteins in leaves. More than 50% of significant proteins in response to ABA were phosphoproteins.

This finding indicated that there is a communication between roots and leaves in response to ABA, which may involve phosphorylation.

CONCLUSIONS

Utilization of proteomics and phosphoproteomics has provided the data that has lead to deeper understanding of ABA responses in both proteins and phosphorylation of those proteins. With the short-term treatment of ABA, we discovered rapid and significant changes in protein abundance in the leaves of roots treated with exogenous ABA. This finding indicated that there is rapid communication between roots and leaves when responding to ABA. ABA decreased the abundance of growth-related and photosynthetic proteins, probably in an effort to reduce leaf area and water loss. Many potential target proteins and phosphoproteins for ABA signaling were identified. Phosphoproteins found in this study were membrane proteins involved in transport and nucleotide binding. These transport proteins may be involved in plant growth and adaptation to water deficits.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Table 4. Common proteins found from transcriptomic, proteomic and phosphoproteomic analysis. The microarray data is from a previous publication\textsuperscript{19}

| Current V1 ID | UniProt ID | Annotation | Microarray | Label-free | TMT | Phosphorylated |
|--------------|------------|------------|------------|------------|-----|---------------|
| VIT_10s0003g02890 | A5BAI4    | Chlorophyll a/b binding protein 3 | −0.045 | 2.133 | 0.188 | 0.934 | Yes |
| VIT_06s0061g00270 | F6GWA8    | Chaperonin 60 subunit alpha 1, chloroplastic | 0.062 | 1.431 | 0.321 | −0.363 | Yes |
| VIT_18s0001g02740 | D9CR63    | Photosystem II 22 kDa | −0.024 | 2.042 | 0.028 | −0.011 | Yes |
| VIT_08s0007g00130 | F6HLD8    | Heat-shock protein 70 | −0.111 | 1.433 | 2.214 | −0.725 |
| VIT_14s0060g00820 | A5AIE0    | Chloroplast stem-loop binding protein of 41 kDa | 0.036 | 1.284 | 0.291 | −0.561 |
| VIT_06s0004g00240 | D7SLM9    | Chaperonin 60 subunit beta 3, chloroplastic | −0.044 | 1.050 | 0.015 | −0.216 |
| VIT_15s0024g00040 | F6JS59    | Photosystem I light harvesting complex gene 3 | 0.090 | 1.705 | −0.385 | 0.234 |

Figure 4. Sequence logos of all phosphorylation sites with Ascores $\geq$ 13 ($P \leq 0.05$). The frequency of each residue present in each data set is proportional to its height. The phosphorylation site is 0 on the $x$ axis flanking by 12 amino-acid residues (at position $-6$ to $+6$). Extracted motifs were shown on the right.

Figure 5. The overlap of proteins found in TMT and label-free method (a), and number of protein identification and quantification from TMT and label-free method (b).
Acknowledgements

This work was funded by the College of Agriculture and Natural Resources at the University of Nevada, Reno with a Nevada Agriculture Experimental Station Multi-State Hatch Grant (Grant # NEV00345).

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