Proteomic Analysis of Environmental Stress Responses in Grapevine

by

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This thesis is presented for the award of the degree of

Doctor of Philosophy

Department of Chemistry and Biomolecular Sciences

Faculty of Science and Engineering

Macquarie University, Sydney, New South Wales, Australia

April 2015

“I am the vine, you are the branches. He that abides in me, and I in him, he bears much fruit: for without me you can do nothing.”

- **Jesus Christ**

Dedicated to my husband Martin William,
and my parents.

Declaration

I certify that this thesis titled ‘Proteomic analysis of environmental stress responses in grapevine’ is my original research. Some parts of this research was achieved in collaboration with other researchers; any form of assistance received from others has been duly acknowledged and their contribution recognised. All sources of information and cited material have been referenced in this thesis. No part of this thesis has been submitted to any other institution as a component of a degree or award. I also certify that this thesis contains no information that has been formerly written or published by anyone else except where due reference is cited in the text.

Iniga Seraphina George

April 2015

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Acknowledgements

The success and final outcome of this thesis required a lot of guidance and support from many people. I am greatly indebted in gratitude to many and will try to thank as many as I can here.

Firstly, I thank my principal supervisor, Professor Paul Haynes for the opportunity to work on this project. His leadership, excellent supervision, knowledgeable guidance in directing my project, constant encouragement and his trust and confidence in me motivated me to accomplish my tasks in time. I am grateful to my co-supervisor, Associate Professor Robert Willows for his instructions and comments on my project whenever I needed it. I gratefully acknowledge both my supervisors for their time, availability, instructions, and insights and also for the freedom to pursue my own thoughts in this project.

Thanks to Professor Brian Atwell for his advice and Muhammad Masood for the help and friendly assistance while working in the greenhouse and plant growth facility. Special and sincere thanks to Dr. Dana Pascovici, for her prompt assistance and great help in data analysis. I also thank Professor Anne Fennell, South Dakota State University, Brookings, USA, for the opportunity to work on collaborative projects in grapevine and Professor Grant Cramer, University of Nevada, Reno, USA, for allowing me to visit his laboratory. Thanks to Dr. Chris Davies, CSIRO Plant Industry, South Australia for starter grape calli. I thank all those whom I have co-authored with, for the opportunity to collaborate and also for their intellectual contribution. I thank Ms. Jane Yang for her kind assistance in regard to higher degree research matters. I thank Catherine Wong, Michelle Kang and Jinxia Ke for administrative assistance.

I extend my gratitude to all my colleagues and friends in the Department of Chemistry and Biomolecular Sciences (CBMS) and the Australian Proteome Analysis Facility

(APAF). Special thanks to Karlie, Mehdi, Gayani, Steve, Sridevi and Samantha from whom I have learnt many things in experimentation. I appreciate Shabnam, Sri, Vignesh, Manveen, Karthik, Katherine, Tony and other past and present PhD students for their motivation and support during my study. Special thanks to Shabnam, Sammi and Manveen for their friendship, which I value very much. I thank all the members of the Haynes' group, Mehdi, David, Sammi, Vineet and Yunqi for being great team mates, and the others in the Biomarker Discovery Lab for the pleasant research environment. I also gratefully acknowledge the help and motivation from my friends outside university, Kanika, Dorji, Ronita, Jude, Rukshana and Cherag and also from church, for their prayers and concern.

My heartfelt thanks to my family; my husband - Martin William who is an epitome of love and patience, my father - Selvam George who is my role model and inspiration, mother - Vasanthi for her care, concern and prayers, sister - Monica, for her long night vigils to give me company me as I wrote this thesis and also for her cheerfulness, parents-in-law, Joseph William and Cecilia, for their constant concern, sacrifice, support and encouragement. I will ever be indebted in gratitude to my immediate family for their unconditional love, sacrifice and help.

I also thank my loving extended family in Sydney, Aunty Regina, Uncle John, Kevin, Aunty Pramila, Uncle Paul, Jemima and Joseph for their love, prayers, support and words of encouragement. I admit my gratefulness to Premi, Mother Mercy and (Late) Uncle Thivian who have been my family when I was away from home, especially during the initial stages of my PhD. Words cannot explain the gratitude I feel towards my family, my backbone of support, for their prayers and patience during my doctoral study.

Above all and most importantly, I would like to thank Jesus Christ, the God Almighty and also my best friend, who gives me the strength to do all things. For with God, all things are possible.

Abstract

Understanding environmental stress responses is essential to improve abiotic stress tolerance in commercially important crops such as grapevine. Erratic abiotic stresses can pose a threat to viticulture practices. Changes in temperature and light exposure, either suddenly or seasonally, are crucial environmental factors that can influence grapevine productivity. The grape genome sequence was released in 2007 and the availability of this genomic information facilitated proteomic investigations to be executed in the *Vitis* species with ease. In this thesis, we aimed to enhance our understanding on biological pathways activated by the impact of abiotic stresses in grapevine, by characterising proteins associated with stress responses.

Firstly, we optimised a sample preparation and fractionation methodology for label-free quantitative shotgun proteomic analysis of grapevine. We then implemented this optimised protocol for all the studies included in this thesis.

Subsequently, we examined the influence of thermal stresses on *Vitis vinifera* and compared protein expression patterns between the control temperature and two different heat and cold stresses. This is the first label-free shotgun proteomic study on grape exposed sudden temperature changes. We demonstrated that proteins involved in phenylpropanoid biosynthesis were more abundant at extreme cold stress and could be cold-responsive proteins. We also observed that sugar metabolism switched between the alternative and classical pathways at thermal stresses.

Next, two hybrid grapevine species - *Vitis riparia* and Seyval, were used to investigate cellular mechanisms associated with photoperiod regulated biological processes of growth cessation and dormancy induction. Several enzymes involved in glycolysis, and dormancy induction were up-regulated in short daylength buds compared to long

daylength buds. We observed active growth as well as greater abundance of phenylpropanoid biosynthesis proteins in long daylength buds.

Finally, we have introduced a new method for validation of quantitative shotgun proteomic data. We developed a protein quantitation false discovery rate and applied it as a noise-level threshold, to establish the significance of the proteomic results in this thesis. All the research studies in this thesis demonstrated the efficacy of label-free proteomics approaches in gaining information on grapevine responses to environmental stresses at the cellular level.

List of Manuscripts

Publication I

Neilson, K. A., **George, I. S.,** Emery, S. J., Muralidharan, S., Mirzaei, M. & Haynes, P. A. Analysis of rice proteins using SDS-PAGE shotgun proteomics. *Methods in Molecular Biology* 2014, *1074*, 289-302.

Publication II

George, I. S., Fennell, A. Y. & Haynes, P. A. Protein identification and quantification from riverbank grape, *Vitis riparia*: comparing SDS-PAGE and FASP-GPF techniques for shotgun proteomic analysis. *Proteomics* 2015, *15*, 3061-3065.

Publication III

George, I. S., Pascovici, D., Mirzaei, M. & Haynes, P. A. Quantitative proteomic analysis of Cabernet Sauvignon grape cells exposed to thermal stresses reveals alterations in sugar and phenylpropanoid metabolism. *Proteomics* 2015, *15*, 3048-3060.

Publication IV

George, I. S. & Haynes, P. A. Current perspectives in proteomic analysis of abiotic stress in grapevines. *Frontiers in Plant Science* 2014, *5*, 1-5.

Publication V

Mirzaei, M., Soltani, N., Sarhadi, E., **George, I. S.,** Neilson, K. A., Pascovici, D., Shahbazian, S., Haynes, P. A., Atwell, B. J. & Salekdeh, G. H. Manipulating root water supply elicits major shifts in the shoot proteome. *Journal of Proteome Research* 2013, *13*, 517-526.

Publication VI

Neilson, K. A., Scafaro, A. P., Chick, J. M., **George, I. S.,** Van Sluyter, S. C., Gygi, S. P., Atwell, B. J. & Haynes, P. A. The influence of signals from chilled roots on the proteome of shoot tissues in rice seedlings. *Proteomics* 2013, *13*, 1922-1933.

Publication VII - Prepared for publication

George, I. S., Fennell, A. Y. & Haynes, P. A. Shotgun proteomic analysis of photoperiod regulated dormancy induction in grapevine. This manuscript is prepared for publication and is expected to be submitted to *Proteomics* by June 2015.

Conference Presentations and Awards

Conference presentations (*Oral presentation)

1. Iniga S. George, Steven C. van Sluyter, Grant R. Cramer and Paul. A. Haynes. 'Proteomic analysis of drought stress in Cabernet Sauvignon grapevines' at the **17th Lorne Proteomics Symposium 2012, Australasian Proteomics Society, Lorne, Victoria, Australia.**

2. Iniga S. George, Anne Y. Fennell, Robert D. Willows and Paul. A. Haynes. 'Shotgun proteomic analysis of grape buds exposed to different photoperiods' at the **5th Annual Grape Research Coordination Network (GRCN) Conference 2013, University of California, Davis, California, United States of America.**

3. Iniga S. George, Dana Pascovici and Paul. A. Haynes. 'Quantitative label-free shotgun proteomic analysis of Cabernet Sauvignon grape cells exposed to hot and cold temperature stresses' at the **62nd American Society for Mass Spectrometry (ASMS) 2014, Baltimore, Maryland, United States of America.**

4. Iniga S. George*, Robert D. Willows and Paul. A. Haynes. 'Quantitative label free shotgun proteomic analysis of a red grapevine variety exposed to heat and cold stresses' at the **International Plant Proteomics Organization (INPPO) 1st World Congress 2014, Hamburg, Germany.**

5. Iniga S. George*, Anne Y. Fennell and Paul. A. Haynes. 'Comparing in-gel and in-solution techniques for shotgun proteomic analysis in riverbank grapes, *Vitis riparia'* at the **2nd Proteomics and Beyond Symposium 2014, Macquarie University, Sydney, Australia.**

6. Iniga S. George*, Dana Pascovici, Mehdi Mirzaei and Paul. A. Haynes. 'Proteomic analysis of Cabernet Sauvignon exposed to thermal stresses reveal changes in phenylpropanoid metabolism' at the **20th Lorne Proteomics Symposium 2015, Australasian Proteomics Society, Lorne, Victoria, Australia.**

Awards

- 2014 Sydney Protein Group (SPG) Lorne Travel Scholarship (AUD 600)
- 2014 International Plant Proteomics Organization (INPPO) Travel Grant (EUR 750)
- 2014 Australian Grape and Wine Authority (AGWA) Australian Government Travel Award (AUD 2500)
- 2014 American Society of Mass Spectrometry (ASMS) Student Travel Stipend 2014 (USD 200)
- 2014 Post Graduate Research Fund (PGRF), Macquarie University (AUD 5000)
- 2013 Grape Wine and Research Development Corporation (GWRDC) Australian Government Travel Award 2013 (AUD 2100)
- 2013 Post Graduate Research Fund (PGRF), Macquarie University (AUD 1900)
- 2012 Runner-up Award in the Three Minute Thesis Competition, Faculty of Science, Macquarie University (AUD 150)
- 2011 International Macquarie University Research Excellence Scholarship (iMQRES) covering a total tuition fee of AUD 117, 980 (based on 2011 rate) and with a living stipend of AUD 25, 392 per annum (tax exempt)

Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
cv	Cultivar
d	Day
DTT	Dithiothreitol
eFASP	Enhanced Filter Aided Sample Preparation
ESI	Electrospray Ionisation
EST	Expressed Sequence Tag
FASP	Filter Aided Sample Preparation
FDR	False Discovery Rate
FW	Fresh Weight
Gn-HCl	Guanidine Hydrochloride
GO	Gene Ontology
GPF	Gas Phase Fractionation
GPM	Global Proteome Machine
HPLC	High Performance Liquid Chromatography
hr	Hour
Hsp	Heat shock protein
IEF-SDS-PAGE	Isoelectric Focusing SDS-PAGE
ICAT	Isotope Coded Affinity Tag
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation
LD	Long daylength
LTQ	Linear Trap Quadrupole

Lys-C	Endoproteinase Lys-C
MALDI	Matrix-Assisted Laser Desorption/Ionisation
m/z	Mass to charge ratio
min	Minutes
MS	Mass Spectrometry
NanoLC-MS/MS	Nanoflow liquid chromatography tandem mass spectrometry
NSAF	Normalised Spectral Abundance Factor
PAGE	Polyacrylamide Gel Electrophoresis
PloGO	Plotting Gene Ontology annotation
PRIDE	PRoteomics IDEntifications
PS-DVB	PolyStyrene-DiVinylBenzene
rpm	Revolutions per minute
SD	Short Daylength
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SILAC	Stable Isotope Labelling by Amino acids in Cell culture
TFE	Trifluroethanol
TMT	Tandem Mass Tags
WEGO	Web Gene Ontology Annotation Plot

CHAPTER 1

Chapter 1

1. Introduction

Each chapter of this thesis contains an introduction detailing the contents within the chapter. Here in chapter 1, I present a broader outlook on proteomic studies in plant responses to different environmental stresses, focusing particularly on grapevines.

1.1 'Change' is constant in this world

'Change' in terms of meteorological conditions is constant on our planet. The climate of our world has changed drastically over whatever timescale one wishes to consider. Variations in wind, temperature and rainfall, among various other factors, are all part of climate change, which includes both alterations in average conditions as well as more frequent occurrences of extreme events. Significant changes, seen as gradual shifts in climatic systems, occur over thousands of years. However, some unpredictable aberrant fluxes over short time spans also contribute to rapid climate change [1]. Temperature fluctuations are caused by natural impacts or anthropogenic factors. Volcanic eruptions or solar radiations are natural factors that are external to the climate regimes, and can affect the climate of the earth [2]. In addition to natural impacts, human beings have also influenced climate change substantially. Human activities have induced the release of greenhouse gases, such as carbon dioxide, a chief fossil fuel combustion product, into the environment. Anthropogenic degradation of the environment by increased amounts of greenhouses gases in the atmosphere has caused an overall warming effect in the last century [3]. Increasing levels of global warming can escalate the probability of severe, irreversible climatic impacts, the consequences of which can alter biological life systems on earth. Global climate change risks are currently high [4], and this represents a severe threat for species extinction and food security caused by extreme events like

glacier meltdowns, floods, storms or prolonged droughts. These events can lead to consequent social effects such as scarcity of food and increased agricultural commodity prices. In the recent past, negative impacts of climate change on crop yields are more prevalent than positive impacts [4]. There is a high probability of extreme weather events occurring more suddenly, vigorously and frequently in the future, and there is a need for biological ecosystems to first tolerate, and then adapt to this ever changing world.

1.2 The importance of plants

Climate change will have many impacts on different species and ecosystems. These include changes in ecosystem processes, species distributions and abundances, and interactions between species [5]. A major portion of the biological ecosystem on earth is made up of terrestrial vegetation. Plants are an essential source of food and metabolic energy for nearly all animals and are crucial for human existence. Plants are essential to ecosystem maintenance as they supply food, both directly and indirectly, and oxygen in the atmosphere as a byproduct of photosynthesis. Besides food and raw materials, plants also provide other products which support human wellbeing such as oil, medicines, fuel, wood, latex, vegetables dyes, resins, clothing, fibres and pigments. Coal and petroleum are fossil fuels of plant origin. Plant foliage has historically aided in the sustenance of all habitats and contributed tremendously to biodiversity on earth. Plants store carbon, and influence climate by eliminating most of the carbon dioxide produced from fossil fuel combustion out of the atmosphere. Global environmental changes emphasise the necessity for higher yielding and better adapting crop plants.

1.3 Abiotic environmental stresses in plants

Stress is any factor which is unfavourable to the growth and development of the organism under examination. Climate change can trigger environmental stresses such as extreme temperature, drought, salinity, flood, heavy rainfall or lack of rainfall, varied photoperiods and other unpredictable weather events. All these issues generally have a negative effect in the agricultural context. The most common abiotic stresses or environmental factors that affect plants globally are temperature, water deficit (drought), and salinity and acidity in soils [6]. Another major limiting factor for crop cultivation is flowering time, which is strongly regulated by daylength (photoperiod) and temperature [7]. Generally, abiotic stresses function as interacting factors and more than one abiotic stress occurs at one time. For example, high temperatures are frequently accompanied by lack of water and increased light radiation, which in turn can affect subsoil mineral toxicity, and this combined effect adversely affects plant growth and metabolism. Abiotic factors pose a major threat to plants crops, as they can severely decrease crop quality and yield. Many crops show low productivity due to temperature stress [8]. For example, rice, a major staple in the world food economy, produces low yields when exposed to heat stress, as rice spikelets are susceptible to damage caused by high temperatures at flowering [9].

Plants, being sessile organisms, are unable to avoid sudden environmental events such as exposure to extreme heat or cold. Thus, they evolve many molecular, biochemical and morphological mechanisms to combat and tolerate non-optimal conditions of growth. Common plant biochemical responses to heat, chilling, drought and salinity include changes in photosynthesis, growth, protein synthesis, hormone metabolism, signalling and cellular defences [10]. Drought induces various physiological and

biochemical plant responses, which include generation of deeper roots, repression of cell growth, conservative use of water, deregulation of photosynthesis, activation of respiration and stomatal closure [11, 12]. Temperature fluctuations can alter growth, sugar metabolism, photosynthesis, gene expression, nutrient uptake and signalling in plants [13-16]. Although the effects of abiotic stresses on plants are mainly negative in nature, certain molecular mechanisms which are involved in combating these stresses can create positive influences in plants, or serve as environmental cues in signalling biochemical processes such as defence mechanisms or dormancy induction. Even though the main plant physiological responses to abiotic stresses are known, their mechanisms remain poorly understood at the molecular level, with the exception of the well-studied ABA signalling pathway. Systems biology approaches have provided a more comprehensive understanding of complex plant responses to various abiotic stresses. Environmental stress responses have been studied using 'omic' technologies in many agriculturally important crops such as rice, wheat, maize and grape, amongst various others [17-22]. While it is beyond the scope of this thesis, it is worth noting that some economically important fungal infections in grapevine, such as *Botrytis cinerea* or *Plasmopara viticola,* are directly related to abiotic stress conditions because establishment and progression of the infection process is dependent on the environment. There are several proteomics analysis papers on these fungal species, notably dealing with their extracellular secretome, or their interactions with grapevines [23].

1.4 Grapevine - genetics, history and diversity

The genus *Vitis* (grapevine) is made up of around 60 to 70 species, in the plant family *Vitaceae. Vitis* is split into two subgenera: *Euvitis* or 'true grapes' and *Muscadinia* or 'Muscadine grapes' [24]. Grapevine has a diploid genome with a size of 475-500 Mb,

consisting of 19 chromosomes [25]. The genotypes of grape are heterozygous and most of the modern cultivated varieties (or cultivars) are self-fertile and hermaphroditic. The *Vitis* genus is of great agronomic value, and is widely distributed and cultivated across most continents, including Europe, North and South America, Australia, Asia and Africa. The cultivation of domesticated grapes began around 6000 to 8000 years ago and the history of winemaking dates back to nearly around the same time [25, 26]. Grapes are a commercially valuable crop globally; they are valued for their economic worth as wine and also because they are considered as a model non-climacteric crop. Grapes are also used for making jam, jelly, raisins, vinegar, and grape juice and are also consumed as fresh fruit. Some of the more notable species of *Vitis* are *Vitis vinifera* (wine grape or European grapevine), *Vitis riparia* (Riverbank grape), *Vitis labrusca* (Fox grapevine), *Vitis aestivalis* (Summer grape), *Vitis rotundifolia* (Muscadine), *Vitis rupestris* (Rock grapevine), *Vitis coignetiae* (Crimson glory vine), *Vitis amurensis* (Amur grape) and *Vitis vulpina* (Frost grape).

Vitis vinifera is the most important, widely grown, and well-studied species, and is used extensively in the wine industry. This species accounts for 90% of the world grape production. Although thousands of distinct *V. vinifera* cultivars exist [25], less than a hundred cultivars make up the vast majority of the worldwide wine market for wine production. Some of the most popular wine grape cultivars are Cabernet Sauvignon, Chardonnay, Pinot Noir, Sauvignon Blanc, Riesling, Merlot, Shiraz, Malbec, Moscato, and Semillon.

In 1890, phylloxera, a primitive aphid that feeds on grapevine, devastated many European vineyards composed of susceptible *Vitis vinifera* species [27]. Grape phylloxera spread to nearly every grape growing region in the world and caused widespread losses. Phylloxera resistant rootstocks were later produced by hybridisation

of many species including *V. labrusca, V. riparia* and some other species native to the host range of phylloxera. In addition to use as phylloxera resistant commercial rootstocks, these hybrids also needed to possess good wine quality attributes. Some of the hybrid and phylloxera resistant *Vitis* cultivars which are commercially valued for wine production include Seyval, Vidal Blanc and Chambourcin.

In Australia, phylloxera was restricted to the state of Victoria and to a few regions around Sydney [28]. Hence, there remain a high percentage of Australian vine plantings on own-roots. These old vines which predate the phylloxera outbreak in Australia, are particularly found in the major grape growing regions of South Australia, including the Barossa Valley and Maclaren Vale [28].

1.5 Wine regions and wine production

Travel, exploration and cultural fondness for wines has led to the wide transport of native grapes all around the world. Vineyards are located in over 90 countries worldwide. However, Italy, Spain and France dominate in vineyard areas, as one-third of all the vineyards worldwide are found in these three countries. Other significant grape producing countries include the United States of America, Australia, China, Turkey, Iran, Argentina, Chile and South Africa. In Australia, there are around 65 wine regions spread across approximately 400,000 acres of land and Australia ranks as the fifth largest exporter of wine by volume to the world.

Wine production is a large scale international industry with production ranging from 252 to 282 million hectolitres over the past decade, with an exceptional increase in 2004, where it edged to over 296 million hectolitres (Figure 1) [29]. The surface area dedicated to vineyards worldwide in 2012 was around 75,280 square kilometres [29, 30]. We examined statistical extracts from the Organisation Internationale de la Vigne

et du Vin (OIV) and observed a constant decline in wine regions and wine production worldwide over the past twelve years [29, 30]. Wine production shows a slow decrease over the past twelve years, while there is a steady increase in worldwide wine consumption (Figure 1). Thus, there exists a possibility of global wine shortages in future. For instance, statistical reports have recorded a world wine production in 2012 at 252 million hectolitres, showing a six percent reduction when compared to 2011, which was also the lowest amount produced in the last ten years. Moreover, OIV reports also state that the total acreage under vine is decreasing substantially to the extent that the combined wine production in Italy, Spain and France has dropped from 62% to 48%, and from 39.1% of total vineyard area to 35.8%, when compared between the years 2000 and 2012. All the data stated above were extracted from StatOIV extracts [29].

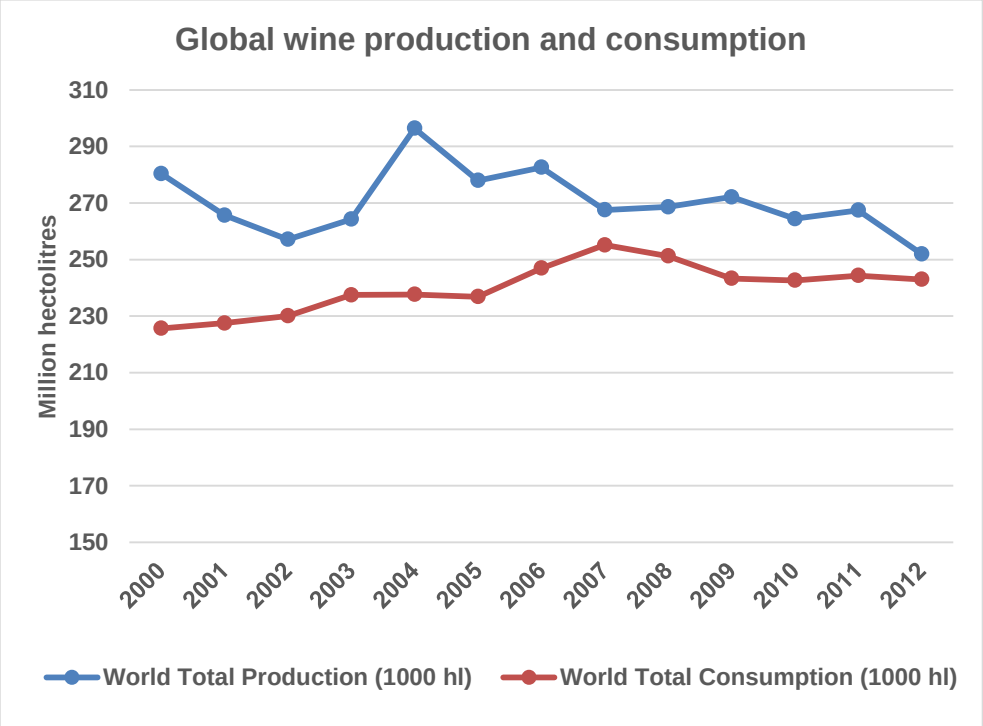

Figure 1. Global wine production and consumption from 2000 - 2012. Statistical data was taken from StatOIV extracts [29]. A gradual decrease is observed in global wine production over the

past decade, with an exception in 2004 where the yield was 296 million hectolitres. On the other hand, global wine consumption trends show an overall increase thus creating a demand for more wine in the future.

Wine-producing regions are distributed across different climatic zones around the world including Mediterranean, temperate, arid, semi-arid, tropical and sub-tropical regions. From 2000 onwards, a reduction in grape production and vineyard area under cultivation in these different climatic zones has been observed, and this is attributed mainly to climate change, temperature fluctuations and poor weather conditions [4, 29]. In the coming years, crops including grapevines will encounter more adverse environments, as there is a greater probability of more frequent and longer heat waves, drought conditions and erratic light exposures. Thus, the ability of food crops and grapevines to sense, withstand, and adapt to climate change is of utmost importance in order to maintain global food security, and to sustain the wine industry. Systems biology approaches have been used to address these issues as they are an excellent platform for characterising plant reactions at the molecular and cellular level. These approaches can also assist in targeted breeding of plants, which are better able to withstand such stressful conditions.

1.6 Proteomics and mass spectrometry

Analyses at the transcriptomic, proteomic and metabolomic levels have been widely used for better understanding of complex cellular networks, physiology and biochemistry related to environmental stresses. Mass spectrometry based proteomics is a well-established, powerful and indispensable tool for high throughput analysis, ranging from single protein identifications to complex multivariate quantitation of differences in protein abundances between cells or whole organisms. The proteome of

any organism is dynamic and easily altered by external environments when compared to its relatively static genome. Thus, quantitative and qualitative analyses of protein expression patterns have been widely employed for studying molecular mechanisms in whole organisms and tissues or cells, when exposed to defined external conditions [31, 32].

Over the past quarter of a century, proteomic technology has improved tremendously, and these developments continue to expand with improvements in sample preparation techniques, pre-fractionation methods, accuracy, resolution, sensitivity, and speed of instrumental analysis, along with concomitant improvements in bioinformatics and software. A fundamental technological breakthrough occurred in the late 1980s with the introduction of electrospray ionisation (ESI) [33] and matrix-assisted laser desorption/ionisation (MALDI) [34] techniques for peptide or protein ionisation methods prior to mass spectrometric analysis. Detailed information on mass spectrometry instrumentation, techniques, innovations and improvements are not elaborated here; the reader is referred to previously published reviews on mass spectrometry for more information [35-39]. Presently, numerous different mass spectrometry technologies and methods exist and these can be used individually or in combination, to achieve optimal results to answer the given scientific questions.

1.7 Proteomic technologies for studying abiotic stress in plants

Several proteomic techniques have been widely used for investigation of molecular level responses to abiotic stress in plants. Conventionally, the well-established two-dimensional gel electrophoresis (2-DE) techniques were commonly used for quantitative proteomic studies in plants. Application of 2-DE has been reported in different abiotic stress studies in diverse plant species ranging from cereal crops in the

grass family to woody trees; examples include cold and salinity stress responses in Arabidopsis, temperature and drought stress in rice, water-deficit in potato, salt stress in grapevine and drought in poplar [40-46]. However, 2-DE techniques have some inherent limitations, especially with regard to hydrophobic and high molecular weight proteins [47], therefore several alternative techniques have been devised to generate higher throughput and more informative data. Shotgun proteomic techniques, such as multidimensional protein identification technology (MudPit) [48], can identify larger numbers of proteins compared to gel based methods. Shotgun techniques also provide greatly improved quantitation, which occurs at the mass spectrometry level rather than by labour-intensive visual comparisons of gel spots. The multiplexed nature of these approaches has facilitated the design of experiments involving comparison of many variables, thus allowing more in-depth information to be produced on stress response pathways at the molecular level. Comparative quantitative proteomics is generally classified into two main approaches of labelled and label-free techniques. Commonly used labelling techniques include stable isotope labels with amino acids in cell culture (SILAC) [49], isotope-coded affinity tags (ICAT) [50], isobaric tags for relative and absolute quantitation (iTRAQ) [51], and tandem mass tags (TMT) [52]. Label-free techniques include spectral counting with normalised spectral abundance factors measurements [53] and signal intensity measurements (also known as area under the curve; AUC) [54-56]. Quantitative proteomic analysis using both labelled and label-free techniques have been employed for discovery of abiotic stress responses in plants. A few examples of such quantitative proteomic investigations include studies on salt stress in Arabidopsis seedlings using SILAC, drought stress in maize using 2-DE and iTRAQ, temperature stress in rice using iTRAQ and TMT, and water-deficit in rice, grapevine,

and peanut, using label-free shotgun techniques and spectral counting [14, 17, 18, 21, 22, 57-59].

1.8 Grapevine proteomics

Grapevine is gaining importance in the scientific community for its economic value and as a model plant. It is one of the oldest cultivated plants [60] and is adaptive to various cultivation environments, as evidenced by its widespread distribution. It is well-established that major biological transformations that affect plant and fruit growth are governed by changes in protein expression, including general metabolism, photosynthesis, cellular defence, production of secondary metabolites and signalling. Grapevine research using proteomics technology has gained enormous momentum in recent years, and it has been employed for understanding complex physiological and biochemical processes. Proteomic findings are improved by the availability of the complete genome sequence of the organism under study for protein identification, regardless of the techniques used. A breakthrough in grapevine research was accomplished with the release of the grape genome in 2007 [61, 62]. This milestone facilitated the initiation of numerous grape proteomic investigations in different avenues of biology. The genetic resources available for grapevine, and the different proteomic grapevine studies are discussed in detail in the following sections.

1.8.1 Grape genomic resources

Genomic resources available for grapevine have expanded greatly in recent years. The International Grape Genome Program (IGGP) was formed in 2001 to develop resources

for grape researchers, and to promote multinational coordination and collaboration (http://www.vitaceae.org/). This program urged the placement of all grape genomic data into major public databases such as NCBI and EMBL-EBI, to provide easy access to grape genomic resources, and also for researchers to download data or to add further value. Grapevine genomics researchers have compiled over 300,000 ESTs in databases from different grapevine varieties [25, 63]. In 2007, a *Vitis vinifera* variety, Pinor Noir PN40024, was fully sequenced by a French-Italian consortium and the grape genome sequence was released [61, 62]. The *V. vinifera* genome sequence was upgraded from the 8X coverage to the 12X coverage in 2010 [64, 65]. The available grape genomic information has led to a surge of high throughput proteomic analysis and a rapid increase in characterisation of targeted grape genes and proteins.

1.8.2 The era of 2D gels

Two dimensional electrophoresis (2-DE) has traditionally been the most extensively used approach for grapevine proteomics, with more than 50% of publications between 2004 and 2015 performed employing the 2-DE technique alone (Table 1 and 2). One of the earliest 2-DE studies was performed in 2004, to examine the physiology of berry ripening in six different cultivars of *V. vinifera* L. cv. Gamay [66]. This study revealed the active role of proteins involved in energy metabolism, defence, and stress response, and the accumulation of dehydrin, invertase, and pathogenesis-related (PR) proteins, such as chitinase and thaumatin-like proteins in ripe berries. Accumulation of PR proteins during berry ripening was confirmed in another 2-DE study in 2007 [67]. Other 2-DE investigations on berries have revealed the key role of UDP-glucose phosphorylase and invertase in sugar loading [66], and established the role of abscisic acid (ABA) [68] and gibberellin (GA) [69] in berry enlargement. Proteome analysis was

also used to uncover a decreased rate of glycolysis and oxidative stress, and increased cytoskeletal rearrangement, during fruit maturation [67].

Protein extraction from developed berries is a challenging task due to high levels of sugar concentration. Two different studies using Cabernet Sauvignon berries have reported the optimised protein solubilisation protocol for 2-DE as a phenol based extraction method, as the results of those studies indicated highest protein yields and greatest spot resolution when phenol extraction was used [70, 71]. Grape skins from red cultivars of Cabernet Sauvignon [72], Barbera [73] and Pinot Noir [74], extracted during different development stages ranging from veraison to full ripening, have also been used for characterisation of proteins involved in fruit ripening using 2-DE.

In addition to berry proteomics, 2-DE approaches were employed for numerous studies on grapes including examination of leaf proteome transformation for alcohol dehydrogenase activity, somatic embryogenesis, response to abiotic and biotic stresses, cultivar comparisons, photoperiod influence on growth cessation, solar irradiation effects on leaves, proteome profiling of leaf apoplastic fluid, herbicide treatment, necrosis, chitosan treatment and the effect of elicitors on grape cell suspensions [45, 75-96]. In addition, 2-DE approaches have also been used for studies in wine, including examination of protein profiling, haze formation and pH effects [97, 98].

Although 2-DE has been traditionally used, this technique has some characteristic limitations including poor representation of proteins, lack of solubility of hydrophobic proteins, poor detection of low abundance proteins, limited reproducibility, laborious experimentation and time consuming nature. Disadvantages of 2-DE, especially gel-to-gel variability, were overcome to some extent by top-down proteomic approaches based on differential in-gel electrophoresis (DIGE) [99]. This technique uses multiplexed fluorescent labelling to allow multiple samples to be loaded in the same gel, thus

minimising gel to gel variability. DIGE was used in studying post-harvest withering, sugar metabolism and organic acid metabolism in berries [100, 101]. DIGE was also used to study the production of trans-resveratrol, a phytoalexin, which a subject of considerable study, due to its reported health benefits when treated with different elicitors such as methyl-beta-cyclodextrin and methyl jasmonate [102].

Conventionally, 2-DE is widely used for grapevine research, but it is known that up to 80% of the proteins being analysed can be lost during 2-DE and only about 20-30% of proteins are detected, especially in plant cells which are rich in membranous structures [47, 103]. Moreover, gel-based techniques are more error prone when compared to MS-based methods [39]. This has led to the emergence of several alternate approaches for quantitative analysis, as exemplified in all the studies described in this thesis.

Table 1. Research findings on grape and wine proteomics from 2005 to 2010, extracted from a review published by Giribaldi et al. in 2010 [60]. Reference of the research publications below are cited in Table 2 and also at appropriate text sections.

Cultivar	Tissue	Technique	Year
Chardonnay	Shoots, roots and leaves	2D gels LC-MS/MS	2005
Chardonnay	Wine	2D gels	2006
Cabernet Sauvignon	Whole clusters	2D gels MALDI-TOF/TOF	2006
Cabernet Sauvignon	Skins	2D gels LC-MS/MS	2007
Nebbiolo	Berries	2D gels MALDI-TOF	2007
Cabernet Sauvignon	Seeds	2D gels LC-MS/MS	2007
Vitis species	Leaves	2D gels MALDI-TOF and LC-MS/MS	2007
Cabernet Sauvignon and Chardonnay	Shoots	2D gels MALDI-TOF/TOF	2007
Chardonnay	Wine	2D gels and immunoblots, LC-MS/MS	2008
Razegui	Stem, roots and leaves	2D gels	2008
Thompson seedless	Callus	2D gels LC-MS/MS	2008
Barbera	Skins	2D gels LC-MS/MS	2008
Raboso Piave, Prosecco and Malvasia Nera	Seeds	MALDI	2008
Cabernet Sauvignon	Grape berry plasma membrane	2D gels MALDI-TOF	2008
Vitis species	Sap	2D gels LC-MS/MS	2009
Arinto	Wine	2D gels	2009
Sauvignon blanc	Wine	SDS-PAGE, Native PAGE, IEF, MALDI-TOF/TOF	2009
Barbera	Cell suspensions	2D gels MALDI TOF and MALDI-TOF/TOF	2009
Cabernet Sauvignon	Skin, flesh and seeds	2D gels MALDI-TOF/TOF	2009
Gamay	Cell suspensions	2D gels MALDI-TOF and LC-MS/MS	2009
Semillon and Sauvignon blanc	Juice	SDS-PAGE LC-MS/MS	2009
Sangiovese and Trebbiano	Skin and flesh	2D immunoblots MALDI-TOF	2009
Pinot Noir, Riesling, Cabernet Sauvignon, Shiraz	Wine	SDS-PAGE LC-MS/MS	2009
Cabernet Sauvignon	Callus	2D gels MALDI-TOF	2009
Semillon and Sauvignon blanc	Juice	LC-MS/MS	2010
Semillon	Juice and wine	SDS-PAGE LC-MS/MS	2010
Chardonnay	Wine	2D gels and SDS-PAGE, MALDI-TOF, LC-MS/MS	2010

Table 2. List of proteomic research articles published from 2007, after the release of the grape genome sequence, till the time of writing of this thesis. Grapevine tissue or product, variety, proteomic technique and search database used in each study are listed.

Grape tissue or product	Variety	Proteomic approach	Year	Databases used for searching	Reference
Berry seeds and skins	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels LC-ESI-MS/MS	2007	*Vitis* protein and EST sequences	Negri et al. [71]
Deseeded berries	*V. vinifera* cv. Nebbiolo Lampia	2D gels MALDI-TOF	2007	Swiss-Prot-TrEMBL	Giribaldi et al. [67]
Berry skins	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels NanoLC-MS/MS	2007	NCBI	Deytieux et al. [72]
Leaves	*V. vinifera* L.	2D gels MALDI-TOF and LC-MS/MS	2007	*Vitis vinifera* entries of Swiss-Prot or TrEMBL or ESTs in GenBank	Sauvage et al. [75]
New shoots with young leaves	*V. vinifera* L. cv. Cabernet Sauvignon and Chardonnay	2D gels MALDI-TOF/TOF	2007	NCBInr and TIGR *Vitis* EST	Vincent et al. [77]
Champagne wine	Chardonnay	2D gels	2007	-	Cilindre et al. [104]
Sparkling wines	Chardonnay, Pinot Noir, Parellada, Macabeu, Xarel.lo	Fast protein liquid chromatography	2007	-	Vanrell et al. [105]
Grape calli	*V. vinifera* cv. Thompson Seedless	2D gels LC-ESI-MS/MS	2008	NCBInr and EST	Marsoni et al. [76]
Berry skins	*V. vinifera* L. cv. Barbera	2D gels LC-ESI-MS/MS	2008	NCBInr	Negri et al. [73]
Young plantlets	*V. vinifera* Razegui	2D gels	2008	NCBI	Jellouli et al. [45]
Seeds	*V. vinifera* cultivars	MALDI/MS	2008	-	Pesavento et al. [106]
Champagne wine	Chardonnay	2D gels NanoLC-MS/MS	2008	NCBInr	Cilindre et al. [82]
Berry plasma membrane	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels MALDI-TOF	2008	NCBInr *Viridiplantae* and Swiss-Prot	Zhang et al. [107]
Calli	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels MALDI-TOF	2009	NCBInr *Viridiplantae* and Swiss-Prot	Zhang et al. [94]
Calli	*V. vinifera* L. cv. Gamay	2D gels MALDI-TOF	2009	NCBInr	Martinez-Esteso et al. [96]
Cell suspensions	*V. vinifera* L. cv. Barbera	2D gels MALDI-TOF, MALDI-TOF-TOF	2009	NCBInr *Viridiplantae*	Ferri et al. [95]
Berry pulp, skin and seed	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels MALDI-TOF/TOF	2009	NCBInr or contigs from *Vitis* gene index version 5.0	Grimplet et al. [108]
Berries	*V. vinifera* cv. Sangiovese and Trebbiano	2D gels MALDI-TOF	2009	NCBInr	Wang et al. [109]

Grape tissue or product	Variety	Proteomic approach	Year	Databases used for searching	Reference
Juice	Semillon and Sauvignon Blanc	SDS-PAGE, NanoLC-MS/MS	2009	NCBI *V. vinifera* protein entries	Van Sluyter et al. [110]
Wine	Portugieser, Riesling, Portugieser Weissherbst	SDS-PAGE, ESI-Q-TOF-MS	2009	Hybrid database with *V. vinifera* sequences from TREMBL, GenBank and RefSeq	Wigand et al. [111]
Wine	Sauvignon	SDS-PAGE, MALDI-TOF/TOF	2009	NCBInr	Esteruelas et al. [112]
Wine	Arinto	2D gels	2009	-	Batista et al. [98]
Juice and wine	Semillon	SDS-PAGE, NanoLC-MS/MS	2009	NCBInr *Viridiplantae*	Marangon et al. [113]
Seeds	Raboso Piave, Raboso Veronese, Marzemina Bianca, *V. vinifera* cv	MALDI-TOF/MS	2010	-	Bertazzo et al. [114]
Xylem sap	Florida Hybrids, *V. vinifera* and *V. rotundifolia* cultivars.	2D gels LC-MS/MS	2010	-	Basha et al. [83]
Wine	Chardonnay	MALDI-TOF, LC-MS/MS	2010	NCBInr	Palmisano et al. [115]
Wine	Valpolicella	SDS-PAGE, MALDI-TOF, nLC-MS/MS	2010	UniprotKB without selecting taxonomy	D'Amato et al. [116]
Shoot tissue	*V. riparia* Michx.	2D gels MALDI-TOF/TOF	2010	Grapevine PN40024, tentative contigs from DFCI Gene Index version 5.0	Victor et al. [89]
Berries	*V. vinifera* cv. Cabernet Sauvignon	2D gels LC-MS/MS	2010	NCBI *V. vinifera* and Uniprot/SwissProt plants	Giribaldi et al. [68]
Leaf tissues	*V. vinifera* cv. Cabernet Sauvignon	iTRAQ	2010	VvGI EST (DFCI Gene Index version 6.0)	Marsh et al. [117]
Buds, leaves, flowers and tendrils	*V. vinifera* L. cv. Sangiovese	SDS-PAGE	2010	Grapevine 8X genome	Parrotta et al. [118]
Young plantlets	*V. vinifera* Razegui	2D gels	2010	-	Jellouli et al. [79]
Juice	Sauvignon Blanc and Semillon	LC-MS/MS	2010	-	Falconer et al. [119]
Wine	Chardonnay	2D gels MALDI-TOF	2010	*Viridiplantae* entries of Swiss-Prot or TrEMBL or ESTs in GenBank	Sauvage et al. [120]

Grape tissue or product	Variety	Proteomic approach	Year	Databases used for searching	Reference
Leaf midribs	*V. vinifera* cv. Nebbiolo	2D gels MALDI-TOF/TOF	2011	Unrestricted NCBI	Margaria et al. [84]
Grape berry exocarp	*V. vinifera* L. cv. Muscat Hamburg	iTRAQ	2011	NCBInr containing all *Vitis*	Martinez-Esteso et al. [121]
Berries	*V. vinifera* cv. Corvina	2D-DIGE	2011	NCBI *Viridiplantae* (Green plants)	Di Carli et al. [100]
Calli	*V. vinifera* L. cv. Prime Seedless	2D gels MALDI-TOF-MS	2011	NCBInr *Viridiplantae* and Swiss-Prot	Zhao et al. [86]
White wine vinegar	Ponti region	SDS-PAGE LC-MS/MS	2011	Uniprot *Viridiplantae* or Swiss-Prot	Di Girolamo et al. [122]
Berries	*V. vinifera* cv. Nebbiolo	2D gels MALDI-TOF/TOF	2011	NCBInr including *V. vinifera*	Giribaldi et al. [123]
Wine	Recioto	SDS-PAGE LC-MS/MS	2011	ESTs *V. vinifera* and Uniprot *Viridiplantae*	D'Amato et al. [124]
Deseeded berries	*V. vinifera* L. cv. Muscat Hamburg	2D-DIGE	2011	NCBInr restricted to *Viridiplantae*	Martinez-Esteso et al. [101]
Cell suspensions	*V. vinifera* L. cv. Gamay	2D-DIGE	2011	NCBInr	Martinez-Esteso et al. [102]
Cell suspensions	*V. vinifera* L. cv. Dauphine	SDS-PAGE MALDI-TOF/MS	2011	NCBI and MSDB plant species	Sharathchandra et al. [125]
Skins	*V. vinifera* L. cv. Pinot Noir	2D gels LC-MS/MS	2011	NCBInr and characterisation using *V. vinifera* genome	Negri et al. [74]
Berry skins	23 varieties of *V. vinifera* and Hybrid Red	MALDI-TOF MS	2012	-	Picariello et al. [126]
Leaves	*V. vinifera* L. cv. Pinot Noir	2D gels LC-MS/MS	2012	NCBInr	Milli et al. [80]
Berries	*V. vinifera* L. Centennial Seedless	2D gels MALDI-TOF, MALDI-TOF/TOF	2012	*Viridiplantae* (Green plants) NCBI	Wang et al. [69]
Green stems	*V. vinifera* L. cv. Chardonnay	2D gels NanoLC-MS.MS, Q-TOF	2012	NCBInr *Viridiplantae*	Spagnolo et al. [127]
Leaves	*V. vinifera* L. cv. Pinot Noir	iTRAQ	2012	Pinot Noir genome, non-redundant Uniprot *Viridiplantae*	Palmieri et al. [128]
Berry mesocarp and exocarp	*V. vinifera* cv. Italia	iTRAQ	2012	*Vitis* species database from NCBI	Melo-Braga et al. [129]
Mature leaves	Florida Hybrids, *V. vinifera* and *V. rotundifolia* cultivars.	2D gels MALDI-TOF	2012	NCBI *Viridiplantae*	Katam et al. [88]

Grape tissue or product	Variety	Proteomic approach	Year	Databases used for searching	Reference
Leaves	*V. vinifera*. L. cv. Sultana	2D gels MALDI-TOF/TOF	2013	NCBInr or Swiss-Prot	Nilo-Poyanco et al. [91]
Cell suspensions	*V. rotundifolia* cv. Monastrell	SDS-PAGE, LC-MS/MS	2013	NCBInr	Belchi-Navarro et al. [130]
Shoot tips	*V. vinifera* cv. Cabernet Sauvignon	Label-free shotgun GPF NanoLC-MS/MS	2013	*V. vinifera* Uniprot, *V. vinifera* IGGP	Cramer et al. [18]
Leaves	*V. vinifera* L. cv. Chardonnay	2D gels MALDI-TOF/TOF	2013	NCBInr	Delaunois et al. [92]
Berry skins	*V. vinifera* 'Jingxiu'	2D gels MALDI-TOF-MS	2013	NCBI *Viridiplantae* (Green plants)	Niu et al. [78]
Leaf midribs	*V. vinifera* L. cv. Barbera	2D gels MALDI-TOF/TOF	2013	NCBI *V. vinifera* and unrestricted NCBI	Margaria et al. [85]
Berry skins	*V. vinifera* L. cv. Cabernet Sauvignon	2D gels LC-MS/MS	2013	*V. vinifera* Uniprot	Pasquier et al. [81]
Berry skins	*V. vinifera* Jingxiu and Muscat of Alexandria progeny	2D gels MALDI-TOF	2013	NCBI *Viridiplantae* (Green plants)	Niu et al. [87]
Berries	*V. labruscana* cv. Kyoho	2D gels MALDI-TOF	2013	NCBInr	Yuan et al. [131]
Deseeded berries	*V. vinifera* L. cv. Muscat Hamburg	iTRAQ	2013	NCBInr	Martinez-Esteso et al. [132]
Leaves	*V. vinifera* cv. Cabernet Sauvignon	iTRAQ	2014	Uniprot Grape	Liu et al. [133]
Berries	*V. rotundifolia* cv. Noble	iTRAQ	2014	Uniprot Grape Data set	Kambiranda et al. [134]
Wine	Chardonnay Champagne	SDS-PAGE, NanoLC-MS/MS	2014	Uniprot *Viridiplantae*	Cilindre et al. [135]
Seeds	White and red *V. vinifera* varieties	SDS-PAGE, LC-MS/MS	2014	UniRef100 database	Gazzola et al. [136]
Berries	*V. labruscana* cv. Kyoho	2D gels MALDI-TOF	2014	NCBInr *Viridiplantae*	Cai et al. [137]
Berries	*V. vinifera* L. cv. Cabernet Sauvignon and Syrah	2D gels MALDI-TOF/TOF	2015	NCBI, nrMSDB restricted to *Viridiplantae* and confirmed using *V. vinifera* Uniprot	Fraige et al. [138]

1.8.3 The renaissance in grapevine proteomics: Emergence of various qualitative and quantitative approaches

Subsequent to the release of the grape genome sequence, several labelled and label-free approaches have been developed and used for more sensitive analysis of the grape proteome. Many studies have utilised iTRAQ to understand a wide array of biological processes in grapevine. These include studies of physiological responses to conditions such as fungal infections with downy and powdery mildew [117, 128], heat stress [133] and ripening events [121, 132, 134, 139].

Along with developments in labelled approaches, improvements in instrumentation have also been implemented, and recent studies include the use of, for example, high resolution MALDI-MS for examining parental relationships and varietal differentiation [106, 114, 126]. Data acquisition using Orbitrap mass spectrometry, including collision-induced dissociation (CID) and higher energy collision-induced dissociation (HCD) peptide ion fragmentation, in conjunction with iTRAQ, was used for profiling berry exocarp proteins. This study is an excellent example of the combined use of advanced instrument methods and isotopic chemical labelling techniques [121]. Two different proteomic studies on cell suspension cultures have established the utility of different shotgun proteomics techniques based on tandem MS (nanoLC-ESI-MS/MS and SDS-PAGE MALDI-TOF/MS), and shown them to be superior to 2-DE gel-based approaches [125, 130].

Label-free techniques are widely used because of their flexibility, adaptability and cost-effectiveness compared to labelled methods. Shotgun proteomics generally generates huge volumes of data and many sophisticated software applications are currently available for such large scale data quantitation [39]. As stated earlier in section 1.7, quantitation based on spectral counting coupled with calculation of normalised spectral

abundance factors (NSAF) [53] have been used in several quantitative studies in plants [21, 22, 58, 140-142]. Spectral counting is one approach to label-free quantitation, but there are a number of other approaches also widely used, as reviewed in Neilson et al. [39]. In peptide spectral counting, relative protein abundances are calculated based on the number of MS/MS spectra which are matched to the analysed proteins. Relative abundances of identified, up-regulated and down-regulated proteins present in different samples, or at different conditions, can be quantitated with ease using NSAF values. The number of peptides identified are normalised based on total spectral counts for the identified proteins in the sample, and the length of the specific identified protein. Shotgun proteomics combined with spectral counting allows investigation of protein expression in various cell types without the limitations of the labelled approaches. Excluding the research work in this thesis, quantitative label-free shotgun proteomic analysis has been used for one other study in grapevine till date [18]. In that study, a label-free shotgun approach based on gas phase fractionation (GPF) and spectral counting was used. In GPF, a sample is repeatedly analysed over multiple smaller mass-to-charge (m/z) ranges. This enables selected ions for collision-induced dissociation to come from a greater number of unique peptides, compared to the ions selected from the wide mass range scan in typical tandem mass spectrometry analysis. Thus, GPF helps to maximise proteome coverage, and can be further optimised by selection of m/z ranges based on theoretical peptides predicted from the grape genome sequence [143].

In addition to technical advancements, 'omic' data integration into functional networks for grapevine has enabled improvements in understanding dynamic processes, analysis of large datasets, and streamlining functional processing, thus leading to significant advances in our understanding of grape biology [85, 144, 145].

Although many grape proteomic studies using various technical approaches have been completed, very few studies have searched the acquired mass spectrometry data against the available grape genome sequence (Table 2). Several studies have used the NCBI non-redundant database (NCBInr) and/or the entire green plants species database for protein identification. These investigations may well be looking at a less than complete picture, which would hamper their understanding of the actual grape proteome. This shortcoming was overcome in this thesis by the use of the Uniprot grape genome sequence for database searching and protein identification for all the studies.

1.8.4 Abiotic and biotic stress response studies in grapevine

Different abiotic and biotic stresses significantly limit the production of grapes across the world. These stresses are generally known to reduce the quality and quantity of both, berries and wine produced.

High-throughput proteomics analyses have been performed in many grapevine varieties challenged with different pathogens, as proteomics permits the characterisation of large numbers of proteins in a given cell to provide an integrated snapshot of molecular stress and adaptation mechanisms. Pathogenesis-related (PR) proteins including thaumatin I, thaumatin II and osmotin-like proteins, were found to be more abundant when grapes were exposed to esca-proper [81, 127], downy mildew [80] and Flavescence dorée phytoplasma infections [84]. Proteomic techniques have been used to understand intricate mechanisms such as plant-mediated resistance in mildew susceptible Pinot Noir, and post-translational modifications including phosphorylation, glycosylation and acetylation in *V. vinifera* cv. Italia exposed to insect attack [128, 129].

One of the earlier studies on abiotic stress response in grape was published in 2005, and included the observation of impaired photosynthesis due to Rubisco fragmentation,

along with stimulation of PR-10 proteins, for cellular defence against herbicide (flumioxazin) treatment [93]. Salinity, water-deficit, temperature and irradiance fluctuations are some of the common abiotic threats to viticulture. Proteomic and transcriptomic analyses have demonstrated differential expression of PR-10 proteins in grapevine cultivar Razegui when exposed to increased salinity [45, 79]. Drought and salinity stresses were investigated in Chardonnay and Cabernet Sauvignon by 2-DE, with proteome expression comparison between the cultivars [77]. The main changes included reduction in photosynthesis, protein synthesis, and shoot elongation. This study was limited by the fact that these responses could not be characterised in detail, as several spots subjected to mass spectrometry were identified either as hypothetical or unknown proteins. In another study, grape berry clusters exposed to well-watered and water-deficit conditions were analysed by 2-DE protein mapping. In that study, major differences were observed in expression profiles of PR proteins, chaperones, and proteins involved in amino acid and phenylpropanoid biosynthesis [108]. More recently, large scale high-throughput analysis generated through shotgun label-free quantitative analysis of Cabernet Sauvignon shoot-tips has indicated that changes in protein metabolism appear to aid in stress acclimation and initiation of antioxidant defences during very early stages of drought [18]. Among abiotic stresses, only water-deficit is known to be beneficial in certain circumstances; for example, carefully monitored water-deficit conditions have been used positively for quality and flavour enhancement in berries [146, 147].

Along with water-deficit, temperature variations accompanied by heat and light inconsistencies are pivotal factors effecting grapevine growth and yield. Although many physiological and anatomical changes have been previously described in grapevine exposed to various biotic and abiotic stresses, molecular data related to protein

expression changes triggered by temperature and light fluctuations, in particular, are still very scarce. Among these, are two publications on Cabernet Sauvignon tissues exposed to heat and light stresses [78, 133]. Niu et al. designed a study using 2-DE protein profiling to understand anthocyanin accumulation in response to sunlight exclusion in berry skins harvested from fruit-set until maturity [78]. In that study, mass spectrometry results were searched against the *Viridiplantae* (Green plants) database, but many identified proteins were either unclassified or of unknown function, which significantly hampered further characterisation. Liu et al. investigated thermotolerance in Cabernet Sauvignon leaves using iTRAQ labelling, and searched mass spectral data against the Uniprot grape database (http://www.uniprot.org/) [133]. Between these two studies, the latter was more effective in interpreting molecular systems associated with heat stress. We investigated molecular responses in grapevine exposed to different temperature stresses of heat and cold using the shotgun label-free proteomic approach in chapter 4 of this thesis. Our study was designed to generate data to enhance our understanding of molecular responses triggered by temperature changes, and to address the limited number of publications of such stress responses in grape.

1.8.5 Photoperiod associated molecular investigations in grapevine

Reproductive development is a crucial and sensitive process in flowering plants. Photoperiod, or daylength, is the duration of light exposure plants receive, and it varies according to time of the year, latitude, seasons and climatic conditions in a particular region. Photoperiod serves as an important environmental signal that programs specific plant development events to match particular environmental conditions [148]. Dormancy induction, bud set and flowering are some of the main biological processes which are controlled by photoperiod in grapevine [149].

Analysis of tubulin isoforms accumulation and changes during grape bud development was one such flowering related proteomic study achieved by 2D immunoblotting [118]. In addition, 2-DE gels have been used to examine complex signals involved in growth cessation and dormancy induction in grapes, when exposed to different photoperiods [89]. In that study, proteins which function in carbohydrate metabolism, peroxidase activity, ascorbate biosynthesis, flavonoid biosynthesis and reductive pentose phosphate cycle were differentially expressed between long and short daylengths. In another 2-DE study, impact of short-term high light intensities was examined in grapevine leaves to understand molecular changes occurring during transition to autotrophy, a process that occurs in springtime when buds burst and young leaves are suddenly exposed to extensive sunlight [91].

Although there are a range of sophisticated techniques, genetic information and relevant software available, significant proteomic publications that interpret molecular mechanisms involved in photoperiod response in *Vitis* species are very few. A more detailed introduction to this topic, and proteomic analysis comparing two genotypes of *Vitis,* exposed to two different photoperiods and harvested at two timepoints, is presented in Chapter 5 of this thesis.

1.8.6 Berry development and fruit proteomics

Research on grape berry development has attracted the interest of many biologists, as viticulturists all over the world are keen on improving cultivation practices to enhance the worth of the final product. Moreover, the key compounds that influence wine quality are produced during berry ripening. One of the initial proteomic studies in this area was designed to analyse the berry mesocarp by 2-DE to survey its biochemical content [66]. Other preliminary proteomic studies centred on optimising extraction protocols, as

grape berries are recalcitrant to protein extraction due to high concentrations of sugars and secondary metabolites, particularly phenolic compounds [70, 71, 109]. As discussed earlier in section 1.8.2, gel-based methods were principally used for berry proteome investigations to study physiological processes, including colouration [87], ripening [67, 72-74, 101] and withering [100].

Changes in cellular defence and general metabolism during ripening were observed in a study of cellular protein expression in berries treated with abscisic acid and gibberellin [68, 69]. Another study reported that lack of sunlight caused inhibition of anthocyanin biosynthesis, which in turn resulted in absence of colour development in berry clusters [78]. Anthocyanin accumulation in berry skins were also profiled to establish varietal differentiation between *V. vinifera,* American hybrid cultivars and Casavecchia cultivars [126]. Berry mesocarp and exocarp have been examined to uncover the role of post-translational modifications in plant responses to infestation by *Lobesia botrana*, the European grapevine moth [129]. In another similar study on pathogen attack, noticeable protein changes were observed in cell structure metabolism of berry pulp, and oxidative stress response in berry skin, when plants were subjected to viral infections [123]. In a recent study, proteomic, transcriptomic and metabolomic data have been integrated for a comprehensive summary of cellular processes underlying ripening and post-harvest drying [145].

Cellular events that occur during storage of harvested berries have also been investigated, with the aim of increasing shelf-life and minimising post-harvest losses. Carbohydrate metabolism was observed to be down-regulated and environmental stress responses were up-regulated as a result of cold-acclimation during cold storage [131]. Additionally, salicylic acid treatment on harvested berries during cold storage showed reduced membrane damage and fruit softening, thus improving shelf-life [137].

Suspension cell cultures have also been used to study specific berry development stages. Common trends in terms of relative protein abundances were recorded between berries and suspension cell cultures [125], establishing that cell suspensions mimic specific berry ripening stages. Proteomic analysis has been used to identify grape seed storage proteins, to provide insights into their polypeptide compositions, as seeds are gaining importance for their nutritional value and functional properties as emulsifying agents [136].

A very recent published study, at the time of writing, described metabolite and proteome changes characterising variety, provenance and ripening process in Syrah and Cabernet Sauvignon [138]. This multivariate approach demonstrated an increase in sugar and anthocyanin content, with a concurrent decrease in organic acid concentration, during ripening.

Among the various grape tissues studied, proteome research on the grape berry has been the most prevalent, with publications covering optimised protein extraction protocols, examination of stress responses, berry maturation and withering processes, storage optimisation, post-translational modifications, data integration into functional networks, as well as dedicated sections on fruit development in two comprehensive reviews by Giribaldi et al. [60] and Palma et al. [63].

1.8.7 Wine proteomics

Proteomic technologies have been utilised in developing suitable biomarkers for wine to address issues related to quality, authenticity and safety. The common objective of most studies which characterise the whole proteome of wine is to attain a unique proteomic signature for the given wine. This enables identification against counterfeit wine, which is a hazard to both, producers and consumers. Righetti and co-workers have

characterised the global proteome of various wine types including red wine, white wine, white-wine vinegar and Champagne [116, 122, 124, 135]. Champagne wine proteins were also studied using 2-DE coupled with tandem mass spectrometry, to differentiate between wines produced from healthy and fungal infected Chardonnay grapes [82].

Haze formation is another problem faced by wine producers. Haze causes turbidity in wine and occurs due to accumulation of proteins such as chitinases and thaumatin-like proteins. The complexity of haze formation has been addressed by numerous proteomic studies, both in white wines such as Semillon, Arinto, and Sauvignon Blanc [98, 110, 112, 113, 119] and red wines such as Portugieser [111]. The influence of fining and riddling agents on final wine quality have also been studied using proteomics [105, 120, 150]. An extensive study on protein glycosylation inspected protein stabilisation and potential allergenic cross-reactivity in Chardonnay white wine by an advanced multiplexed proteomic approach [115]. Yeast domestication in relation to winemaking was also studied by observing the genetic variability of proteomes between two strains of winemaking yeast [151].

This brief section on wine proteomics is included as part of the introduction although it is not directly relevant to the context of this thesis. It cannot really be avoided, because a majority of grapes are grown for the purpose of producing wine. Hence, only a brief discussion on wine proteomics is pointed out here and it is not elaborated upon in depth. Published reviews on different mass spectrometric techniques that have been applied to study wine proteins are available for further reading [152, 153].

1.9 Concluding remarks and research objectives

Proteomic technologies have been widely used in studying protein expression signatures in various plant species including grapevine. Dynamic changes in protein profiles

influenced by the impact of environmental stresses can be identified and quantified by using sophisticated mass spectrometric instrumentation and multiplexed analyses. Technical advancements and availability of genome sequence data continue to expand, enabling greater understanding and more available insights into specific proteins and molecular mechanisms involved in stress response in plants. Many proteomic studies executed prior to, and even some after, the release of the grape genome sequence, are restricted by the drawback of using the NCBI non-redundant, Green plants, or grape EST databases for analyses. This limitation was avoided in all grapevine studies in this thesis, as the available complete grape genome sequence was used for database searching and protein identification. Moreover, two different abiotic stresses of temperature and photoperiod changes, which are not well studied in the *Vitis* species, were characterised in this thesis. Shotgun label-free techniques were implemented for all grapevine proteomic analysis, as this approach is proven to be reliable, reproducible, efficient and cost-effective. Spectral counting using normalised spectral abundance factors was preferred for quantitation as it is simple, fast and highly reproducible.

The objectives of this research were to optimise and implement a platform for proteomic analysis of grapevine employing the quantitative label-free approach based on spectral counting. This optimised method was subsequently used to characterise proteins and pathways altered by environmental stresses of different temperature and light exposure treatments in grapevine. A brief summation of the contents of each chapter in the thesis is as follows:

Chapter 1 outlines the background information, history of proteomic studies in grapevine, and justification for conducting this research.

Chapter 2 comprises the materials and methods used in this thesis. This chapter contains the methods used for the proteomic analysis of grapevine, as well as one publication (publication I) detailing the method used for proteomic analysis of rice.

In chapter 3, the label-free shotgun proteomic approach for grapevine was tested and optimised through a comparative study using two different separation techniques based on in-gel and in-solution digestion, with concurrent quantitation. We aimed to find the method which would identify the maximum number of proteins, and generate the most biologically relevant and useful information. This chapter is presented as a research article (publication II).

In chapter 4, we report on using the quantitative label-free shotgun approach developed in chapter 3, for the proteomic analysis of grape cells exposed to the environmental stress of differential temperature treatments. This was a multi-variant experimental setup involving analysis of biological triplicates of cells maintained at control temperatures, and then subjected to treatment with moderate and extreme cold stress, and moderate and extreme heat stress. The aims were to investigate and identify protein changes in Cabernet Sauvignon suspension cell cultures exposed to sudden thermal stresses, and to determine which metabolic pathways were specifically altered by changes in temperature. This chapter is presented as a research article (publication III).

In chapter 5, the influence of another environmental stress of daylength variation was studied. Here, we investigated photoperiod regulated dormancy induction in grapevines exposed to different photoperiods. The objectives were to identify and quantify proteomic changes in a complex study consisting of two different genotypes of *Vitis*, exposed to two different photoperiods of long daylength and short daylength, and harvested at two different timepoints of 28 days and 42 days after budding. The primary aim of protein identification and quantification was to enhance our understanding of

signalling networks associated with dormancy induction and growth cessation. This chapter is written in a format of a manuscript prepared for publication (publication VII). Our collaborators on this project have analysed the same samples using a transcriptomics approach, and their data will be incorporated into a subsequent manuscript for publication, but is not included in this thesis.

Chapter 6 was designed as a validation experiment for quantitation based on spectral counting, and underpins the work described in the other chapters of this thesis. Protein expression changes in a same - same control experiment were measured by spectral counting to determine protein quantitation false discovery rates (FDRs). Biological triplicates of Cabernet Sauvignon cell cultures grown at 26°C were compared against another set of biological triplicates of Cabernet Sauvignon cell cultures grown at the same growth conditions, to observe the number of differentially expressed proteins. There has been plenty of literature published regarding false discovery in protein identification, but almost none regarding false discovery in protein quantitation. This chapter is written in the format of a thesis chapter. The results of this chapter will be used, along with additional data on other biological materials, for publication in future.

Chapter 7 concludes the findings of this thesis and also includes publication IV, which is a short perspective article that discusses the current developments in grapevine proteomics and its role in addressing abiotic stress. It also highlights some existing limitations of grape proteomic studies and identifies areas with promising scope for potential further research.

Along with studies on grape, two quantitative proteomic studies of environmental stresses of drought and temperature in rice were performed during the course of my studies. In these two rice studies, a label-free shotgun proteomic approach was followed in publication V, while a novel instrumental approach using triple stage mass

spectrometry with labelled tandem mass tags (TMT) was used in publication VI for protein quantitation in response to chilling effects. Publications V and VI are attached as appendices 1 and 2.

Supporting information files for all the publications and thesis chapters are available on the DVD attached to this thesis.

CHAPTER 2

Chapter 2

2. Materials and Methods

This chapter is divided into two sections. The first section describes the general materials and methods used for all the proteomic studies in this thesis. In this section, I have elaborated on the workflow followed for extraction of proteins from grapevine, in-solution digestion, label-free shotgun proteomic analysis, database searching with information on genome sequence used and quantitation. This general methodology was employed in chapters 3, 4, 5 and 6 in this thesis. Specific protocols describing the growth of samples and protein extractions are stated in depth in the respective publications or thesis chapters.

The second section of this chapter comprises of one publication (publication I). This published article is the methodology which reports the common workflow for in-gel digestion and label-free shotgun proteomics analysis for plant tissues, with particular reference to rice. We have described steps involved in protein extraction from plant tissue, SDS-PAGE, in-gel trypsin digestion, mass spectrometry analysis and database searching. This publication is included in this thesis because the proteomic pipeline described here was employed in chapter 3 for in-gel digestion of grape leaves and in publication V (appendix 1) for in-gel digestion of rice shoots, in preparation for shotgun proteomic analysis. I estimate my contribution to this publication as approximately 35% of the writing and preparation.

2.1 Methodology for in-solution digestion and shotgun proteomic analysis of grapevine samples

2.1.1 Protein extraction

Approximately 1g (fresh weight) of plant tissue (cells/leaf/shoot) was used for protein extractions. For each condition under examination, three biological replicates were used for proteomic analysis in all studies in this thesis. Proteins were extracted using Gn-HCl extraction buffer (6M Gn-HCl, 1% N-lauroylsarcosine, 10mM EDTA, 0.1M tricine, 5% β-mercaptoethanol) for all studies in this thesis, with the exception of chapter 5 where the phenol extraction protocol as described by Vincent et al. was used [70]. Protein were precipitated using chloroform-methanol [154] and concentrations were measured by the Pierce BCA protein assay (Thermo, San Jose, CA).

2.1.2 Filter aided sample preparation

For gel-free shotgun proteomics, protein pellets were digested in-solution by a Filter Aided Sample Preparation (FASP) method using Lys-C and trypsin [155]. Protein extracts were dissolved in 200µL 50% TFE, 0.1M NH_4HCO_3, 50mM DTT, heated (50°C, 20min) and concentrated to 20µL in Amicon Ultra 0.5mL 30K ultrafiltration devices (Millipore). An aliquot of 100µL 50% TFE, 0.1M NH_4HCO_3, 50mM iodoacetamide was added, incubated in the dark for 1hr at room temperature and centrifuged (14,000g, 45min). Alkylated proteins were washed using 200µL of 50% TFE, 0.1M NH_4HCO_3 (four times), centrifuged (14,000g, 45min), and the flow through was discarded. To the ~20µL retentates in the ultrafiltration devices, 1µL of 0.25µg/µL Lys-C (Sigma) and 24µL of 50% TFE, 0.1M NH_4HCO_3 was added and incubated overnight at 30°C. After Lys-C digestion, trypsin digestion was performed by addition

of 2.5µL of 1µg/µL trypsin (Promega), 350µL 20% acetonitrile (ACN), 50mM NH_4HCO_3 and incubated at 37°C for 5hr. The reaction was stopped by addition of 50% formic acid (10µL), and resulting peptides were centrifuged into new ultrafiltration receptacles (14,000g, 45min). This was followed by two rinses of the ultrafiltration devices using 100µL 50% ACN, 2% formic acid and centrifugation (14,000g, 45min). Each extract was dried in a Speedvac to near dryness and reconstituted with 60µL 2% TFE, 2% formic acid.

2.1.3 Gas phase fractionation

Mass ranges for gas phase fractionation (GPF) were calculated in silico for the *Vitis vinifera* genome from UniProtKB (65,328 entries, March 2013) as described in [143]. The four optimised mass ranges which were calculated were: the low mass range 400-506 amu, the low-medium mass range of 501-658 amu, the medium-high mass range of 653-913 amu and the high mass range of 908-1600 amu. Charge states of +2 and +3, and fixed cysteine modification of carbamidomethylation were used for calculation of these four mass ranges between 400-1600 amu.

2.1.4 Tandem mass spectrometry

Each FASP digest (10µL) of each biological replicate was injected as four fractions and scanned over the four calculated m/z ranges using a Velos Pro linear ion trap mass spectrometer (Thermo). Reversed phase columns were packed to approximately 8cm (100µm id) with Magic C18AQ resin (200Å, 5µm, Michrom Bioresources, California) in a fused silica capillary with an integrated electrospray tip. A pre-column was packed with PS-DVB (3cm, 100µM id, Agilent). An electrospray voltage of 1.8kV was used via a liquid junction upstream of the C18 column and samples were injected using an

Easy-nLC II nanoflow high pressure liquid chromatography system (Thermo). Peptides were washed with Buffer A (2% v/v ACN, 0.1% v/v formic acid) at 500nl/min, 7min, eluted with 0-50% Buffer B (99.9% v/v ACN, 0.1% v/v formic acid) at 500nl/min over 168min and washed with 95% Buffer B at 500nl/min for 5min. Spectra were acquired for 180min for each fraction, totalling 12hr per biological replicate. Automated peak recognition, MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy and dynamic exclusion duration of 90s were performed using Xcalibur software, Version 2.06 (Thermo).

2.1.5 Protein identification

Raw mass spectrometry analysis data were converted to mzXML files using the ReAdW program. The mzXML files were searched against a *V. vinifera* genome sequence database and processed using the global proteome machine (GPM) software, Version 2.1.1, X!Tandem algorithm (http://www.thegpm.org). For each experiment, the four FASP fractions of each sample were processed sequentially and merged to generate output files of protein identifications with protein log (*e*) values less than -1. GPM search parameters included fragment mass error of 0.4 Da for peptide identification, complete modification of carbamidomethylation of cysteine, and potential oxidation of methionine and tryptophan. MS/MS spectra were searched against the *V. vinifera* database with additional searching against the reverse sequence database to evaluate false discovery rates (FDR). Information of all identified peptides and proteins were deposited in the ProteomeXchange Consortium via the PRIDE repository (http://proteomecentral.proteomeexchange.org), with different PRIDE accession identity numbers as stated in the respective chapters.

2.1.6 Data processing and statistical analysis

The GPM outputs obtained from three biological replicates from each sample were combined to produce a single output file for each condition. The final output file of each condition contained only the proteins that were reproducibly identified in all three biological replicates of that condition, and the total number of spectral counts in the three biological replicates was a minimum of five. This filtering excluded reversed database hits and contaminants, and also transformed the low stringency protein identification data of individual replicates into high stringency datasets of reproducibly identified proteins present in each condition. Protein and peptide false discovery rates (FDRs) were calculated using reversed database searching. The protein FDR was calculated by dividing the (total number of reverse protein hits in the list) by the (total number of proteins in the list) x 100. The peptide FDR was calculated by [2 x (total number of reverse peptide hits in the list/ total number of peptides in the list) x 100]. Additional statistical filtering that was used for analysis are detailed in the respective thesis chapters.

Protein abundances were calculated using normal spectral abundance factors (NSAF) with an addition of a spectral fraction of 0.5 to all spectral counts to compensate for null values and permit log transformation for further statistical analyses [53, 156]. The NSAF value for a protein *k* was calculated by diving the number of spectral counts (SpC, the total number of MS/MS spectra) identifying the protein (*k*) by the length of the protein (L), divided by the sum of SpC/L for all proteins (N) in the experiment [53]. NSAF values were used as measures of protein abundances.

Student *t*-tests were performed to identify proteins that were up-regulated and down-regulated in the proteins reproducibly identified in the different conditions. Two-sample unpaired *t*-tests were run on log transformed NSAF data, and proteins with a p-value

<0.05 were considered to be differentially expressed between conditions. The general method of data processing used is described here in this section. Detailed information on data processing and statistical analysis used in the specific chapters is included in the respective chapters.

2.1.7 Functional annotations

Gene Ontology (GO) information was extracted from the UniProt database (uniprot.org) and matched to the lists of identified proteins. In-house software developed using the R statistical programming framework (http://www.r-project.org/), and the gene ontology and annotation program PloGO [157] were used to summarise the GO annotations. Proteins were classified based on their biological processes using Web Gene Ontology Annotation Plot (WEGO) (wego.genomics.org.cn). GO annotations for each biological category of interest were summarised from a list of selected biological process GO categories for each comparison (presence/absence or up-regulated/down-regulated proteins at a particular condition compared to another condition). Proteins with known biological processes were classified at GO level 5 with 'biological process' as the ontology type. NSAF abundance data were averaged for three biological replicates, summed and plotted to obtain an understanding of the overall protein abundance change in the different conditions for GO biological process categories of interest.

2.2 Methodology for in-gel digestion and shotgun proteomic analysis of plant tissues

2.2.1 Synopsis of Publication I: Analysis of rice proteins using SDS-PAGE shotgun proteomics

This paper describes the protocol for extraction of proteins from plant tissue with particular reference to rice leaves, followed by SDS-PAGE, in-gel trypsin digestion, tandem mass spectrometry using NanoLC-MS/MS and database searching using the GPM software. It also describes the method used for quantitation based on spectral counting, with information on the software tools utilised and statistical analysis followed. I estimate my contribution to this paper as approximately 35% of the writing and preparation.

2.2.2 Publication I

Chapter 21

Analysis of Rice Proteins Using SDS-PAGE Shotgun Proteomics

Karlie A. Neilson, Iniga S. George, Samantha J. Emery, Sridevi Muralidharan, Mehdi Mirzaei, and Paul A. Haynes

Abstract

In this chapter we describe the workflow used in our laboratory to analyze rice leaf samples using label-free shotgun proteomics based on SDS-PAGE fractionation of proteins. Rice proteomics has benefitted substantially from successful execution of shotgun proteomics techniques. We describe steps on how to proceed starting from rice protein extraction, SDS-PAGE, in-gel protein digestion with trypsin, nanoLC-MS/MS, and database searching using the GPM. Data from these experiments can be used for spectral counting, where simultaneous quantitation of several thousand proteins can be obtained.

Key words Shotgun proteomics, Rice, Label-free, Plant proteomics, SDS-PAGE

Abbreviations

2-DE	Two-dimensional electrophoresis
ACN	Acetonitrile
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DTT	Dithiothreitol
FDR	False discovery rate
GO	Gene ontology
GPM	Global proteome machine
IAA	Iodoacetamide
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
NSAF	Normalized spectral abundance factor
RP	Reversed phase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
WEGO	Web gene ontology annotation plot

Jesus V. Jorrin-Novo et al. (eds.), *Plant Proteomics: Methods and Protocols*, Methods in Molecular Biology, vol. 1072, DOI 10.1007/978-1-62703-631-3_21,

1 Introduction

To over half the world's population, rice is life. In 2011, rice was harvested from approximately 160 million hectares of land with over 460 million tons of milled rice produced and over 456 million tons consumed, according to the United States Department of Agriculture (as reported by IRRI at http://ricestat.irri.org, accessed 27 April 2012). Prices for rice have risen to record highs due to global demand in recent years, with rice constituting 19 % of caloric intake worldwide [1]. In addition to its nutritional and economic importance, rice has become an attractive model system for cereal genomic research because of its relatively small genome (~43 Mb), a high degree of genomic synteny with other cereal crop plants, availability of tens of thousands of searchable insertion lines (e.g., T-DNA, Tos17, Ds), compatibility with genetic transformation, and availability of a sequenced genome that contains approximately 32,000 genes [2]. However, at the time of sequencing, it was estimated that one-third of rice proteins had no known function [3]. One of the major challenges in rice research is to fully annotate the genome with a functional description for each protein to provide a better understanding of rice traits.

Proteomics involves the study of proteins encoded by the genome in a cell, tissue, or organism at a given time or under a particular set of environmental conditions. The study of proteomics is substantially more complex than genomics; the proteome is dynamic and varies with environmental stresses or cellular cues and, furthermore, proteins can be altered by numerous cellular processes such as posttranslational modifications, splicing, degradation, and proteolysis. Traditionally, proteomics has been performed using a two-dimensional electrophoresis (2-DE) gel approach, but with the development of techniques such as multidimensional protein identification technology (MudPIT) [4–7], proteomics has seen rapid growth in shotgun techniques. This shift has been encouraged by changes in mass spectrometry (MS) instrumentation, and driven by the need to analyze many more proteins at a time. Shotgun proteomics is based on the identification of proteins from a complex mixture after separation in at least two dimensions, at either the protein or the peptide level, prior to analysis by tandem MS (MS/MS).

Rice proteomics research has progressed rapidly over the past decade. Increasing proteome coverage and improving genome annotation continue to be a major concern [8, 9], and identification of posttranslational modifications has emerged as an area of intense research [10]. Cataloguing rice proteins is a necessary task; however, the field of proteomics has moved beyond simple protein identification and is now driven to accurately and reliably quantify the differences in protein abundance [11–13]. Plant proteomics research has followed this trend accordingly; quantitation of

differentially expressed proteins between two or more conditions dominates the rice proteomics field [14]. The use of quantitative shotgun proteomics has increased in prevalence and has been used in rice to analyze the effects of abiotic stress in comparative studies [15–18], and for revealing the molecular mechanisms of rice development [19–21].

Despite a recent surge in shotgun techniques observed in the literature, many rice proteomics studies continue to be carried out with the use of 2-DE gels (*see* ref. 14 for a recent review and testament to the technique). This technique is compatible with analysis of plant proteins as the denaturing buffers used for protein separation are well suited to plant protein extraction, proteins can be highly resolved, and protein identification can be performed with simple MS instrumentation and software.

In this chapter we present details of a method, known variously as a "slice and dice" or GeLC-MS/MS experiment, that can be used to simultaneously identify and quantify thousands of proteins at a time without laborious sample handling and visual comparisons of protein spots. This method is executed by separating proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in-gel digestion of proteins with trypsin, and subsequent extraction of peptides, followed by reversed-phase (RP) separation and direct elution of peptides onto a tandem mass spectrometer [22, 23].

2 Materials

All solutions should be prepared using MilliQ water and highest mass spectrometric grade analytical reagents. Prepared solutions are to be stored at room temperature, unless otherwise indicated. Appropriate waste disposal regulations should be strictly followed. All solutions and samples must be carefully handled to prevent keratin contamination.

1. Extraction buffer was prepared with 8 M urea, 100 mM Tris–HCl, pH 8.5, and 1 % Triton X-100.
2. SDS sample buffer (5× stock solution) was prepared with 6.25 mL 1 M Tris–HCl, pH 6.8, 2 g glycerol, 2.3 g SDS, 617.22 mg dithiothreitol (DTT), 1 mL 5 % (w/v) bromophenol blue, and MilliQ water to 20 mL. Sample buffer was diluted to 2× with MilliQ water.
3. SDS running buffer (10× stock solution) was prepared to 1 L with 144.135 g glycine, 30.285 g Tris, 10 g SDS, and MilliQ water. The stock solution was diluted to 1× with MilliQ water before use.
4. Urea, SDS, Tris, glycine, and Triton X-100 were from Sigma.
5. Bicinchoninic acid (BCA) reagents were from Pierce.

6. DTT, iodoacetamide (IAA), 10 % Tris–HCl precast gels, and Coomassie brilliant Blue G-250 were from Bio-Rad.
7. Trypsin was of Promega sequencing grade.
8. Formic acid was of Fluka 98 % mass spectrometry grade.
9. Acetonitrile was of Merck Lichrosolv liquid chromatography grade.
10. nanoLC-MS/MS was performed using an LTQ-XL linear ion trap mass spectrometer (Thermo, San Jose, CA).
11. Reversed phase C18 columns were packed in-house to approximately 7 cm (100 μm id) using 100 Å, 5 μm Zorbax C18 resin from Agilent Technologies, CA.
12. Spectrum files are converted to .mzxmL files prior to database search using Readw.exe, available for free download from http://sourceforge.net/projects/sashimi/files/ReAdW%20%28Xcalibur%20converter%29/.
13. XTandem of the Global Proteome Machine software version 2.1.1 is freely available for download from http://www.thegpm.org/tandem.
14. The Scrappy program is available as a series of R modules which are available for download from http://www.proteomecommons.org.

3 Methods

The workflow detailed below is a simple and effective methodology for a shotgun proteomics experiment that can be routinely used to analyze tissue samples from almost any organism, including recalcitrant tissues (Fig. 1). SDS-PAGE is a fundamental and robust method for protein fractionation and is an amenable technique for sample preparation prior to MS analysis.

This experiment may be performed for both qualitative and quantitative assessments of the proteome of any organism. In-gel digestion can be performed over 2 days once proteins have been separated electrophoretically, and three replicates of 16 fractions can be analyzed over 2½ days of machine analysis time on the mass spectrometer. This means that the workflow can be completed in less than a week for three replicates, and just over a week for a control-versus-treatment experiment of six replicates in total. This approach has been used in several published quantitative proteomic experiments in rice, where the results generated have been analyzed using quantitative tools such as Scrappy [15–17, 24]. The workflow is flexible, in that a variety of protein extraction and MS/MS analysis methods may be applied. The workflow below is written for rice leaves, but can be readily adapted to other tissues including cell cultures, roots, and seeds.

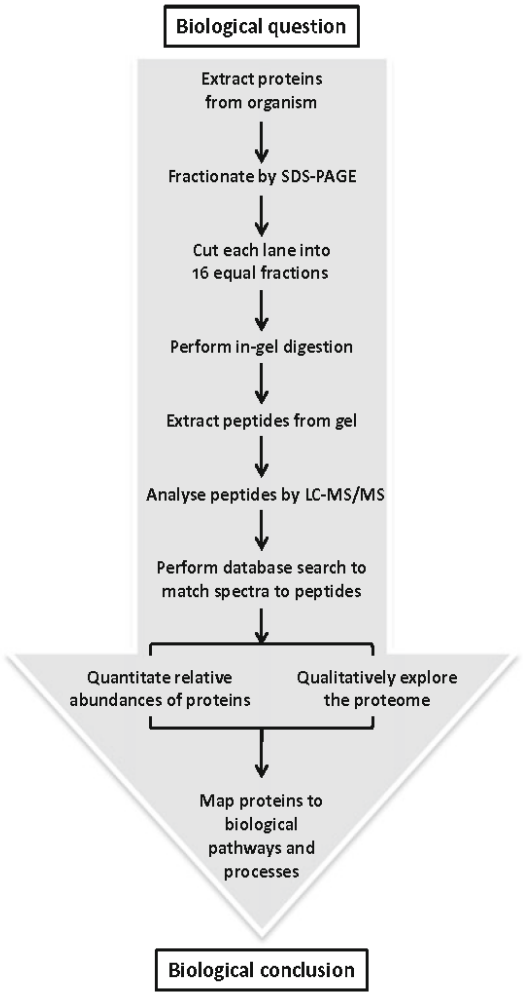

Fig. 1 Schematic diagram of SDS-PAGE shotgun proteomics workflow

3.1 Protein Extraction Protocol

1. The midsection of rice leaves were harvested from rice (*Oryza sativa* cv. Nipponbare) plants grown in a greenhouse.
2. The leaf material was ground into a fine powder under liquid nitrogen with a chilled mortar and pestle (*see* **Note 1**). After the nitrogen is evaporated from the powder (it should appear dry), approximately 100 mg aliquots of the fine leaf powder were weighed out into prechilled tubes using a chilled spatula. The leaf material can be stored at −80 °C if not used immediately.
3. The ground leaf material plus 2 mL of extraction buffer (8 M urea in 100 mM Tris–HCl, pH 8.5, with 1 % (v/v) Triton X-100) was subjected to probe sonication on ice for 3 × 20-s pulses.
4. The extract was centrifuged at 17,000 × *g* for 10 min at 4 °C and supernatant removed to a clean tube (*see* **Note 2**). Repeat if required to remove all debris from the extract.

5. Ice-cold 100 % (v/v) trichloroacetic acid (TCA) was added to the supernatant in a fresh tube to a final concentration of 25 % (v/v). The solution was vortexed briefly, and proteins were precipitated overnight at −20 °C.
6. The protein suspension was centrifuged at 17,000×*g* for 10 min at 4 °C to pellet the precipitate. The supernatant was removed and the protein pellet was washed twice with 850 μL ice-cold acetone.
7. The protein pellet was air dried for 5 min until the acetone evaporated.
8. The protein was solubilized in 100 μL of 2× SDS sample buffer, without DTT or bromophenol blue, and a BCA assay was performed to determine the concentration of the protein sample using bovine serum albumin (BSA) as a standard (*see* **Note 3**).
9. 1 M stock solution of DTT was added to 100 μg of protein extract to give a final concentration of 40 mM DTT. A trace of bromophenol blue was added, and the mixture was heated at 75 °C for 5 min before fractionation by SDS-PAGE using a 10 % precast gel and 1× SDS running buffer. Proteins were electrophoresed at 70 V for 15 min, followed by 160 V for 50 min.
10. The gel was lightly stained with Coomassie blue to provide a visual aid for subsequent handling, although this is not strictly necessary.

3.2 In-Gel Digestion Procedure

This procedure was originally adapted from Shevchenko et al. [25].

1. The stained gel was placed on a clean glass plate, and the lane containing the protein extract was excised from the gel using a clean scalpel (*see* **Note 4**). The lane was then cut into 16 equal-size fractions from top to bottom of the gel, and each of the 16 gel bands was cut into smaller fractions (*see* **Note 5**) (Fig. 2).
2. The 16 groups of gel pieces were transferred to 0.65 mL polypropylene tubes or 96-well plate and excess water removed. The gel pieces were washed briefly with 100 mM NH_4HCO_3 to ensure correct pH of gel pieces.
3. The gel pieces were destained with 200 μL 50 % (v/v) acetonitrile (ACN) and 50 % (v/v) 50 mM NH_4HCO_3 by vortexing, and then incubating for 10 min at room temperature. The liquid was removed, and then this step was repeated. The gel pieces should be clear and free of stain at this stage. If the gel pieces are still stained, then repeat the above washing as necessary. However, it is allowable to proceed with traces of stain, as it will be removed during further washing steps (*see* **Note 6**).
4. The gel pieces were washed for 5 min with 200 μL 50 % (v/v) ACN and 50 % (v/v) 50 mM NH_4HCO_3, and then 5 min with

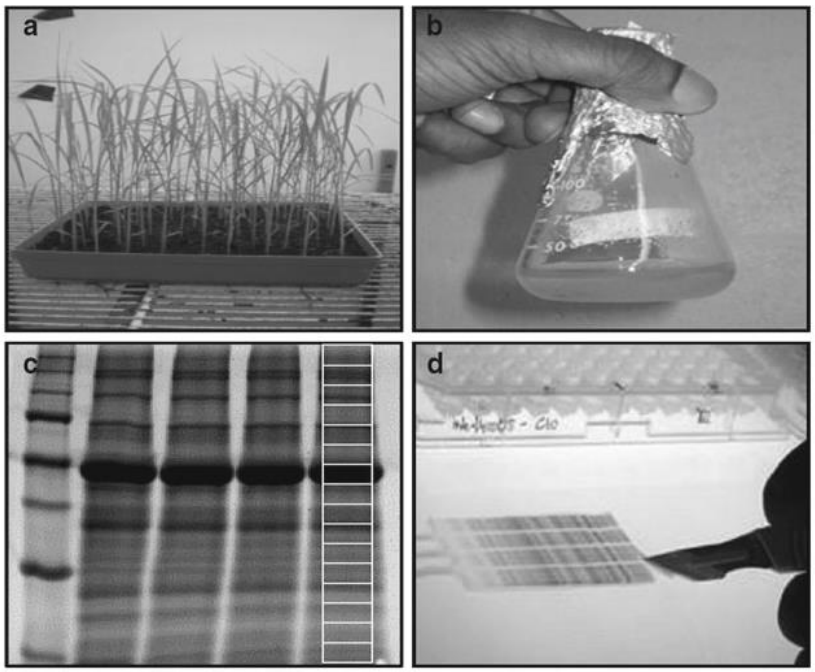

Fig. 2 Proteins are extracted from (**a**) rice leaves, stems, or roots, or (**b**) rice cell cultures and separated by SDS-PAGE. (**c**) After visualizing with a protein stain, (**d**) lanes are sliced into 16 equal fractions and transferred to a 96-well plate (or microcentrifuge tubes) for in-gel digestion

100 % (v/v) ACN to dehydrate gel pieces. The samples were vortexed during these incubations.

5. The ACN was removed and the gel pieces allowed to either air-dry on bench or in fume hood for 10 min, or evaporated briefly in a vacuum centrifuge without heat. The gel pieces should be noticeably shrunken and white.
6. The gel pieces were rehydrated with 50 μL 10 mM DTT in 50 mM NH_4HCO_3 and vortexed to mix. The tubes were briefly centrifuged and proteins were reduced for 60 min at 37 °C.
7. The gel pieces were cooled to room temperature, DTT solution was removed, and 50 μL of 55 mM IAA in 50 mM NH_4HCO_3 was added and vortexed to mix. The tubes were briefly centrifuged and proteins were alkylated for 45 min in the dark at room temperature.
8. The IAA solution was removed and the gel pieces were washed with 100 μL of 100 mM NH_4HCO_3 for 5 min with vortexing, and then washed twice with 50 % (v/v) ACN and 50 % (v/v) 50 mM NH_4HCO_3 for 5 min with vortexing.
9. The gel pieces were dehydrated with 100 μL ACN as in **steps 4** and **5**. Again, the gel pieces should be noticeably shrunken and probably white.

10. Trypsin solution was prepared in the buffer provided (Promega) to 1 μg/μL, and then 5 μL of the solution was added to 395 μL 50 mM NH_4HCO_3. This should be enough for 20 digestions at a concentration of 12.5 ng/μL. An adequate volume of trypsin solution was added to cover the gel bands. This volume will vary but is usually around 20 μL and up to 50 μL for large gel bands.
11. The gel pieces were rehydrated at 4 °C for at least 30 min in trypsin solution. Incubation must be kept cool so as to allow as much active trypsin to be absorbed into the gel as possible before auto-digestion occurs (*see* **Note 7**).
12. The tubes were centrifuged briefly, and an additional 25 μL 50 mM NH_4HCO_3 was added to cover gel pieces.
13. Proteins were digested overnight at 37 °C, or for a minimum of 4 h.
14. The digest solution supernatant (if any) was transferred into a clean 0.65 mL tube.
15. Peptides were extracted from the gel pieces with 30 μL (or enough to cover) of 50 % ACN (v/v) and 2 % (v/v) formic acid and incubated for 20 min with vortexing at room temperature. The tubes were centrifuged briefly, and the supernatant was removed and combined with initial digest solution supernatant. For large pieces of gel, use more liquid where required.
16. **Step 15** was repeated to give a combined peptide extract volume of around 60–100 μL (*see* **Note 8**). For large gel slices, use slightly larger volumes, repeat extraction a third time, and combine all extracts.
17. The extracted digests were vortexed, centrifuged briefly, and then concentrated in a vacuum centrifuge to approximately 5 μL each. Do not dry completely if possible. The peptides were reconstituted to a volume of 10 μL with 1 % (v/v) formic acid. If peptides are reduced to dryness, reconstitute peptides in 10 μL of 1 % (v/v) formic acid (*see* **Note 9**).
18. The 10 μL extracts were centrifuged at 17,000 × *g* for at least 15 min to pellet any microparticulates. The supernatant was very carefully transferred to a fresh 0.65 mL polypropylene tube, or into a 96-well plate. The sample is now ready for analysis by LC-MS/MS.

3.3 nanoLC-MS/MS of Gel Fractions

1. Each of the 16 fractions prepared was analyzed sequentially using a nanoLC-MS/MS system, employing an LTQ-XL ion-trap mass spectrometer (Thermo, San Jose, CA).
2. Reversed phase columns were packed in-house to approximately 7 cm (100 μm i.d.) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies, CA, USA) in a fused silica capillary with an integrated electrospray tip (*see* **Note 10**).

3. A 1.8 kV electrospray voltage was applied via a gold-electrode liquid junction upstream of the C18 column.
4. Samples were injected onto the C18 column using a Surveyor autosampler (Thermo, San Jose, CA).
5. Each sample was loaded onto the C18 column followed by an initial wash step with buffer A (5 % (v/v) ACN, 0.1 % (v/v) formic acid) for 10 min at 1 μL/min.
6. Peptides were subsequently eluted from the C18 column with 0–50 % buffer B (95 % (v/v) ACN, 0.1 % (v/v) formic acid) over a 30-min linear gradient at 500 nL/min followed by 50–95 % buffer B over 5 min at 500 nL/min and 5 min was with 95 %B prior to column re-equilibration (*see* **Note 11**).
7. The column eluate was directed into the nanospray ionization source of the mass spectrometer.
8. Spectra were scanned over the range 400–1,500 amu. Automated peak recognition, dynamic exclusion (90 s), and tandem MS of the top six most intense precursor ions at 40 % normalization collision energy were performed using Xcalibur software (Thermo).

3.4 Protein and Peptide Identification

1. The set of 16 data files from one experiment were acquired in the proprietary .RAW format. These were first converted to .mzxmL format using the freeware Readw.exe program (*see* **Note 12**).
2. The set of 16 .mzxmL data files from a given sample were then placed into one directory and peptide-to-spectrum matching was performed using the XTandem algorithm. We use the Global Proteome Machine (GPM) software [26], which is freely available and runs the XTandem Tornado version. Searching the set of .mzxmL files stored in a directory enables the user to choose for a single combined summary output file to be created in addition to all 16 individual result files (*see* **Note 13**).
3. The combined protein and peptide identification output file for all 16 gel slices was then exported to an Excel spreadsheet. This contains six columns of data, with the headers Identifier, log(*I*), rI, log(*e*), pI, and Mr (kDa). Two other headers are included, Description and Annotated Domains, but will only have entries automatically provided if this information is available within the searched database. The Excel file is then exported to comma-separated value format, which is then compatible with input into the Scrappy software described elsewhere [27].

An example of the output produced by this approach is provided in Table 1. Included in this abridged table are the first 20 proteins identified from a rice leaf extract, sorted by the confidence

Table 1
Table of results from a GPM search showing the top 20 proteins identified in a previous study, sorted by log(*e*) value

Identifier	log(*I*)	rI	log(*e*)	pI	Mr (kDa)	Description
gi|11466795|	9.36	3,512	−1,288	6.22	52.8	RuBisCO large subunit
gi|115468792|	9.17	3,085	−1,057.5	6.95	48	RuBisCO large subunit
gi|115440423|	8.56	1,324	−838.3	8.14	172.7	ATPase delta/epsilon subunit
gi|115481654|	8.65	914	−802.8	5.95	84	ATP synthase beta chain
gi|11466794|	8.65	932	−795.2	5.47	54	ATP synthase CF1 beta subunit
gi|115439533|	7.82	224	−671.8	6.51	111.4	Glycine dehydrogenase
gi|115487804|	9.13	1,678	−634.5	9.01	59	RuBisCO large subunit
gi|115473843|	7.68	182	−590.1	6.04	125.6	Glutamate synthase
gi|115478691|	7.75	727	−560.6	5.14	28	Chlorophyll A–B-binding protein
gi|115438250|	7.72	712	−515.7	5.29	27.5	Chlorophyll A–B-binding protein
gi|11466784|	8.45	844	−508.3	5.95	55.6	ATP synthase CF1 alpha subunit
gi|115439621|	7.59	643	−505.4	5.29	27.9	Chlorophyll A–B-binding protein
gi|115468926|	7.73	191	−501.9	5.98	96.9	Glycine cleavage system P-protein
gi|115466224|	7.98	377	−495.3	5.44	73.4	Thiamine pyrophosphate family, transketolase
gi|115486823|	8.43	562	−477.3	5.86	47.9	Ribulose-bisphosphate carboxylase activase
gi|115487910|	7.24	186	−450.5	6.62	102	Clp protease ATP-binding subunit
gi|115436780|	8.1	300	−426.3	6.08	34.8	Manganese-stabilizing protein
gi|115488160|	7.49	153	−423.7	5.12	61.1	GroEL-like type I chaperonin
gi|115438793|	8.41	564	−371.3	8.78	30	Carbonic anhydrase
gi|11466786|	7.96	339	−363.4	6.63	82.5	Photosystem I P700 chlorophyll a apoprotein A2

The Identifier indicates the gene identifier number associated with the protein, and will depend on the origin of the FASTA database, log(*I*) represents the log sum intensity of the spectra acquired, rI is the number of redundant peptides identified for the protein (used in spectral counting quantitation), log(*e*) is the log expectation value that the protein match was random, pI is the isoelectric point of the protein, and Mr (kDa) indicates the mass of the protein

score, log(*e*). From this single experiment, which was first published as part of a larger data set in Gammulla et al. [16], 760 proteins were identified to be common to three replicates at 0.79 % false discovery rate (FDR) with an average of 37,469 peptides at 0.21 % FDR.

3.5 Beyond Protein Identification: Quantitation and Extraction of Biological Relevance

Quantitative proteomics can be divided into two categories: label-free methods and the use of isotope labelling. Label-free proteomics has several advantages over labelling techniques: all organisms and sample types may be analyzed, the number of samples that can be compared is not limited, and data analysis is comparatively straightforward and does not require the use of specific software. However, the volume of data accumulated in a global, label-free proteomics study, such as in a GeLC-MS/MS experiment, can be overwhelming. An important goal in functional proteomics is to globally profile changes in protein abundances in biological systems and also provide a snapshot of the protein expression state in response to biological perturbations. The eventual outcome of any quantitative proteomics study is to draw a biological conclusion from the large volume of data acquired. This can be systematically achieved by measuring the protein abundance differences between proteins from two or more conditions, applying statistical tests for significance, and visualizing results in a biological context (*see* ref. 28 for a recent review).

We analyze our data by spectral counting, specifically by calculating normalized spectral abundance factors (NSAFs) [29] for each protein in a data set. This calculation takes into consideration that the number of spectra identified for a protein will be dependent on the length of that protein. This calculation is incorporated in a series of freely available R modules assembled in the form of the Scrappy program [27]. Spectral counting using NSAFs has been demonstrated to be an accurate method for calculating relative abundances of proteins between two or more samples [30–32]. In a quantitative study comparing spectral counting using NSAFs with isobaric labelled peptides, we found that a much greater volume of differentially expressed proteins were identified with the label-free method, although the two methods yielded similar biological conclusions [33].

Extracting biological relevance from large-scale proteomics data sets is a challenge in both label-free and labelled proteomic studies. Numerous tools exist to functionally categorize protein identification data and map proteins to biological processes. In our experience, we have found it useful to begin functional analysis by accumulating Gene Ontology (GO) annotations [34] for proteins identified in the data set, followed by functional categorization using Web Gene Ontology Annotation Plot (WEGO) [35]. WEGO maps GO annotations to either functional pathways or processes and calculates a p-value for category enrichment. Quantitative analysis of functional categories can be performed using PloGO, a freely available, open-source tool [24]; rather than simply summing the number of proteins in a category, the sum of NSAF values can be used to estimate the relative abundance of proteins in a pathway or a process. This information can be used to highlight areas of the proteome that are most responsive to a stress or a specific treatment and hence, that particular area may be studied in further detail.

4 Conclusion

Label-free shotgun proteomics based on SDS-PAGE fractionation of proteins from rice is a simple, robust, and versatile technique. The method can be used with proteins extracted from any tissue type as varying extraction methods can be applied and data can be collected for qualitative or quantitative purposes. In our experience, the technique is highly reproducible for the identification and quantitation of large protein data sets using spectral counting, although other methods of label-free quantitation can be applied. The range of achievable outcomes of label-free quantitative proteomics in the field of systems biology is bound to expand with improvements in instrumentation and computer software.

5 Notes

1. During protein extraction, keep falcon tubes, scissors, and spatula on dry ice. Wipe mortar, pestle, and spatula clean with a lint-free tissue between samples.
2. Chill the centrifuge and rotor to 4 °C before starting protein extraction.
3. DTT and bromophenol blue are not compatible with a BCA assay and so were added after measuring protein concentration. SDS needs to be diluted to <5 % to be compatible with BCA reagents.
4. Spray enough water on the plate to stop the gel drying out. Wipe down all surfaces before starting with a lint-free cloth in methanol, work in a hood if possible, and wear gloves and sleeve protectors. Cover hair and beards, and take all available steps to avoid keratin contamination.
5. It is not necessary to contain noticeable bands within a single fraction; it is more important to make the gel fractions equal sized and reproducible across replicates. This may be done by dividing the gel into two fractions, and then each two fractions into four fractions until 16 fractions are obtained. This may be achieved by simply cutting each fraction down the middle for a total of four rounds.
6. Treat gels stained with Sypro ruby, Deep Purple, or other fluorescent stains the same as a Coomassie-stained gel.
7. Allow trypsin to incubate with the gel pieces on ice for 20–30 min.
8. For the most complete extraction of peptides, peptides may be extracted in sequential steps with the first in 1 % (v/v) formic acid, the second in 50 % (v/v) ACN, and the third in 90 % (v/v) ACN (each diluted in water).

9. Dried or reconstituted peptides can be stored at −80 °C for as long as required.
10. nanoLC columns can also be purchased from suppliers such as Michrom or New Objective.
11. It is not required to completely eliminate carryover between injections as all the samples in a given set will be combined for subsequent analysis.
12. Our experimental design consists of 16 SDS-PAGE gel slices from each of the three biological replicates of a given sample. This can be completed in 2½ days of mass spectrometric analysis time.
13. We use the "mudpit combine" option in GPM searches to produce a unified output file from the individual search result files.

Acknowledgements

KAN, ISG, and SM acknowledge support in the form of iMQRES awards. SJE acknowledges support in the form of an APA scholarship. PAH acknowledges funding support from the Australian Research Council and wishes to thank Gayani Gammulla for providing images.

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CHAPTER 3

Chapter 3

3. Protein identification and quantification from riverbank grape, *Vitis riparia*: Comparing SDS-PAGE and FASP-GPF techniques for shotgun proteomic analysis

3.1 Synopsis of Publication II

The aim of this experiment was to establish an optimal platform for label-free quantitation and shotgun proteomic analysis of grapevine, so that the same protocol could be adopted for the remaining chapters in this thesis. This publication reports the comparison between two different fractionation and sample preparation techniques of in-gel digestion and in-solution digestion performed in grapevine shoots belonging to a *Vitis* species; *Vitis riparia* or riverbank grape. The primary aim of this study was to identify that technique which would generate more reproducible, high-throughput proteomic data when quantified based on spectral counting.

V. riparia is recognised as a commercial rootstock, and is used in hybrid scion, rootstock breeding for phylloxera resistance, low temperature tolerance and early ripening studies. The *V. riparia* species was analysed in this chapter because the second aim of this study was to observe the efficacy of cross-species protein identification using the *Vitis vinifera* database.

This study was performed with the objective of method optimisation. We compared the gel-based and in-solution digestion protocols to assess their advantages in the design of future grapevine proteomics experiments included in this thesis. This thesis chapter is presented as a publication written in the form of a technical draft.

3.2 Key Findings

Quantitative comparisons of the total and unique number of proteins identified by each technique indicated that Filter Aided Sample Preparation (FASP) coupled with Gas Phase Fractionation (GPF) proved to be a better method than SDS-PAGE for shotgun proteomics in grapevine. There was a 24% increase in the total number of reproducibly identified proteins when FASP-GPF was used. Thus, this method was chosen for all the grapevine studies included in this thesis.

Database searching results demonstrated that cross species peptide spectra searches against the *V. vinifera* database allowed protein identification in *V. riparia*, confirming that cross species analysis can provide biologically meaningful results.

We also compared our results to a dataset of differentially expressed proteins of the same samples previously identified by 2D-gel separation and found that shotgun proteomics could cover most of the proteins identified by 2D-gel separations.

In conclusion, we established that FASP-GPF is the optimum method for shotgun proteomics of grapevine samples when compared to SDS-PAGE, as it considerably increased the throughput and protein identification across biological replicates.

3.3 Contributions

I performed 90% of all the experimental work which included protein extractions from *V. riparia* leaves, determination of protein concentrations, digestion by two alternative techniques of in-gel method using SDS-PAGE and in-solution method using FASP, mass spectrometry analysis, database searching and data analysis. *V. riparia* plants were grown and provided by Professor Anne. Y. Fennell from South Dakota State University, United States of America. I was responsible for 95% of the result interpretation and

manuscript writing. This research was conducted under the supervision of Professor Paul. A. Haynes.

3.4 Publication II

TECHNICAL BRIEF

Protein identification and quantification from riverbank grape, *Vitis riparia*: Comparing SDS-PAGE and FASP-GPF techniques for shotgun proteomic analysis

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Protein sample preparation optimisation is critical for establishing reproducible high throughput proteomic analysis. In this study, two different fractionation sample preparation techniques (in-gel digestion and in-solution digestion) for shotgun proteomics were used to quantitatively compare proteins identified in *Vitis riparia* leaf samples. The total number of proteins and peptides identified were compared between filter aided sample preparation (FASP) coupled with gas phase fractionation (GPF) and SDS-PAGE methods. There was a 24% increase in the total number of reproducibly identified proteins when FASP-GPF was used. FASP-GPF is more reproducible, less expensive and a better method than SDS-PAGE for shotgun proteomics of grapevine samples as it significantly increases protein identification across biological replicates. Total peptide and protein information from the two fractionation techniques is available in PRIDE with the identifier PXD001399 (http://proteomecentral.proteomexchange.org/dataset/PXD001399).

Received: February 25, 2015
Revised: March 20, 2015
Accepted: April 24, 2015

Keywords:
Filter Aided Sample Preparation (FASP) / Gas Phase Fractionation (GPF) / Grapevine / Label-free quantitative shotgun proteomics / Plant proteomics / *Vitis riparia*

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Shotgun proteomics is a widely used technique to identify proteins from complex mixtures by analysing individual peptides. Shotgun proteomics has evolved from simple single protein identifications by analysing individual peptides to elaborate quantitative proteomics where relative protein abundance differences between samples can be differentiated with accuracy, reproducibility and high throughput. In shotgun proteomics, label-free quantitative approaches [1–4] are used as an alternative to labelled approaches like SILAC, ICAT, iTRAQ and TMT [5–8]. Recent studies indicate that label-free quantitation is less expensive and more versatile than labelled quantitative proteomics [9]. Ongoing improvements in shotgun proteomics are attributed to advancements in sample preparation techniques, mass spectrometry instrumentation and bioinformatics. A standard shotgun proteomics experiment workflow includes sample preparation, protein or peptide fractionation, protein or peptide separation, mass detection in a mass spectrometer, database searching and data analysis. Reproducible high throughput proteomic analysis is dependent upon selecting an optimal sample preparation technique. Two sample preparation methods that have been employed regularly for plant proteomic studies, including grapevine [1, 10, 11], are SDS-PAGE followed by in-gel digestion, and in-solution digestion followed by filter aided sample preparation (FASP) [12]. In-gel digestion protocols employ 1D SDS-PAGE that is a protein separation technique, based on molecular weight separation. FASP is an in-solution digestion technique which employs commercially available spin filters (microcentrifuge devices) for sample clean-up and protein digestion [12]. The FASP protocol can be enhanced (eFASP) using different

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Abbreviations: **FASP**, filter aided sample preparation; **GPF**, gas phase fractionation; **TFE**, 2,2,2-Trifluoroethanol

combinations of reagents during digestion [13] or coupled with gas phase fractionation (GPF) peptide separation in the mass spectrometer [14] to improve proteomic coverage. GPF utilises the resolving power of a mass spectrometer to separate different series of ions in different mass ranges. Grapevine proteomics has been studied using gel and liquid protein separation methods including 2D SDS-PAGE and FASP in different grapevine species like *Vitis vinifera* and *Vitis riparia* [1, 10, 11, 15] and iTRAQ in *V. vinifera* [16] and *Vitis rotundifolia* [17].

In the current shotgun proteomic study, we performed a comparison of two different sample preparation methods of gel and liquid fractionation techniques (SDS-PAGE and FASP-GPF) using *V. riparia* leaf proteins, with concurrent label-free quantitation. *V. riparia*, commonly known as riverbank grape, is a species native to the North American continent. It has been used extensively as a commercial rootstock and in hybrid scion and rootstock breeding for phylloxera resistance, low temperature tolerance, early ripening and ease of propagation. This study investigated the efficacy of cross-species protein identification using a *V. vinifera* database and quantified the differences in protein number and identities that are obtained using gel and liquid fractionation techniques.

Spur-pruned, potted, 2–6 year old *V. riparia* Michx plants were removed from cold storage, roots pruned, repotted and placed in the greenhouse for growth under 15 h daylength and 25/20°C day/night temperatures. Shoots were trained vertically and fully expanded leaves from the fifth node of the shoot were harvested after 40 days of growth. Proteins were extracted from three biological replicates of fully expanded leaves (4 and 5 apical node). Approximately 1 g (fresh weight) of leaf samples were ground in liquid nitrogen, suspended in extraction buffer (6 M Gn-HCl, 1% N-lauroylsarcosine, 10 mM EDTA, 0.1 M tricine, 5% β-mercaptoethanol), followed by 5 min sonication, 10 min incubation at 75°C water bath and 6 min centrifugation at 11 000 g. Proteins were precipitated using methanol-chloroform [18]. Protein concentration was determined by the Pierce BCA protein assay (Thermo, San Jose, CA, USA). Protein concentrations of the three biological replicates ranged from 2.68 to 2.98 mg/mL.

Aliquots of 200 μg of protein were used for both gel-based and liquid fractionation techniques. Analyses were conducted using three biological replicates for each technique. For SDS-PAGE fractionation, protein pellets were dissolved in SDS sample buffer, separated on 10% Tris-HCl precast BioRad gel at 75 V (20 min) and 160 V (40 min), fixed for 1 h, stained with colloidal Coomassie (15 min) and destained in water overnight. In-gel digestion was performed as described in [19]. Individual lanes of each of the three biological replicates were cut into 16 approximately equal slices, destained with 100 mM NH_4HCO_3, washed with 50% ACN/100 mM NH_4HCO_3 and dehydrated with 100% ACN. Samples were reduced (10 mM DTT) and alkylated (50 mM iodoacetamide) for 1 h in the dark, and digested with 20 μL of trypsin (12.5 ng/mL) at 37°C (overnight). Peptides were extracted with 50% ACN/2% formic acid, evaporated to dryness in a vacuum centrifuge, and reconstituted with 2% formic acid.

FASP-GPF fractionation was conducted by digesting protein pellets in-solution as described in [20]. Proteins were dissolved in 200 μL of 50% Trifluoroethanol (TFE), 0.1 M NH_4HCO_3 and 50 mM DTT and concentrated to 20 μL in Amicon Ultra 0.5 mL ultrafiltration devices (10 K cut-off, Millipore). An aliquot of 100 μL 50% TFE, 0.1 M NH_4HCO_3, 50 mM iodoacetamide was added and the mixture was incubated in the dark (1 h) and then centrifuged (14 000 g, 45 min). Alkylated proteins were washed with 200 μL 50% TFE, 0.1 M NH_4HCO_3 and centrifuged (14 000 g, 45 min). To the ~20 μL retentates left in the ultrafiltration devices, 1 μL 0.25 μg/μL Lys-C (Sigma), and 24 μL 50% TFE and 0.1 M NH_4HCO_3 was added and incubated overnight at 30°C. Trypsin digestion was conducted using 2.5 μL 1 μg/μL trypsin (Promega), 350 μL 20% ACN and 50 mM NH_4HCO_3 and incubated at 37°C (5 h). Trypsin digestion was stopped with 10 μL 50% formic acid and the peptides were centrifuged into new ultrafiltration receptacles (14 000 g, 45 min). This was followed by two rinses of the ultrafiltration devices using 150 μL 50% ACN, 2% formic acid and centrifugation repeats. Each extract was evaporated to dryness in a vacuum centrifuge and reconstituted with 2% TFE, 2% formic acid.

Tryptic digests from the 1D gel slices and the FASP extraction were analysed using an LTQ Velos Pro linear ion-trap mass spectrometer (Thermo). Chromatography was performed in reversed phase columns packed in-house to approximately 8 cm (100 μm I.D.) with Magic C18AQ resin (200Å, 5 μm, MichromBioresources, CA) in a fused silica capillary with an integrated electrospray tip and the pre-column was packed with PS-DVB (3 cm, 100 μM id, Agilent). A 1.8 kV electrospray voltage was applied via a liquid junction upstream of the C18 column and samples were injected using an Easy-nLC II (Thermo). Each of the 16 fractions of each biological replicate from the 1D gels were washed with Buffer A (2% v/v ACN, 0.1% v/v formic acid) at 150 μL/min (4 min), 0–50% Buffer B (99.9% v/v ACN, 0.1% v/v formic acid) for 75 μL/min (34 min) and with 95% Buffer B at 150 μL/min (7 min). Each of the 16 fractions was run for 45 min in the mass spectrometer, totalling 12 h per biological replicate. Spectra was scanned in the positive mode over a mass range of 400–1600 amu. MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy and dynamic exclusion duration of 90 s were automatically selected for MS/MS in each MS run. FASP peptide extracts were washed with Buffer A at 500 nL/min (7 min), eluted with 0–50% Buffer B at 500 nL/min (168 min) and washed with 95% Buffer B at 500 nL/min (5 min). An aliquot of 10 μL of FASP digest of each biological replicate was injected four times and scanned in the positive mode in four mass ranges of 400–506, 501–658, 653–913 and 908–1600 amu. Mass ranges were calculated as described in [21] using the *V. vinifera* genome as a reference sequence. Spectra were acquired for 180 min for each fraction, totalling 12 h per biological replicate. Xcalibur software (Version 2.06, Thermo) was used for automated peak

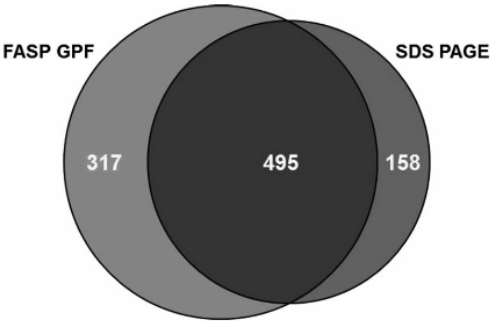

Figure 1. Proportional Venn diagram showing the number of unique and common proteins reproducibly identified by LC–MS/MS after SDS-PAGE and FASP-GPF fractionation methods.

recognition, MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy and dynamic exclusion duration of 90 s. Spectra were searched against the UniProtKB *V. vinifera* genome (65 328 entries, March 2013) using the global proteome machine software (Version 2.1.1) [22, 23]. Parent ion tolerance of 2 Da and fragment mass error of 0.4 Da were used for peptide identification. Complete modification of carbamidomethylation of cysteine and potential modifications of oxidation of methionine and tryptophan were considered. Peptide and protein information from the three biological replicates of the each of the two different fractionation techniques used is available in the ProteomeXchange Consortium via PRIDE [24] with identifier PXD001399. An individual protein was retained in the final data set of reproducibly identified proteins of each method only if the protein was identified in all three biological replicates (i.e., leaf material harvested from three different plants) and the total number of spectral counts in the three biological replicates was a minimum of five. Protein and peptide false discovery rate (FDR) was calculated as described in [2].

A total of 970 non-redundant proteins were identified from the two different methods (Fig. 1). Table 1 summarises the protein and peptide data identified by SDS-PAGE and FASP-GPF methods. The number of proteins identified across the three biological replicates was highly consistent in each method with a standard error of 4.3% and 9.3%, consistent with the standardised 200 μg protein loading. The protein and peptide FDR was <2% and <0.5%, respectively, indicating that the data set was of high quality and further filtering was not required. There was a 24% increase in the total number of proteins identified reproducibly by FASP-GPF (812 proteins) when compared to the in-gel digestion approach (653 proteins). It should be noted that the peptide numbers indicated in Table 1 are low stringency numbers which include substantial noise, and these are shown only as an indication of experimental consistency. Figure 1 shows that an increased number of unique proteins were reproducibly identified by FASP-GPF (317 proteins) in comparison with SDS-PAGE (158 proteins), while 495 proteins were common to both methods. The number of total proteins and unique proteins identified using SDS-PAGE and FASP-GPF were sorted using database theoretical molecular weight values and are shown in Fig. 2A and B. FASP-GPF identified a greater number of total and unique proteins across all molecular mass ranges. Figure 2B shows that there was greater than a threefold increase in number of unique proteins identified by FASP-GPF in the 0–20, 40–60, 80–100 and 100–120 kDa mass ranges and an almost twofold increase in unique peptides identified in the 60–80 kDa mass range in comparison to SDS-PAGE fractionation. In particular, different translation initiation factors, chaperonins, 40S ribosomal proteins, histone proteins and proteases were uniquely identified by FASP-GPF. The lists of proteins identified reproducibly from the FASP-GPF and SDS-PAGE fractionation methods are summarised in Supporting Information Table 1 (columns include protein identity, peptide counts in each replicate and predicted molecular mass). In addition, we compared the proteins identified in this study to the differentially expressed proteins from *V. riparia* shoot tips studied by Victor et al. [15]. We found that approximately 79% of the differentially expressed proteins identified by 2D-gel separation were found in our data sets from the SDS-PAGE and FASP-GPF *V. riparia* leaf samples. This confirms the idea that shotgun proteomics can cover most of the proteins that can be identified by 2D-gel separations. It must also be noted that there are proteins uniquely identified by each technique.

Table 1. Summary of protein and peptide identification data for *V. riparia* leaf samples using SDS-PAGE and FASP-GPF fractionation procedures

Method	Low stringency number of proteins identified			High stringency number of proteins common to all three replicates	Protein FDR[b] (%)	Peptide FDR (%)	Protein RSD[c] (%)
	R1[a]	R2[a]	R3[a]				
SDS-PAGE	1712	1770	1864	653	1.22	0.18	±4.3
FASP-GPF	2084	1868	1735	812	0.25	0.15	±9.3

a) R1, R2 and R3 denote biological replicates 1, 2 and 3, for proteins.
b) FDR, false discovery rate.
c) RSD, relative standard deviation for number of proteins in samples ($n = 3$).

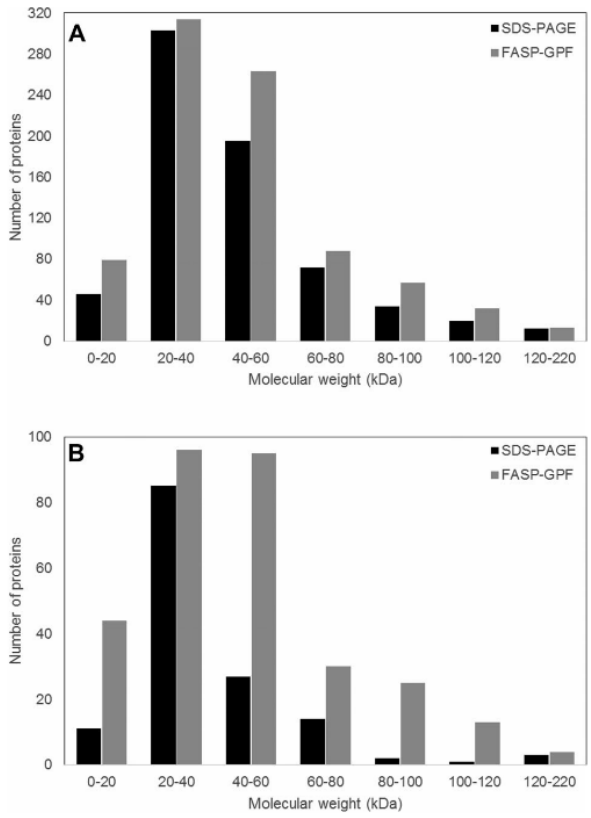

Figure 2. Proteins identified by shotgun proteomics, separated by theoretical molecular weights. (A) The total number of proteins and (B) unique number of proteins, which were identified reproducibly in all three replicates of SDS-PAGE and FASP-GPF fractionation procedures.

The average count of peptides identified in each technique was calculated by dividing the sum of the averaged total peptides identifying a protein by the number of proteins detected using that technique. It was found to be 38 and 16 for SDS-PAGE and FASP-GPF, respectively. This shows that many peptides were redundant when the SDS-PAGE protocol was used and this could be a reason for less protein identities in SDS-PAGE when compared to FASP-GPF. We also compared the total and unique peptides identified in each biological replicate using each of the methods of SDS-PAGE and FASP-GPF. Approximately 1000 more unique peptides were seen in each replicate using FASP-GPF when compared to SDS-PAGE. FASP-GPF identified more non-redundant peptides over the four different mass ranges thus generating a greater number of protein hits. For example, RuBisCO (Uniprot accession number P56648) was identified by both methods, but the average number of peptides identified by the FASP-GPF for RuBisCO was 472 (with 82 unique peptides) while the average number of peptides in the SDS-PAGE method was 1922 (with 65 unique peptides) for the same protein.

In addition to the medium (gel or liquid) used in fractionation, digestion efficiency is also of crucial importance in identifying individual proteins from complex mixtures. In our protocols, SDS and TFE were used for protein solubilisation. SDS is an extensively used detergent but it can interfere with MS analysis [25]. It is critical to remove SDS prior to MS to avoid damaging effects. In our FASP protocol, another common solute, TFE, was used as a denaturant. TFE is MS compatible, improves protein identification and does not have as many disadvantages as SDS [20, 26]. The SDS-PAGE method is relatively time-consuming and more susceptible to keratin contamination during the gel 'slice and dice' steps of sample processing and handling. Use of spin columns in FASP minimises sample handling and keratin contamination. Although SDS-PAGE is a well-established technique for plant shotgun proteomics, FASP-GPF proved to be an improved method for grapevine shotgun proteomics. Another study we performed in our laboratory recently using the protozoan parasite *Giardia duodenalis* similarly demonstrated that FASP-GPF performed better than SDS-PAGE fractionation for shotgun proteomics [21]. FASP works well when sample amount is not limited, as in the current study, and has also been shown to adapt well for analysis of nanogram amounts of protein [27, 28].

Using SDS-PAGE and FASP-GPF, the qualitative and quantitative comparisons of the total and unique number of proteins identified, and the distribution of proteins based on molecular weight and reproducibility, indicate that FASP-GPF fractionation performs better for shotgun proteomics in grapevine. These results also demonstrate that cross species peptide spectra searches against the *V. vinifera* database allow protein identification in *V. riparia*. We conclude that FASP-GPF is a better method than SDS-PAGE for shotgun proteomics of grapevine samples as it considerably increases the throughput and protein identification across biological replicates.

The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [24]: dataset identifier PXD001399. PAH acknowledges support from the Australian Research Council Training Centre for Molecular Technology in the Food Industry. AYF acknowledges support from the National Science Foundation Program DBI0604755. ISG acknowledges support from Macquarie University in the form of the iMQRES scholarship and a travel grant from the Grape and Wine Research and Development Corporation (GWRDC).

The authors have declared no conflict of interest.

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CHAPTER 4

Chapter 4

4. Quantitative proteomic analysis of Cabernet Sauvignon grape cells exposed to thermal stresses reveals alterations in sugar and phenylpropanoid metabolism

4.1 Synopsis of Publication III

This research article investigated cellular responses in grape at the protein level when exposed to sudden temperature changes. This experiment was designed to mirror environmental changes, with particular reference to unexpected temperature fluctuations which are inclined to be more frequent in future. This chapter is presented as a published research article and reports the proteomic analysis of grape cells exposed to different temperatures stresses of heat and cold. We compared protein changes between *Vitis vinifera* cv. Cabernet Sauvignon suspension cell cultures grown at a control temperature of 26° and at four different thermal stresses of 10°C, 18°C, 34°C and 42°C. Samples used in this study were processed by the in-solution FASP method coupled with GPF which was substantiated as the optimal technique for grapevine proteomic analysis in chapter 3. Spectral counting, using normalized spectral abundance factors was employed to generate statistically significant quantitative proteomic information. We aimed to identify proteins and biochemical pathways involved in heat and cold stress responses in grape cells.

4.2 Key Findings

We have presented evidence for differential protein expression at thermal stresses in grapevine. We found nine proteins involved in the phenylpropanoid pathway to be more

abundant at an extreme cold stress of 10°C, indicating that some proteins involved in phenylpropanoid metabolism and anthocyanin synthesis were cold-responsive.

We also observed that sucrose metabolism displayed switching between alternative and classical pathways during thermal stress temperatures. In addition, we were able to provide a putative functional annotation for many of the identified proteins that were labelled as putative uncharacterised proteins. This publication provides valuable information into the specific regulatory pathways and signalling networks involved in grapevine responses to temperature stresses.

4.3 Contributions

I performed 100% of all the experimental work pertaining to the establishment of grape suspension cultures, induction of temperature stresses, growth curve measurements, protein extractions from harvested cells, in-solution trypsin digestion using the FASP protocol, peptide extractions, mass spectrometry analysis for biological triplicates of cells harvested from each of the five different temperatures, database searching and data analysis. I was responsible for 95% of the manuscript writing. Assistance for statistical analysis and result interpretation was provided by Dana Pascovici and Mehdi Mirzaei. This research was conducted under the supervision of Professor Paul. A. Haynes.

4.4 Publication III

DOI 10.1002/pmic.201400541 *Proteomics* 2015, *15*, 3048–3060

RESEARCH ARTICLE

Quantitative proteomic analysis of cabernet sauvignon grape cells exposed to thermal stresses reveals alterations in sugar and phenylpropanoid metabolism

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Grapes (*Vitis vinifera*) are a valuable fruit crop and wine production is a major industry. Global warming and expanded range of cultivation will expose grapes to more temperature stresses in future. Our study investigated protein level responses to abiotic stresses, with particular reference to proteomic changes induced by the impact of four different temperature stress regimes, including both hot and cold temperatures, on cultured grape cells. Cabernet Sauvignon cell suspension cultures grown at 26°C were subjected to 14 h of exposure to 34 and 42°C for heat stress, and 18 and 10°C for cold stress. Cells from the five temperatures were harvested in biological triplicates and label-free quantitative shotgun proteomic analysis was performed. A total of 2042 non-redundant proteins were identified from the five temperature points. Fifty-five proteins were only detected in extreme heat stress conditions (42°C) and 53 proteins were only detected at extreme cold stress conditions (10°C). Gene Ontology (GO) annotations of differentially expressed proteins provided insights into the metabolic pathways that are involved in temperature stress in grape cells. Sugar metabolism displayed switching between alternative and classical pathways during temperature stresses. Additionally, nine proteins involved in the phenylpropanoid pathway were greatly increased in abundance at extreme cold stress, and were thus found to be cold-responsive proteins. All MS data have been deposited in the ProteomeXchange with identifier PXD000977 (http://proteomecentral.proteomexchange.org/dataset/PXD000977).

Received: November 17, 2014
Revised: April 13, 2015
Accepted: May 7, 2015

Keywords:
Abiotic stress / Grape / Plant proteomics / Shotgun proteomics / Temperature stress / Vitis vinifera

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Grapes are an important economic fruit crop and are widely cultivated for their high commercial value as a fruit, or in juice, distilled spirits and wine. Wine production is a significant industry with a global production of 265 million hectolitres in 2011. There is a possibility of a wine shortage in future due to increase in wine consumption, without a proportionate increase in grape production [1]. Abiotic stresses like drought, salinity and temperature fluctuations, typical of climate change, result in low harvests of grape crops worldwide [2]. Among abiotic stresses, only water deficit is known to be beneficial in terms of wine quality [2], as carefully monitored water-deficit conditions have been used for quality and flavour enhancement in berries [3, 4]. Sudden temperature fluctuations pose a threat to successful grape cultivation. Although a moderately cool temperature with a minimum threshold of 10°C is considered to be the base temperature for breaking bud dormancy [5, 6], very low temperatures can damage developing buds and shoots [5], and freezing can wipe out the entire grape harvest of a season. Low temperatures can also

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Significance of the study

In this study, we report on proteome level changes observed in Cabernet Sauvignon grape cells exposed to sudden temperature stresses. Cells were grown at control temperature and then switched to conditions of either moderate heat stress, extreme heat stress, moderate cold stress, or extreme cold stress. More than 2000 proteins were identified and quantified using spectral counting of the entire dataset. Several biochemical pathways were clearly altered in response to stress, including the switching between alternative and classical pathways of sugar metabolism, and the up-regulation of the phenylpropanoid biosynthesis pathway in response to cold stress.

alter carbon metabolism and severely deregulate photosynthesis in grapevine inflorescences [7, 8]. High temperatures can impede physiological processes in grapevines [5], alter grape quality due to accumulation of increased must sugar content in fruit [9], and lead to poor quality grapes with low yields. Previous studies have shown that harvesting in warm temperatures adversely affects the quality of grapes of the Cabernet Sauvignon and Chardonnay varieties [6]. Till date, no positive effect of temperature stress on either wine quality or yield has been recorded in grapevine.

Our planet is rapidly growing hotter, with an estimated increase in global temperatures by 2 to 5°C expected to occur during the 21st century [10]. Global warming is predicted to be accompanied by extreme temperature events which are more frequent in occurrence and more powerful in magnitude. These extreme weather events and temperature shifts are expected to increase in future, creating an inimical environment for grape crops, as many viticultural practices, such as grape harvest dates, are heavily influenced by climate and weather. Understanding the molecular basis of cold and heat stress responses in grapevine is an initial step toward development of cultivars which can survive these temperature changes.

Grapevine (*Vitis vinifera* L.) was established as a non-climacteric model species after the availability of the grape genome sequence in 2007 [11, 12]. The release of the grape genome sequence spurred the initiation of several transcriptomic and proteomic studies of stress responses in grapevine.

Mass spectrometry based proteomics has successfully been used to study varietal differentiation [13, 14], disease resistance [15, 16], bud development [17], berry development [18–20], salt stress [14, 21], water stress [14, 22, 23], herbicide treatment [24] and different photoperiods [25] in grapevine. However, there have as yet been very few proteomic studies with respect to temperature stress in grapevine. Proteomic changes in response to hot or cold stress have, however, been studied in many other plant species such as flax [26], Arabidopsis [27], rice [28, 29] and wheat [30]. Temperature stress in grapevine has been characterised at the transcriptomic level [31–33], but transcriptomic studies do not always correlate with proteomic studies [34]. Hence, there is a need for proteomic analysis of temperature stress responses in grapevine in order to better understand the molecular processes involved.

Quantitative shotgun quantitative proteomics is a powerful tool for analysis of complex mixtures, facilitating the study of changes in protein abundance, protein interactions and modifications. Improvements in label-free quantitative proteomic approaches have made them more versatile and dependable [35], and a valuable alternative to label-based approaches like ICAT [36], SILAC [37] and iTRAQ [38]. Proteomic analysis of grapevine has previously been performed mainly using the 2D gel based approach [14, 16, 20, 23, 25, 39], with a few exceptions where the iTRAQ approach was used [18, 40, 41]. Although the 2D gel based approach has been traditionally used for grapevine proteomic research, it has certain limitations including poor representation of proteins, lack of solubility of hydrophobic proteins, difficulty in distinguishing between protein isoforms, poor detection of low abundance proteins and limited reproducibility. It has also been reported that 2DE techniques are more error prone, and identify significantly fewer proteins, when compared to mass spectrometry based shotgun methods [35, 42]. Label-free quantitative shotgun proteomics presents an excellent alternate approach for quantitative analyses to investigate global protein identification and expression, and systematically profile complex proteomes in grapevine cells.

Undifferentiated cells, as suspension cultured cells, have been successfully used in identifying novel stress response proteins in the study of differential metabolic response of rice exposed to hot and cold temperatures using label-free shotgun proteomics [28]. Grape cell cultures have previously been used to investigate proteomic changes in relation to ripening related processes [43], somatic embryogenesis [44], necrosis [39], response to elicitors, and response to chitosan treatment [45, 46]. Specific responses to induced treatments and common trends in terms of relative protein abundances were recorded in these studies comparing seedlings and suspension cell cultures. Proteomic analysis of cell suspensions have also been observed to accurately mimic specific berry development stages in *V. vinifera* cv. Dauphine [43].

We report here the first shotgun label-free quantitative proteomic analysis of cultured Cabernet Sauvignon grape cells exposed to low and high temperatures. This experiment was designed to study the differentially expressed proteins in grape cells exposed to four different temperature stresses of 10, 18, 34 and 42°C, when the control temperature was 26°C. This experiment aimed to reveal the cellular responses to sudden temperature changes, mirroring unexpected temperature fluctuations which are prone to occur more frequently in the future in vineyard regions across the world. Further aims were to identify novel thermal stress response proteins, and characterise the differentially expressed proteins and pathways, as a means of understanding their function at a molecular level. In this study, we analysed the grape cell

proteome using the Filter Aided Sample Preparation method (FASP) [47–49] coupled with gas phase fractionation (GPF) [50]. Spectral counting [51] using normalised spectral abundance factors (NSAF) [52] was employed to generate statistically significant quantitative proteomic information.

2 Materials and methods

2.1 Growth and establishment of Cabernet Sauvignon suspension cells cultures

Cabernet Sauvignon grape callus cultures were maintained on Grape Cormier Medium (GCM), which comprised of Gamborgs B5 media (Sigma, St. Louis, MO, USA) with minimal organics supplemented with 30 g/L sucrose, 0.25 g/L casein hydrolysate, 0.93 μM kinetin, 0.54 μM naphthaleneacetic acid (NAA) and 0.8% (w/v) agar and incubated in darkness at 26°C [53]. The callus was subcultured at 21 day intervals. Approximately 2 g of subcultured friable callus was inoculated into 250 mL flasks containing 35 mL of same culture medium outlined above, but without agar and incubated on a rotary shaker (110 rpm, 26°C). Cell suspensions were maintained by transferring 5 mL of a seven-day-old culture into 35 mL of fresh medium. Growth curve measurements were taken after four weeks of subculturing, when the cells were homogenous and synchronised. The growth curve of the cell suspension cultures were determined by measuring the fresh weight (FW) of cells at 24 h intervals, over a 15 day period. Growth curve measurements were performed in biological triplicates and until cells reached the stationary phase.

2.2 Imposition of temperature stress on grape suspension cell cultures

The specific time to induce temperature stress on the grape suspension cell cultures was determined based on their growth curve. After subculturing, cell suspensions were grown at 26°C for six days till they reached mid-log phase and transferred into incubators pre-set at 10, 18, 34 and 42°C for a 14 h stress, on a rotary shaker (110 rpm). A 14 h stress was chosen based on FW and cell viability measurements. The control cultures were grown continuously at 26°C for seven days after subculturing. Biological triplicates of all suspension cell cultures were harvested using cell strainers (BD Falcon, USA) after the stress period and cells were stored at –80°C.

2.3 Protein extraction

Approximately 1 g (FW) of cells was suspended in ice-cold 80% acetone, 10 mM $Na_2S_2O_5$ at –20°C for 12 h, centrifuged (11 000 *g*, 10 min, –4°C), and the supernatant discarded. Proteins were extracted from cell pellets using 6 M Gn-HCl, 1% N-lauroylsarcosine, 10 mM EDTA, 0.1 M tricine, 5% β-mercaptoethanol, followed by sonication (5 min), incubation (75°C water bath, 10 min) and centrifugation (11 000 *g*, 6 min). Proteins were precipitated using chloroform–methanol [54]. Protein concentrations were measured by the Pierce BCA protein assay (Thermo, San Jose, CA).

2.4 In-solution digestion and peptide extraction

In preparation for gel-free shotgun proteomics, protein pellets were digested in-solution by a modified FASP method using Lys-C and trypsin [47]. Protein extracts (250 μg) were dissolved in 200 μL 50% TFE, 0.1 M NH_4HCO_3, 50 mM DTT and concentrated to 20 μL in Amicon Ultra 0.5 mL 30K ultrafiltration devices (Millipore). Then 100 μL 50% TFE, 0.1 M NH_4HCO_3, 50 mM iodoacetamide were added, incubated in the dark (1 h, room temperature) and centrifuged (14 000 *g*, 45 min). Alkylated proteins were washed four times using 200 μL of 50% TFE, 0.1 M NH_4HCO_3, centrifuged as above and the flow through was discarded. To the ~20 μL retentates in the ultrafiltration devices, 1 μL of 0.25 μg/μL Lys-C (Sigma) and 24 μL of 50% TFE, 0.1 M NH_4HCO_3 was added and incubated (30°C, overnight). Trypsin digestion was performed by addition of 2.5 μL of 1 μg/μL trypsin (Promega), 350 μL 20% acetonitrile (ACN), 50 mM NH_4HCO_3 and incubation (37°C, 5 h). Digestion was stopped with 10 μL 50% formic acid and resulting peptides were centrifuged into new ultrafiltration receptacles (14 000 *g*, 45 min). This was followed by two rinses of the ultrafiltration devices using 150 μL 50% ACN, 2% formic acid and centrifugation as above. Each extract was dried in a Speedvac to dryness and reconstituted with 60 μL 2% TFE, 2% formic acid.

2.5 Gas phase fractionation

Mass ranges for GPF were calculated in silico [50, 55]. The *V. vinifera* genome from UniProtKB with 65 328 entries (March 2013) was used to calculate the optimised mass ranges based on theoretical trypsin digestion. Charge states of +2, +3 and modification of carbamidomethyl were incorporated for calculation of four mass ranges between 400–1600 amu. The optimised *m/z* ranges used were 400–506, 501–658, 653–913 and 908–1600 amu.

2.6 Nano LC-MS/MS

Each of the biological triplicate sets of FASP digests of the five different temperature points were analysed using an LTQ Velos Pro linear ion trap mass spectrometer (Thermo, San Jose, CA). Chromatography was performed in reversed phase columns packed in-house to approximately 8 cm (100 μm id) with Magic C18AQ resin (200 Å, 5 μm, Michrom Bioresources, California) in a fused silica capillary with an

integrated electrospray tip, coupled to pre-columns packed with PS-DVB resin (3 cm, 100 μM id, Agilent). A 1.8 kV electrospray voltage was applied via a liquid junction upstream of the C18 column and samples were injected using an Easy-nLC II (Thermo). Peptides were initially washed with Buffer A (2% v/v ACN, 0.1% v/v formic acid) at 500 nL/min for 7 min and eluted with 0–50% Buffer B (99.9% v/v ACN, 0.1% v/v formic acid) at 500 nL/min for 168 min, followed by a wash step with 95% Buffer B at 500 nL/min for 5 min. An aliquot of 10 μL of each sample was injected four times into the mass spectrometer and scanned in the four mass ranges described earlier. Spectra was acquired for 180 min for each fraction, totalling 12 h per sample. Automated peak recognition, MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy and dynamic exclusion duration of 90 s [56] were performed using Xcalibur software (Version 2.06, Thermo).

2.7 Database search and protein identification

Raw files obtained from nano LC-MS/MS were converted to mzXML files and searched against the UniProtKB *V. vinifera* genome (65 328 entries, March 2013) using the global proteome machine software, Version 2.1.1 (http://www.thegpm.org) [57, 58] and the X!Tandem algorithm. Spectra were also searched against a reversed sequence database for estimation of false discovery rates (FDR). The four fractions of each replicate were processed sequentially, with output files for each fraction, followed by generation of a non-redundant merged output file for protein and peptide identifications with log (*e*) values less than –1. Parent ion tolerance of 2 Da and fragment ion mass error of 0.4 Da were used for peptide identification. Complete modification of carbamidomethylation of cysteine and potential modifications of oxidation of methionine and tryptophan were considered. Complete peptide and protein information from the triplicate analyses of the five temperature points is available in the ProteomeXchange Consortium via the PRIDE partner repository [59] with the dataset identifier PXD000977.

2.8 Data processing and quantitative analysis

The 15 lists of proteins obtained from triplicate analyses of five different temperature points were filtered using two criteria. A protein was retained as a valid hit in the final dataset if (i) the protein was identified in two out of three biological replicates of at least one temperature point, and (ii) the total number of spectral counts in triplicates of at least one temperature point was a minimum of five. This transforms the low-stringency protein identification data from individual replicate analyses into a single high-stringency dataset of reproducibly identified proteins present at each temperature point.

The protein FDR was calculated for each list of proteins using (total number of reverse protein hits in the list/total number of proteins in the list) x 100 and the peptide FDR was calculated using 2 x (total number of reverse peptide hits in the list/total number of peptides in the list) x 100 [28].

Protein abundance was calculated using NSAF [52], with an addition of a spectral fraction of 0.5 to all spectral counts to compensate for null values and enable log transformation for subsequent statistical analyses [28]. Summed NSAF values were used as a measure of relative protein abundance.

Additional statistical filtering was applied to the two datasets of proteins which were identified using the above criteria as being detected only at either extreme cold stress or extreme heat stress, because these were the focus of further investigation and discussion in the manuscript. The raw spectral counts were summed across the three biological replicates for each temperature, and the spectral counts present in the extreme stress temperature samples were transformed into a *Z*-score value.

For a set of protein abundance values (P) numbered one through n across n experiments (in this case five temperatures), the *Z*-score is calculated using:

$$Z\text{−score} = (\text{abundance}(P) - \text{mean abundance}(P_1 \ldots P_n)) / \text{SD}(P_1 \ldots P_n).$$

Z-score transformation has been shown to be useful in minimising distortion in quantitative datasets caused by occasional experimental artefacts. For a normal dataset, calculated *Z*-scores are clustered around the mean, so retaining only those proteins with a *Z*-score equal to plus or minus one standard deviation either side of the mean value can be used to remove statistical outliers [60]. This reduced the number of proteins considered for further investigation by approximately 14%. This type of statistical filtering is only applicable when comparing one set of measurements against other sets of measurements within a coherent dataset.

2.9 Statistical analysis

A series of *t*-tests were performed to identify proteins that were up-regulated and down-regulated in the proteins reproducibly identified in the different conditions. Two-sample unpaired *t*-tests were run on log transformed NSAF values, and proteins with a p-value < 0.05 were considered to be differentially expressed.

2.10 Gene ontology information and functional annotation

GO information was extracted from the UniProt database (uniprot.org) and matched to the list of reproducibly identified proteins. In-house software developed using

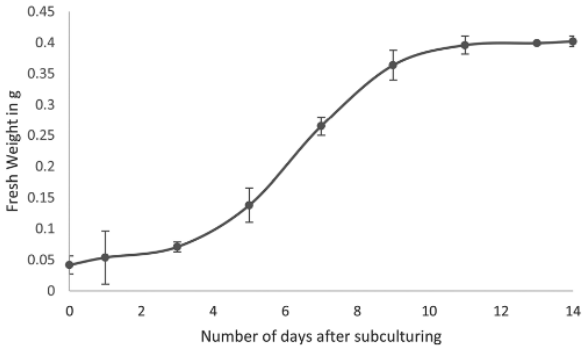

Figure 1. Growth pattern of Cabernet Sauvignon suspension cell cultures grown at control temperature of 26°C. Cultures subjected to the four different temperature stresses were grown at 26°C for six days after subculturing and transferred to stress temperatures for 14 h. Each point represents an average of three biological replicates.

the R statistical programming framework (http://www.r-project.org/) and plotting gene ontology and annotation (PloGO) program was used to summarise the GO annotation [61]. Proteins were then classified based on their biological processes using Web Gene Ontology Annotation Plot (WEGO) (wego.genomics.org.cn) [62]. The available GO annotation for each category of interest was summarised from a list of selected biological process GO categories, for the results of each comparison (up-regulated or down-regulated proteins). WEGO classification with GO level 5 with biological processes as ontology type was used to classify proteins with known biological processes. For GO categories of interest, NSAF abundance data were summed and plotted to obtain an understanding of overall protein abundance change in different conditions for biological process categories.

3 Results and discussion

3.1 Growth of Cabernet Sauvignon cell cultures

The growth profile determined by change in FW of Cabernet Sauvignon suspension cell cultures grown at the control temperature of 26°C is shown in Fig. 1. The growth curve shows a steady growth rate from the third day to the ninth day, with the cell pellet weight almost doubling on the sixth day. The growth rate is seen to subsequently decrease after the eighth day. From this curve, the sixth day after subculturing was selected as the optimum time to induce thermal stress, as it was in the middle of the log phase of growth of cells. Subsequent cultures were grown at 26°C for six days and then subjected to thermal stress for 14 h before harvesting. The effect of thermal stress on growth of cell cultures was determined by FW and cell viability measurements. A relative decreased growth of 60% was observed at extreme temperatures of 10 and 42°C after 14 h of stress. Approximately 80% of cells were viable after 14 h of stress in all four temperatures. Growth curve measurements are summarised in Supporting Information Table 1.

3.2 Quantitative analysis of label-free shotgun proteomic data

A total of 2042 non-redundant proteins were reproducibly identified from five temperature points. Table 1 summarises the protein and peptide data of the cells grown at different temperatures. The number of proteins reproducibly identified ranged from 1491 at the control temperature to 1604 from the cells grown at 10°C. The number of low-stringency peptides counted in each nano LC-MS/MS analysis was very consistent across the five temperature treatments, with an average standard error of approximately 5%, consistent with the standardised 250 μg protein loading. For the high-stringency identified proteins, peptide FDR ranged from 0.13 to 0.17% across the five temperatures, and the protein FDR values at each temperature were less than 1%, indicating that the dataset was of sufficiently high stringency that no further filtering was needed (Supporting Information Table 2 contains information on proteins reproducibly identified in each replicate at all five temperatures).

3.3 Presence/absence of proteins at different temperatures

The 2042 non-redundant reproducibly identified proteins were sorted based on their presence or absence at each temperature (Fig. 2). Proteins identified in all five temperatures formed the largest category with 1003 proteins. The other prominent treatment-specific protein groups were 63 proteins exclusive to extreme cold stress (10°C), and 62 proteins exclusive to extreme heat stress (42°C). A total of 77 proteins were detected in all of the temperature stress conditions but not at the control, while 34 proteins were detected only in the control temperature. A total of 79 proteins were detected in all temperatures except 42°C, and 59 proteins were seen in all temperatures except 10°C. Taken together, these results confirm that grape cells respond distinctly to different conditions by expression of different proteins during heat and cold stress. The proteins detected only at either extreme cold stress or extreme heat stress were further filtered based on *Z*-score values and are discussed in Sections 3.4 and 3.5. This secondary filtering based on *Z*-score values removed statistical outliers and reduced the numbers to 53 proteins identified exclusively at extreme cold stress conditions (10°C) and 55 proteins identified only in extreme heat stress conditions (42°C).

Table 1. Summary of protein and peptide identification data of Cabernet Sauvignon cell suspension cultures grown at control (26°C) and stress temperatures (10, 18, 34 and 42°C)

Condition	Low stringency redundant count of peptides			Average number of peptides (±%RSD[b])	Number of reproducibly identified proteins[c]	Protein FDR[d] (%)	Peptide FDR (%)
	R1[a]	R2	R3				
10°C	30502	30342	31369	30738 ± 1.8	1604	0.69	0.16
18°C	27583	25390	26676	26550 ± 4.2	1591	0.57	0.15
26°C	25715	24376	21353	23815 ± 9.4	1491	0.47	0.15
34°C	24022	26243	26088	25451 ± 4.9	1540	0.52	0.17
42°C	33303	34402	37670	35125 ± 6.5	1540	0.78	0.13

a) R1, R2 and R3 denote replicate 1, replicate 2 and replicate 3 respectively.
b) RSD, relative standard deviation for number of low stringency peptides identified (expressed as percentage of the average).
c) Defined as those proteins present in two out of three replicates at a given temperature point with a minimum spectral count of five.
d) FDR, false discovery rate.

3.4 Proteins detected only at extreme cold stress (10°C)

Fifty-three proteins were detected exclusively at 10°C, as summarised in Supporting Information Table 3. Proteins involved in DNA synthesis, translational initiation and RNA splicing were observed and these included translation initiation factors, helicases, replication factors and DNA polymerases. Histone and actin proteins, which are fundamental elements of cytoskeletal development and cell division, were also found. Enzymes stilbene synthase (STS), phenylalanine ammonia-lyase (PAL), flavanone-3-hydroxylase and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, which are involved in phenylpropanoid biosynthesis, were detected in this category and are discussed in detail in Section 3.7. Many glycosyltransferases (A5B349, A5BE79 and A5BIH9) were identified. Glycosyltransferases have many functions in plants, but the majority are known to be involved in biosynthesis of polysaccharides and glycoproteins leading to modifications in the plant cell wall [63]. Response to stress category proteins, including, for example, Q43237, caffeoyl-CoA O-methyltransferase, which are involved in the reinforcement of the plant cell wall and in response to wounds or pathogens, were found. Plant cell wall modifications have

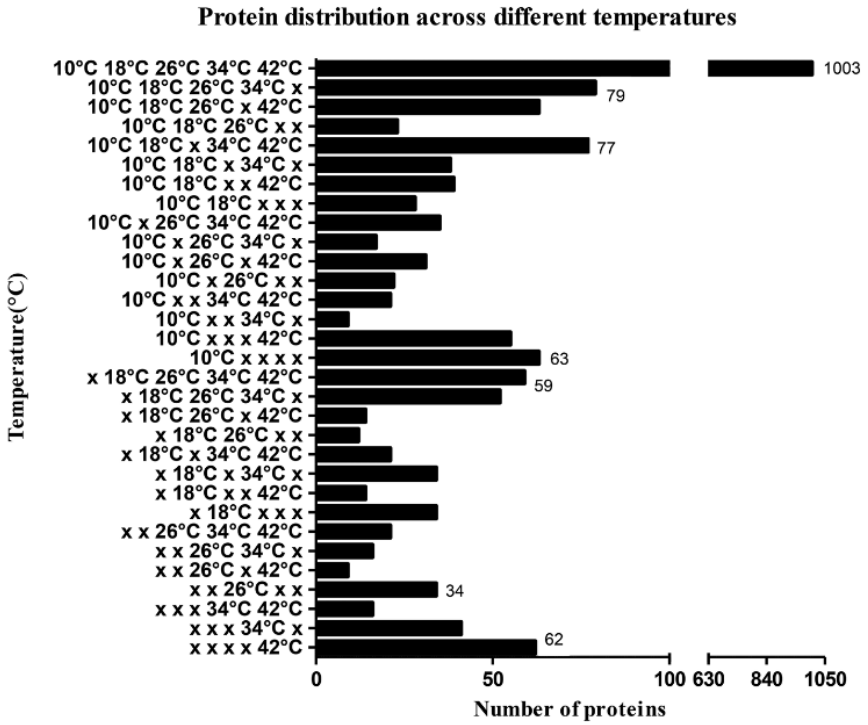

Figure 2. Classification of 2042 non-redundant proteins into 31 categories based on the presence and absence of proteins in five different temperatures. The vertical axis (*y*-axis) represents temperature combinations of 10, 18, 26, 34 and 42°C, '*x*' denotes absence of proteins at a given temperature and 31 different categories are shown from top to the bottom. The number of proteins found in combinations which are stated in the manuscript are indicated at the end of the respective data point.

been observed earlier in response to extreme hot and cold stress in rice [28]. Aspartate aminotransferases D7SUD7 and D7TBL7 which are key enzymes that function in the biosynthesis of amino acids and are known to regulate carbon and nitrogen metabolism in plants were observed [63]. Aldehyde dehydrogenase was another enzyme abundant at 10°C. Overexpression of aldehyde dehydrogenase genes have demonstrated an improved abiotic tolerance and protection against oxidative stress in Arabidopsis [64]. Other proteins found in this group were responsible for ubiquitination and proteolysis. This indicates that cell response to cold stress involved both protein biosynthesis as well as breakdown. Putative uncharacterised proteins A5AXH5 and D7SW22 found in this category were of unknown function and may now be considered as thermal stress responsive proteins, mainly cold stress.

3.5 Proteins detected only at extreme heat stress (42°C)

The total number of proteins (1540) identified at 42°C was approximately the same as the number of proteins identified in each of the other temperatures (Table 1). This pattern of protein species abundance is very different to that observed in a similar study in rice cell cultures, where growth at extreme heat stress caused a sharp reduction in the number of reproducibly identified proteins detected, indicative of a metabolic shutdown occurring in the cells [28].

Fifty-five proteins were exclusively identified at 42°C, as summarised in Supporting Information Table 4. Heat-shock proteins formed the vast majority in this cohort of identified proteins. Heat-shock proteins; HSP20, HSP101, HSP70 and class II HSPs of various molecular weights, along with chaperone proteins were detected at 42°C. HSPs are generally associated with heat stress response and assist in protein folding [30], and have been observed to be up-regulated at the transcript level in response to elevated temperatures in grapevine [65]. This indicated that the impact of heat stress involved complex responses in grapevine, which included changes in protein folding and proteolysis. Casein lytic proteinase B4 (ClpB4) which belongs to the heat-shock protein 100 family was also found. ClpB4 functions in degradation of misfolded proteins which accumulate during thermal stress. These proteins also assist in protein aggregate resolubilisation [28, 66]. Some proteins involved in proteolysis were observed in this category, but were few in number. This helps to explain the observation that the number of proteins identified under extreme heat stress was similar to the number of proteins found at each of the other temperatures. Heat stress also induced expression of proteins, like theoredoxin which participates in redox reactions and several secondary stresses such as oxidative stress. Chitinase class III protein, which is known to function in abiotic stress responses in Arabidopsis, was found in this category [67].

Table 2. Proteins differentially expressed between the five different temperatures

	Control (26°C) vs. moderate hot stress (34°C)	Control (26°C) vs. extreme hot stress (42°C)
Down-regulated	132	369
Up-regulated	88	116
Unchanged	1081	831
	Control (26°C) vs. moderate cold stress (18°C)	**Control (26°C) vs. extreme cold stress (10°C)**
Down-regulated	77	170
Up-regulated	95	145
Unchanged	1131	1030

3.6 Comparison of differences in protein abundances between control and temperature stresses

Student *t*-test analyses were performed for four different comparisons to observe the statistical significance of fold-changes of proteins. Quantitative comparisons were undertaken between control and both moderate and extreme temperatures. The number of up-regulated, down-regulated and unchanged proteins of the four different temperature stresses versus the control is summarised in Table 2. Extreme temperatures (42 and 10°C) had a more drastic influence on the number of up- or down-regulated proteins when compared to moderate stress temperatures (34 and 18°C). Approximately a 25 and 40% increase were observed in the number of up-regulated proteins in extreme heat and cold stresses when compared to their moderate parallels.

Protein abundances were compared and classified for proteins with known biological processes categorised according to WEGO for proteins up- or down-regulated at the extreme temperatures when compared to the control and illustrated in Figs. 3 and 4. An increase in abundance was observed at 10°C in the up-regulated proteins involved in metabolic processes, biosynthetic processes, glycolysis, transport, lipid metabolism and secondary metabolism (Fig. 3). In contrast, a large increase in abundance was observed at 42°C in the down-regulated proteins involved in the same biological process categories (Fig. 4). Most of the categories of up- and down-regulated proteins displayed a mirror image in differences in protein abundance profiles; the proteins that were up-regulated at 10°C relative to control were down-regulated at 42°C.

3.7 Phenylpropanoid biosynthesis pathway

The differences in protein abundances reported in this study reflect the complex signals and pathways activated due to the sudden impact of temperatures changes. Extreme cold of 10°C triggered a dramatic increase in the

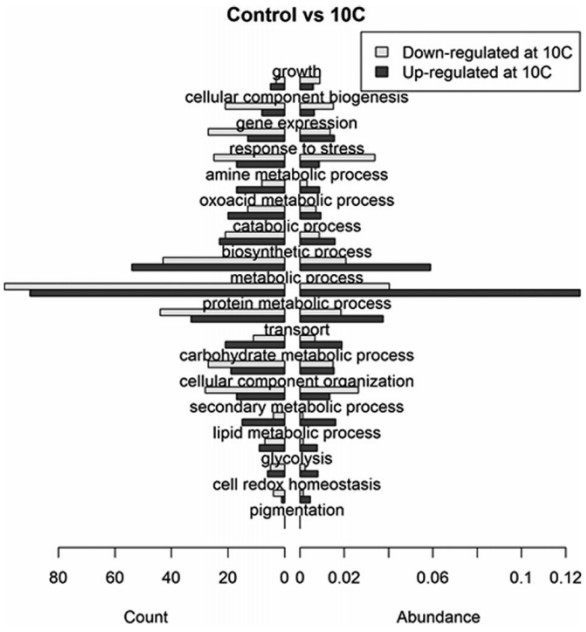

Figure 3. Comparison of the number and abundance of differentially expressed proteins between cells grown at control (26°C) and extreme cold (10°C). The up-regulated and down-regulated proteins were classified into different biological process categories using GO ontology annotations.

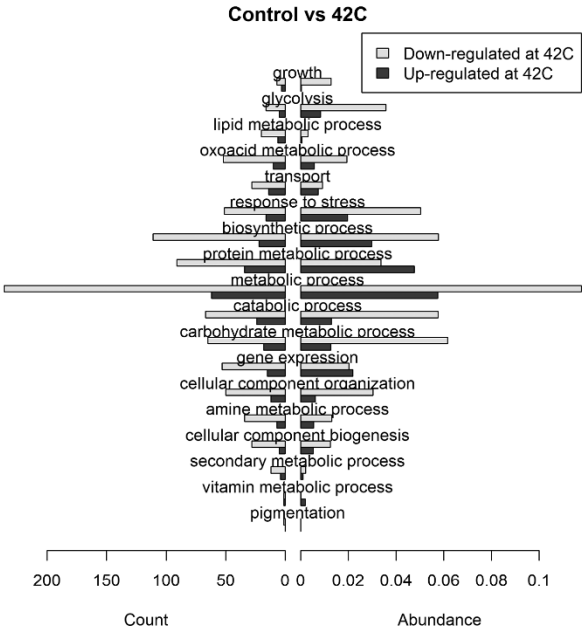

Figure 4. Comparison of the number and abundance of differentially expressed proteins between cells grown at control (26°C) and extreme heat (42°C). The up-regulated and down-regulated proteins were classified into different biological process categories using GO ontology annotations.

abundance of proteins that function in general phenylpropanoid metabolism, which leads to the production of anthocyanin, stilbene, flavonoids and lignin. Previous studies at the transcript level on Arabidopsis [68], maize [69] and pepper [70] have established that low temperatures enhance transcript abundances of genes involved in the general phenylpropanoid and anthocyanin pathways. Our experiments confirmed that this response was also observed at the proteomic level in grapevine. The proteins in this pathway that had higher NSAF values displaying high abundance at the extreme cold stress of 10°C were 3-Deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP) synthase (B9VU85), PAL (A5BPT8), cinnamate-4-hydroxylase (C4H) (A5BRL4), O-methyltransferase (F6HM50), STS (P51070), chalcone synthase (CHS) (Q8W3P5), aspartate aminotransferases, anthocyanin synthase (A2ICC9), anthocyanin reductase (D7U6G6) and chalcone flavanone isomerase (A5ANT9). The general phenylpropanoid pathway is illustrated in Fig. 5, highlighting the enzymes that were greatly up-regulated at 10°C when compared to the other four temperatures. NSAF values of all identified proteins are given in Supporting Information Table 5 worksheet 1 and worksheet 2 which contain the list of NSAF values for the above nine proteins. DAHP synthase is an intermediate in the production of aromatic amino acids such as phenylalanine. It is the first enzyme in the shikimate pathway and thus regulates the amount of carbon entering the pathway. PAL was up-regulated at 10°C. PAL catalyses the non-oxidative deamination of phenylalanine to trans-cinnamate, which is an important regulatory point between primary and secondary metabolism. PAL has been extensively studied with respect to plant responses to abiotic and biotic stresses [68,70]. PAL is involved in the first committing step in the general phenylpropanoid pathway, and also has a role in biosynthesis of various polyphenol compounds which lead to synthesis of lignin and flavonoids in plants. STS, which is involved in stilbene biosynthesis, and CHS, which is involved in anthocyanin biosynthesis, were also up-regulated at 10°C. Stilbenes are phenylpropanoids produced by many plants species including grapevine. A recent study on stilbenes in grapevine has confirmed their functional role important in defence response [71]. Resveratrol, a stilbene usually found in red wine, is known for inducing health benefits for humans, when red wine is consumed in moderation. Our results suggest that exposure to low temperatures could cause an increase in production of such compounds of pharmacological value. C4H and O-methyltransferase were up-regulated at 10°C, while anthocyanin reductase and anthocyanin synthase were up-regulated at both the extreme stresses of 42 and 10°C. Anthocyanin reductase participates in flavonoid biosynthesis and is involved in production of proanthocyanidin.

Anthocyanin and flavonoids are synthesised for defence response in plants to detoxify the ROS produced due to the impact of low temperature stress. Cell wall components are modified by the production of lignin as protection. The high abundance of proteins involved in the phenylpropanoid pathway at extreme cold stress reveals a correlation to previous

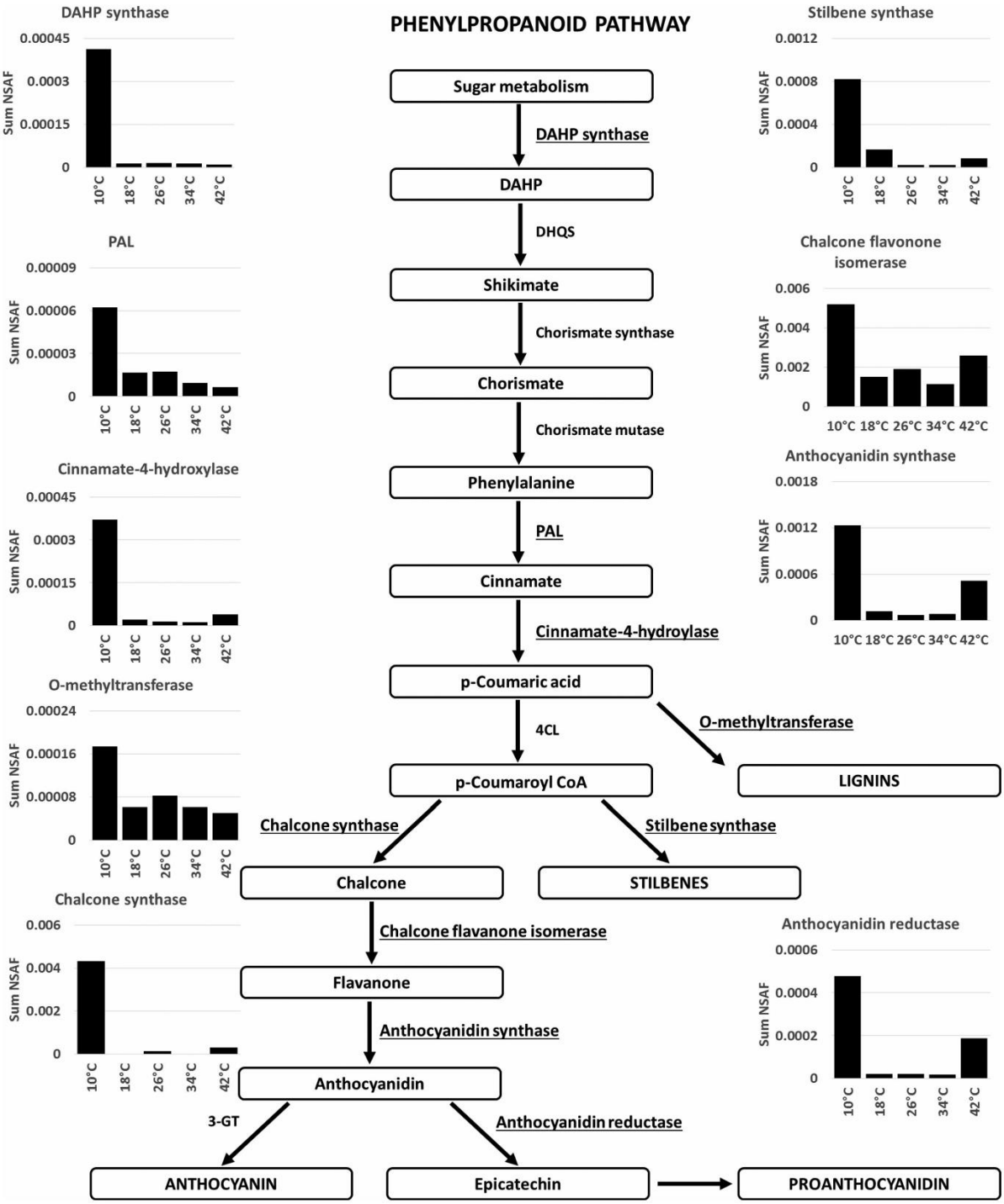

Figure 5. Change in abundance in enzymes involved in the general phenylpropanoid pathway at the various temperature of 10, 18, 26, 34 and 42°C. The enzymes of interest are underlined and their sum NSAF values are calculated and plotted.

studies at the transcriptomic level [68–70]. These proteins are clearly cold-responsive proteins, the expression of which characterises molecular responses to low temperatures in plant cells. Production of secondary metabolites like phenolic acids, flavonoids, lignin and stilbenes are known to be responsible for plant defence mechanisms against secondary attacks by insects or infectious diseases. A sudden temperature shock of 10°C triggered the increase in production of these compounds and this may be an initial defence response. Perhaps if the temperature stress was induced for a longer period of time, there would have been a decrease in secondary metabolite production and this would have made the plants more vulnerable to secondary attacks.

3.8 Sugar metabolism

The grape suspension cell cultures analysed in this study were grown in Grape Cormier Medium which contained sucrose as the main carbon source. It is already established through a study in cultured sycamore cells that sucrose breakdown occurs in two different pathways involving different enzymes [72]. Sucrose breakdown occurs either via the invertase pathway (classical) or the sucrose synthase pathway (alternative). In the classical pathway, glucose and fructose are formed as products from sucrose when it is cleaved by the enzyme invertase. In the alternative pathway, UDP glucose and fructose are formed as products from sucrose when it is cleaved by the enzyme sucrose synthase. UDP glucose is then converted to glucose-1-phosphate by the enzyme UDP-glucose pyrophosphorylase, leading to glycolysis. Putative uncharacterised protein E0CR04 showed homology to UDP-sugar pyrophosphorylase and was seen at all five temperatures (Supporting Information Table 5, worksheet 3). This suggests that grape cells mainly employed the alternative pathway for sucrose breakdown. However, the enzymes with homology to invertases were expressed abundantly at heat and cold temperature stresses. Invertases F6GY06, F6HAU0 and Q3L7K5 were significantly more abundant at thermal stresses compared to control and also more abundant at 10°C when compared to 42°C (Supporting Information Table 5, worksheet 3). Thus, our results establish that grape cells employ either the invertase pathway or the sucrose synthase pathway, depending on their growth temperature. Similar findings have been observed in rice suspension cultures exposed to thermal stress [28]. The impact of exposure to thermal stresses could be a trigger for the cell to opt for sucrose breakdown via the classical pathway. The regulation of sucrose between the two pathways varies according to the availability of sucrose, growth stage, tissue function and the plant species [72–74]. Invertases have been proposed to function in response to cold and to channel sucrose into catabolism in contrast to sucrose synthase which channels sucrose into anabolism [74]. Sugars regulate cell growth by modulation of gene and protein expression, and thus sugar metabolism could be involved as either a protective mechanism or defence response in cells exposed to various thermal stresses.

3.9 Comparison to other proteomics studies of temperature stressed plant cells

Many studies on proteomic response of plant cells to temperature stress have been performed using 2DE. A study on shotgun proteomic analysis of a rice cultivar indicated upregulation of reactive oxygen scavenging and chaperone activity at extreme heat stress [75], as was observed in this study. In a very similar experimental design to this study, a clear switching between the classical and alternative pathway of sugar metabolism in response to extreme temperatures was observed in cultured rice cells [28]. Rice cells were cultured in suspensions at a control temperature of 28°C in a sucrose based medium and subjected to a three day exposure to two low temperature and two high temperature stresses differing by increments of 8°C. The differences in the experimental design was that in this study we used grape cells, the time of stress exposure was reduced (14 h) and the temperature points were different.

In two recent studies, Arabidopsis plant cells were exposed to different temperature stresses for proteomic analysis. In one study, the 2D DIGE approach was used to study Arabidopsis seedlings impacted with heat stress [76] while in the second study, differential protein expression pattern was observed during cold acclimation [77]. The first study indicated protein folding (chaperone) and cellular metabolism as the main stress response to heat-shock treatment. In the latter study, a comparative transcriptomic and proteomic analysis was performed to understand the mechanism of cold regulation and indicated that translational regulation could determine protein levels in stress conditions in plants.

4 Concluding remarks

The objective of this study was to identify proteins and biochemical pathways involved in heat and cold stress responses in grape cells. This analysis was done through protein expression in grape cell suspension cultures of the Cabernet Sauvignon variety exposed to four different temperatures stresses that ranged from very cold to very hot temperatures. Label-free quantitation using NSAF values was employed to explore differential protein expression patterns in response to temperature stress. Many of the identified proteins were labelled as putative uncharacterised proteins and we were able to provide a putative functional annotation for them after intensive manual searches in various databases, and also by association with GO annotations for known proteins. In nature, abiotic stresses generally occur as interacting factors and temperature stresses are often associated with drought or higher light intensities. To withstand and combat these stresses, plants employ alternate defence mechanisms such as enhanced water uptake through vascular systems. Such

responses are not possible in cell suspension cultures, which is one limitation of this study. However, specific molecular responses related to sugar metabolism and phenylpropanoid biosynthesis were clearly identified in grape cells exposed to thermal stresses. Our quantitative shotgun proteomics experiment provides molecular insights into thermal stress tolerance mechanisms in plants, and valuable information for further studies, such as confirmatory experiments to validate the protein expression changes. We have presented evidence for differential protein expression and regulation of thermal stress response proteins, and also for proteins involved in the phenylpropanoid biosynthesis pathway and carbohydrate metabolism. This study provides valuable insights into the specific regulatory and signalling networks involved in plant responses to thermal stress.

The MS proteomics data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [78]: dataset identifier PXD000977. PAH acknowledges support from the Australian Research Council Training Centre for Molecular Technology in the Food Industry and thanks Tony Cosgriff for continued support and encouragement. ISG acknowledges support from Macquarie University in the form of the iMQRES and PGRF scholarships. ISG thanks Chris Davies from CSIRO, for starter callus samples, Muhammad Masood for help at the plant growth facility and Martin William for continued encouragement.

The authors have declared no conflict of interest.

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CHAPTER 5

Chapter 5

5. Shotgun proteomic analysis of photoperiod regulated dormancy induction in grapevine

5.1 Synopsis of Publication VII (manuscript prepared for submission)

This chapter is written in a format as a manuscript prepared for publication. It reports protein analysis of photoperiod regulated dormancy induction in grapevines exposed to different photoperiods. In this study, protein changes were identified and quantified in two different genotypes of *Vitis,* exposed to two different photoperiods of long daylength and short daylength, and harvested at two different time points of shoot growth and axillary bud development. The primary aim of protein identification and quantification was to enhance our understanding of signalling networks associated with dormancy induction and growth cessation. This study provides valuable insights into proteomic analysis of bud dormancy induction in a short daylength and paradormancy maintenance in a long daylength in grapevine.

5.2 Key Findings

This study provided detailed mechanistic insights into signalling systems that initiate growth cessation and dormancy induction in grapevine. Proteomic analysis revealed genotype specific patterns of protein expression in the F2-110 genotype. Protein changes in the photoperiod comparison indicated that the short daylength treatment, especially at a 28 day time point, could contribute to dormancy induction.

We have demonstrated that long daylength shoots continue to grow and produce new shoots and leaves, while short daylength shoot tips maybe be involved in dormancy

induction. We have illustrated that various enzymes involved in glycolysis and dormancy induction were up-regulated in short daylength buds compared to long daylength. We also observed greater abundance of phenylpropanoid biosynthesis proteins at long daylengths, which may be a result of active growth in long daylength buds. Proteomic analysis also revealed genotype specific patterns of protein expression in the *V. riparia* F2-110 genotype.

5.3 Contributions

I executed 85% of all the experimental work which included protein extractions from the two different grapevine genotypes, which were exposed to two different photoperiods and harvested at two different time points. I calculated protein concentrations, digested proteins by the FASP in-solution method, performed all the mass spectrometry analysis, database searching, and data analysis. I interpreted the results and was responsible for 95% of the writing and manuscript preparation. This research was conducted under the supervision of Professor Paul. A. Haynes. We collaborated with Professor Anne. Y. Fennell from South Dakota State University, United States of America, who was in charge of growing the plant samples, exposing them to different photoperiods, harvesting the shoots and providing biological replicates of each condition for this research project.

5.4 Publication VII (manuscript prepared for submission)

Shotgun proteomic analysis of photoperiod regulated dormancy induction in grapevine.

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Abstract

The environmental regulation of bud dormancy varies among the diverse genotypes in grapevine. Certain grapevine genotypes become dormant in response to decreasing photoperiod and others require low temperature or both environmental cues to induce dormancy. This study used a proteomic approach to gain an understanding of the underlying molecular events involved in bud dormancy commitment. Two F_2 siblings (F2-110 and F2-040), with differences in photoperiod induced dormancy responsiveness were subjected to long day (LD, 15 hr, paradormancy maintenance or dormancy inhibition) or short day (SD, 13 hr, dormancy commitment) treatments. Proteins were extracted at two different time points of 28 days and 42 days of photoperiod exposure, from three biological replicates, and label-free quantitative shotgun proteomic analysis was performed. A total of 1577 non-redundant proteins were identified in the combined dataset of the eight different conditions (data available via ProteomeXchange with identifier PXD001627). Protein extracts were digested using Filter Aided Sample Preparation (FASP) coupled with gas phase fractionation (GPF). On average, approximately 930 proteins were identified in each replicate. Differential expression of proteins present in the F2-110 genotype after SD treatment indicated a genotype specific pattern of protein expression, as well as differential expression in response to photoperiod, especially at the 28 day time point. This provides detailed insights into molecular level events which occur during dormancy induction in grapevine. Increased abundance of photosystem proteins and phenylpropanoid biosynthesis pathway proteins were observed in LD buds as a result of active growth in LD buds, while buds became dormant at SD. Glycolysis, oxidative stress and various enzymes involved in endodormancy were abundant in SD buds.

1. Introduction

Grapes are grown all over the world and are gaining more prominence as an economically important fruit crop. Wine regions worldwide are spread across various climatic zones, contributing to the diversity of viticulture. Grapevines (*Vitis* spp.) grown in continental regions with temperate climates are generally exposed to characteristic cold and dry winters, which induce winter dormancy. Growth cessation and winter dormancy is a complex biological process programmed in response to annual environmental cues. This biological process is a significant aspect in breeding new grapevine cultivars suitable for continental climates. Daylength, temperature and water availability are some vital abiotic environmental signals for flowering, growth synchronization and dormancy induction in grapevine [1]. Daylength, also referred to as photoperiod, is the length of light exposure to plants. Daylength changes throughout the year according to geographic latitudes and seasons. It is one of the key environmental cues that grapevines employ to recognize seasonal changes and also to trigger various other biological processes, such as leaf growth, axillary branching, flowering, stem elongation or bud endodormancy [2, 3]. Cold acclimation and dormancy induction in grapevines native to temperate climates are often activated by exposure to short photoperiods [4]. *Vitis riparia* is a grapevine species adapted to temperate climates and is a native to North America. *V. riparia* is phylloxera resistant and is extensively used as a commercial rootstock, in rootstock breeding, and in hybrid scions. 'Seyval' is a hybrid wine cultivar derived from a complex hybridization of *Vitis vinifera* and *Vitis rupestris*, and is also phylloxera resistant. Gene expression and floral development during exposure to long and short photoperiods were investigated previously in *V. riparia* grapevine species and hybrid *Vitis* cultivar 'Seyval' via transcriptomic analysis [3, 5]. Growth cessation, shoot tip abscission and bud endodormancy are induced in *V. riparia*

by short photoperiod (SD), and are evident after 28 days, whereas growth is maintained and buds are paradormant in long photoperiods (LD) [6]. Genetic model systems have been used to exhibit differences in dormancy induction, and for identification of regulatory mechanisms involved in grape bud dormancy for breeding and mapping programs [1, 7]. Although the influence of photoperiod induced differential gene expression has been studied in different *Vitis* species by transcriptomic analysis [2, 3, 5, 8], the proteomics approach has been applied to shoots, but not to explore bud dormancy in grapevine [4]. Mass spectrometry based proteomics has aided in studying molecular networks and biological processes to enhance understanding of established transcriptomic studies, and also to discover information that is beyond the reach of transcriptomics.

Proteomics has been previously employed in a few studies in grapevine, where dormancy related biological processes such as bud development [9], and effects of different photoperiods [4] were investigated using two-dimensional (2D) gel electrophoresis. Gel based techniques have been predominantly used for proteomic studies in grapevine [9-14], with a few exceptions which involve shotgun proteomics techniques such as iTRAQ [15-18] and label-free quantitation techniques [19]. Many published proteomic studies are limited by the fact that they did not have access to a complete grape genome sequence; rather, they used very large databases compiled from all available plant species [10, 13-15, 18], or *Vitis* expressed sequence tags (ESTs) [11, 12] for protein identification. These approaches work reasonably well, but do not always present a complete picture. The grape genome sequence was released in 2007 [20, 21] and searching mass spectra against this sequence information provides better quality proteomic results.

Previous studies have documented the influence of phytohormones on seed and bud dormancy. Abscisic acid regulates dormancy and dormancy release stimuli in *Vitis vinifera* buds [22]. Another study had reported that mitochondrial activity, hypoxic conditions, ethylene metabolism and cell enlargement during bud dormancy release may be instrumental in understanding the dormancy-release mechanism [23]. Moreover, both quantitative proteomics and transcriptomic approaches have been employed to better understand the molecular basis of bud dormancy. This includes investigation of differential protein expression during dormancy induction, dormancy, and dormancy break in other plants such as poplar, Japanese apricot flower and tree peony (*Paeonia suffruticosa*) [24-26].

Shotgun quantitative proteomics is a powerful and widely used tool for protein identification from complex mixtures, including identification of the presence and absence of proteins, and differences in protein abundance, between samples. Quantitative shotgun proteomics has been used to study protein interactions in Cabernet Sauvignon grapevines exposed to water deficit conditions [19] and extreme hot and cold temperatures (Publication III). Shotgun label-free proteomics is renowned for its accuracy, reproducibility, high throughput [27] and is cheaper, less time-consuming, and less labour intensive, when compared to 2D gel based proteomic approaches.

We report the first shotgun label-free quantitative proteomic analysis of two F_2 siblings, F2-110 and F2-040, (generated from the self of F_1 from a cross between *Vitis riparia* and the hybrid *Vitis* cultivar 'Seyval') exposed to two different photoperiods of long day (LD, 15 hr, paradormancy maintenance or endodormancy inhibiting) and short day (SD, 13 hr, endodormancy inducing). In this study, we analysed the grape shoot proteome of two different grapevine genotypes, harvested after exposure to two different photoperiods, using Filter Aided Sample Preparation (FASP) [28, 29] coupled

with gas phase fractionation (GPF) [30]. It has been previously reported that FASP-GPF is an optimised fractionation technique for quantitative shotgun proteomics in grapevine (Publication II). Quantitation was based on spectral counting using normalised spectral abundance factors (NSAF) [31, 32]. Examining the molecular processes which are triggered by exposure to different photoperiods will enhance our understanding on the signalling networks involved in dormancy induction and growth cessation, and aid in identification of potential molecular markers that could facilitate selective breeding of grapevine cultivars that can adapt to continental climates.

2. Materials and Methods

2.1 Growth of plant material and imposition of photoperiod treatments

A mapping population of 141 individuals of F_2 hybrids were developed by selfing single F_1 plants from a cross between a North American grapevine species *Vitis riparia* (USDA PI 588289) and a hybrid *Vitis* cultivar 'Seyval' (Seyve-Villard 5-276) as described in [1]. Two individuals from this population, F2-110 and F2-040, each representing the photoperiod response phenotype most like one or the other grandparent (*V. riparia* or Seyval) for dormancy induction were selected and propagated for this study. One hundred and twenty plants each of two different genotypes; F2-110 ('*V. riparia* like') and F2-040 ('Seyval like') were generated. Potted, spur-pruned, 2- to 6-year-old vines of F2-110 and F2-040 were removed from cold storage and grown in long photoperiod (LD, 15 h) at 25/20 ± 3ºC day/night temperatures with 600–1,400 mol m^{-2} s^{-1} photosynthetic photon flux in a climate-controlled unshaded glass greenhouse (En Tech Control Systems Inc., Montrose, Minn.) in Brookings, South Dakota (44.3 N). Vines were grown in 19L pots at 1 pot per 0.4 m^2 with four shoots trained vertically. When the grapevines reached 12-15 nodes (30 days post bud break), they were

randomized into two groups for photoperiod treatments: LD or SD (15h, paradormancy maintenance or dormancy inhibition and 13h, dormancy induction, respectively). Five days after randomization (35 days post bud break), the differential photoperiod was imposed. Plants continued with LD and SD photoperiod treatment imposed under the same temperature conditions. SD was imposed using an automated white-covered black-out system (Van Rijn Enterprises Ltd, Grassie, Ontario). Each experimental unit was composed of ten vines and there were three replicates/plots for each genotype and time point in each photoperiod treatment. Buds were harvested for each experimental unit replicate between 8:30 and 11:30 a.m., at 28 and 42 days of the LD or SD treatments. Buds were harvested from nodes 3 to 12 from the shoot base. The buds were immediately frozen in liquid nitrogen and stored at -80ºC for future protein extraction. A total of 24 samples resulted (i.e. three biological replicates of F2-110 LD, F2-110 SD, F2-040 LD, F2-040 SD harvested at 28 d and 42 d respectively).

2.2 Protein extraction and protein assay

Approximately 1 g (fresh weight) of bud samples were ground in liquid nitrogen and proteins were extracted using the phenol-extraction protocol as described in [13]. Proteins were precipitated using methanol-chloroform [33]. Protein concentration was determined by the Pierce BCA protein assay (Thermo, San Jose, CA).

2.3 In-solution digestion and peptide extraction

Protein pellets were digested in-solution by a modified Filter Aided Sample Preparation (FASP) method as described in [28] and (Publication II). Protein extracts (250µg) were dissolved in 200µL 50% TFE, 0.1M NH_4HCO_3, 50mM DTT, heated (50°C, 20 min) and concentrated to 20µL in Amicon Ultra 0.5mL 30K ultrafiltration devices (Millipore).

An aliquot of 100µL 50% TFE, 0.1M NH_4HCO_3, 50mM iodoacetamide was added, incubated in the dark for 1 hr at room temperature and centrifuged (14,000g, 45 min). Alkylated proteins were washed using 200µL of 50% TFE, 0.1M NH_4HCO_3 (four times), centrifuged (14,000g, 45 min), and the flow through was discarded. To the ~20µL retentates in the ultrafiltration devices, 1µL of 0.25µg/µL Lys-C (Sigma) and 24µL of 50% TFE, 0.1M NH_4HCO_3 was added and incubated overnight at 30°C. Trypsin digestion followed Lys-C digestion by addition of 2.5µL of 1µg/µL trypsin (Promega), 350µL 20% acetonitrile (ACN), 50mM NH_4HCO_3 and incubation at 37°C for 8 hr. The reaction was stopped with 10µL 50% formic acid and resulting peptides were centrifuged into new ultrafiltration receptacles (14,000g, 45 min). This was followed by two rinses of the ultrafiltration devices using 100µL 50% ACN, 2% formic acid and centrifugation (14,000g, 45 min). Each extract was dried in a Speedvac to near dryness and reconstituted with 60µL 2% TFE, 2% formic acid.

2.4 Gas phase fractionation and nano LC-MS/MS

Mass ranges for gas phase fractionation (GPF) [30] were calculated using the online GPF calculator as described in [34] and (Publication III). The *Vitis vinifera* genome from UniProtKB (65,328 entries, March 2013) was used to calculate the optimised mass ranges based on theoretical trypsin digestion of all available grape protein sequences. Charge states of +2 and +3, and fixed cysteine modification of carbamidomethylation were used for calculation of four mass ranges between 400-1600 amu. The calculated m/z ranges were 400-506, 501-658, 653-913 and 908-1600 amu. Each FASP digest (10µL) of each biological replicate of each sample was injected as four fractions and scanned in the calculated four m/z ranges using a Velos Pro linear ion trap mass spectrometer (Thermo). Reversed phase columns were packed in-house to

approximately 8cm (100µm id) with Magic C18AQ resin (200Å, 5µm, Michrom Bioresources, California) in a fused silica capillary with an integrated electrospray tip. A pre-column was packed with PS-DVB (3cm, 100µM id, Agilent). An electrospray voltage of 1.8kV was used via a liquid junction upstream of the C18 column and samples were injected using an Easy-nLC II nanoflow high pressure liquid chromatography system (Thermo). Peptides were washed with Buffer A (2% v/v ACN, 0.1% v/v formic acid) at 500nl/min, 7 min, eluted with 0-50% Buffer B (99.9% v/v ACN, 0.1% v/v formic acid) at 500nl/min over 168 min and washed with 95% Buffer B at 500nl/min for 5 min. Spectra were acquired for 180 min for each fraction, totalling 12 hr per biological replicate, with overall 288 hr of data acquisition for the 24 samples. Automated peak recognition, MS/MS of the top nine most intense precursor ions at 35% normalisation collision energy and dynamic exclusion duration of 90 s [35] were performed using Xcalibur software, Version 2.06 (Thermo).

2.5 Protein identification

Raw files were converted to mzXML files, searched against a *V. vinifera* genome sequence database (65,328 entries, UniProtKB, March 2013) and processed using the global proteome machine (GPM) software (version 2.1.1, X!Tandem algorithm, freeware at http://www.thegpm.org) [36]. The four FASP fractions of each replicate were processed consecutively for each condition and merged to generate non-redundant output files of protein identifications with protein log (*e*) values less than -1, for each fraction. GPM search parameters included fragment mass error of 0.4 Da for peptide identification, complete modification of carbamidomethylation of cysteine, and potential oxidation of methionine and tryptophan. MS/MS spectra were searched against the *V. vinifera* database with additional searching against the reverse sequence database

to evaluate false discovery rates (FDR) [32]. Information for all identified peptides and proteins is available in the ProteomeXchange Consortium via the PRIDE repository [37] with the identifier PXD001627 (http://proteomecentral.proteomeexchange.org).

2.6 Data processing and filtering for high stringency datasets

The GPM outputs obtained from three biological replicates from each condition were combined to produce a single output file for each condition. The final output file of each condition contained only the proteins that were reproducibly identified in all three biological replicates of that condition, and the total number of spectral counts in the three biological replicates was a minimum of five. This transformed the low stringency protein identification data of individual replicates into high stringency datasets of reproducibly identified proteins present in each condition. Protein and peptide false discovery rates (FDRs) were calculated using the reversed database as described in [38]. Protein FDR = [(total number of reverse protein hits in the list/total number of proteins in the list) x 100] and peptide FDR = [2 x (total number of reverse peptide hits in the list/ total number of peptides in the list) x 100].

2.7 Quantitative proteomic and statistical analysis

Protein abundances were calculated using normal spectral abundance factors (NSAF), with an addition of a spectral fraction of 0.5 to all spectral counts to compensate for null values and permit log transformation for further statistical analyses [39]. The NSAF value for a protein *k* was calculated by diving the number of spectral counts (SpC, the total number of MS/MS spectra) identifying the protein (*k*) by the length of the protein (L), divided by the sum of SpC/L for all proteins (N) in the experiment. Corresponding NSAF values were used as measures of protein abundances. Student *t*-tests were

performed to identify proteins that were up-regulated and down-regulated in the different conditions. Two-sample unpaired *t*-tests were run on log transformed NSAF data, and proteins with a p-value <0.05 were considered to be differentially expressed between conditions. A protein was considered to be unique to a certain condition if spectra were present across three biological replicates in that condition while absent in one or more replicates in the other condition that it is compared against.

2.8 Gene Ontology information and functional annotation

Gene Ontology (GO) information was extracted from the UniProt database (uniprot.org) and matched to the list of identified proteins. In-house software developed using the R statistical programming framework (http://www.r-project.org/) and plotting gene ontology and annotation program (PloGO) was used to summarise the GO annotations [40]. Proteins were classified based on their biological processes using Web Gene Ontology Annotation Plot (WEGO) (wego.genomics.org.cn) [41]. GO annotations for each category of interest was summarised from a list of selected biological process GO categories for each comparison (up-regulated or down-regulated proteins). Proteins with known biological processes were classified using GO level 5 with biological process as the ontology type. NSAF abundance data were averaged for three biological replicates, summed and plotted to obtain an understanding of the overall protein abundance change in the different conditions for GO biological process categories of interest.

2.9 Qualitative and quantitative comparisons between the two genotypes, photoperiods and time points

Five different qualitative and quantitative comparisons were made of differentially expressed proteins between the two genotypes, photoperiod treatments and time points. The comparisons selected for further analyses were:

(i) Same genotype and different photoperiods

a. F2-110 LD vs. F2-110 SD at 28 d

b. F2-110 LD vs. F2-110 SD at 42 d

(ii) Different genotypes and same photoperiod

a. F2-110 SD vs. F2-040 SD at 28 d

b. F2-110 SD vs. F2-040 SD at 42 d

(iii) Same genotype, same photoperiod but different time points

F2-110 SD at 28 d vs. F2-110 SD at 42 d

Five separate student *t*-tests were performed between the above five comparisons to determine the statistical significance in fold changes of identified proteins and to investigate the up-regulated and down-regulated proteins and their associated pathways for each comparison. Protein expression data is described for one condition relative to the other condition. For example, in the comparison between F2-110 LD buds relative to F2-110 SD buds of same age, up-regulated in F2-110 LD = down-regulated in F2-110 SD and vice versa.

3. Results and Discussion

3.1 Shotgun proteomic data analysis

A total of 1577 non-redundant proteins were identified in the combined dataset from the eight conditions of F2-110 LD, F2-110 SD, F2-040 LD and F2-040 SD harvested at 28 d and 42 d, respectively. The number of proteins and peptides identified in the two different genotypes, at two different photoperiods, harvested after 28 d (4 weeks) are summarised in Table 1. The number of proteins and peptides identified in the two different genotypes, at two different photoperiods, harvested after 42 d (6 weeks) are summarised in Table 2. On average, around 2000 proteins (low-stringency number of proteins from Tables 1 and 2) were identified in all conditions with an average protein standard error of 3.9%. The number of low stringency peptides in each nanoLC-MS/MS run were comparable across all replicates with an average standard error of 6.6%, consistent with the 250 µg protein loading for each replicate. The high stringency number of reproducibly identified proteins ranged from 801 to 969 (Table 1) and 917 to 1037 (Table 2) across replicates harvested at 28 d and 42 d, respectively. Supporting Information Table 1 contains the list of 1577 non-redundant proteins identified in three biological replicates of the combined dataset in the conditions of F2-110 LD, F2-110 SD, F2-040 LD and F2-040 SD harvested at 28 d and 42 d. Supporting Information Table 2 summarises the list of proteins identified in each replicate, of each condition, with information on the peptide counts, total number of proteins identified and sum of peptides.

3.2 Presence and absence of proteins in the different genotypes, photoperiods and time points based on the five different comparisons

The proteins that were reproducibly identified in each condition were grouped so that only two different conditions (of either genotype or photoperiod or time point) were compared at a given time for further analysis. Five different comparisons were chosen (as explained in section 2.9) to investigate the presence/absence of proteins and their differential expression in terms of abundance. A total of 234 and 154 proteins were uniquely detected in the same genotype after 28 d of LD or SD, while a total of 199 and 225 proteins were uniquely detected after 42 d of exposure to LD and SD in F2-110 genotype buds (Table 3). Buds of the two different genotypes (F2-110 and F2-040) exposed to the same photoperiod (SD) treatment were compared and a total of 194 and 258 proteins were exclusively detected in F2-110 and F2-040 respectively, at 28 d of SD. Similarly at 42 d of SD, 237 and 185 proteins were exclusively identified from F2-110 and F2-040, respectively. A total of 154 proteins were uniquely detected in F2-110 SD plants harvested at 28 d, compared to 251 proteins uniquely detected at 42 d. The lists of proteins uniquely detected in each condition with annotation and spectral counts are given in Supporting Information Tables 3, 4 and 5.

3.3 Quantitative comparisons of differentially expressed proteins

The comparison between LD and SD of the F2-110 genotype was performed to study the protein changes due to the impact of shorter daylength. The proteins identified as being significantly differentially expressed ($p<0.05$) between the different conditions in the five comparisons are summarised in Table 4. At 28 d, 65 proteins were down-regulated and 66 were up-regulated in the F2-110 genotype, while 95 proteins were down-regulated and 58 proteins were up-regulated at 42 d of SD. A total of 105 and 111 proteins were significantly down-regulated in the F2-110 genotype when compared to the F2-040 genotype at 28 d and 42 d, respectively. Eighty five proteins were more

abundant in F2-040 genotype at 28 d and 65 were more abundant at 42 d. In the time point comparison of 28 d vs 42 d, 110 proteins were down-regulated and 72 were up-regulated at the 42 d time point in F2-110 genotype exposed to SD. Lists of up-regulated and down-regulated proteins identified in the five comparisons along with their p-values and average NSAF values are summarised in Supporting Information Table 6 (i. same genotype and different photoperiod), Supporting Information Table 7 (ii. different genotypes and same photoperiod) and Supporting Information Table 8 (iii. different time points). The proteins that were up or down-regulated in this study were also compared with the differentially expressed proteins identified in a similar study on *V. riparia* shoot tips exposed to LD (15 h) and SD (13 h) treatments investigated by 2DE [4]. Approximately 80% of the proteins identified in the previous 2DE based study were also found in this study, which indicated that we have captured most of the previously available information. However, in this study we identified a total of around 930 proteins compared to around 780 in the previous study [4], so this approach has yielded a considerable amount of new information.

3.4 Identification of differentially expressed proteins between photoperiod treatments LD and SD in F2-110 genotype

The up-regulated and down-regulated proteins between LD and SD, at 28 d and 42 d, were classified based on their biological process categories and their numbers and abundances were compared (Figure 1A and 1B). Significant differences were observed in proteins involved in carbohydrate metabolism, glycolysis, catabolic processes and response to oxidative stress, all of which were more abundant at SD when compared to LD, at both time points. Proteins involved in proteolysis, cellular component assembly, amino acid metabolism, photosynthesis and transport were more in number and

abundance in LD plants. Proteins that function in cellular component assembly were of two-fold greater abundance at LD 28 d (Figure 1A) and increased by twenty-fold at LD 42 d (Figure 1B). Proteins involved in organelle organisation were almost equal in number and abundance at LD 28 d, but showed six-fold increase in abundance at LD 42 d. The notable changes of abundances in categories that have relevant biological significance in relation to dormancy are discussed in further detail in the following sections in the comparison between LD and SD of the F2-110 genotype. Supporting Information Table 6 contains details on the up and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold changes for the comparison between same genotype and different photoperiods (i.e. comparison i).

3.4.1 Carbohydrate metabolism and glycolysis

Major changes in abundance were detected in proteins that function in carbohydrate metabolism and carbon assimilation in relation to exposure to LD and SD photoperiods. Export of carbohydrates to sink tissues is a necessary requirement for floral induction, as sucrose is required to fuel metabolism in the growing inflorescences. Many proteins involved in the Calvin Benson cycle, tricarboxylic acid (TCA) cycle and glycolysis were up-regulated at SD. The enzymes triose-phosphate-isomerase (A5BV65), glyceraldehyde-3-phosphate dehydrogenase (F6GSG7, F6HG44, A5AGZ1), phosphoglycerate kinase (A5CAF6), fructose-bisphosphate aldolase (A5B118) and transketolase (F6I1P0), all of which are involved in the Calvin Benson cycle, were up-regulated at SD, at either 28 d or 42 d. Proteins in the TCA cycle, that were up-regulated at SD 28 d, were aconitase (D7TEL2), citrate synthase (D7TMQ2), succinate semialdehyde dehydrogenase (F6H9T6) and acyl-coenzyme A oxidase (F6H4X3), while malate dehydrogenase (A5BPU3) was up-regulated at 42 d SD. Genes involved in

the TCA cycle have been reported to be in higher number in soybean during SD [42]. Quantitative differences in TCA cycle organic acids, sucrose and carbohydrate levels preceding floral transition stages have also been detected in maize by transcriptomic and metabolomic analyses [43].

In this study, we observed twelve enzymes that function in glycolysis to be more abundant at SD when compared to LD. These enzymes are illustrated in Figure 2 and their NSAF values are plotted. The twelve identified glycolytic enzymes are enolase (D7T227), phosphoglycerate kinase (A5CAF6), two different pyruvate kinases (F6I5U5, F6HVY1), phosphoglycerate mutase (C5DB50), three different types of glyceraldehyde-3-phosphate dehydrogenase (F6GSG7, F6HG44 and A5AGZ1), triose-phosphate-isomerase (A5BV65), fructose-bisphosphate aldolase (A5B118), phosphofructokinase (F6I7K1) and phosphoglucomutase (D7T1T9). Triose-phosphate-isomerase (A5BV65) was extremely up-regulated with nearly a fifty fold increase at 28 d SD. Glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase and fructose-bisphosphate aldolase have been previously identified as up-regulated at SD 28 d in a similar study, where *V. riparia* was investigated by 2D electrophoresis [4]. We also found that a large number of enzymes involved in starch accumulation were present at SD, similar to earlier studies in grapevine and tobacco [4, 44]. In another study, Arabidopsis grown at a 12 hr SD photoperiod showed high levels of glycolytic enzymes [45]. Two previous studies, one of which was performed at the transcript level in grapevine, suggested that the temporary induction of glycolysis could be a response to dormancy-induction stimuli [23, 46]. These results suggest that increased carbohydrate metabolism and greater abundance of glycolytic enzymes could lead to surplus carbon accumulation at SD.

Many photosynthetic proteins, such as photosystem II subunit proteins (A5B1D3, F6H8B4), light harvesting complex of photosystem II (F6GVX0) and light harvesting chlorophyll B-binding protein (F6H2E4), were more abundant at LD, while we observed down-regulation of photosynthesis at SD. This is similar to results from another grapevine study where photosynthesis related genes were down-regulated at SD [3], indicating that their down-regulation is part of the metabolic reprogramming during bud dormancy transitioning.

3.4.2 Phenylpropanoid biosynthesis

Proteins associated with phenylpropanoid metabolism were present at greater abundance at LD compared to SD at 42 d (Figure 1B). Anthocyanidin synthase (A2ICC9), chalcone-flavonone-isomerase-2 (A5ANT9) and putative flavanone 3-hydroxylase (A5ANR7) were up-regulated at LD 42 d. Anthocyanidin synthase (A2ICC9) showed almost forty fold increase at LD. Two of these proteins, anthocyanidin synthase and chalcone-flavonone-isomerase-2 (A2ICC9 and A5ANT9), were identified as up-regulated in our previous study using Cabernet Sauvignon grape cells exposed to extreme cold stress of 10°C (Publication III). Chalcone synthase, chalcone isomerase and anthocyanidin synthase (ANS) were also abundant at LD in *V. riparia* analysed by 2-DE [4]. The phenylpropanoid metabolic pathway is involved in the synthesis of phenolic compounds and various secondary plants products, including lignin. Production of secondary metabolites is known to be involved in cell wall metabolism and plant defence mechanisms against insects or infectious diseases. Phenylpropanoid biosynthesis has been observed to increase at LD in other plant species such as soybean [47] and Arabidopsis [48]. Greater phenylpropanoid abundance

is associated with cell wall metabolism which may be a result of active growth in LD buds.

3.4.3 Cytoskeletal development

Proteins associated with cellular component assembly were up-regulated at LD when compared to SD, at both 28 d and 42 d harvests (Figure 1A and 1B). Proteins in this category included cytoskeletal proteins, histone proteins and proteasome components. Microtubules, especially tubulins and actins, are fundamental elements of cytoskeletal development, cell division and plant growth. Tubulins are also known to be involved in signalling, abiotic stress and defence responses. Very recently, tubulins have been established as a marker for response to stress factors and abiotic stresses in grapevine suspension cells of the *V. rupestris* species [49]. We observed five tubulins to be up-regulated at LD (i.e. down-regulated in SD). The abundance pattern of the five tubulins (F6GUV7, F6HLZ6, F6HKY1, F6HUD5, F6HBQ3) and two histone proteins (A5B4J3 and D7T3I0) are shown in Figure 3. Actin proteins (A5BN09, A5AL11) and protein fate deciding units, such as proteasome domain proteins (F6HT17, D7U564 and D7SHA5) and ubiquitin conjugating enzyme (D7TVC2), were also more abundant at LD. We observed increased abundance of tubulins and proteasome subunits at LD, which was consistent with a previous study using 2-DE [4]. Short photoperiod is probably sensed as an abiotic stress in grapevine, as tubulins were down-regulated at SD. Tubulins have been previously observed to be down-regulated in drought conditions in rice roots [50]. Cell division, growth, light sensing and signalling are plant life cycle processes that are regulated by ubiquitin and 26S proteasome proteins [51], both of which were abundant at LD, indicating that all the above processes would occur at a higher rate in actively growing LD shoot tips. We also identified three isoforms of

aquaporins (aquaporin PIP- 2-1; Q5PXH0, aquaporin PIP2; Q2HZF5 and aquaporin TIP2-1; Q0MX09) as down-regulated at SD (28 d). Differential expression of aquaporins have been reported in water-deficit in rice [52] and cold stress in cotton [53]. Aquaporins also function in cell division [54] and floral organelle development [55].

3.4.4 Heat shock proteins

Many proteins involved in heat shock responses were abundant at SD compared to LD, as illustrated in Figure 4. Sixteen heat shock proteins (HSPs) of class II, HSP20, HSP70 and chaperones were significantly up-regulated at SD. Four of the identified HSPs (F6H3Q8, F6H3Q4, F6H3R0 and A5AQ47) displayed more than a ten-fold increased abundance at SD. Although the exposure of light and heat was for a shorter duration in the SD treatment, more heat shock proteins were abundant at SD, suggesting that SD is sensed as a signal for other biological processes such as dormancy induction or transition from the vegetative phase into the reproductive phase. In addition to their role in heat stress response, HSPs are known to function as molecular chaperones to regulate protein folding. Protein folding is associated with many processes such as seed germination, breaking of dormancy and growth resumption. HSPs expression and function have been confirmed in plant development processes including pollen and seed development [56]. HSP accumulation has also been observed during growth in seeds, seed pods and flowers, and during seed maturation in maize [57-59]. It has also been reported that heat shock proteins could be an alternative stimuli involved in grape-bud dormancy release [23].

3.4.5 Unknown proteins up-regulated at LD

Four proteins of unknown function were more abundant at LD (Figure 5); these were identified with Uniprot accessions F6HUX3, A5C9K6, D7SPM6 (LD 28 d) and F6HBC6 (LD 42 d). These four proteins should now be considered as long daylength responsive proteins as they were found abundant at LD in the F2-110 genotype. These unknown proteins may function in light sensing responses, temperature stress and in growth. Characterisation of these unknown proteins could provide important insights into molecular responses to photoperiod, as well as aid in developing potential markers that would facilitate selective breeding of grapevine cultivars. These proteins are listed in Supporting Information Table 6, and are found in worksheets named down-regulated in SD (42 d) and down-regulated in SD (28 d).

3.5 Identification of differentially expressed proteins between different genotypes of F2-110 and F2-040 of the short photoperiod treatment

Up- and down-regulated proteins between the two genotype comparisons were classified based on biological process categories and their numbers and abundances are plotted in Figure 6A (28 d) and 6B (42 d). Proteins in catabolic process, glycolysis, photosynthesis and cellular component assembly were more abundant at 28 d (Figure 6A) in the F2-110 genotype, which is more responsive to SD. It was noted that photosynthesis and glycolysis were abundant at the F2-110 genotype, both at 28 and 42 d. Photosystem II light harvesting complex, chlorophyll A/B binding proteins, light harvesting chlorophyll proteins, magnesium chelatase and peptidyl-prolyl-cis-trans-isomerase were abundant in the F2-110 genotype. Supporting Information Table 7 contains details of the up- and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold changes for the comparison between different genotypes exposed to the same photoperiod SD treatment (i.e. comparison ii).

3.5.1 Cellular component assembly

A contrasting difference in abundance patterns was observed in cellular component assembly proteins at 28 d and 42 d, where more proteins (greater number and abundance) of this category were present in the F2-110 genotype at 28 d (Figure 6A), while more proteins were abundant in the F2-040 genotype at 42 d (Figure 6B). The proteins in this category, that were abundant in F2-040 (42 d) included tubulins, histone and proteasome complex proteins. Nine alpha- and beta-chain tubulins, and a histone protein were significantly up-regulated in the F2-040 genotype, and their abundances are shown in Figure 7. The role of tubulins and proteasome components in cellular organisation, cytoskeletal development and protein fate were discussed earlier in section 3.4.3. Plant life cycle development processes such as cell division and growth, as well as protein fate deciding processes such as proteolysis, were more abundant in SD buds of the F2-040 genotype, especially after 42 days of treatment; this represents a distinct genotype-specific protein expression pattern.

3.6 Comparison of different time points F2-110 SD at 28 days vs. F2-110 SD at 42 days

In this comparison, the differential expression of proteins, described in terms of protein abundances, reflect the complex signals and pathways activated at different time points in SD buds of the F2-110 genotype. The abundance of the up- and down-regulated proteins were compared between 28 d and 42 d in the F2-110 genotype buds exposed to SD, and the results are illustrated in Figure 8. It was observed that most biological categories such as proteolysis, organelle organisation, cellular component assembly, amino acid metabolism, response to oxidative stress, catabolism, gene expression,

biosynthesis processes, phenylpropanoid metabolism, signal transduction and transport were up-regulated at 28 d. Increased abundance of oxidative stress proteins, transport and cellular trafficking proteins has been reported to function in the regulation and execution of grape-bud dormancy release [23]. A more detailed explanation on dormancy induction and oxidative stress are described in the next section. Supporting Information Table 8 contains details of the up- and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold change for the comparison between different time points of 28 d and 42 d, in F2-110 genotype exposed to SD (i.e. comparison iii).

3.6.1 Endodormancy and paradormancy

In grapevine, the switch to endodormancy ('true dormancy') requires the attainment of a quiescent phase, followed by signals within the bud leading to dormancy release [8, 46]. The scheme of bud endodormancy transitions in temperate perennial plants and the enzymes involved in these processes are illustrated in Figure 9A. Many enzymes involved in endodormancy induction and paradormancy maintenance processes were identified in the F2-110 genotype at SD. Moreover, we also observed that many of the identified enzymes were more abundant in the buds harvested at the SD 28 d time point (Figure 9C). Phytochrome (B9U4G3) was highly abundant at SD (Figure 9B), similar to a transcriptomic study where high levels were detected at SD in grapevine leaves [2]. Exposure to short photoperiods could function as a trigger for over expression of phytochrome, thus leading to endodormancy. Phytochrome gene expression is generally regulated by the circadian clock (in Arabidopsis and other herbaceous plants) but it is known to be regulated by photoperiod in grapevine [2]. Apart from mediating dehydration, a characteristic feature during dormancy development, phytochrome also

functions in directing the expression of the flowering locus in *Vitis* species, and in determining whether the meristem should commit to enter dormancy [7, 46, 60].

Enzymes including superoxide dismutase (A5B1Y5), glutathione-S-transferase (A5C352, A5AG54) ascorbate peroxidase (D7SKR5), sucrose synthase (F6HGZ9), alcohol dehydrogenase (D7U461, D7UBZ3), phosphoenolpyruvate carboxylase (F6H2N7) and glutathione peroxidase (D7TW03) were abundant in 28 d SD buds, while different types of alcohol dehydrogenase (F6I0F6), superoxide dismutase (O65199), glutathione-S-transferase (F6HPH1) and ascorbate peroxide (F6I106), all of which are involved in dormancy release, were abundant at 42 d SD (Figure 9C). Transcripts of alcohol dehydrogenases and sucrose synthase are considered markers for bud endodormancy [46]. The functional significance of sucrose synthase during grape bud-endodormancy release, especially in low oxygen conditions, is associated with the up-regulation of glycolysis [61]. Our study confirms this fact, as sucrose synthase (Figure 9C) and enzymes involved in glycolysis (section 3.4.1) were detected in high abundance after SD treatment. Sucrose synthase up-regulation has also been recorded in response to water deprivation [62]. In our study, we identified high levels of phytochrome at SD, which could initiate dehydration, and subsequent up-regulation of sucrose synthase to trigger endodormancy. Coordinated induction of sucrose synthase and alcohol dehydrogenase are known signals for bud burst [46, 61]. We observed that peroxidases and alcohol dehydrogenases were expressed as oxidative stress responses (abundant at SD). Superoxide dismutases and peroxidases are known to react with reactive oxygen species as part of antioxidant protection systems, while ascorbate peroxidases regenerate antioxidants. Redox reactions play an important role in endodormancy and bud break in temperate perennial plants [46]. Physiological examination, floral development and gene expression studies have suggested that SD has a potential role in

dormancy initiation and transition in grapevine [3, 8]. Our proteomic study confirmed that SD has a regulatory role in bud endodormancy, and provided further insights into molecular events involved in paradormancy maintenance and dormancy induction in *Vitis*.

4. Concluding Remarks

The objective of this study was to identify proteins and biochemical pathways involved in paradormancy maintenance and dormancy induction in grapevine. Proteomic investigation using label-free quantitation with NSAF values for abundance calculation was employed to study the molecular processes in grapevine shoots of different genotypes, exposed to different photoperiods and harvested at different time points. This study provided detailed mechanistic insights into signalling systems that are involved in paradormancy maintenance and dormancy induction. Greater abundance of phenylpropanoid biosynthesis proteins was observed in LD paradormant buds, and this could be associated with cell wall metabolism which may be a result of active growth in LD buds. This corresponded to responses found in LD *V. riparia* shoot tips [4]. In addition, photosystem proteins were up-regulated in LD buds, while SD showed a down-regulation of photosynthesis indicating metabolic reprogramming during dormancy induction. Moreover, glycolysis, oxidative stress and various enzymes involved in dormancy induction were up-regulated in SD buds compared to LD. It is noted that a similar response was found under SD in *V. riparia* shoot tips suggesting that changes in glycolysis are involved in both growth cessation and dormancy induction processes [4]. Both genotypes, F2-110 and F2-040 responded to photoperiod. The molecular basis of photoperiod regulated dormancy induction was well characterised at the protein level in the SD F2-110 genotype at 28 d and 42 d.

Development of grapevines that adapt to climate is a great challenge for breeding programs and our research gives valuable information for additional research, such as confirmatory experiments to validate the protein expression changes. Since these experiments were obtained using a grapevine mapping population, it would also be possible to combine proteomics and quantitative genetics with the aim of correlating observed changes in protein expression with protein quantity loci (PQLs), as has been pursued in other plant species [63-65]. This study has produced a better understanding of the proteomic control of dormancy induction, and will provide useful future directions for identification of potential markers that will facilitate selective breeding programs in grapevines.

Acknowledgements

PAH acknowledges the support from the Australian Research Council (ARC) through the Food Omics Research Centre. Anne Y. Fennell acknowledges support from the National Science Foundation (NSF) Program DBI0604755. ISG acknowledges support from Macquarie University for funding through the iMQRES scholarship and from travel grants from the Postgraduate Research Fund (PGRF) and Grape and Wine Research Development Corporation (GWRDC). The authors thank Dana Pascovici, Supakan Rattanakon and Shila Shahbazian for help during statistical analysis, data analysis and protein extractions respectively.

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Supporting Information Tables

Supporting Information Table 1. List of 1577 non-redundant proteins identified in three biological replicates of combined dataset in the conditions of F2-110 LD, F2-110 SD, F2-040 LD and F2-040 SD, harvested at 28 d and 42 d respectively, with the number of peptides identified in each replicate.

Supporting Information Table 2. List of proteins identified in each replicate, of each condition, with information on the peptide counts, total number of proteins identified and sum of peptides in each replicate.

Supporting Information Table 3. List of proteins uniquely identified in the same genotype (F2-110) and in different photoperiod treatments (LD and SD), at two time points (28 d and 42 d).

Supporting Information Table 4. List of proteins uniquely identified in the different genotypes (F2-110 and F2-040) and in same photoperiod treatment (SD), at two time points (28 d and 42 d).

Supporting Information Table 5. List of proteins uniquely identified at two time points (28 d and 42 d) in the F2-110 genotype at SD.

Supporting Information Table 6. List of up and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold change for the comparison between same genotype F2-110 and different photoperiods (i.e. comparison i), at 28 d and 42 d.

Supporting Information Table 7. List of up and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold change for the comparison between different genotypes exposed to the same photoperiod SD treatment (i.e. comparison ii), at 28 d and 42 d.

Supporting Information Table 8. List of up and down-regulated proteins, annotations, NSAF values, spectral counts, p-values and fold change for the comparison between different time points of 28 d and 42 d in the F2-110 genotype exposed to SD (i.e. comparison iii).

Figure Legends

Figure 1. Quantitative and qualitative comparison of significantly differentially expressed proteins obtained from two *t*-test comparisons of **A**. F2-110 LD vs F2-110 SD at 28 d and **B**. F2-110 LD vs F2-110 SD at 42 d. Up and down-regulated proteins in F2-110 SD relative to F2-110 LD were classified into 15 biological process categories using PloGO annotation. The number of proteins in each category and the corresponding abundance (sum NSAF values) were plotted adjacently. Protein expression data is described for F2-110 SD relative to LD (i.e up-regulated in SD = down-regulated in LD and vice versa).

Figure 2. Changes in abundance of enzymes involved in glycolysis in SD and LD buds of the F2-110 genotype. Higher NSAF value corresponds to higher abundance. The average NSAF was calculated for each protein from the three biological replicates of that condition. Nine enzymes which were up-regulated in SD are highlighted in the glycolysis pathway.

Figure 3. Abundance of cytoskeletal proteins in the comparison between LD and SD treatments, in the F2-110 genotype buds. Five tubulins and two histone proteins were up-regulated at LD.

Figure 4. Abundance of sixteen heat shock family proteins in SD compared to LD buds of the F2-110 genotype.

Figure 5. Four unknown proteins were up-regulated in LD F2-110 compared to SD. Average NSAF was calculated for each protein by averaging the NSAF values of the three biological replicates of that condition.

Figure 6. Quantitative and qualitative comparison of significantly differentially expressed proteins obtained from two *t*-test comparisons of **A**. F2-110 SD vs F2-040 SD at 28 d and **B**. F2-110 SD vs F2-040 SD at 42 d. Up and down-regulated bud proteins in F2-110 SD relative to F2-040 SD were classified into 14 biological process categories using PloGO annotation. The number of proteins in each category and the corresponding abundance (sum NSAF values) were plotted adjacently.

Figure 7. Abundance of cytoskeletal proteins expressed in the comparison between F2-110 and F2-040 genotypes exposed to short photoperiod (SD). Nine tubulins and a histone protein were up-regulated in the F2-040 genotype.

Figure 8. Quantitative and qualitative comparison of significantly differentially expressed proteins obtained from a *t*-test comparison of F2-110 SD 28 d vs F2-110 Sd 42 d. Up and down-regulated proteins at 28 d were classified into 15 biological process categories using PloGO annotation. The number of proteins in each category and the corresponding abundance (sum NSAF values) were plotted adjacently.

Figure 9. A. Schematic representation of enzymes involved in processes from bud differentiation until bud burst in grapevine. **B.** High abundance of phytochrome in SD buds compared to LD, calculated by the average NSAF values in three biological

replicates of LD and SD of the F2-110 genotype. **C.** Abundance of enzymes involved in dormancy induction in the F2-110 SD buds in the different time point comparison (28 and 42 days). The representation of this biological cycle in plants was derived from [41].

Figure 1

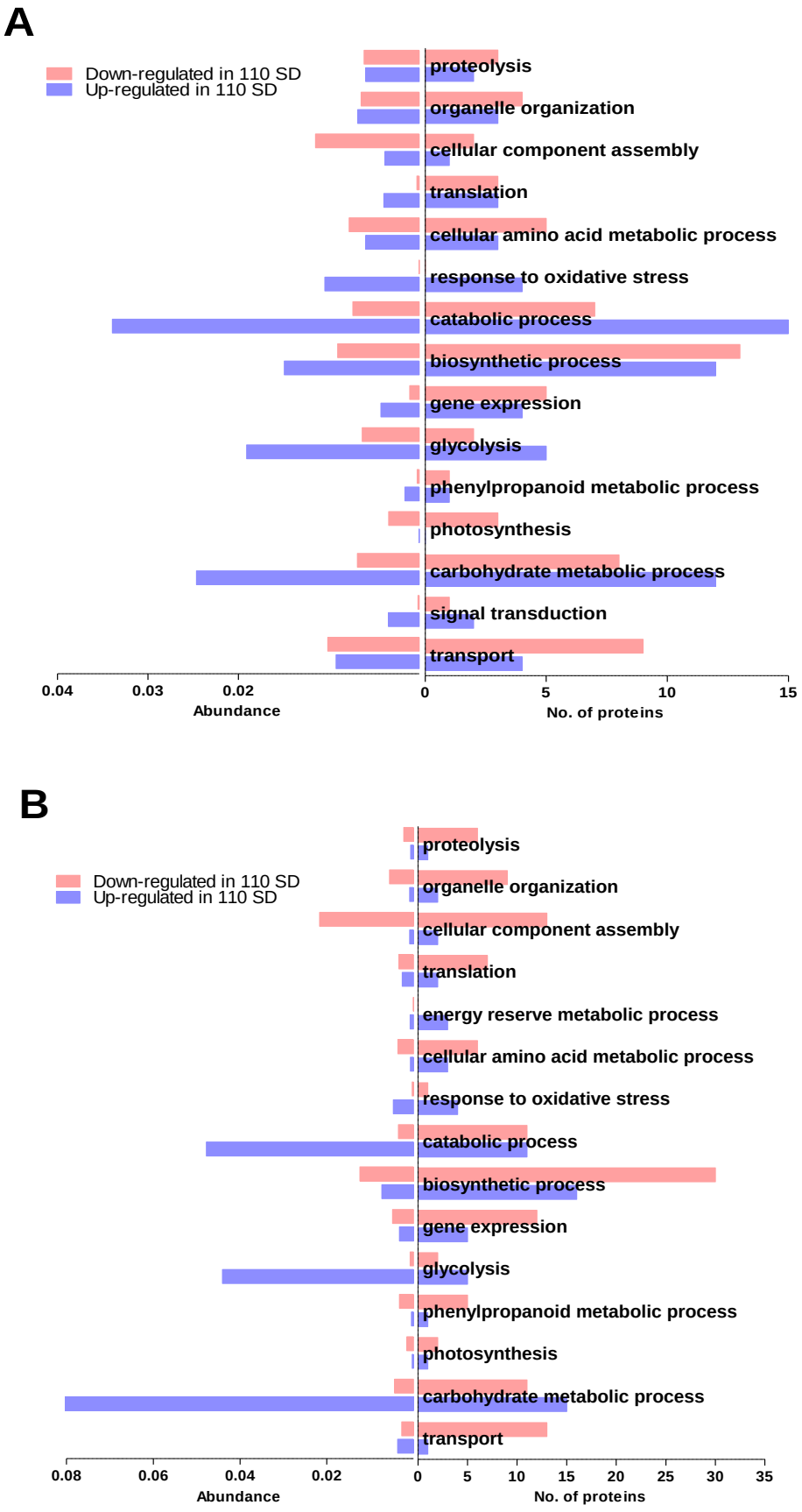

A
Down-regulated in 110 SD
Up-regulated in 110 SD
proteolysis
organelle organization
cellular component assembly
translation
cellular amino acid metabolic process
response to oxidative stress
catabolic process
biosynthetic process
gene expression
glycolysis
phenylpropanoid metabolic process
photosynthesis
carbohydrate metabolic process
signal transduction
transport
0.04
0.03
0.02
Abundance
0
5
10
15
No. of proteins
B
Down-regulated in 110 SD
Up-regulated in 110 SD
proteolysis
organelle organization
cellular component assembly
translation
energy reserve metabolic process
cellular amino acid metabolic process
response to oxidative stress
catabolic process
biosynthetic process
gene expression
glycolysis
phenylpropanoid metabolic process
photosynthesis
carbohydrate metabolic process
transport
0.08
0.06
0.04
0.02
Abundance
0
5
10
15
20
25
30
35
No. of proteins

Figure 2

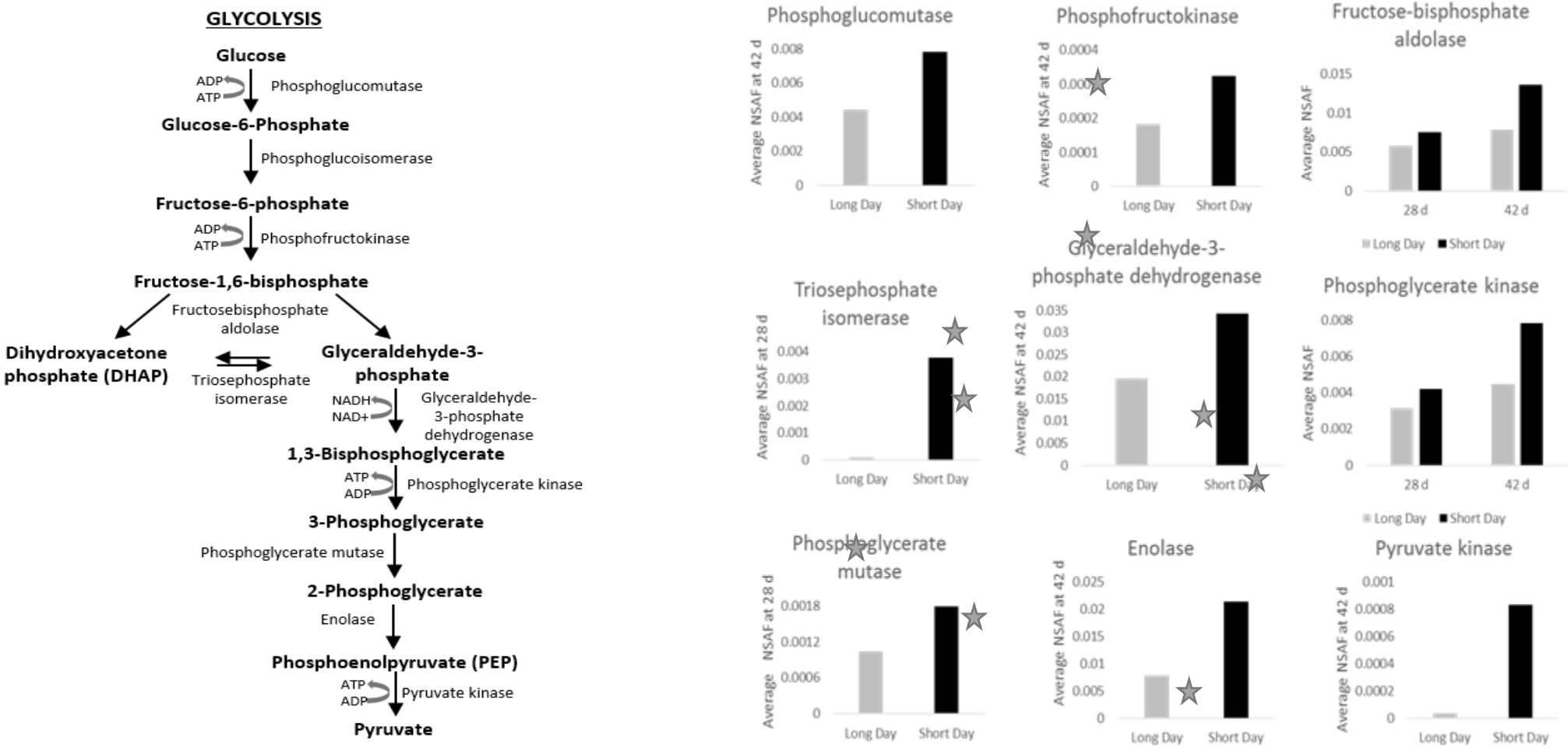

GLYCOLYSIS
Glucose
ADP
ATP
Phosphoglucomutase
Glucose-6-Phosphate
Phosphoglucoisomerase
Fructose-6-phosphate
ADP
ATP
Phosphofructokinase
Fructose-1,6-bisphosphate
Fructosebisphosphate aldolase
Dihydroxyacetone phosphate (DHAP)
Triosephosphate isomerase
Glyceraldehyde-3-phosphate
NADH
NAD+
Glyceraldehyde-3-phosphate dehydrogenase
1,3-Bisphosphoglycerate
ATP
ADP
Phosphoglycerate kinase
3-Phosphoglycerate
Phosphoglycerate mutase
2-Phosphoglycerate
Enolase
Phosphoenolpyruvate (PEP)
ATP
ADP
Pyruvate kinase
Pyruvate
Phosphoglucomutase
Average NSAF at 42 d
Long Day
Short Day
Phosphofructokinase
Average NSAF at 42 d
Long Day
Short Day
Fructose-bisphosphate aldolase
Average NSAF
28 d
42 d
Long Day
Short Day
Triosephosphate isomerase
Avarage NSAF at 28 d
Long Day
Short Day
Glyceraldehyde-3-phosphate dehydrogenase
Average NSAF at 42 d
Long Day
Short Day
Phosphoglycerate kinase
Average NSAF
28 d
42 d
Long Day
Short Day
Phosphoglycerate mutase
Average NSAF at 28 d
Long Day
Short Day
Enolase
Average NSAF at 42 d
Long Day
Short Day
Pyruvate kinase
Average NSAF at 42 d
Long Day
Short Day

Figure 3

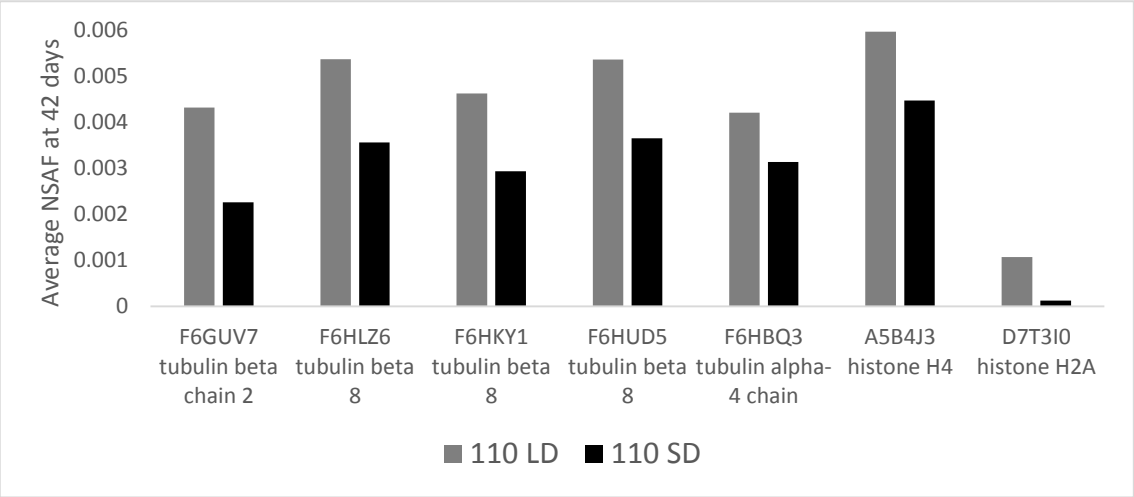


Figure 4

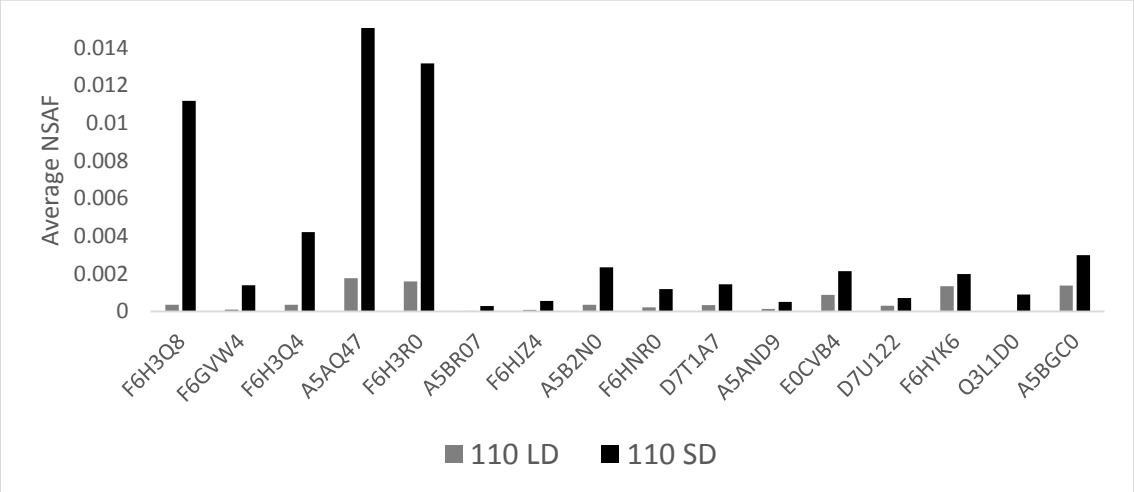


Figure 5

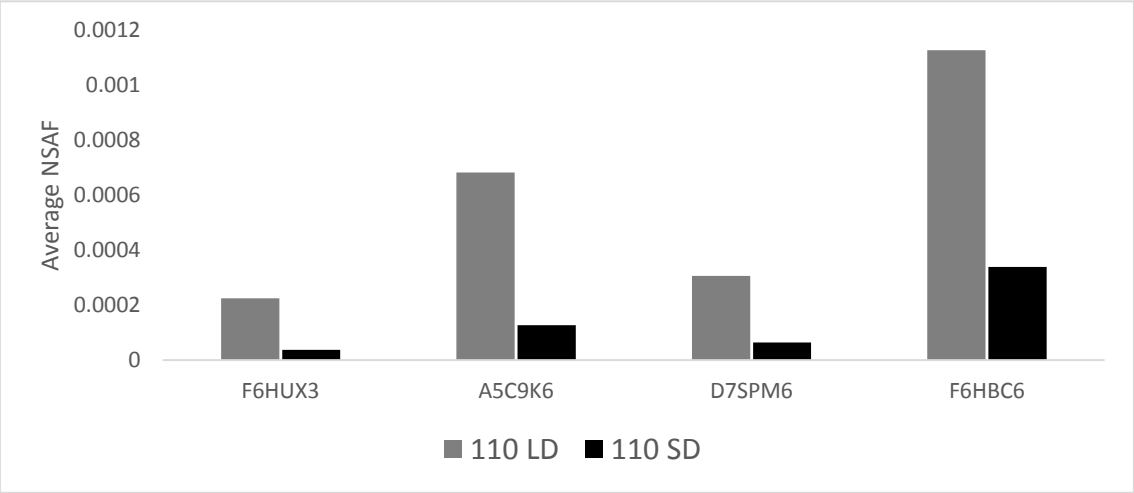

0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
Average NSAF
F6HUX3
A5C9K6
D7SPM6
F6HBC6
110 LD
110 SD

Figure 6

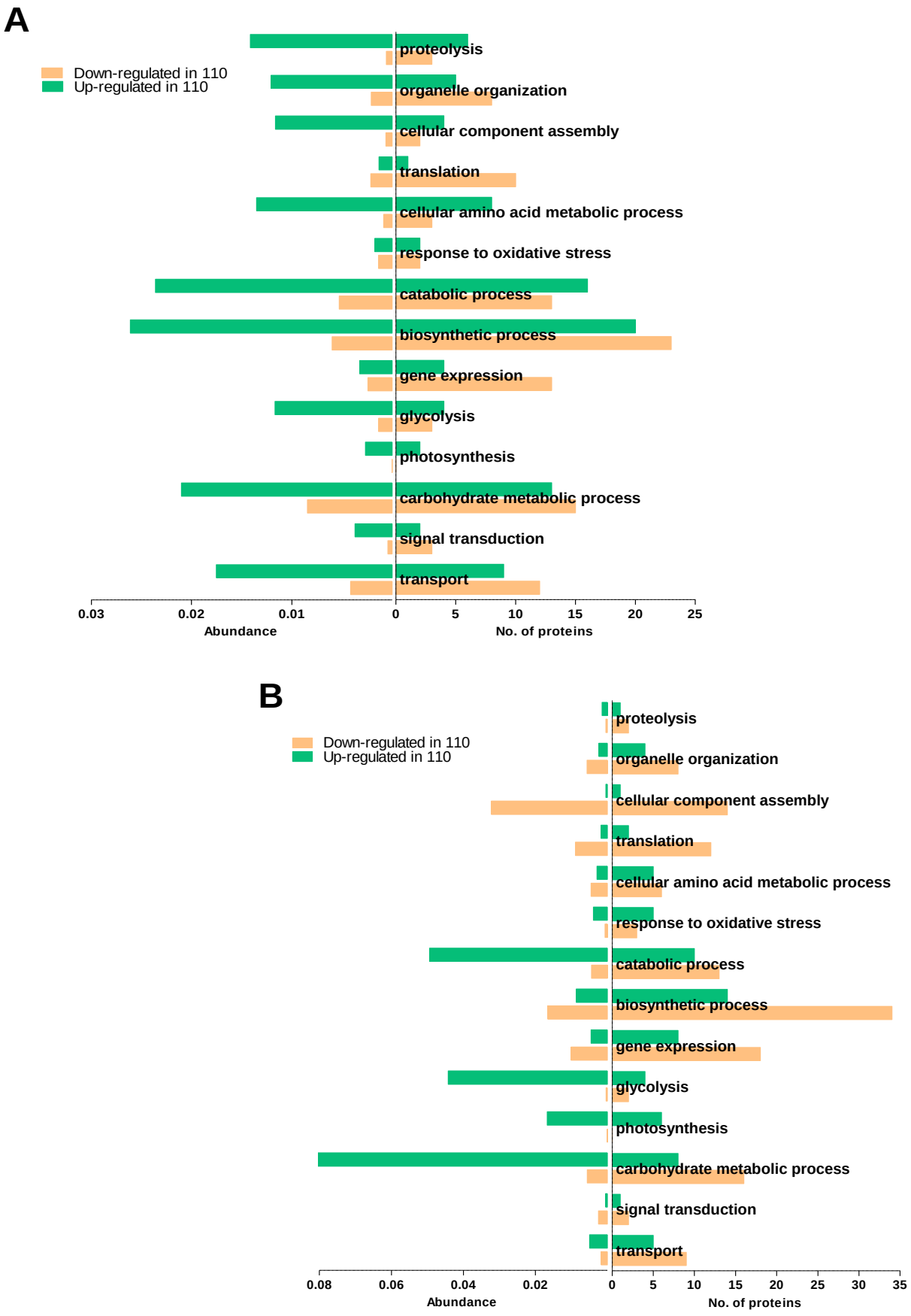

A
Down-regulated in 110
Up-regulated in 110
proteolysis
organelle organization
cellular component assembly
translation
cellular amino acid metabolic process
response to oxidative stress
catabolic process
biosynthetic process
gene expression
glycolysis
photosynthesis
carbohydrate metabolic process
signal transduction
transport
0.03
0.02
0.01
0
5
10
15
20
25
Abundance
No. of proteins
B
Down-regulated in 110
Up-regulated in 110
proteolysis
organelle organization
cellular component assembly
translation
cellular amino acid metabolic process
response to oxidative stress
catabolic process
biosynthetic process
gene expression
glycolysis
photosynthesis
carbohydrate metabolic process
signal transduction
transport
0.08
0.06
0.04
0.02
0
5
10
15
20
25
30
35
Abundance
No. of proteins

Figure 7

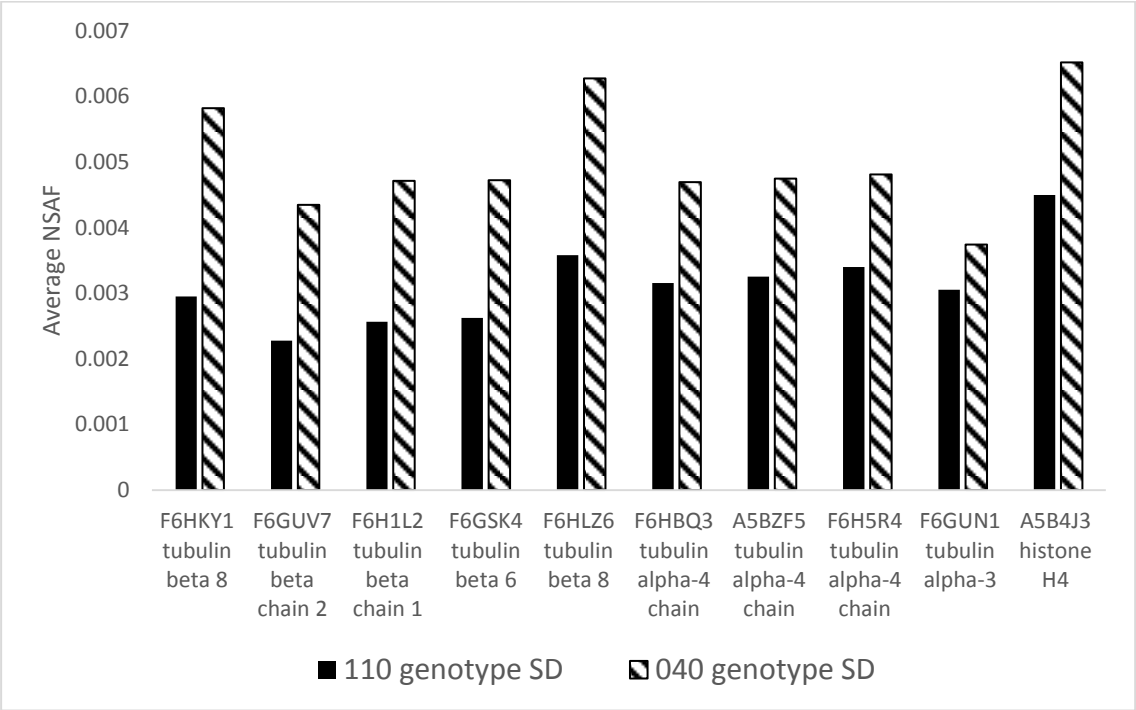
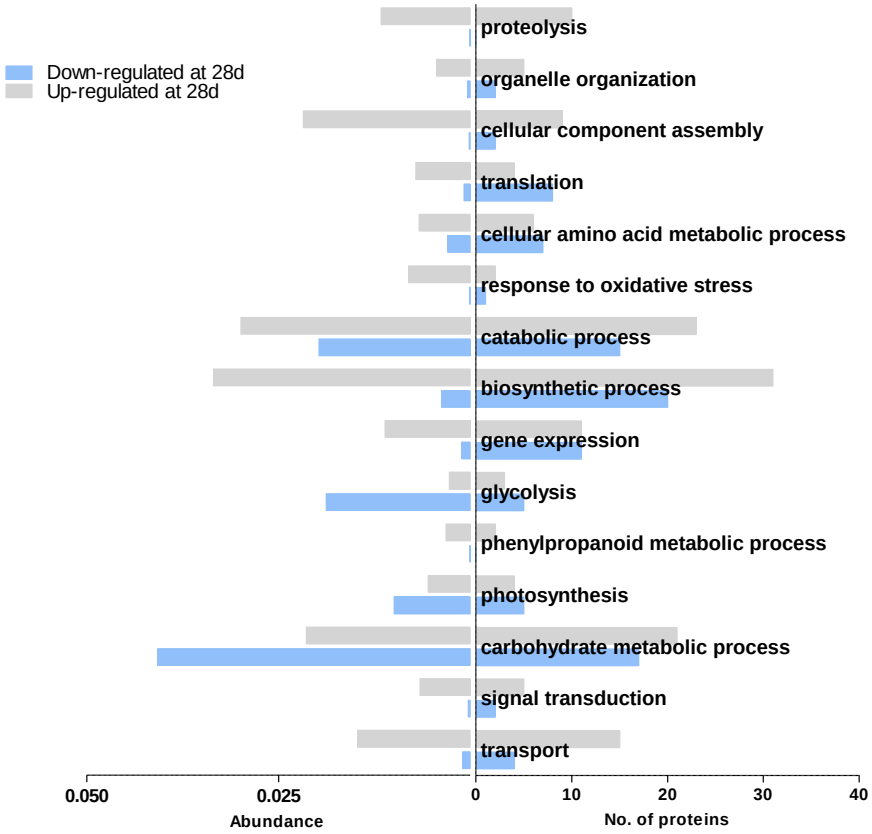

0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Average NSAF
F6HKY1 tubulin beta 8
F6GUV7 tubulin beta chain 2
F6H1L2 tubulin beta chain 1
F6GSK4 tubulin beta 6
F6HLZ6 tubulin beta 8
F6HBQ3 tubulin alpha-4 chain
A5BZF5 tubulin alpha-4 chain
F6H5R4 tubulin alpha-4 chain
F6GUN1 tubulin alpha-3
A5B4J3 histone H4
110 genotype SD
040 genotype SD

Figure 8


Down-regulated at 28d
Up-regulated at 28d
proteolysis
organelle organization
cellular component assembly
translation
cellular amino acid metabolic process
response to oxidative stress
catabolic process
biosynthetic process
gene expression
glycolysis
phenylpropanoid metabolic process
photosynthesis
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signal transduction
transport
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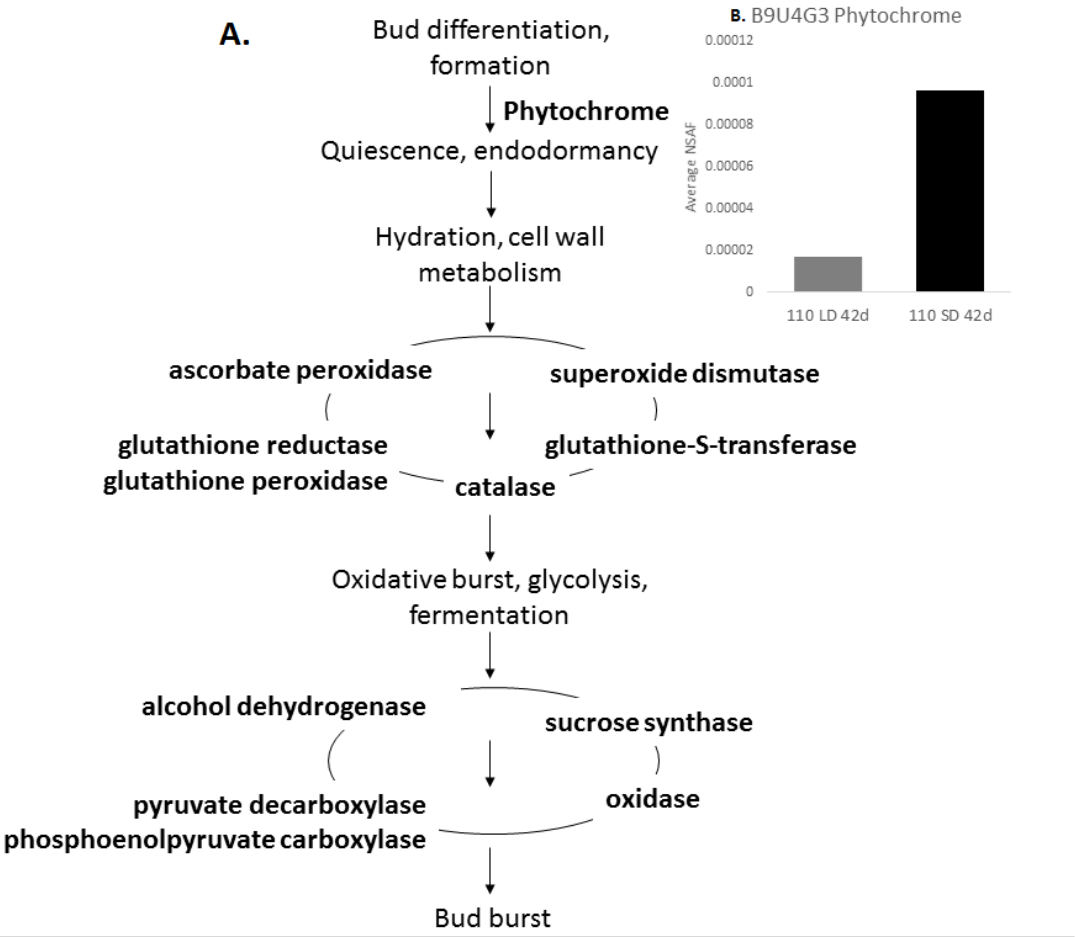
10
20
30
40
Abundance
No. of proteins

Figure 9


A.
Bud differentiation, formation
Phytochrome
Quiescence, endodormancy
Hydration, cell wall metabolism
ascorbate peroxidase
superoxide dismutase
glutathione reductase
glutathione peroxidase
glutathione-S-transferase
catalase
Oxidative burst, glycolysis, fermentation
alcohol dehydrogenase
sucrose synthase
pyruvate decarboxylase
phosphoenolpyruvate carboxylase
oxidase
Bud burst
B. B9U4G3 Phytochrome
Average NSAF
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
110 LD 42d
110 SD 42d

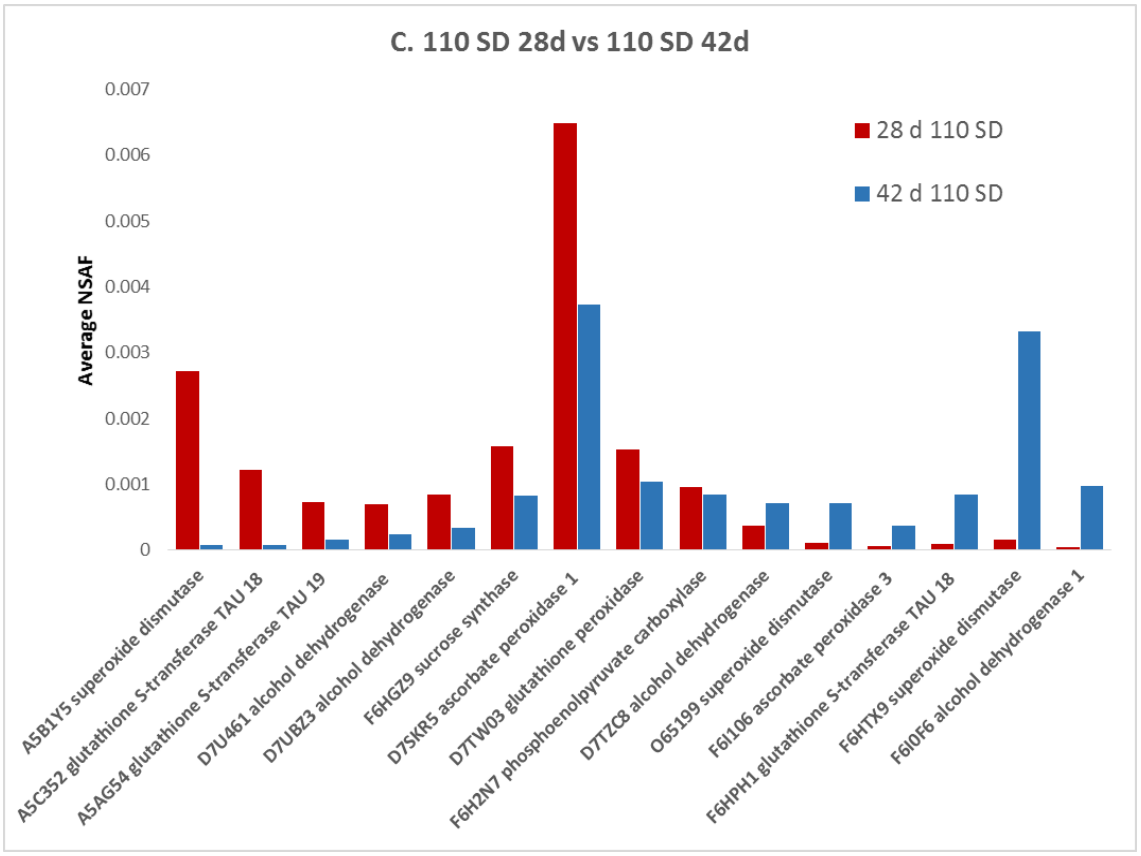

C. 110 SD 28d vs 110 SD 42d
Average NSAF
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
28 d 110 SD
42 d 110 SD
A5B1Y5 superoxide dismutase
A5C352 glutathione S-transferase TAU 18
A5AG54 glutathione S-transferase TAU 19
D7U461 alcohol dehydrogenase
D7UBZ3 alcohol dehydrogenase
F6HGZ9 sucrose synthase
D7SKR5 ascorbate peroxidase 1
D7TW03 glutathione peroxidase
F6H2N7 phosphoenolpyruvate carboxylase
D7TZC8 alcohol dehydrogenase
O65199 superoxide dismutase
F6I106 ascorbate peroxidase 3
F6HPH1 glutathione S-transferase TAU 18
F6HTX9 superoxide dismutase
F6I0F6 alcohol dehydrogenase 1

Table 1: Summary of protein and peptide identification data of F2-110 and F2-040 genotypes exposed to LD and SD photoperiods harvested after 28 days.

Genotype and photoperiod treatment (28 d)	Low stringency number of proteins identified			Low stringency count of peptides			Low stringency peptide FDR[b] (%)			Protein RSD[c] (%)	Peptide RSD (%)	High stringency number of proteins common to all three replicates
	R1[a]	R2	R3	R1	R2	R3	R1	R2	R3			
F2-110 LD 28 d	2111	2108	2071	11141	10977	11057	2.55	2.51	2.46	1.06	0.74	969
F2-110 SD 28 d	1961	2012	1806	10960	10702	9009	2.23	2.54	2.66	5.57	10.37	872
F2-040 LD 28 d	1815	1998	1804	9338	10349	9109	2.18	2.51	2.02	5.82	6.87	801
F2-040 SD 28 d	2000	2042	2151	10365	11130	11621	2.82	1.94	2.05	3.78	5.73	940

[a)] R1, R2 and R3 denote biological replicates 1, 2 and 3, respectively for proteins and redundant peptides and peptide FDR.

[b)] FDR, false discovery rate.

[c)] RSD, relative standard deviation for number of proteins and peptides in samples (n=3).

Table 2: Summary of protein and peptide identification data of F2-110 and F2-040 genotypes exposed to LD and SD photoperiods harvested after 42 days.

Genotype and photoperiod treatment (42 d)	Low stringency number of proteins identified			Low stringency count of peptides			Low stringency peptide FDR[b] (%)			Protein RSD[c] (%)	Peptide RSD (%)	High stringency number of proteins common to all three replicates
	R1[a]	R2	R3	R1	R2	R3	R1	R2	R3			
F2-110 LD 42 d	1977	2141	2171	11051	11158	11216	2.24	2.22	2.51	4.98	0.75	943
F2-110 SD 42 d	2185	2119	1939	14754	14386	11053	2.29	2.20	2.59	6.12	15.22	975
F2-040 LD 42 d	2289	2257	2186	13654	13713	12121	2.46	2.74	2.95	2.35	6.86	1037
F2-040 SD 42 d	2019	2053	2067	10371	10296	11528	2.47	2.68	1.99	1.21	6.44	917

[a)] R1, R2 and R3 denote biological replicates 1, 2 and 3, respectively for proteins, redundant peptides and peptide FDR.

[b)] FDR, false discovery rate.

[c)] RSD, relative standard deviation for number of proteins and peptides in samples (n=3).

Table 3: Classification of non-redundant proteins based on the presence and absence of proteins in the different genotypes, photoperiods and time points based on the five different comparisons. For each comparison, three biological replicates of one condition were compared against the other condition. A protein was considered to be unique to a certain condition if spectra were present across three biological replicates in that condition, while absent in one or more replicates in the other condition that it is compared against.

i. Same genotype, different photoperiods	
a. 28 days	F2-110 LD vs. F2-110 SD
Proteins detected in F2-110 LD only	234
Proteins detected in F2-110 SD only	154
b. 42 days	F2-110 LD vs. F2-110 SD
Proteins detected in F2-110 LD only	199
Proteins detected in F2-110 SD only	225
ii. **Different genotypes, same photoperiod**	
a. 28 days	F2-110 SD vs. F2-40 SD
Proteins detected in F2-110 SD only	194
Proteins detected in F2-40 SD only	258
b. 42 days	F2-110 SD vs. F2-40 SD
Proteins detected in F2-110 SD only	237
Proteins detected in F2-40 SD only	185
iii. Same genotype, same photoperiod, different time points	
F2-110 SD 28 days vs. F2-110 SD 42 days	
Proteins uniquely present in F2-110 SD - 28 days	154
Proteins uniquely present in F2-110 SD - 42 days	251

Table 4: Proteins differentially expressed between the two photoperiod treatments and two genotypes at two time points of 28 days and 42 days. **A**. Protein expression data is described for SD buds relative to LD buds of same age (i.e up-regulated in SD = down-regulated in LD and vice versa). **B.** Protein expression data is described for F2-110 buds relative to F2-040 buds of same age (i.e up-regulated in F2-110 = down-regulated in F2-040 and vice versa). C. Protein expression data is described for F2-110 SD buds harvested at 42 days relative to F2-110 SD buds harvested at 28 days (i.e up-regulated in F2-110 SD 42 d = down-regulated in F2-110 SD 28 d and vice versa).

A. 28 days	F2-110 LD vs. F2-110 SD	F2-110 SD vs. F2-040 SD
Down-regulated in F2-110 SD	65	105
Up-regulated in F2-110 SD	66	85
Unchanged	919	887
B. 42 days	F2-110 LD vs. F2-110 SD	F2-110 SD vs. F2-040 SD
Down-regulated in F2-110 SD	95	111
Up-regulated in F2-110 SD	58	65
Unchanged	974	931
C. Time point comparison	F2-110 SD 28 days vs. F2-110 SD 42 days	
Down-regulated in F2-110 SD 42 d	110	
Up-regulated in F2-110 SD 42 d	72	
Unchanged	891	

CHAPTER 6

Chapter 6

6. A new approach for validation: measurement of protein-fold changes by spectral counting to determine protein quantitation false discovery rates

6.1 Synopsis

All the previous chapters in this thesis are a culmination of studies employing quantitative label-free shotgun proteomics to investigate differential expression of proteins in grapevine exposed to various conditions such as temperature stresses (chapter 4) or photoperiod variations (chapter 5). In this thesis chapter, we introduce a new approach for validation of these proteomic experiments. We have investigated protein fold-changes in biological triplicates of Cabernet Sauvignon grape cells grown at a control temperature of 26°C against biological triplicates of another set of Cabernet Sauvignon grape cells grown at the same control temperature of 26°C. This experiment was devised to estimate the protein quantitation, rather than protein identification, false discovery rate in the control-control samples, and compare it to Cabernet Sauvignon grape cells grown at different temperature stresses (chapter 4) with the aim of validating our previous results. We measured a noise threshold level (protein quantitation false discovery rate) from the control-control comparison and evaluated whether the protein-fold changes in our previous experiments exceeded this cut-off level. This experiment was developed with the aim of supporting the results described in the other chapters in this thesis. In summary, we observed that our previous results did indeed report significant protein fold-changes, but there was a significant level of noise present in the data.

I was responsible for growing the grape suspension cells, protein extractions, mass spectrometry analysis, data analysis and result interpretation. I estimate my contribution

to be around 95% of the work in this chapter, which involved experimental work and data analysis prior to writing this thesis chapter. This data will be used for publication in future when we have additional data with similar analyses performed in different organisms.

6.2 Introduction

Mass spectrometric analysis by shotgun proteomics has been routinely employed for large-scale protein identifications from complex biological mixtures and has evolved over the years to become less descriptive and more quantitative. Of the many available methods of quantitation, spectral counting is one of the extensively used approaches for examining differential protein expression patterns [53, 58, 158-160]. Quantitative shotgun proteomic experiments based on spectral counting aim for the compilation of a set of reliable protein identifications covering the proteome as extensively as possible, as well as assessment of the validity of these identifications by protein false discovery rate (FDR) estimations. Over the last few years, several statistical tools and strategies have been developed for protein FDR estimations [161-163]. One such statistical tool is the Scrappy program which was developed in our laboratory in 2012, is freely available, and allows effective and reproducible quantitation based on normalised spectral abundance factor (NSAF) values [156]. In all the studies included in this thesis, we utilised NSAF values in conjunction with the Scrappy program for label-free quantitation [53, 156]. A function of one of the modules in Scrappy is to sort through two different biological samples under analysis, and produce a high stringency dataset which lists the differentially expressed proteins (i.e. up-regulated or down-regulated) and unchanged proteins identified, and quantify them between the two samples being compared. We employed this approach in Scrappy for obtaining highly reproducible

datasets and for calculating fold-changes in several previous publications [21, 22, 159, 164-166], as well as in chapters 4 and 5 in this thesis.

It is known that proteomic variations between biological samples can sometimes be reported due to false positive fold-changes caused by various factors, or can be hidden for proteins identified with low abundances. The field has gradually changed so that now it is considered a standard practice to make some attempt to verify the proteomic differences for identified proteins observed by label-free quantitation through orthogonal tools such as polymerase chain reaction (PCR), multiple reaction monitoring (MRM) or Western blotting [167, 168]. We considered Western blotting as a means of validating our previous results in this thesis, however, Western blotting using grapevine samples is limited in utility by the lack of availability of sufficiently specific antibodies. We also assessed that targeted quantitative mass spectrometric analysis, such as MRM, required a level of method development which was beyond the scope and time-frame of this PhD study and thesis.

In this chapter, we present a probabilistic framework that addresses the concern of validation of proteomic results. We employed a same - same control experiment where protein fold-changes were calculated between biological samples grown and processed in identical conditions. This control versus control experiment was designed to determine protein quantitation FDRs, as measured by spectral counting. By definition, these two samples should be very nearly identical and any protein fold changes uncovered can be considered as false discoveries, attributable to a variety of (mainly technical) reasons.

In this study, we examined the number of ‘differentially expressed’ proteins in biological triplicates of Cabernet Sauvignon cell cultures grown at 26°C compared against another set of biological triplicates of Cabernet Sauvignon cell cultures grown

under identical conditions. This data was used to estimate a protein quantitation FDR in the form of a control-control noise threshold level. This threshold level was used to analyse if the results from chapter 4 (control versus temperatures stresses) are significant protein fold-changes rather than noise. This experiment was performed with the aim of supporting the results described in the other chapters in this thesis.

6.3 Materials and Methods

6.3.1 Cell cultures

Cabernet Sauvignon grape cells were established and subcultured as suspension cell cultures on Grape Cormier Medium (GCM) as described in chapter 4. For this experiment, six biological replicates of suspension cell cultures were grown using identical growth conditions and growth media components at 26°C (control). Cells were harvested for the six biological replicates using cell strainers (BD Falcon, USA) and stored at -80°C.

6.3.2 Protein extraction, digestion and peptide extraction

Approximately 1 g fresh weight of cells from six biological replicates were used for protein extraction with 6 M Gn-HCl, 1% N-lauroylsarcosine, 10 mM EDTA, 0.1 M tricine, 5% β-mercaptoethanol, followed by sonication (5 min), incubation (75°C water bath, 10 min) and centrifugation (11,000 g, 6 min) as described in chapter 4. Proteins were precipitated using methanol-chloroform [154], and their concentrations were measured by the Pierce BCA protein assay (Thermo, San Jose, CA). The protocols used for protein extraction, in-solution digestion using FASP [155] and peptide extraction are described in chapter 4.

Aliquots (250 µg) of protein extracts from each of the six biological replicates were dissolved in 200 µL 50% TFE, 0.1 M NH_4HCO_3, 50 mM DTT and concentrated to 20 µL in Amicon Ultra 0.5 mL 30K ultrafiltration devices (Millipore). An aliquot of 100 µL 50% TFE, 0.1 M NH_4HCO_3, 50 mM iodoacetamide was added, incubated in the dark (1 hr, room temperature) and centrifuged (14,000 g, 45 min). Alkylated proteins were washed four times using 200 µL of 50% TFE, 0.1 M NH_4HCO_3, centrifuged as above, and the flow through was discarded. To the ~20 µL retentates in the ultrafiltration devices, 1 µL of 0.25 µg/µL Lys-C (Sigma) and 24 µL of 50% TFE, 0.1 M NH_4HCO_3 was added and incubated (30°C, overnight). Trypsin digestion was performed by addition of 2.5 µL of 1 µg/µL trypsin (Promega), 350 µL 20% acetonitrile (ACN), 50 mM NH_4HCO_3 and incubation (37°C, 5 hr). Enzymatic digestion was stopped with 10 µL 50% formic acid and the resulting peptides were centrifuged into new ultrafiltration receptacles (14,000 g, 45 min), followed by two rinses of the ultrafiltration devices using 150 µL 50% ACN, 2% formic acid and centrifugation as above. Each extract was dried in a Speedvac and reconstituted with 60 µL 2% TFE, 2% formic acid.

6.3.3 Gas phase fractionation, chromatography and tandem MS/MS

As described in chapter 4, the *V. vinifera* genome from UniProtKB with 65,328 entries (March 2013), charge states of +2, +3 and modification of carbamidomethylation were used to calculate the optimised mass ranges of 400-506, 501-658, 653-913 and 908-1600 amu based on theoretical trypsin digestion [143].

An aliquot of 10 µL of each FASP digest of each biological replicate was injected as four fractions and scanned in the four calculated m/z ranges using a Velos Pro linear ion trap mass spectrometer (Thermo). Reversed phase columns packed in-house (~8 cm,

100 µm id) with Magic C18AQ resin (200 Å, 5 µm, Michrom Bioresources, California) in a fused silica capillary with an integrated electrospray tip and a pre-column packed with PS-DVB (3 cm, 100 µM id, Agilent) were used. Spray voltage of 1.8kV via a liquid junction upstream of the C18 column was used and samples were injected using an Easy-nLC II nanoflow high pressure liquid chromatography system (Thermo). Peptides were washed with Buffer A (2 % v/v ACN, 0.1 % v/v formic acid) at 500 nl/min, 7 min, eluted with 0-50 % Buffer B (99.9 % v/v ACN, 0.1 % v/v formic acid) at 500 nl/min over 168 min and washed with 95 % Buffer B at 500 nl/min for 5 min. Spectra were acquired for 180 min for each fraction, totalling 12 hr per biological replicate. Automated peak recognition, MS/MS of the top nine most intense precursor ions at 35% normalised collision energy and dynamic exclusion duration of 90 s were performed using Xcalibur software, Version 2.06 (Thermo).

6.3.4 Protein identification and data processing

Database searching using GPM software (version 2.1.1, X!Tandem algorithm) [169] was performed as described in chapter 4, with the same *V. vinifera* genome sequence containing 65,328 entries, from UniProtKB (March 2013). Raw files were converted to mzXML files and processed using GPM. The four FASP fractions of each replicate were processed consecutively and merged to generate one non-redundant output file of protein identifications with protein log (*e*) value less than -1. GPM search parameters included fragment mass error of 0.4 Da for peptide identification, complete modification of carbamidomethylation of cysteine, and potential oxidation of methionine and tryptophan. MS/MS spectra were also searched with additional searching against the reverse sequence database. Each output file for each replicate was of low stringency and was converted to a highly stringent dataset by further processing.

For a protein to be considered as a valid hit in a one set of control condition, it had to fulfil the two requirements of:

(i) the protein must be present in all three biological replicates of one condition and

(ii) the total spectral counts for the protein from all three biological replicates of that condition must be a minimum of five. Thus, low stringency data was converted to high stringency data by retaining only protein identifications which fulfilled these two criteria.

6.3.5 Control - control experiment and statistical analysis

This control-control experiment was designed to determine the amount of noise present within biological replicates grown in the same growth conditions. This noise can affect the fold-changes reported for identified proteins in experiments comparing one sample against another. In order to avoid bias in the results caused by one experiment possibly being slightly different from the others, the low stringency output files from the six biological replicates were re-sorted into each of the ten possible different combinations to obtain comparisons of all possible combinations of one set of triplicates versus the other triplicate set.

The six biological control replicates were labelled as Control 1 to Control 6. The ten different combinations that were used for analysis are:

Combination 1: Controls 1, 2, 3 compared to Controls 4, 5, 6

Combination 2: Controls 1, 2, 4 compared to Controls 3, 5, 6

Combination 3: Controls 1, 2, 5 compared to Controls 3, 4, 6

Combination 4: Controls 1, 2, 6 compared to Controls 3, 4, 5

Combination 5: Controls 1, 3, 4 compared to Controls 2, 5, 6

Combination 6: Controls 1, 3, 5 compared to Controls 2, 4, 6

Combination 7: Controls 1, 3, 6 compared to Controls 2, 4, 5

Combination 8: Controls 1, 4, 5 compared to Controls 2, 3, 6

Combination 9: Controls 1, 4, 6 compared to Controls 2, 3, 5

Combination 10: Controls 1, 5, 6 compared to Controls 2, 3, 4

Protein abundances were calculated using normal spectral abundance factors (NSAF) [53], with an addition of a spectral fraction of 0.5 to all spectral counts to compensate for null values and permit log transformation for further statistical analyses [156].

Student *t*-tests were performed to identify proteins that were up-regulated and down-regulated in the proteins reproducibly identified in the two sets of control conditions, for all ten combinations. Two-sample unpaired *t*-tests were run on log transformed NSAF data, and proteins with a p-value <0.05 were considered to be differentially expressed between the ten combinations. The number of up-regulated, down-regulated and unchanged proteins were recorded in each *t*-test analysis.

The total spectral count cut-off (in triplicates) was chosen to be a minimum of five in this experiment, to keep it as a constant parameter for comparison; data analyses in chapters 4 and 5 of this thesis were also performed with a minimum spectral cut-off value of five. The percentage of up-regulated and down-regulated proteins was calculated by dividing the sum of up-regulated and down-regulated proteins by the total number of proteins identified in each of the ten combinations. These percentages obtained from the ten combinations were averaged to obtain the false discovery rate for protein quantitation for this experiment (represented as a percentage).

$$\text{Protein quantitation FDR} = \frac{\text{Sum of up-regulated and down-regulated proteins}}{\text{Total number of proteins identified}} * 100$$

6.3.6 Minimum spectral count analysis

We repeated the two-sample unpaired *t*-tests for the ten combinations another seven times, by altering the minimum number of total spectral cut-off (in triplicates) to 3, 4, 6, 7, 8, 9 and 10. This was performed to identify the number of up-regulated, down-regulated and unchanged protein in each of the ten combinations, when the minimum spectral cut-off value ranged from three to ten peptides. The average number of up-regulated, down-regulated and unchanged proteins were calculated from the ten combinations and protein quantitation FDRs were calculated for each spectral cut-off value.

6.3.7 Comparison of false discovery rates of protein quantitation of control versus control and control versus temperature stresses

The average false discovery rate of protein quantitation obtained from the control-control experiment (26°C) was compared to false discovery rates of protein quantitation calculated for *t*-test results extracted from chapter 4. Protein quantitation FDRs were calculated for four different comparisons of control versus moderate hot (34°C), control versus extreme hot (42°C), control versus moderate cold (18°C) and control versus extreme cold (10°C). This analysis was performed to observe if the newly developed protein quantitation false discovery rate threshold (from control-control) was lower than the protein quantitation false discovery rates obtained from samples grown at different temperature conditions.

6.4 Results and Discussion

6.4.1 Protein identification and *t*-test comparisons

The low stringency number of proteins and peptides identified from each of the six biological replicates of Cabernet Sauvignon grape suspension cells grown at 26°C are shown in Table 1. On average, there were approximately 2900 proteins and 18000

peptides identified in each of the control replicates, indicating consistency in each LC-MS/MS run, in accordance with the standardised 250 µg of protein loading. The minor differences in protein numbers between replicates could be due to biological variability between the control samples grown in different culture flasks, or could be caused by a number of different technical variations. The average protein and peptide relative standard deviations were 4.7% and 7.3% respectively; both less than 10%, which is the usual benchmark we use for acceptable reproducibility in our laboratory. Supporting Information Table 1 contains the lists of proteins identified in each of the six replicates of the control samples along with their annotations and total number of peptides.

The low stringency files obtained from the six replicates were sorted into sets of triplicates in ten combinations, and concurrently converted to high stringency data, as stated in section 6.3.5, to observe fold-changes between the control sample sets. Table 2 represents the number of unchanged, up-regulated and down-regulated proteins identified in the ten combinations of control-control. For comparative purposes the table also includes data reproduced from chapter 4 - comparisons of control temperature (26°) to the different temperature stresses of 10°C, 18°C, 34°C and 42°C.

Sample	Low stringency number of proteins	Low stringency number of peptides
Control 1	3157	20199
Control 2	3109	19151
Control 3	2983	17234
Control 4	2777	18006
Control 5	3015	20188
Control 6	2895	17289
Average	2990	18678
RSD (±%)	4.7%	7.3%

Table 1. Summary of protein and peptide identification data of six biological replicates of Cabernet Sauvignon cell suspension cultures grown at control (26°C).

t-test comparisons	Unchanged proteins	Up-regulated proteins	Down-regulated proteins	Up/down regulation (%)

6.4.2 Determination of protein quantitation FDR

The control-control experiment was designed to determine the protein quantitation FDR by the measurement of protein fold-changes calculated by spectral counting. This analysis was based on the assumption that very few proteins should be differentially expressed between the control-control comparisons. However, we observed that some proteins were significantly differentially expressed ($p<0.05$) in the ten combinations of control-control analysis. We also observed that the number of up-regulated, down-regulated and unchanged proteins were different for each combination (Table 2). This differential expression pattern of proteins in the ten combinations was utilised to calculate the protein quantitation FDR for each permutation as stated in section 6.3.5 and was expressed as a percentage (Table 2). The protein quantitation FDR ranged from 3.4% to 9.4% for the control samples in the combinations from 1-10. On average, approximately 30 proteins were down-regulated and 40 proteins were up-regulated between the control samples while around 1100 protein remained unchanged. From this data, the average value of protein quantitation FDR was calculated to be 6%. This 6% limit was therefore considered as the threshold of noise for further analysis and comparison to our previous results.

Combination 1	1080	75	37	9.40
Combination 2	1083	51	23	6.40
Combination 3	1124	33	23	4.75
Combination 4	1086	51	19	6.06
Combination 5	1100	27	30	4.93
Combination 6	1118	17	22	3.37
Combination 7	1102	35	25	5.16
Combination 8	1075	37	52	7.65
Combination 9	1109	36	36	6.10
Combination 10	1105	35	35	5.96
Average control vs control	**1098**	**40**	**30**	**6**
Control vs moderate hot (34°C)	1081	88	132	16.91
Control vs extreme hot (42°C)	831	116	369	36.85
Control vs moderate cold (18°C)	1131	95	77	13.20
Control vs extreme cold (10°C)	1030	145	170	23.42

Table 2. Student *t*-tests results indicating number of unchanged and significantly differentially expressed proteins (up-regulated or down-regulated proteins with a p-value <0.05), for the ten control-control combinations and in the comparisons of control to temperature stresses of heat and cold. The protein quantitation false discovery rate is shown in the last column as a percentage of up or down regulation.

The number of proteins that were statistically significantly changed, and the number of proteins that were unchanged in the different control-control combinations, as analysed by two-sample unpaired *t*-tests, are illustrated in Figure 1. The last segment in the figure is an average of up-regulated, down-regulated and unchanged proteins, calculated from the ten permutations.

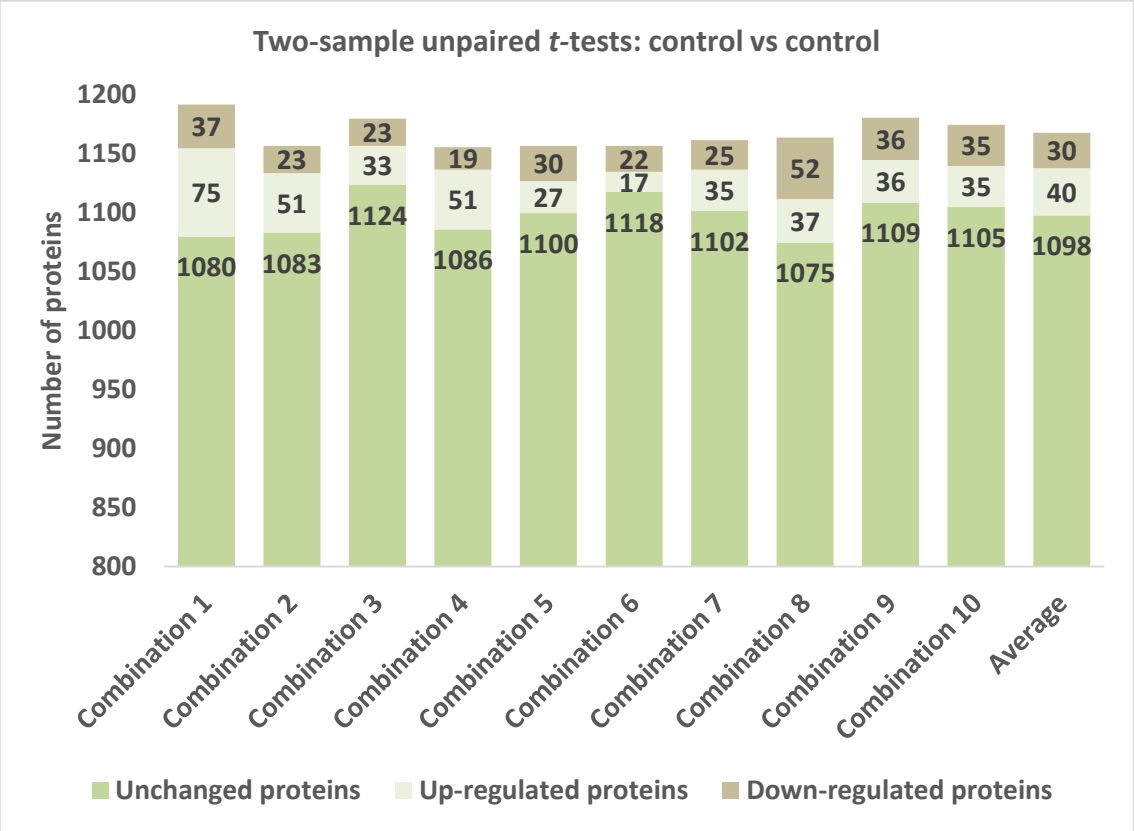


Figure 1. Proteins classified as unchanged, up-regulated and down-regulated from each two-sample *t*-test for the ten different combinations, with a minimal spectral cut-off value of five. The average number of unchanged, up-regulated and down-regulated proteins from the ten combinations are shown as the last data bar termed 'Average'.

6.4.3 Comparison of protein quantitation FDRs calculated from different minimum spectral cut-off values

The entire analysis of the control-control experimental data as shown in Figure 1 was repeated another seven times, each time applying a different minimum spectral cut-off value from three to ten, as stated in section 6.3.6. The average numbers of down-regulated, up-regulated and unchanged proteins of the ten combinations for each spectral cut-off value are shown in Figure 3. Protein quantitation FDRs were calculated as before for each of the cut-off value analyses and graphed as a percentage in Figure 4.

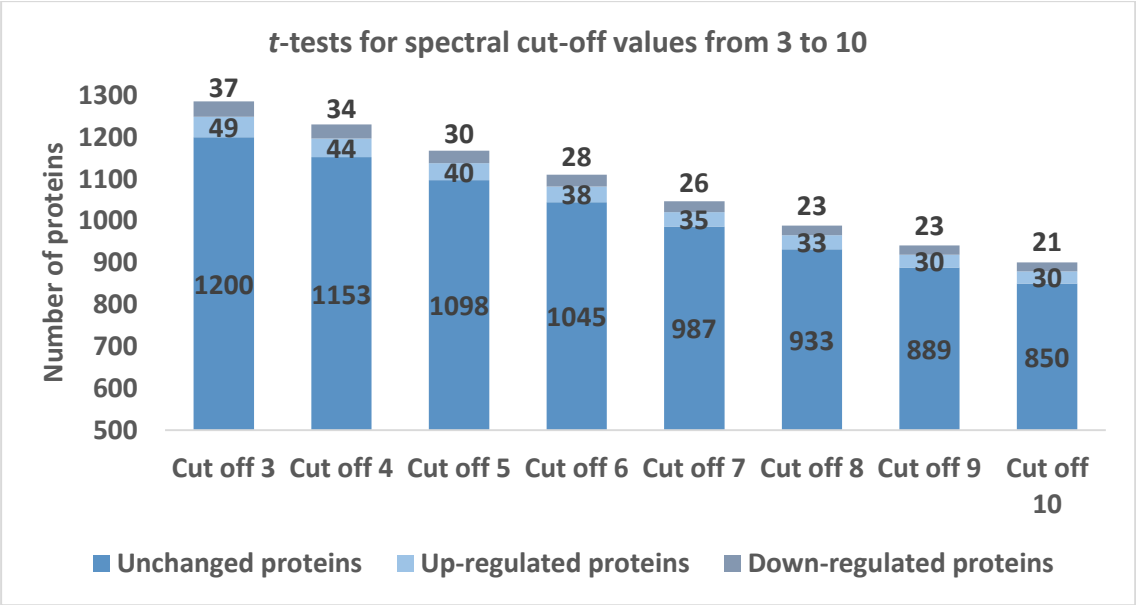


Figure 3. Total protein identifications from control-control experiment using averages of up-regulated, down-regulated and unchanged proteins from the ten combinations where the minimum spectral cut-off value ranged from three to ten.

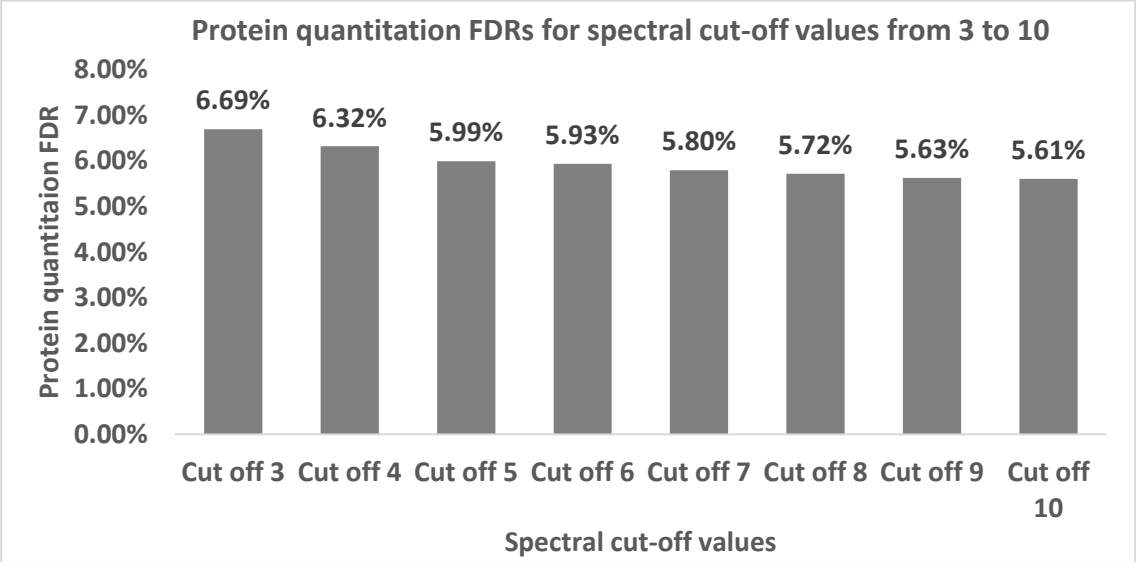


Figure 4. Protein quantitation FDRs (expressed as a percentage) for each minimum spectral cut-off value from three to ten, calculated from the average number of up-regulated, down-regulated and unchanged proteins from the ten combinations.

It was observed that the total number of proteins identified ranged from 901 to 1286 (Figure 3) and the protein quantitation FDR ranged from 5.61% to 6.69% (Figure 4) between the cut-off values from 10 to 3. The percentage of proteins that were reported as differentially expressed in the control-control experiment reduced from 6.69% to

5.61% when the spectral cut-off value was increased from 3 to 10. We observed that the highest cut-off value of 10 did not greatly reduce the noise in the protein quantitation data but led to a loss of 385 proteins in the final dataset. There was an improvement in noise level when the spectral cut-off value was increased, but this increase was considered minimal against the cost of loss of a high number of proteins in the final dataset. The decrease in protein quantitation FDR between cut-off values 4 and 5 produced a change of 0.33% difference in fold proportion (Figure 4) and with a loss of 55 unchanged proteins (Figure 3). We observed that cut-off value 5 provided a suitable balance between stringency and FDR error when analysing fold changes. Thus, spectral cut-off value 5, which was used in the previous chapters of this thesis, was considered valid in terms of stringency.

Supporting Information Table 1 contains a worksheet labelled *t*-test analysis, which states the number of up-regulated, down-regulated and unchanged proteins in each of the ten combinations, for each analysis of minimum spectral cut-off value from three to ten, along with the calculated protein quantitation FDRs.

6.4.4 Control versus temperature stresses

We next examined how the determined noise threshold of 6% might be used for validating our previous proteomic data. We used this value to check our results, by analysing whether our previous results obtained from comparing control to heat and cold temperature stresses showed biologically relevant fold-changes as opposed to false-positive fold changes. The number of proteins identified as up- and down-regulated in the Cabernet Sauvignon cell suspension cultures grown at four different temperatures of 10°C, 18°C, 34°C and 42°C compared to the control are shown in table 2 (results extracted from chapter 4 of this thesis). The proportion of all proteins

reporting a fold-change compared to the control, against all proteins identified in a certain condition was higher than the noise threshold of 6% for all control versus temperature stress comparisons. The percentage of differentially expressed proteins ranged from a minimum of 13.2% (moderate cold) to 36.9% (extreme hot). These data are also presented graphically in Figure 5. From this figure, it is clear that all control versus temperature stress comparisons showed levels of differential protein expression that were greater than the noise threshold of 6%.

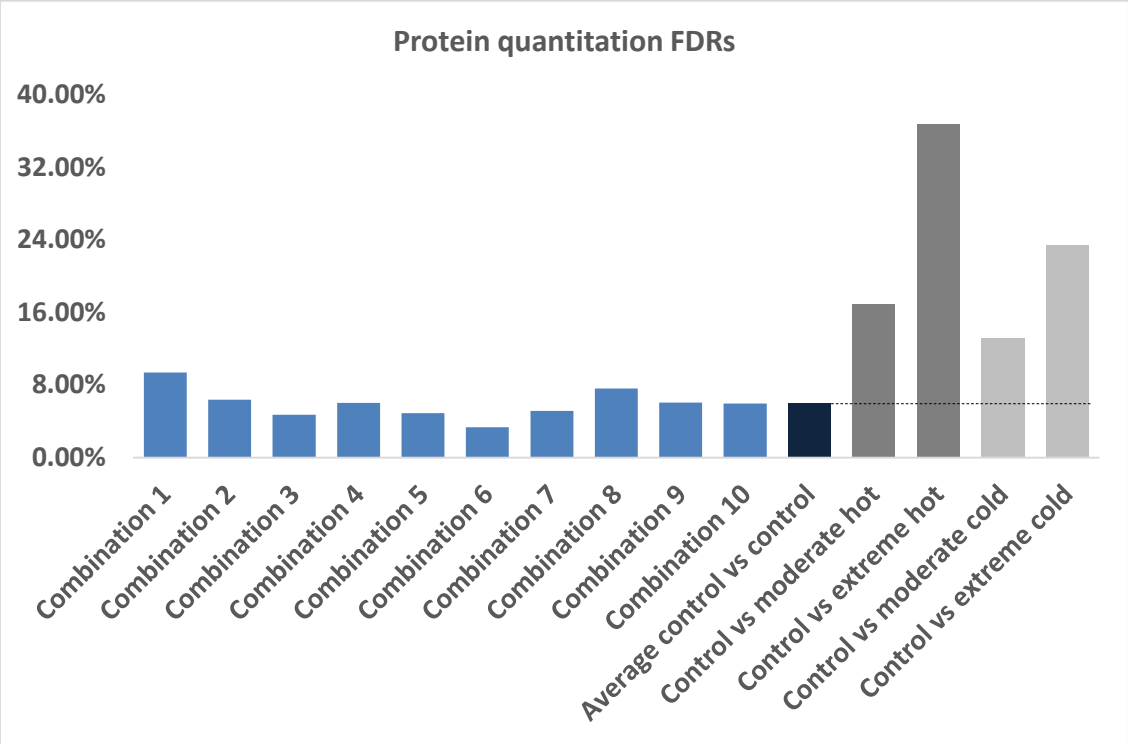


Figure 5. Comparison of protein quantitation FDRs of control-control noise cut-off level (6%) to the four temperature stresses. Combinations 1-10 are the various control-control combinations. Average control vs control is the noise cut-off level. Dark grey data bars = protein FDRs for heat stress, light grey data bars = protein FDRs for cold stress. Protein fold-changes in all the temperature stresses were more than the noise. Proportion of changed versus total proteins were compared against the control-control with the minimal spectral cut-off of five.

6.4.5 Discussion of results

The results observed in this chapter were very unexpected. Firstly, we did not expect that the detectable noise level in our protein quantitation data would be as high as 5 to 6%, when the protein identification false discovery rates in the datasets are all well below 1%. Secondly, the data presented in Figure 4 seem to contradict the accepted wisdom in the field. We have clearly shown that increasing the number of peptides required for identification, all the way up to a value of 10 across three experiments, does not significantly impact on the protein quantitation noise level. We expected to see the protein quantitation false discovery rate reduce to a negligible level by increasing the minimum number of peptides used, but that did not happen. The noise level appears to reach a plateau at about 5.5%, while the number of proteins retained in the dataset decreases sharply, by as much as 33%. We have no specific explanation for how this occurs, but we can speculate that this is somehow related to noise level inherent in data dependent sampling techniques. However, taking a more optimistic view of things, the results presented in the previous chapters in this thesis are thus, at least partially, validated though the statistical analysis approach presented in this chapter. We have demonstrated that the number of proteins identified as significantly differently expressed in all the temperature stresses compared to control (chapter 4) is greater than the determined noise threshold. This is especially obvious for the 37% and 24% of proteins observed with significant fold changes in response to extreme heat and cold conditions.

This may seem like a fairly simple idea, because in many ways it is. However, when we began investigating protein quantitation false discovery rates, we were surprised to learn how few published studies actually incorporate a true control-control experiment. It is not clear whether this is caused by resource limitations, such as, the cost of expensive labelling reagents or additional instrument time, or whether it is because it was not

considered worthwhile. In our experience, this approach helped greatly with understanding the parameters needed for statistically rigorous data analysis in our other studies, and we believe it would be an excellent idea to include this as a standard feature in our future experiments. It is also worth noting that this type of validation of the whole dataset avoids the obvious statistical significance problems involved in 'validating' large-scale proteomics datasets by performing orthogonal experiments on a small number of proteins.

These experiments have also opened up an additional avenue of further investigation. In addition to the work described in this chapter, preliminary work on same - same control experiments has been performed in our laboratory in several other species. Surprisingly, a false discovery rate of around 5 to 6% at the protein quantitation level has been observed quite consistently. This is true not only for spectral counting based label-free quantitation, but also for some experiments using isotopic chemical labelling based quantitation.

We have begun investigating whether additional statistical analysis, or other parametric filtering, could be used to reduce the protein quantitation false discovery rate without significantly increasing the protein identification false negative rate. For example, we did attempt to use both Bonferroni and Benjamini-Hochberg adjusted P values [170, 171] on some of the data analysed in the course of this chapter. We found that the 'corrected' values contained very little noise, but we had also eliminated virtually all of the differences between the control and temperature stress treated samples. Clearly, these corrections are not specific or subtle enough for this kind of data. The development of refined statistical analysis techniques is ongoing in our laboratory and will likely lead to publications in the near future, but completing it is beyond the scope of this thesis.

6.5 Concluding Remarks

This study was designed on the theme of development of a new type of biological validation method in the form of a control-control threshold, expressed as protein quantitation false discovery rate. We produced a numerical measure for the protein quantitation noise level that is inherent in our method of protein processing and data analysis. This relatively simple experiment, which can be applied to biological material harvested from any species, demonstrated that our previous experiments in grape cell temperature stress have involved the measurement of biologically relevant protein fold-changes in addition to a measurable amount of false positive fold changes derived from experimental noise. Application of an empirically determined noise threshold to all proteomic data is a direct, economical and quick method to ensure the validity of proteomic results. It is also applicable to the analysis of any type of biological sample, so it avoids the problem referred to earlier, of lack of availability of antibodies for Western blotting. In future, we plan to execute similar control-control experiments, as were implemented in this grapevine study, in a variety of different organisms to investigate the scope of this approach for use as a validation tool. Studies are underway in our laboratory, for example, on different plant species including rice and barley, the protozoan parasite *Giardia duodenalis,* honeybee brain and wing tissue, and various different regions of rat brain tissue.

CHAPTER 7

Chapter 7

7. Conclusions and future directions

7.1 Conclusions

In the first section of this chapter, I have drawn conclusions for all the studies in this thesis and included a note on future directions of this research. In the second section of this chapter, I have included a published article (publication IV) on the current perspectives of proteomic analysis in grapevine.

Proteomics is a post-genomic technology that has experienced great expansion in its use for investigating cell biology processes. A breakthrough in grape research was attained when the grape genome was sequenced. This event represented a significant landmark in the escalation of proteomic studies in grape. These advancements have facilitated the research conducted in this thesis. Taken together, the experimental components of this research illustrate the immense potential of molecular level discoveries that can be achieved through modern proteomic technology. This thesis is a collection of studies that established a platform for optimal shotgun proteomic analysis of grapevine (chapter 3), investigated the differential proteomic responses in various *Vitis* species upon exposure to two different environmental stresses (chapters 4 and 5) and introduced a new method for validation of observed results (chapter 6).

We identified and quantified dynamic changes in protein profiles influenced by the impact of environmental stresses, using sophisticated mass spectrometric instrumentation and a label-free quantitation approach. We employed shotgun quantitative proteomic analysis technique for all the studies presented here, because it is economical, reliable and efficacious in the exploration of biological networks. It must be noted that the proteomic methodology followed in this thesis is only one of the

numerous choices available in terms of sample preparation methods, instrumentation and technology, for proteomic studies in plants as well as other species.

In the first experimental chapter (chapter 3), we established an optimal method for label-free shotgun proteomics of grapevine samples and demonstrated that in-solution digestion by filter-aided sample preparation coupled with gas phase fractionation provided high-throughput high quality results. This optimised method was subsequently used to characterise proteins and pathways changed by environmental stresses of differential temperature and light treatment in grapevine, as described in chapters 4 and 5, respectively.

In the next experimental chapter (chapter 4), we observed the differential display of protein expression patterns at two different heat and cold stresses in cell cultures of the Cabernet Sauvignon cultivar of the *V. vinifera* genotype. In the comparisons of protein expressions at 10°C, 18°C, 34°C and 42°C to the control temperature of 26°C, we observed that sucrose metabolism displayed switching between alternative and classical pathways during thermal stress temperatures. Another significant alteration was observed in phenylpropanoid metabolism. We observed that nine proteins involved in the phenylpropanoid pathway were more abundant at the extreme cold stress of 10°C, indicating that they were cold-responsive. This study added considerable information to the knowledge pool of proteomic analysis of temperature stress in grapevines, which is currently limited in the number of available publications.

In the following experimental chapter (chapter 5), we performed a detailed investigation into signalling systems and mechanisms that trigger growth cessation and dormancy induction in grapevine. The environmental factor examined in this study was varied light exposures (daylength treatments), which was selected with the aim of studying biological processes related to flowering. Our results illustrated that various enzymes

involved in glycolysis and dormancy induction were up-regulated in short daylength buds which were exposed to 13 hours of light, as compared to long daylength buds which were exposed to 15 hours of light. We have provided evidence that long daylength buds continue to grow, while dormancy was induced in short daylength buds. We also observed genotype specific patterns of protein expression in the *V. riparia* (F2-110) genotype when compared to the other hybrid genotype, Seyval (F2-040). The findings of this research improved our understanding on molecular processes involved in endodormancy.

In the last experimental chapter (chapter 6), we introduced a new approach for validation of quantitative label-free shotgun proteomics studies, based on generating a protein quantitation false discovery rate. We investigated protein fold-changes in the same samples utilised in chapter 4, but also performed additional experiments involving analysis of Cabernet Sauvignon cells grown at a control temperature of 26°C, against another set of biological replicates of the same samples. We detected a noise threshold level from the control-control comparison and evaluated that the observed protein-fold changes in our previous experiments were above this cut-off level, thus providing some validation of our previous results.

One additional unexpected outcome from chapters 4 to 6 was that we were able to provide a putative functional annotation for many of the identified proteins that were labelled as putative uncharacterised proteins. This information will undoubtedly be useful in subsequent studies performed in our laboratory.

7.2 Future directions

The work described in this thesis has added valuable insights at the molecular level into abiotic stress responses in grape, and also provided a basis for future work that will

broaden our knowledge of environmental stress responses in plants. There are a number of direct extensions of the research work in this thesis which could be implemented to help build our understanding of grapevine responsiveness to varying environmental factors. These studies could generate molecular level information for use in targeted breeding of grapevines to generate varieties that will better withstand stressful ecological conditions. In order to further the progress of the research in this thesis, future studies in the following directions could be valuable:

- A complementary study to investigate changes triggered by thermal stresses in mature grapevines or in grapevines harvested at different developmental stages. Designing an experiment along similar lines to the setup described in chapter 4 would enable protein identification and quantification of expression patterns in multiple dimensions, and such a study would facilitate a direct comparison between how mature plants and cell suspension cultures respond to exposure to the same temperature stresses. This would be slightly more complicated to control in mature plants than in cell cultures, because the study design would need to take into account diurnal temperature and light intensity variations and fluctuations.
- One of the major finding in chapter 4 in the study of grape cells exposed to thermal stresses, was notable alterations in phenylpropanoid metabolism. We observed that phenylpropanoid biosynthesis was up-regulated at extreme low temperatures. One obvious follow-up experiment would be to validate these observations by an orthogonal protein quantitation approach. Future directions could include analysis of the resulting phenolic compounds by developing a method for the preparation and extraction of the identified phenolic compounds based on their chemical properties, polarity and the different proportions of flavonoids.

- In addition, it would be also be informative to investigate the expression patterns of proteins in different grapevine tissues, such as roots, to assess water up-take or signalling, or shoots, to investigate changes in photosynthesis, and other physiological changes in response to thermal stresses. In the case of leaf tissue, RuBisCo depletion would likely need to be introduced into the experimental protocol in order to avoid overshadowing of lower abundance proteins.
- It would also be highly informative to assess the correlation between differential responses to various abiotic stresses by examining other environmental stresses such as drought and salinity in grapevine. Identifying a set of core proteins involved in a common stress response would provide high value information for use in a selective breeding program.
- Comparative quantitative proteomic analysis could be performed in other grapevine varieties to observe genotype specific properties in response to ecological stresses or to study developmentally important processes. The species *Vitis vinifera* (chapters 4 and 6) and *Vitis riparia* hybrids (chapters 3 and 5) were used for analyses in this thesis. Proteomic investigations in different species such as *Vitis rotundifolia, Vitis rupestris,* or in other commercially important *Vitis vinifera* cultivars, such as Chardonnay, Shiraz, Pinot Noir or Semillon, could be performed for comparative studies and also to identify a stress response signature unique to specific genotypes.
- Another dimension to this type of research would be to perform studies that were focused more on wine, rather than grapes. As an example, one could investigate protein differences in stored grape samples used in preparation of different vintages exposed to different environmental stresses during the growing season. This information could be interrogated to determine which biological process, that could

directly or indirectly influence the taste and flavour of wine, is activated by that particular environmental fluctuation, in the vintage under examination.

- The body of work presented in this thesis demonstrates the proteomic discoveries in grapevine accomplished through the shotgun label-free approach. Alternative labelled proteomic approaches using tandem mass tags (TMT) and isobaric tags for relative and absolute quantitation (iTRAQ) could be employed to add complementary information that would be valuable in understanding grapevine cell biology, as these techniques are orthogonal to label-free approaches.
- Complementary information could also be acquired by various other 'omics' techniques, such as transcriptomics or metabolomics, to catalogue and correlate the global molecular changes caused by stress responses, to provide a broader snapshot of the overall responses at the cellular level.

In this thesis, we have provided evidence of differential protein expression when grape cells and plants are exposed to different environmental stresses, but there is still a lot of research that is required to produce grapevines that can tolerate harsh ecological stresses and adapt to changing climatic conditions. Also, more research is needed to establish standard protocols for molecular breeding programs and to relate molecular studies to the final end product which reaches the markets for consumption (i.e. wine). We consider that the experiments and results described in this thesis are essential initial steps along this trail.

7.3 Synopsis of Publication IV: Current perspectives in proteomic analysis of abiotic stress in grapevines

This publication is a perspective article which discusses some of the current research on abiotic stresses in grapevine, with particular reference to the recent developments in grapevine proteomics. A short note on abiotic stress studies in other plant species is also mentioned in this article. We have also briefly discussed the recent advancements in data analysis software and listed some current limitations of proteomic studies in grapevine, primarily highlighting on the fact that protein sequences in grape database repositories need much more functional annotations to help obtain better results, and a more comprehensive understanding of biological responses. We have also identified areas with promising scope for future research in grape proteomics. I estimate my contribution to this paper as approximately 95% of the writing and preparation, with assistance from Professor Paul. A. Haynes.

7.4 Publication IV

frontiers in
PLANT SCIENCE

PERSPECTIVE ARTICLE
published: 08 December 2014
doi: 10.3389/fpls.2014.00686

Current perspectives in proteomic analysis of abiotic stress in Grapevines

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Grapes are an important crop plant which forms the basis of a globally important industry. Grape and wine production is particularly vulnerable to environmental and climatic fluctuations, which makes it essential for us to develop a greater understanding of the molecular level responses of grape plants to various abiotic stresses. The completion of the initial grape genome sequence in 2007 has led to a significant increase in research on grapes using proteomics approaches. In this article, we discuss some of the current research on abiotic stress in grapevines, in the context of abiotic stress research in other plant species. We also highlight some of the current limitations in grapevine proteomics and identify areas with promising scope for potential future research.

Keywords: grape, proteomics, grapevine, abiotic stress

INTRODUCTION

Grapes are a valuable fruit crop and wine production is a globally important industry with 265 million hectoliters of wine produced in 2011 (www.oiv.int) (2012). Grapevine production can be hampered by influential abiotic stresses like drought, climate fluctuations, and salinity. These factors pose a direct threat to viticulture practices. Global warming reports estimate an increase in temperature by 2–5°C by the end of the twenty-first century (Salinger, 2005), along with higher probability of stronger, more powerful, and more frequent climate fluctuations. The future beholds a warmer and more arid planet with sudden temperature fluctuations, caused by either natural or anthropogenic impacts. Global warming can lead to desertification, drought and intense soil salinity, all of which adversely affect grapevine quality and quantity. Studies have reported that abiotic stresses can impact wine grape production by decreasing yield and lowering quality of grapes produced (Jones et al., 2005; Cramer, 2010; Hannah et al., 2013). There is a forecast estimated decrease of up to 73% of surface land area suitable for viticulture in the main wine producing regions of the world by 2050 (Hannah et al., 2013). Although vineyards in some areas are adjusting to climate acclimation (Van Leeuwen et al., 2013), there is a need to develop environmentally sustainable crops without compromising on productivity and quality. This has driven much research into studies on plant responses to abiotic stresses. Proteomics using state of the art mass spectrometry is a powerful and promising tool to study molecular mechanisms and biological traits in plants. Grapevine responses to abiotic stresses like salt stress (Vincent et al., 2007; Jellouli et al., 2008), drought (Vincent et al., 2007; Grimplet et al., 2009b; Cramer et al., 2013), and temperature (Liu et al., 2014) have been effectively investigated. This short article will discuss the developments in grapevine proteomics, consider its current role in unraveling insights in molecular responses to abiotic stresses, and briefly discuss the current limitations of proteomic studies in grapevines.

DEVELOPMENTS IN PROTEOMICS

Proteomic analysis techniques are constantly developing, with continuing improvements in sensitivity, resolution, accuracy, and speed of analysis. Advances in sample preparation techniques, mass spectrometry instrumentation and bioinformatics tools have paved the way for high throughput analysis. There have been great advancements in this field over the past two decades and these developments continue to expand, thus enhancing our understanding of molecular systems. In the past, sample preparation techniques using both in-gel digestion and in-solution digestion have been employed in proteomics studies on grape. Proteomic responses have been studied in tissues of grape berry (Sarry et al., 2004; Vincent et al., 2006; Grimplet et al., 2009b; Giribaldi et al., 2010; Martinez-Esteso et al., 2011b), leaf (Sauvage et al., 2007; Jellouli et al., 2010b; Delaunois et al., 2013; Nilo-Poyanco et al., 2013; Liu et al., 2014), stem (Jellouli et al., 2008), root (Castro et al., 2005; Jellouli et al., 2010b), shoot (Vincent et al., 2007; Cramer et al., 2013), and cell cultures (Martinez-Esteso et al., 2009, 2011c). Previously, two-dimensional gel electrophoresis techniques were mainly used (Vincent et al., 2007; Jellouli et al., 2008, 2010a; Grimplet et al., 2009b; Giribaldi and Giuffrida, 2010), but these are now being replaced by shotgun proteomics techniques including iTRAQ and TMT (Martinez-Esteso et al., 2011a; Liu et al., 2014), or label-free quantitation methods (Cramer et al., 2013), using ever more sophisticated mass spectrometers. Mass spectrometry instrumentation has evolved over the years from basic time-of-flight tandem mass spectrometers to multiplexed hybrid mass spectrometers. Instruments have become faster and more sensitive, with concomitant increases in resolution, thus generating far more data at higher accuracy. To keep up with these

advancements, and the tremendous amount of data acquired, statistical software used to analyse mass spectrometry results, including from grapevine studies, has also been the subject of intense development. Statisticians, mathematicians and computer scientists have made efforts to create new and user friendly databases and algorithms to help understand molecular mechanisms. Consequently, the sequencing of the grape genome in 2007 (Jaillon et al., 2007; Velasco et al., 2007) represented a major breakthrough transition in grapevine proteomic research. The use of the *Vitis vinifera* genome sequence, containing approximately 30,000 genes, in database searches provided more reliable results than could be produced previously. Previous studies on grape have generally used the NCBI non-redundant database or EST contigs for identifying proteins (Marsh et al., 2010; Martinez-Esteso et al., 2011a) which works reasonably well but produces data which often represents an incomplete picture.

ABIOTIC STRESS STUDIES IN DIFFERENT SPECIES

It is essential to produce sustainable plant varieties that adapt to climate variability, and to develop a broad spectrum of abiotic stress tolerant crops. Environmental factors influence dynamic changes in plants, often caused by either single or joint effects of numerous abiotic stress responsive pathways, that can be well characterized at the global level using high-throughput proteomic approaches. Proteomics has been successfully used to study abiotic stress responses in a wide range of plants like Arabidopsis (Rocco et al., 2013; Vialaret et al., 2014), rice (Neilson et al., 2013; Mirzaei et al., 2014), maize (Benesova et al., 2012), and poplar (Zhang et al., 2010), among many others, all of which have genomes that have been sequenced. This approach has also been employed for biomarker discovery in plant species with incomplete genome sequences, like peanut (Kottapalli et al., 2013), mango (Renuse et al., 2012), and even rare species like Pachycladon (Mirzaei et al., 2011), an Alpine species endemic to New Zealand. Among *Vitis vinifera* cultivars, proteomic studies prior to the sequencing of the grape genome relied on searching mass spectra against NCBI non-redundant protein databases or ESTs (Sarry et al., 2004; Vincent et al., 2006). The process of using mass spectrometers to identify proteins by cross species peptide identification is difficult, but it has become easier with the development of more high accuracy mass spectrometers.

Abiotic stress responses have been investigated in grapevine varieties of Chardonnay (Castro et al., 2005; Vincent et al., 2007), Tunisian Razegui (Jellouli et al., 2008), Cabernet Sauvignon (Vincent et al., 2007; Grimplet et al., 2009b; Cramer et al., 2013; Liu et al., 2014), and Pinot Noir (Negri et al., 2011). Grapevines have developed several adaptive approaches at the cellular and metabolic levels to mitigate, and recuperate from, the destructive effects of hostile environmental conditions. General responses include differential regulation of sugar metabolism, signaling, growth, protein synthesis, and hormone metabolism. As an example, we have observed changes in phenylpropanoid biosynthesis in Cabernet Sauvignon cells exposed to temperature stress (George et al., 2014). Osmotic stress response is the most common response to harsh environments (Cramer, 2010). Proteomics has aided in the study of differential expression of single proteins, global expression patterns, and association with regulatory pathways, and has also been used to substantiate and complement transcriptomic and metabolomic studies (Cramer et al., 2007; Zamboni et al., 2010). For example, a strong correlation was observed between transcriptomic and proteomic data in the investigation of biotic stress response to trunk diseases in green stems of Chardonnay (Spagnolo et al., 2012).

In order to better understand the metabolic changes involved in stress responses in both vegetative and reproductive parts of grapevines, and how dynamic the adaptative responses are in such situations, better experiments are needed. Ideally, if funding permitted, one would design experiments including sampling of all tissues—roots, shoots, leaves, and berries—at different developmental stages, including berry growth, veraison and ripening, and under various environmental conditions at different locations. Such an exhaustive study would be an invaluable resource for the grapevine research community, especially if it was expanded to include transcriptomic and metabolomic analysis in addition to the proteomic data sets.

CURRENT LIMITATIONS OF GRAPEVINE PROTEOMICS AND FUTURE SCOPE

With the genome sequences of various plants being completed and released regularly, it is becoming easier to examine the biological pathways that trigger plant protein responses. Studies in grape have increased exponentially since 2007, after the release of the grape genome sequence data. A vast amount of knowledge has been obtained from these studies on various defense mechanisms and biological pathways triggered by external factors, including both biotic, and abiotic stresses. Investigations have been performed on different varieties ranging from the widely recognized *Vitis vinifera* cultivars like Cabernet Sauvignon and Chardonnay (Vincent et al., 2007), to other species like *Vitis riparia* (Victor et al., 2010) and *Vitis rotundifolia* Michx Muscadine (Kambiranda et al., 2014). Despite these advances, detailed understanding of proteins and protein families which are essential for stress responses are still limited. The available grape genome sequence was based on analyses of only one *Vitis vinifera* variety, Pinot Noir PN40024. Most studies so far have used only the Pinot Noir genome sequence as the reference genome. It is well known that a significant amount of transcript and protein sequences are either species specific or cultivar specific and hence may not be well represented within the Pinot Noir genome. This may lead to incompleteness in protein identification when studying other grape varieties or species. Thus, there is a clear need for sequencing of more cultivars, such as the commercially important Cabernet Sauvignon, and more related species such as *V. rotundifolia* and *V. riparia*. There are now hundreds of genome sequences available for different ecotypes of Arabidopsis (Weigel and Mott, 2009), and with the continued rapid developments in gene sequencing technologies we would hope that in the near future we will also see the publication of complete genome sequence information data for many different varieties of grapevine.

A critical challenge in grapevine proteomics is to infer biological meaning from the huge amount of mass spectrometry data acquired. The general procedure for the study of plant-environment interactions includes protein identification,

protein characterization (including function annotation), construction of identified proteins into a biological network, characterization of differential protein expression under stress conditions, and assimilation of all the above into a linking framework. The initial step for this type of workflow is to identify and annotate proteins, and integrate them into the biological context.

In order to illustrate some of the current difficulties in this process, we surveyed grapevine protein sequences in NCBI and Uniprot, using the simple keyword "*Vitis vinifera*." We found 161926 sequences in NCBI and 65548 sequences in Uniprot, which is indicative of a high level of redundancy and repetition, particularly in the NCBI database. We examined the entries in the Uniprot database in more detail. **Table 1** shows the number of protein entries for different grapevine species found in the Uniprot database, along with an indication of how many of these are still uncharacterized. Most protein entries in the database are unreviewed, which means that no additional supporting information has been presented for them. Moreover, 78% of the protein entries in the *Vitis vinifera* database are listed as "putative uncharacterized proteins." Hence, proteomic study is severely limited by the lack of better quality annotations.

In previous studies, since the roles of many individual proteins were not well defined, their biological functions were inferred from homologous proteins from other species. This task is tedious and time consuming, and produces less than complete protein identification data. Although grapevine does not have a well-established database like PPDB (Sun et al., 2009) (which is dedicated exclusively for *Arabidopsis thaliana* and *Zea mays* research), there is a basic database developed uniquely for grapevine molecular network study called VitisNet (Grimplet et al., 2009a). VitisNet was developed from the combination of *Vitis vinifera* (cv. Pinot Noir PN40024) genome sequencing project data, and ESTs from the *Vitis* genus, and is very useful for annotating grapevine proteins (Grimplet et al., 2012). PlantPReS (not yet published) (http://proteome.ir/PlantStress.aspx) is a freely available plant stress protein database which integrates different plant proteomic responses to stress studies. It currently has 83 plant species and is inclusive of *Vitis vinifera*. This database is still under construction, but the data that have been incorporated so far have proved useful in annotating grapevine proteins identified in proteomics experiments.

There is a pressing need to enable the integration of large datasets, streamline biological functional processing, and improve the understanding of dynamic processes in systems biology experiments in grapevines. At the moment, software packages available for this purpose are mainly designed to work with mammalian systems. It is to be hoped that in the future more software is available that is specifically designed to function with plant protein and genome sequences, including grapevines.

Table 1 | Number of protein entries in the UniprotKB database for different grapevine species.

Species	UniprotKB entries	No. of reviewed proteins	No. of unreviewed proteins	No. of putative uncharacterized proteins
Vitis vinifera	65548	206	65342	50775
Vitis rupestris	536	3	533	1
Vitis labrusca	195	1	194	5
Vitis riparia	178	2	176	2
Vitis vulpina	167	2	165	2
Vitis amurensis	155	0	155	0
Vitis rotundifolia	111	2	109	0
Vitis aestivalis	66	3	63	0
Vitis coignetiae	7	0	7	0

CONCLUSION

Proteomics is a powerful tool for molecular level discovery of biological networks in grapevine. Plant species with completely sequenced genomes, smaller genome sizes and well annotated libraries are easier to study and understand; grapevines remain a challenge. Recent advancements in mass spectrometry and proteomic techniques, coupled with the availability of complete genome sequences and improvements in bioinformatics tools, are continually strengthening this field of study. Research in this area, however, needs to be further accelerated by sequencing more grapevine varieties and different cultivars. Protein sequences in database repositories need much more functional annotation, which will help obtain better results and a more comprehensive understanding of biological responses. Proteomics has an important role to play in the future in helping to understand at the molecular level how grapevines respond to the many challenges they face.

ACKNOWLEDGMENTS

Paul A. Haynes acknowledges support from the Australian Research Council for the Food Omics Research Centre and thanks Scott Ireland for continued support and encouragement. Iniga S. George acknowledges support from Macquarie University in the form of an iMQRES scholarship, and travel grants from the International Plant Proteomics Organization (INPPO) and the Australian Grape and Wine Authority, (AGWA).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 October 2014; accepted: 18 November 2014; published online: 08 December 2014.

Citation: George IS and Haynes PA (2014) Current perspectives in proteomic analysis of abiotic stress in Grapevines. Front. Plant Sci. 5:686. doi: 10.3389/fpls.2014.00686

This article was submitted to Plant Proteomics, a section of the journal Frontiers in Plant Science.

Appendix

Two publications (publication V and VI) are attached as appendix 1 and 2. Studies in grapevine proteomics were the main focal points of my PhD research. However, I was also involved in two rice proteomic studies which had a thematically related aim of examining whether environmental stresses when applied to the roots could alter global protein expression in the shoots. We investigated drought and temperature stress in rice by the quantitative proteomic approach during the course of my studies.

We employed the label-free shotgun proteomic approach to examine drought responsiveness in rice shoots (publication V) which is included in appendix 1. I estimate my contribution to this research as 10% of the experimental work and 35% of the result interpretation and manuscript writing.

We identified and quantified proteins which were differentially expressed in response to chilling effect in rice using triple stage mass spectrometry with labelled tandem mass tags (TMT) in publication VI, which is included in appendix 2. I estimate my contribution to this research as 40% of the experimental work and 10% of the result interpretation and manuscript writing.

Appendix 1

Journal of proteome research

Article

pubs.acs.org/jpr

Manipulating Root Water Supply Elicits Major Shifts in the Shoot Proteome

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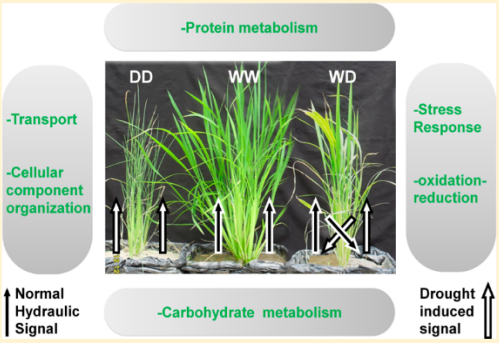
Supporting Information

ABSTRACT: Substantial reductions in yield caused by drought stress can occur when parts of the root system experience water deficit even though other parts have sufficient access to soil water. To identify proteins associated to drought signaling, rice (*Oryza sativa* L. cv. IR64.) plants were transplanted into plastic pots with an internal wall dividing each pot into two equal compartments, allowing for equal distribution of soil and the root system between these compartments. The following treatments were applied: either both compartments were watered daily ("wet" roots), or water was withheld from both compartments ("dry" roots), or water was withheld from only one of the two compartments in each pot ("wet" and "dry" roots). The substantial differences in physiological parameters of different growth conditions were accompanied by differential changes in protein abundances. Label-free quantitative shotgun proteomics have resulted in identification of 1383 reproducible proteins across all three conditions. Differentially expressed proteins were categorized within 17 functional groups. The patterns observed were interesting in that in some categories such as protein metabolism and oxidation−reduction, substantial numbers of proteins were most abundant when leaves were receiving signals from "wet" and "dry" roots. In yet other categories such as transport, several key transporters were surprisingly abundant in leaves supported by partially or completely droughted root systems, especially plasma membrane and vacuolar transporters. Stress-related proteins behaved very consistently by increasing in droughted plants but notably some proteins were most abundant when roots of the same plant were growing in both wet and dry soils. Changes in carbohydrate-processing proteins were consistent with the passive accumulation of soluble sugars in shoots under drought, with hydrolysis of sucrose and starch synthesis both enhanced. These results suggest that drought signals are complex interactions and not simply the additive effect of water supply to the roots.



KEYWORDS: *rice, shotgun proteomics, label-free, abiotic stress, drought, signaling, shoot, root*

1. INTRODUCTION

Water limitation is a major constraint for global agriculture. Whereas inadequate water permanently affects 28% of the world's soils, another 47% are subject to intermittent water deficits because of shallow soils or mineral imbalances.[1] Consequently, most civilizations have had an acute interest in improving the ability of plants to use water efficiently through selection of water-efficient crops. Plants avoid drought by various mechanisms, including generating deep roots, storing water in bulky tissues, and adapting their life cycles to cope with weather patterns characterized by intermittent rainfall.[2] However, cell-level responses to drought remain poorly understood.

Drought results from a shortage of water in the root zone, which decreases crop yield. Grain crops trade water for CO_2 by opening their stomata and then convert the photosynthetic products to marketable yield. Therefore, assessment of stomatal behavior is essential for effective evaluation of drought performance. These concepts were summarized by Passioura

Received: July 5, 2013
Published: November 25, 2013

relation.[3] Plants sense and respond to soil drying by regulating shoot processes such as stomatal conductance, hence mitigating shoot transpiration.[4] While this regulation is achieved through stomatal control, genes that encode acclimation events must also be critical.[5] It is well-known that drought stress can reduce plant yield, even when part of the root system is adequately supplied with water.[6] This is presumably because of changes in export of root-borne signals such as abscisic acid and cytokinins,[7] with signaling between root systems of droughted and non-droughted plants in the same soil even possible.[8] However, the nature of root-borne signaling remains difficult to establish with certainty because of the complexity of the chemistry, with sap pH, hormones, and mineral nutrient all potential factors. It is well established that protein turnover is extensively altered in shoots of plants subjected to drought.[9] Apart from signals that elicit a shoot-specific response, drought induces a major disruption to plant carbohydrate allocation. Hence we used downstream gene-level responses in this study to analyze regulation of abiotic stress signals. The mechanistic basis of long-distance signaling in plants in drying soil has been researched using only a relatively small number of cereals and legume species. In this study, we use rice, a drought-sensitive species.

Recently, we applied quantitative label-free shotgun proteomics approach to analyze rice root response to partial root zone drying (PRD).[10] We exposed rice seedlings to PRD, resulting in a substantial decrease in shoot dry weight, presumably by root-to-shoot communication. By analyzing proteins in well-watered root tissues and adjacent water deficit roots, we provided quantitative evidence that the water supplied was able to alter gene expression remotely. It was shown that proteins involved in oxidation−reduction reactions and transport were also highly dependent upon drought signals, with the latter largely absent in roots receiving a drought signal, while oxidation−reduction proteins were strongly present during drought.[10]

In the current study, we analyzed the proteome pattern of rice leaves in this PRD system using a label-free shotgun proteomics approach. We observed that differences in leaf physiological parameters were accompanied by differential accumulation of proteins related to transport, cellular component organization, and protein metabolism. We also report on proteins associated with oxidative stress and stress response. Finally, we describe changes in key proteins involved in carbohydrate metabolism.

2. MATERIALS AND METHODS

2.1. Plant Material and Sampling

Seeds of *Oryza sativa* L. cv. IR64 were obtained from the International Rice Research Institute (Los Baños, The Philippines). The seeds were surface sterilized, pregerminated for 3 d, and grown on Yoshida culture solution[11] for 10 days prior to transplantation into rectangular pots (measuring 26 cm × 20 cm) and 15 cm deep. Pots had an internal wall that divided them into two equal volumes of soil which were watered, either identically or differentially. A mixture of loam, sand, and clay (1:2:1) was added to within 5 cm of the top of the pot. The roots of each seedling were distributed equally between the two parts of the pot by training seminal roots of small seedlings into each half and plants watered normally for 14 days after transplantation. Then, three watering treatments were imposed for 14 d, with three replicates in a randomized strategy. The three treatments were: plants watered daily (Well-Watered, WW); same as WW plants for 14 d after transplanting, then watering was stopped in both halves of the pot (Droughted plants, DD); same as WW plants for 14 d after transplanting, then watering was stopped in one-half of each pot, producing a root system that is partially wet and partially dry (Split-root plants, WD). Experiments were carried out in a glasshouse during the spring-summer season at 36°N latitude using natural illumination in a glasshouse. Urea (2.73 g), Solophos (1.84 g), and muriate of potash (1.04 g) were added to each pot, as sources of nitrogen, phosphate, and potassium, respectively. Shoots from three individual pots were sampled to form three replicates. Proteomic analysis was executed in a randomized design with three replicates.

2.2. Physiological Measurements

Total shoot dry weight was measured after desiccation in a drying oven at 70 °C for 48 h. Stomatal conductance was measured at the center of the abaxial side of the first fully expanded leaves using a dynamic diffusion porometer (AP4, Delta-T Devices Ltd., Cambridge, U.K.). All analyses were performed using three biologically independent replicates and one-way analysis of variance (ANOVA, $p < 0.05$) using MSTATC software (Michigan State University, East Lansing, MI).

2.3. Sucrose, Glucose, And Fructose Measurements

Leaf samples were harvested and immediately stored in liquid nitrogen and then lyophilized. Samples (100 mg) were ground to a fine powder with a mortar and pestle and were extracted with 1.5 mL of boiling 80% ethanol (v/v) for 30 min with gentle shaking. The washing step with ethanol was performed several times in order to complete removal of residual glucose. The homogenates were centrifuged at 10 000*g* at 4 °C for 10 min. The supernatant was removed at 40 °C in a vacuum rotary evaporator (Rotavapor RE 111, Büchi, Switzerland), resolubilized in 10 mL of distilled water, and added to 9% (w/v) $Ba(SO_4)_2$ and 5% (w/v) $Zn(SO_4)_2$. Samples were centrifuged at 10 000*g* for 15 min and sucrose, glucose, and fructose determined by HPLC according to Wilson et al.,[12] with some modifications.

The stored aliquot from the general extraction and preparation (see above) was evaporated in a vacuum rotary evaporator at 30 °C and 180 rpm, and 1 mL of pure water was then added. The final extract was filtered with a syringe-head filter with a pore size 0.45 lm (Xpertek, Sartorius). The extract was injected into an HPLC (Knauer Corp., Germany) with a pulseless pump (Wellchrom K-1001, Germany) using a 0.7 mL min^{-1} flow rate and HPLC grade water (pH = 2.5) as the mobile phase and a RI detector (K2300, Germany). A Eurokat column (300 mm × 8 mm) and precolumn ((30 mm × 8 mm) (Knauer Corp., Germany) was used for the separation of sucrose, glucose, and fructose, and the carbohydrates were quantified using 5-point calibration curves, ranging from 1 to 100 μmol L^{-1}.

2.4. TCA-Acetone Protein Extraction and Separation by SDS-PAGE

Freeze-dried shoot powder (50 mg) was suspended in 1200 μL of 10% TCA in acetone, 0.07% β-mercaptoethanol, and incubated for 45 min at −20 °C. The pellet that was collected from the extract after centrifugation at 16 000*g* for 30 min was washed with 600 μL of 100% acetone and centrifuged once again at 16 000*g* for 30 min. This washing step was repeated

three times, and the colorless pellet was lyophilized in a vacuum centrifuge. Protein pellets were solubilized in sodium dodecyl sulfate (SDS) sample buffer, and Bradford assay was performed for protein quantification. Then, 120 μg of protein was loaded in each well and separated on 10% bis-Tris polyacrylamide gels at 150 V for 1 h. Proteins were visualized by colloidal Coomassie Blue staining. Gels were washed twice in water for 10 min per wash.

2.5. Trypsin In-Gel Digestion

Each gel lane was cut into 16 equal pieces, and each piece was further sliced into smaller pieces and placed into a well of a 96-well plate. Gel pieces were washed briefly with 100 mM NH_4HCO_3 and with 200 μL of ACN (50%)/100 mM NH_4HCO_3 (50%) for 10 min twice, before dehydration with 100% ACN. Samples were air-dried, reduced with 50 μL of 10 mM DTT/50 mM NH_4HCO_3 for 1 h at 37 °C, and alkylated with 50 μL of 50 mM iodoacetamide/50 mM NH_4HCO_3 for 1 h at room temperature in the dark. They were briefly washed with 100 mM NH_4HCO_3 and 200 μL of ACN (50%)/100 mM NH_4HCO_3 (50%) for 10 min, dehydrated with 100% ACN, and air-dried. Samples were then digested with 20 μL of trypsin (12.5 ng/mL 50 mM NH_4HCO_3) for 30 min on ice and at 37 °C overnight. Peptides resultant from trypsin digestion of proteins samples were extracted two times with 30 μL of ACN (50%)/formic acid (2%), dried, vacuum centrifuged, and reconstituted to 10 μL with 2% formic acid.

2.6. Nanoflow Liquid Chromatography–Tandem Mass Spectrometry

Extracted SDS-PAGE gel tryptic digests were analyzed by nanoLC–MS/MS using an LTQ-XL ion-trap mass spectrometer (Thermo, San Jose, CA). Reversed-phase chromatography columns were packed in-house to approximately 7 cm in 100 μm i.d. tubing using 100 Å, 5 μM Zorbax C18 resin (Agilent Technologies, Santa Clara, CA) in a fused silica capillary with an integrated electrospray tip. An electrospray voltage of 1.8 kV was applied via a liquid junction upstream of the C18 column. A Surveyor autosampler (Thermo, San Jose, CA) was used to inject samples onto the C18 column. Each sample was loaded onto the C18 column, washed with buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) for 10 min at 1 μL/min, eluted with 0–50% buffer B (95% (v/v) ACN, 0.1% (v/v) formic acid) over 58 min at 500 nL/min followed by 50–95% buffer B over 5 min at 500 nL/min. Peptides eluted from the column were directed into the nanospray ionization source of the mass spectrometer and scanned in the spectral range 400–1500 amu. Tandem MS of the top six most intense precursor ions at 35% normalization collision energy using dynamic exclusion with a 90 s window was performed using Xcalibur software version 2.06 (Thermo, San Jose, CA).

2.7. Protein Identification

The result raw files were converted to mzXML format and processed through the global proteome machine (GPM) software (version 2.1.1) of the X!Tandem algorithm (freely available at http://www.thegpm.org). The 16 gel fractions were processed serially for each experiment, and the output files were generated as nonredundant, merged files with protein identifications with log (e) values less than −1, for each individual gel fraction. A protein database compiled from NCBI *O. sativa* with 26 938 protein sequences (August 2011) was used in GPM to search the tandem mass spectra; the database also included common trypsin and human peptide contaminants. False discovery rates (FDR) were evaluated by searching against a reversed sequence database. Search parameters included MS and MS/MS tolerances of ±2 Da and ±0.2 Da, respectively, carbamidomethylation of cysteine as fixed modifications, oxidation of methionine as variable modifications, and tolerance of two missed tryptic cleavages and K/R-P cleavages.

2.8. Data Processing and Quality Control

The GPM outputs from each of the three replicates with protein identification were combined to produce a single shotgun proteomic analysis output file for each treatment. The contaminants and reversed database hits were excluded. In each treatment, the final data set contained the proteins that were common in each of the three replicates and had a total spectral count of at least six for at least one condition.[13,14] The protein false discovery rate was calculated using the reverse database, hence protein FDR = {(no. reverse proteins identified)/(total protein identifications)} × 100.[15] Peptide false discovery rate was calculated as peptide FDR = {2 × (no. reverse peptide identifications)/(total peptide identifications)} × 100.[16]

2.9. Quantitative Proteomic Analysis

Normalized spectral abundance factors (NSAF) were used to calculate protein abundance data. The number of spectral counts identifying the protein was divided by the estimated length of the protein for each protein, k, in sample i. $NSAF_i$ values for each sample i were obtained by normalizing $SpC_k/length_k$ values to the total by dividing by the sum $(SpC_k/length_k)$ over all proteins. The average of the NSAF values for all replicates was used as a measure of protein abundance to plot overall protein abundance for a particular condition. A spectral fraction of 0.5 was added to the total spectral counts for each protein to compensate for null values and allow for log transformation of the NSAF data prior to statistical analysis.[17]

2.10. Statistical Analysis

As noted previously, only the proteins that were present in all three replicates for at least one condition were included in the data set. Log transformed NSAF data was used for the 2-sample unpaired t tests and the proteins with a t test p-value <0.05 were considered to be differentially expressed. The resulting sets of up- and down-regulated proteins were subsequently functionally annotated. An analysis of variance was also performed to identify proteins changing in abundance among those proteins present reproducibly in all three conditions. The analysis was performed on log-transformed NSAF data and proteins with an ANOVA p-value less than 0.05 were considered to show a significant change between the different experimental conditions.

2.11. Functional Annotation

Functional annotation was obtained as gene ontology annotations from the Uniprot database and matched against the identified proteins. In-house software developed using the R statistical programming framework (http://www.r-project.org/) was used for processing the gene ontology annotation.[10,17] These differentially expressed proteins from each comparison test were further categorized based on their biological process into 17 different categories, using the PloGO R package.[18] The numbers of proteins in each GO category of interest were plotted side-by-side separately for the up- and down-regulated proteins.

dx.doi.org/10.1021/pr400696u | J. Proteome Res. 2014, 13, 517–526

3. RESULTS

3.1. Physiological and Soluble Sugar Concentrations in Shoots

Water stress significantly reduced leaf dry weights of WD plants by 31% and that of DD plants by 42% (Figure 1A). This was accompanied by decreases in stomatal conductance 7 and 14 days after initiation of water stress (Figure 1B). Soluble sugars increased steadily at the end of the 14 day-drought period, with the shoots of DD plants having the highest soluble carbohydrate concentrations. Sucrose, glucose, and fructose rose by 30%, 70%, and 330%, respectively, in shoots of DD plants compared to WW plants (Figure 1C).

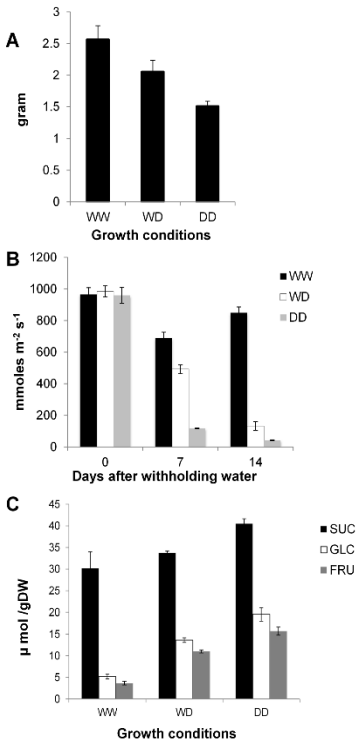


Figure 1. Physiological characteristics of shoots taken from WW, WD, and DD plants: (A) shoot dry weights; (B) stomatal conductance; (C) sucrose (SUC), glucose (GLC), and fructose (FRU) levels from leaves in all three conditions. WW indicates well-watered plants, WD indicates split-rooted plants, and DD indicates water-deprived plants.

3.2. Analysis of Label-Free Shotgun Proteomics

There were 1383 nonredundant proteins reproducibly identified, distributed across three treatments. We identified the highest number of reproducible proteins in WW plants (1108), while fewer reproducible proteins were identified in WD and DD plants (971 and 998, respectively). The number of peptides initially identified in each nanoLC–MS/MS run was found to be consistent among all replicates and treatments, with an overall relative standard deviation of 11% across the entire experimental data set. Calculated levels of peptide FDR ranged from 0.12 to 0.27% while protein FDR was less than 1.2% (Table 1). Low levels of peptide and protein FDR among each run indicate that the data did not require any further filtering after combining. Supporting Information Table S1 contains details of all the proteins identified in each condition, including their raw peptide counts and NSAF values.

3.3. Differentially Expressed Proteins among Three Watering Treatments

For the 685 proteins present reproducibly in all three treatments, the log NSAF values were verified as being normally distributed and then an ANOVA was undertaken. Overall, 143 proteins were identified as showing significant difference in expression at the 0.05 confidence level. After multiple testing, adjustment of the *p*-values was performed using the FDR method as implemented in the p.adjust method of the R stats package. These proteins are listed in Supporting Information Table S2, sorted by *p*-value.

3.4. Pairwise Comparisons

In pairwise comparisons of well-watered plants (WW, as reference) to the other two conditions, WD plants showed down-regulation of 109 proteins and up-regulation of 136 proteins, while 1012 proteins did not show any significant changes. DD plants reversed this pattern, with a higher number of down-regulated proteins, 140, as compared to the number of up-regulated proteins, 130, and 1021 proteins unchanged in expression.

Figure 2 reveals that the categories containing the highest number of up-regulated proteins are those associated with carbohydrate metabolism, generation of precursor metabolites and energy, transport, and oxidation–reduction. However, in five biological categories, no proteins were up-regulated. Of 16 biological processes in which proteins were down-regulated, the highest numbers of proteins were associated with biosynthetic processes, protein metabolism, and transport.

4. DISCUSSION

Physiological observations clearly show that the effects of water deficit on rice shoots can be ameliorated by providing half the root system with a water supply (the so-called "split-root system"). This allowed us to analyze shoots which are hydrated but receiving signals arriving from droughted portion of roots. By analyzing the proteome of these leaf tissues, we can begin to separate the complex effects of drought on gene expression in the shoots. The two watering treatments we used elicit notional signals from roots in wet and dry soil. By using the split-root configuration, these signals can interact with each other to

Table 1. Summary of Numbers of Proteins Identified

	no. of identified proteins[a]			redundant count of peptides[a]					
growth condition	R1	R2	R3	R1	R2	R3	no. of proteins[b]	protein FDR[c](%)	peptide FDR[c] (%)
WW	2815	2755	2572	38 860	43 614	35 978	1108	0.63	0.1
WD	2394	2475	2408	39 525	35 740	33 158	971	1.2	0.27
DD	2436	2435	2489	32 396	30 877	34 003	998	0.8	0.14

[a]R1, R2, and R3 denote replicate 1, replicate 2, and replicate 3, respectively. [b]Number of proteins common to all 3 replicates. [c]FDR = False discovery rate.

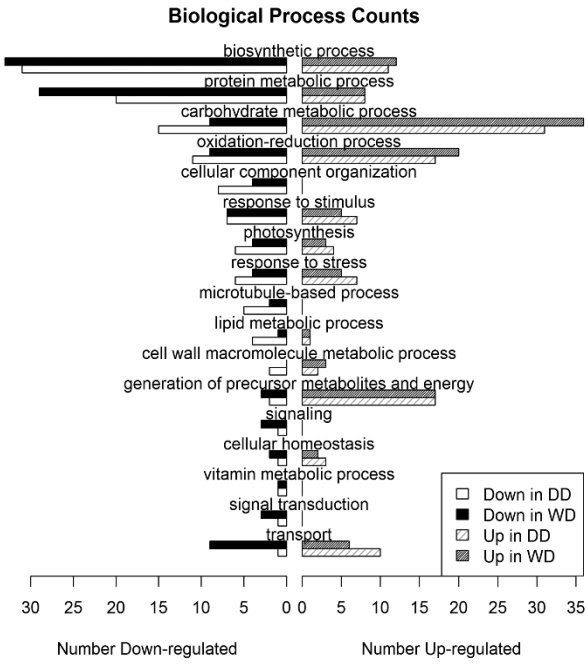


Figure 2. Qualitative comparison of differentially expressed proteins obtained from each *t* test comparison (WW vs WD and WW vs DD). Up-regulated and down-regulated proteins from each test were classified into 17 different biological process categories. Using PloGO ontology annotation (number of proteins in each biological process) was used to plot the up- and down-regulated proteins side-by-side.

produce a unique proteomic signature in WD shoots which is not intermediate between WW and DD plants. The identity of the signals is itself complex;[19] positive signals might include hormones such as cytokinins (from "wet" roots) and ABA (from "dry" roots), while negative signals affect plant metabolism through disruption of normal transport (e.g., reduced carbohydrate export to roots). Differential expression of individual proteins and, more importantly, families of proteins can in this way be used to infer signaling induced by drought. On the basis of background knowledge of the physiology of drought response in cereals and the data obtained from *t*-test analysis, we chose six functional categories as key elements of the drought response: (1) oxidation−reduction, and stress-related proteins, as broadly representative of drought-induced signaling responses; (2) transport, protein metabolism, and cellular component organization represent processes that might be suppressed by drought. From these changes, we ultimately hope to provide candidate marker genes for breeding programs. Carbohydrate metabolism is then analyzed because it is a major metabolic *consequence* of drought as exemplified by the dramatic accumulation of soluble sugars (Figure 1A).

4.1. Transport

Proteins associated with membrane transport fulfill a wide range of biological functions such as cell signaling, hydraulics, and cellular homeostasis. In our study, changes in the expression patterns of transport proteins were nonuniform; about half were up-regulated while the remainder were down-regulated (Table 2A).

Several key transporters were surprisingly abundant in leaves supported by partially or completely droughted root systems, especially plasma membrane and vacuolar transporters (Table 2A). They are required for normal cell function and become critical in stresses such as anaerobiosis, drought, cold, salinity, anoxia, and acidosis. Their functional role is in solute homeostasis and facilitation of vesicle fusion.[20] Vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase) is also an electrogenic proton pump located on the tonoplast but in this case it is energized by pyrophosphate (cf. ATP); it is known to be associated with abiotic stress, such as cold stress,[21] salinity, anoxia,[22] and drought.[23] Interestingly, H^+-ATPases located on the plasma membrane follow a similar expression pattern to the V-PPase, with the highest abundance in leaf cells when receiving drought signals from roots. H^+-ATPases are the key pumps responsible for establishment of cellular membrane potential in plants by proton extrusion to the apoplast, and are therefore critical for the response of plants to various environmental stresses.[24]

Even more marked drought-induced up-regulation was seen for ferritin, where there was no change in WD plants, presumably because the hydraulic signal from "wet" roots abolished the drought signal. Ferritin plays a role in development as well as in defense response to different abiotic stresses such as heat and salt stress.[25] Along with the mitochondrial translocase and hexose transporter, ferritin appears to be a potential marker for drought stress.

Aquaporins are water channel proteins belonging to the major intrinsic protein (MIP) superfamily and are therefore important factors in regulation of tissue hydraulics,[26] mesophyll conductance,[27] and specifically for events which occur during drought. In addition, aquaporins are also involved in many other basic physiological processes including seed germination, reproductive growth, cell elongation and stomatal movement. Differential expression of aquaporins in tissues subjected to various abiotic stresses, especially drought, has been reported previously.[28] However, it is unknown whether long-distance signals from roots can induce aquaporin expression in shoots, particularly xylem-borne signals coming from droughted roots.

In this study, two aquaporins were identified, probably aquaporin PIP2-2 and probably aquaporin PIP2-6. Both proteins were more abundant in DD and WW compared with WD plants. This finding confirms the expression pattern of aquaporins in our previous drought study on vegetative rice plants, where nine distinct aquaporins were highly expressed in both control and extreme drought conditions but expressed at lower levels during mild drought and the recovery period after drought.[13] The underlying reason for this differential pattern of expression under two contrasting physiological conditions is not clear; aquaporins might play different roles in well-watered plants from those in severe drought, with the former requiring rapid water influx to hydrate expanding cells while the latter requires maximum osmotic water inflow for survival.

A number of important transport proteins were found to be down-regulated in response to drought signals coming from dry roots, even when shoots were well hydrated (positive drought signals). Among these proteins, Coatomer subunit beta-2 and subunit beta-1 were strongly down-regulated in both WD and DD conditions, which is similar to the expression previously reported in chickpea roots in response to drought stress.[29] Similarly, DEAD-box ATP-dependent RNA helicase 12 was down-regulated in both WD and DD. The majority of helicases belong to the DEAD-box protein superfamily, which are

Table 2. List of Differentially Expressed Proteins under Three Different Watering Regimens and Comparison in Their Relative Abundance (Sum NSAF) Across All Treatments Shown in Bars[a]

A

Transport		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115482814|	DEAD-box ATP-dependent RNA helicase 12	
gi|115436002|	Coatomer subunit beta-2	
gi|115484427|	Coatomer subunit beta-1	
gi|115457384|	Probable aquaporin PIP2-6	
gi|115465862|	Plastocyanin	
gi|115472957|	Putative karyopherin-beta 3 variant	
gi|115483604|	Putative carnitine/acylcarnitine translocase	
gi|115436368|	Putative small GTP-binding protein	
gi|115456519|	Ras-related protein Rab11C	
gi|115447423|	Probable aquaporin PIP2-2	
gi|115472727|	Cytochrome b6-f complex iron-sulfur subunit	
gi|115486898|	Ferritin	
gi|115470493|	Succinate dehydrogenase	
gi|115461036|	Plasma membrane ATPase	
gi|115468606|	Vacuolar H+-translocating inorganic pyrophosphatase (V-PPase)	
gi|115468606|	Vacuolar ATPase B subunit	
gi|115452567|	Mitochondrial import inner membrane translocase subunit	
gi|115445159|	Putative hexose transporter	

B

Protein metabolic process		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115449027|	Putative isoleucyl-tRNA synthetase	
gi|115462139|	Histidine--tRNA ligase	
gi|115456525|	Ribosomal protein S6 family	
gi|115463659|	Ribosomal protein L1	
gi|115456525|	Putative plastid ribosomal protein S6	
gi|115463659|	Ribosomal protein L1	
gi|115483552|	Eukaryotic translation initiation factor 3 subunit 9	
gi|115455471|	Eukaryotic translation initiation factor 5A-2	
gi|115464481|	Os05g0477300 protein	
gi|115473507|	Os07g0628700 protein	
gi|115435066|	protein density regulated protein drp1	
gi|115454745|	Valyl-tRNA synthetase	
gi|115483552|	Eukaryotic translation initiation factor 3 subunit 9	
gi|115488938|	Elongation factor Ts	
gi|115440757|	40S ribosomal protein S26 (S31)	
gi|115487104|	40S ribosomal protein S16	
gi|115456089|	40S ribosomal protein S2	
gi|115456215|	40S ribosomal protein S23	
gi|115481984|	40S ribosomal protein S17-4	
gi|115445399|	50S ribosomal protein L21	
gi|115470885|	Putative 60S ribosomal protein L4/L1	
gi|115455427|	Putative ribosomal protein L13a	
gi|115456942|	Os04g0121100 protein	
gi|115465521|	Putative DegP protease	
gi|115457870|	FK506-binding protein	

Protein metabolic process		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115446187|	Putative peptidylprolyl isomerase	
gi|115440455|	Subtilisin-like serine proteinase	
gi|115441285|	Putative subtilisin-like protease	
gi|115458440|	Os04g0430700 protein	
gi|115464789|	Os05g0508300 protein	
gi|115446435|	ATP-dependent Clp protease ATP-binding subunit	
gi|115464933|	Heat shock protein 101	
gi|115462469|	Proteasome subunit beta type	
gi|115480019|	Proteasome subunit beta type 1	
gi|115462469|	Proteasome subunit beta type	

C

cellular component organization		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115440571|	Tubulin beta-4 chain	
gi|115450157|	Tubulin beta-2 chain	
gi|115455701|	Tubulin beta-7 chain	
gi|115469470|	Tubulin beta-3 chain	
gi|115472953|	Tubulin alpha-1 chain	
gi|115444453|	Tubulin beta-5 chain	

D

Stress response		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115456239|	Actin-depolymerizing factor 3 (OsADF3) (ADF-3)	
gi|115449075|	18.6 kDa class III heat shock protein	
gi|115452113|	17.4 kDa class I heat shock protein 3	
gi|115452123|	17.4 kDa class I heat shock protein 2	
gi|115451881|	Small heat shock protein	
gi|115464933|	Heat shock protein 101	
gi|115480399|	Drought-induced S-like ribonuclease	

E

oxidation reduction		Sum NSAF Value
Identifier	Description	WW WD DD
gi|115473931|	Superoxide dismutase [Cu-Zn]	
gi|115443787|	Catalase isozyme A (CAT-A)	
gi|115446541|	2-Cys peroxiredoxin BAS1	
gi|115449517|	Glutathione reductase (GR)	
gi|115489048|	Lipoxygenase	
gi|115454943|	NADH-ubiquinone oxidoreductase 75 kDa subunit	
gi|115470551|	Aldo/keto reductase family-like protein	
gi|115484681|	Alcohol dehydrogenase 1	
gi|115477633|	Putative sorbitol dehydrogenase	
gi|115472727|	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	
gi|115447759|	Glutathione peroxidase	
gi|115463191|	Superoxide dismutase [Mn]	
gi|115445513|	Peroxisomal fatty acid beta-oxidation multifunctional protein	
gi|115450109|	Os03g0101600 protein	
gi|115470493|	Succinate dehydrogenase	
gi|115439655|	Malic enzyme (Fragment)	

[a]Additionally, their relative abundance (sum NSAF) was compared across all treatments as shown in bars in five different biological processes (A) transport, (B) protein metabolic process, (C) cellular component organization, (D) stress response, and (E) oxidation−reduction.

required for normal growth as well as regulation of some stress-induced pathways.[30] Similarly, photosynthetic proteins such as plastocyanin, an electron carrier from cytochrome f in the cytochrome b6/*f* complex Photosystem I, responded to signals from roots in dry soil.[31] Plastocyanin is critical to cyclic photophosphorylation and therefore is likely to have a role in regulating electron transport rate under situations where carbon assimilation and NADPH demand are reduced, thus protecting PSI against excess reactive oxygen production.[32]

4.2. Protein Metabolism

Alterations in protein biosynthesis and proteolysis are critical for the acclimation to abiotic stresses such as water deficits.[33] At early stages of water stress, synthesis of stress-response proteins increases but if the stress becomes deleterious, growth ceases and senescence is triggered, which includes proteolytic breakdown of photosynthetic proteins. This was observed very distinctly among the "protein metabolic process" proteins where a large number of translation factors (eukaryotic translation elongation factors), proteins in the ribosomal family which are involved in protein synthesis, initiation factors, elongation factors, and tRNA synthetases were down-regulated in both the WD and DD plants when compared with WW plants (Table 2B). Eukaryotic initiation factors (eIF) are proteins involved in the initiation phase of eukaryotic translation and function by forming a complex with the 40S ribosomal subunit promoting ribosomal scanning of mRNA and joining with the 60S ribosomal subunit to create the 80S ribosome. We observed that proteins of the 40S ribosomal

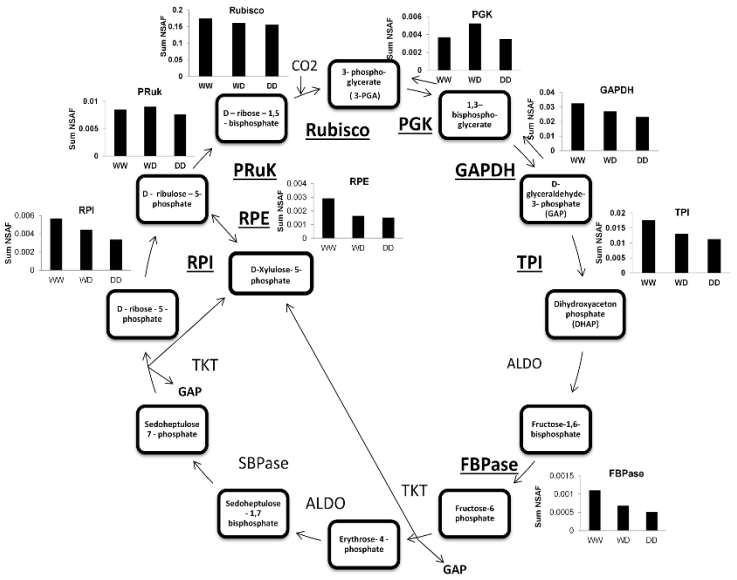


Figure 3. Changes in abundance of Calvin Cycle enzymes in leaves of WW, WD, and DD plants. Sum NSAF value for each protein is the sum of the biological triplicates with higher NSAF corresponding to higher abundance.

family, namely, 40S ribosomal protein S2, 40S ribosomal protein S16, 40S ribosomal protein S23, 40S ribosomal protein S26, and 40S ribosomal protein S17-4 were significantly down-regulated in DD plants and even more strongly down-regulated in WD plants. In addition, eukaryotic translation elongation factor 3 subunit 9 and elongation factor Ts were also significantly down-regulated in WD and, to a lesser degree, in DD plants. Lowered levels of a majority of ribosomal proteins in WD and DD plants suggests that protein synthesis was inhibited in both conditions; however, the impact was, paradoxically, often more severe in plants with only half the roots droughted. This suggests that a strong positive signal arriving at the leaves from droughted roots was enhanced when combined with sap from well-watered roots; ABA delivery in WD plants could fulfill this role. A lower abundance of ribosomal proteins was previously reported when rice cell cultures were grown at more than 36 °C.[34]

Interestingly, proteins involved in proteolysis were very specifically up-regulated in WD plants, indicating that signals from dry roots interacted with hydrated shoots. These included three proteases, putative peptidylprolyl isomerase, subtilisin-like serine protease, and putative subtilisin-like protease. None was up-regulated substantially when roots were droughted. In contrast, a few proteins were up-regulated approximately in proportion to the strength of the drought impact. These included three proteasome subunits and ATP-dependent Clp protease ATP-binding subunit, all being potential drought markers. These are proposed to play a role in turnover of the proteome, including ribosomal proteins, initiation factors, and elongation factors.

4.3. Cellular Component Organization

All the statistically significant changes in tubulins were a down-regulation caused by part or all the root system being droughted (Table 2C). While these proteins are thought of as housekeeping proteins and are thus used as internal controls for various expression studies, including on water stressed tissues,[35,36] differential expression of these proteins in Table 2B suggests a dynamic role of tubulins in abiotic stresses. Tubulins are questionable as housekeeping genes, reconfirming our earlier drought study on rice roots.[10] In addition to these reports, the down-regulation of tubulins was also reported in two taxa and advanced-generation hybrids of *Piriqueta cistoides spp.* in response to prolonged drought stress.[37]

4.4. Stress Response

Among stress-related proteins, five heat shock proteins were identified (Table 2D), namely, putative 17.8 kDa class II heat shock protein, small heat shock protein, 17.4 kDa class I heat shock protein 3, 17.4 kDa class I heat shock protein 2, and heat shock protein 101. Interestingly, four heat shock proteins showed a similar trend; that is, they were most abundant when roots were fully droughted, with a dose-dependent reduction in WD plants, suggesting that Hsp expression is triggered by a drought signal, most probably ABA.[38] Hsps are molecular chaperones and their role is to retain and protect the unfolded state of newly synthesized proteins in order to inhibit them from misfolding or aggregating.[39] Thus they are probably protective, even in a highly drought-sensitive plant such as japonica rice. Hsps/chaperones were initially thought to be differentially expressed only in response to heat stress; however, differentiation in expression of these proteins has also been found in recent studies on various other environmental stresses such as drought,[40] salt,[41] and cold.[42] Differential expressions of Hsps from various studies have proven that they may be an important element in adaptation or protection of plants against different stimuli by restoration of cellular homeostasis.

We also identified actin-depolymerizing factor 3 (ADF-3), and found that it was highly expressed in WD and DD conditions but absent in well-watered plants. Actin-depolymerizing factor is known as a vital element for osmoregulation under osmotic stress and part of the defense response against abiotic stresses such as water deficit, salinity, and cold.[43] In addition, we found drought-induced S-like ribonuclease protein is significantly up-regulated when roots were droughted. Up-regulation of this protein was previously reported in various environmental stresses such as drought stress,[44−46] cold,[47,48] and salt stress.[49]

dx.doi.org/10.1021/pr400696u | J. Proteome Res. 2014, 13, 517−526

4.5. Oxidation–Reduction

Water stress deficits inhibit photosynthesis through stomatal closure although the greater effect on growth of sinks commonly causes carbohydrate accumulation when water is withheld.[50] Therefore, surplus energy produced due to continuing excitation of the light harvesting complexes causes increased production of reactive oxygen species (ROS) in chloroplasts and can directly impair many processes including the function of photosystem (PS) II.[51] Among differentially expressed proteins found in this study in the oxidation–reduction category, we identified five antioxidant enzymes (Table 2E). Many protective enzymes, including catalase isoenzyme A, glutathione reductase, and 2-Cys peroxiredoxin BAS1, were especially abundant when only half the roots were droughted, reminiscent of the phenomenon reported where the partial drought induced a distinct gene expression pattern (section 3.2 above). Glutathione peroxidase was highly expressed only when the entire root system was droughted; this presumably works in concert with catalases to detoxify peroxide. Superoxide dismutase, a critical enzyme in plants for avoidance of oxidative stress, was found in two functionally contrasting forms; superoxide dismutase [Mn] rose in proportion to the severity of the drought while superoxide dismutase [Cu–Zn] appeared to play a constitutive role in fully watered plants; these opposing expression patterns were also found for superoxide dismutases in rice cultivars in response to drought.[44,45]

4.6. Carbohydrate Metabolism

The patterns of differential expression of proteins reported above reflect complex signals emanating from droughted roots, some of which can be overridden when shoots are hydrated (WD plants), others not. The effect of drought in the root system on carbohydrate levels in shoots is highly predictable because of the imbalance which develops between photo assimilate synthesis and utilization[52] (Figure 1A). This experiment gave us the opportunity to test whether this secondary response to drought was accompanied by specific changes in the enzymes associated with carbohydrate metabolism.

Even though soluble carbohydrates accumulated, photosynthesis was probably suppressed by stomatal closure, potentially causing a feedback on Calvin Cycle activity. Accordingly, we detected a proportional reduction in the expression of a majority of enzymes associated with carbon fixation (Calvin Cycle) in DD and WD treatments (Figure 3). The proteins identified included ribulose bisphosphate carboxylase large chain (Rubisco), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TPI), fructose-1,6-bisphosphatase (FBPase), ribulose-phosphate 3-epimerase (RPE), ribose-5-phosphate isomerase (RPI), and phosphoribulokinase (PRuk).

Reducing sugars accumulated when plants were droughted. Sucrose is known to modulate expression of a large number of metabolic genes through sugar signaling pathways.[53] Hence in drought, sucrose could have a direct impact on protein levels. We therefore concentrated on three key enzymes: sucrose synthase, invertase, and sucrose phosphate synthase. Sucrose phosphate synthase, which consumes hexoses to form sucrose, was unaffected by watering treatments. However, both degradative enzymes, namely, invertase and sucrose synthase, were up-regulated, possibly by a drought signal arising in the roots or a passive accumulation of sucrose in the shoots. These possibilities could be tested experimentally, for example, by chilling roots where both enzymes also increased 2-fold when the roots were under stress.[54]

High sucrose levels in droughted plants would have a favorable effect on acclimation to drought by acting as an osmoprotectant, thus preventing protein denaturation and maintaining cellular membrane integrity. Sucrose also contributes to osmotic adjustment in cereals. In addition, sucrose may also play a role as a water replacement to sustain membrane phospholipids in the liquid-crystalline phase and to avoid structural changes in soluble protein.[55] Proteins associated with starch metabolism were also found to be up-regulated in drought conditions. ADP-glucose pyrophosphorylase and granule-bound glycogen/starch synthase were especially abundant in fully droughted plants, and the amount of each enzyme was proportional to the water supply, with the metabolic machinery required for starch synthesis largely absent. Even more stark was the down-regulation of the starch branching enzyme when plants were well-watered (Figure 4).

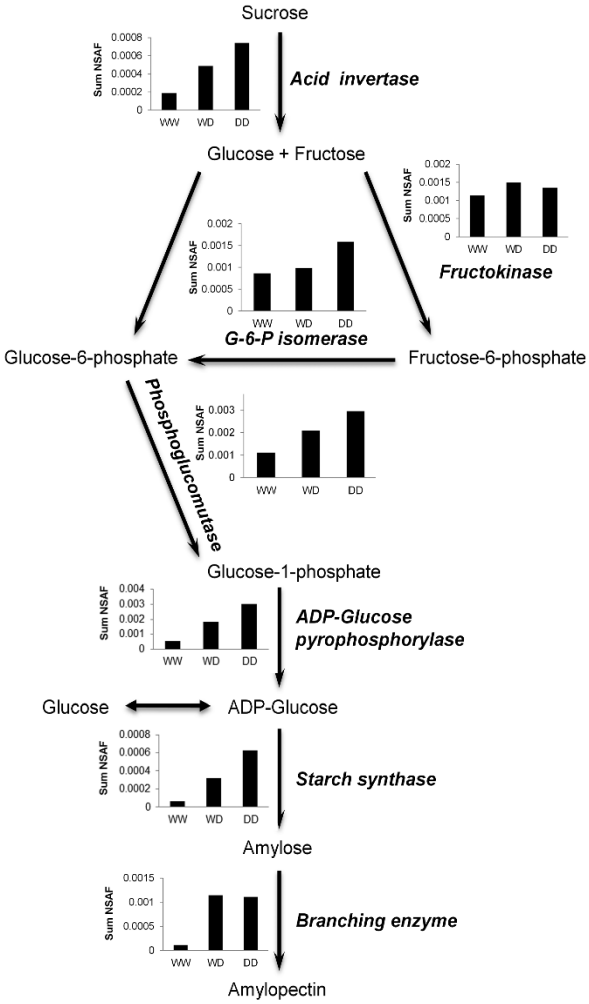


Figure 4. Pathways of carbohydrate transformation in leaves, showing sucrose degradation and starch synthesis with the key enzymes responsible depicted. Histograms show the abundance of all proteins which were differentially expressed ($p = 0.05$) in WD and DD plants compared to WW plants.

5. CONCLUSIONS

This study expanded on the theme of an earlier paper[10] dealing with the proteome of roots in dry soil. We now report on changes in the proteome of shoots of these plants to infer the impact of soil drying on shoot metabolism. By dividing the root system into separate compartments, we could compare fully droughted plants with well-watered controls as well as partially droughted plants in which "wet" and "dry" root-borne signals interact. The patterns observed are interesting in that in some categories, proteins are consistently down-regulated by drought as might be expected (e.g., cellular component organization) whereas in other categories, substantial numbers of proteins are most abundant when leaves are receiving signals from both "wet" and "dry" roots (e.g., protein metabolism, oxidation−reduction). In yet other categories, drought consistently up- or down-regulated proteins (e.g., transport). This may provide scope for selection of markers specific to various drought regimes.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Table S1: List of the reproducible proteins identified in each condition (WW, WD, and DD), including their raw peptide counts and corresponding NSAF value. Supplementary Table S2: Details of 143 significantly differentially expressed proteins across three conditions using ANOVA including their *p*-value and biological classification of differentially expressed proteins (up-, down-regulated, and unchanged), obtained from *t*-test analysis of WW versus WD and DD plants. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with ProteomeXchange accession: PXD000319. Username: review90470. Password: JFE5HLdE

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

P.A.H. acknowledges funding support from Macquarie University and the Australian Research Council and wishes to thank Henry Holmes for continued support and encouragement. G.H.S acknowledges funding support from Agricultural Biotechnology Research Institute of Iran.

■ ABBREVIATIONS

ABA, abscisic acid; ACN, acetonitrile; ADF, actin-depolymerizing factor; DD, droughted plants; DTT, dithiothreitol; FDR, false discovery rate; GO, gene ontology; GPM, global proteome machine; Hsp, heat shock protein; nanoLC−MS/MS, nanoflow liquid chromatography−tandem mass spectrometry; NSAF, normalized spectral abundance factor; PloGO, Plotting Gene Ontology annotation; PRD, partial root zone drying; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SpC, spectral count; TCA, tricarboxylic acid; V-PPase, Vacuolar H^+-translocating inorganic pyrophosphatase; WD, split-root plants; WW, well-watered plants

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Appendix 2

DOI 10.1002/pmic.201200475 *Proteomics* 2013, *13*, 1922–1933

RESEARCH ARTICLE

The influence of signals from chilled roots on the proteome of shoot tissues in rice seedlings

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Low root temperature causes a decrease in water uptake, which leads to mineral and nutrient deficiencies with potentially decreased root and shoot growth. Differential temperature effects in plants have been studied extensively, however, the effect of root chilling on the global protein expression in shoots has not been explored. In this study, we imposed chilling temperatures on roots of rice plants while maintaining shoots at optimum atmospheric temperature. Shoot materials (growing zones and leaves) were harvested at five points over a time course of four days, including a two-day recovery period. Proteins were quantified by tandem mass tags and triple stage MS, using a method developed to overcome ratio compression in isobaric-labelled quantitation. Over 3000 proteins in each of the tissues were quantified by multiple peptides. Proteins significantly differentially expressed as compared with the control included abscisic acid-responsive and drought-associated proteins. The data also contained evidence of a possible induction of a sugar signalling pathway.

Received: October 18, 2012
Revised: December 6, 2012
Accepted: February 7, 2013

Keywords:
Plant proteomics / Rice / Root chilling / Signalling / Tandem mass tags

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1 Introduction

Rice faces multiple abiotic stresses and is the subject of extensive research [1–4]. As a tropical grass, it is particularly susceptible to low temperatures at all stages of growth, especially during fertilization [5]. However, less obvious chilling injury can occur during vegetative growth and is especially likely as the growing range of the crop increases with human food demand, and extends into marginal areas. Specifically, root chilling is possible during the early daylight hours when the soil is slower to warm than the atmosphere and evaporative cooling near the root growing zone can be considerable. Roots exposed to chilling have diminished water uptake due to increased root hydraulic resistance [6] and decreased membrane permeability [7]. This leads to reduced water and nutrient uptake, with potentially decreased root and shoot growth [8].

The impact of root chilling on physiological function has stimulated extensive studies of events in the shoot tissues of mainly perennial species. These studies concentrate on biomass allocation [9], allocation of nutrients and carbohydrates [10], shoot water relations [11] and, in the case of rice, nutrient uptake and growth [8]. Gene expression in leaves of plants subjected to root chilling treatment has been examined in wheat [12] but, to our knowledge, a proteomic investigation of the effects of chilled roots on shoots maintained at optimum temperature has not been performed.

This study addresses a fundamental question of plant development because it provides a means of perturbing the root-shoot functional equilibrium by depressing metabolic activity in the roots without directly affecting the shoots. In that root chilling induces a complex set of tissue responses [13, 14],

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Abbreviations: ABA, abscisic acid; **FDR**, false discovery rate; **LOX**, Lipoxygenase; **MS3**, triple stage MS; **TMT**, tandem mass tag

Colour Online: See the article online to view Fig. 2 in colour.

detailed global proteomic studies are a way to address the effect of transmitted signals from the chilled cells to untreated tissues, in this case, shoots. The nature of these long-distance signals and the genes and proteins they interact with remains one of the greatest puzzles in plant biology. It is well established that reduced respiration in chilled roots conserves carbohydrates, causing an imbalance of photoassimilate levels. Accordingly, in young cotton plants, soluble carbohydrates accumulated in the leaves when roots were chilled [15]. Sucrose is more than a metabolic resource for plants, acting as a signal in development and thus a potential means of communicating a constraint on root metabolism in the shoots [16].

Abscisic acid (ABA) is also an effective long-distance signal, which is known to increase in response to cold stress as well as other abiotic perturbations [17–19]. In differential temperature studies where roots have been chilled, ABA increases in xylem exudates and enhances tolerance by inducing stomatal closure to reduce water loss [20]. It has also been shown that turgor pressure in chilled root plants was maintained in plants pre-treated with ABA [21], suggesting that ABA has a key role in hydraulic function.

Proteomics has been employed extensively in the analysis of cold stress in plants [22] and recently to elucidate long-distance drought signalling in a split root experiment [23]. However, this is the first attempt at using the technique to identify proteins that respond to transmitted chilling-induced signals. The majority of plant proteomics studies have been executed using a 2DE approach [1]. However, the field of proteomics has moved beyond laborious, visual spot volume comparisons toward accurately and reliably quantifying proteins at the MS level [24]. In the current study, we employed tandem mass tags (TMTs) [25], which enabled simultaneous identification and multiplexed quantitation of thousands of proteins. Quantitation of isobaric-labelled peptides has come under scrutiny in recent years for compression of signal ratios. This problem has been ameliorated with the development of a method, which we employed in this study, for isolating target reporter ions from concomitant contaminating, near-isobaric ions using triple stage MS (MS3) [26].

In the present study, the roots of rice plants grown hydroponically were subjected to chilling temperature of 15°C in a time course experiment extending over 4 days and including a 48-h recovery period. To analyse the long-distance effects on growth and protein expression in shoots, leaf elongation rates were measured, and proteins from the shoot growing zones and leaves of plants were analysed by TMT labelling followed by protein identification and MS3-based quantitation.

2 Materials and methods

2.1 Growth and treatment conditions

Seedlings of a relatively cold-tolerant rice cultivar (*Oryza sativa* L. cv. Nipponbare) were germinated in soil and transferred to a hydroponic solution at the 2–3 leaf stage. The hydroponic solution contained 0.09 mM $(NH_4)_2SO_4$, 0.05 mM KH_2PO_4, 0.05 mM KNO_3, 0.03 mM K_2SO_4, 0.06 mM $Ca(NO_3)_2$, 0.07 mM $MgSO_4$, 0.11 mM Fe-EDTA, 4.6 μM $MnSO_4$, 0.3 μM $ZnSO_4$ and 0.3 μM $CuSO_4$ according to [27]. Plants were grown in a temperature-controlled glasshouse with a 12-h photoperiod and day/night temperatures of 29/18°C until the 6–7 leaf stage. After 4 h of daylight, four-fifths of the plants were transferred to a separate hydroponic solution in a foam-insulated container for root chilling treatment. Chilling was achieved using a temperature-controlled recirculating pump with 14°C water passed through copper pipes placed at the bottom of the container. The temperature of the hydroponic solution was decreased gradually from 29°C to 15 ± 2°C over approximately 3 h, so as to reduce shock injury to the plants, and remained at this temperature for the duration of the treatment. The roots were the only part of the plant, which came in contact with the chilled hydroponic solution and, as air temperature remained at 29/18°C, the shoot growing zones and leaves of the treated plants remained at optimum growth temperatures.

The shoot growing zones, laminas of all leaves and entire root systems of plants were harvested 0, 8, 24 and 48 h after initiation of root chilling. The temperature of the chilled roots was then gradually brought back to air temperature and the plants were allowed to recover for two days. At 96 h after the initial treatment, tissues were harvested from the remaining plants as a recovery time point.

2.2 Leaf length measurement

The length of the seventh emerging leaf from control and treated plants was measured in four plants at each harvest and additionally at 72 h after initiation of root chilling. Leaf elongation rates were calculated by dividing changes in leaf length by elapsed time. A Student's *t*-test was performed between leaf elongation rates of leaves from control plants and leaves from the chilled roots plants over the treatment period (0–48 h) and over the two-day recovery period (48–96 h).

2.3 Protein extraction

Harvested plant material was immediately frozen in liquid nitrogen and stored at -80°C until required. Proteins were extracted from the shoot growing zones and leaves by first grinding the tissue to a fine powder in a mortar and pestle in the presence of liquid nitrogen. To 1 g of powdered tissue, 5 mL of cold extraction buffer (8 M guanidine-HCl, 10 mM Tris-HCl, pH 8.0, 1% w/v *N*-laurylsarcosine, 1% w/v polyvinyl polypyrrolidone, 1 mM sodium metabisulfite, 1% v/v β-mercaptoethanol, protease inhibitors (Roche), 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM PMSF) was added and probe sonicated on ice for 3 × 10 s pulses at 60% amplitude. The extracts were incubated at 37°C

for 1 h with occasional vortexing followed by centrifugation at 5000 × *g* for 10 min. The supernatant for each extract was divided into three equal volumes of approximately 1.5 mL, and proteins were precipitated with chloroform and methanol [28]. At room temperature, 6.5 mL methanol and 1.5 mL chloroform were added to the protein extract and mixed by inversion, then 4.5 mL of water was added and mixed by inversion. Samples were incubated at room temperature for 15 min then centrifuged at 5000 × *g* for 5 min. The samples were separated into three phases: the upper phase was removed carefully and discarded, and 4.5 mL methanol was added to the inter- and lower phases. The extract was vortexed and centrifuged at 5000 × *g* for 5 min. The supernatant was removed and the pellet was washed twice with ice-cold 100% v/v methanol and once with 90% v/v methanol containing 5 mM sodium metabisulfite.

2.4 Lys-C digestion and TMT reaction

Protein pellets were solubilized in 2 mL of 8 M urea and a BCA assay (Thermo Scientific, Rockford, IL) was performed to determine protein concentration. Urea was diluted to 3 M with 50 mM Tris-HCl, pH 7.5 and proteins were digested with 1:80 enzyme/protein ratio of Lys-C (Wako, Japan) at 37°C overnight. Lys-C digestion was terminated with 1% v/v final concentration of formic acid and peptides were desalted with C_{18} SPE (Sep-Pak, Waters, Milford, MA). Peptides were dried in a vacuum centrifuge and resuspended in 200 mM Na-HEPES, pH 8.5. For each time point, 100 μg of digested peptides were labelled with reagents from a 6-plex TMT kit (Thermo Scientific) according to [26]. The 0 h time point was labelled twice to make up a 6-plex, such that the labels TMT-126, -127, -128, -129, -130 and -131 were reacted with peptides from the samples 0, 8, 24, 48, 96 and 0 h, respectively, for each of the shoot growing zone and leaf samples. TMT reagents were resuspended in 40 μL of anhydrous ACN and 0.2 mg of reagents was combined with each of the 100 μL peptide aliquots. Samples were incubated at room temperature for 1 h and the reaction was quenched with the addition of hydroxylamine to a final concentration of 0.3% v/v for 15 min at room temperature. The TMT-labelled samples were mixed in equal ratios, diluted with 1% v/v formic acid, desalted by C_{18} SPE and dried in a vacuum centrifuge.

2.5 Strong cation exchange chromatography

Samples were fractionated by strong cation exchange chromatography using an Agilent 1100 quaternary HPLC pump with a PolyLC polysulfoethyl aspartamide column (200 mm × 4.6 mm, 5 μm, 200 Å; PolyLC, Columbia, MD). The column was equilibrated with Buffer A (7 mM KH_2PO_4, pH 2.6, 30% v/v ACN), which was also used for sample resuspension, sample injection and peptide adsorption to the column. Peptide elution was achieved with a 60 min gradient to 100% Buffer B (7 mM KH_2PO_4, 350 mM KCl, pH 2.6, 30% v/v ACN), at a flow rate of 0.9 mL/min. Peptides were collected in 1.5 min increments and dried in a vacuum centrifuge. Fractions were resuspended in 1% v/v formic acid and, based on the intensity from the strong cation exchange chromatographic UV trace, combined into a total of 20 fractions and 15 fractions for peptides from the shoot growing zone and leaf samples, respectively. These fractions were desalted by C_{18} SPE and dried in a vacuum centrifuge.

2.6 nano-LC-MS3

Peptides were analysed using LC coupled to MS3 (LC-MS3), which reduces TMT signal ratio compression as described previously [26]. The shoot growing zone and leaf TMT-labelled samples were each analysed using an LTQ Orbitrap Velos (Thermo Fischer Scientific, San Jose, CA) equipped with a Famos autosampler (LC Packings) and an Agilent 1100 binary HPLC pump (Agilent Technologies, CA). For both samples, peptides were separated on a microcapillary column (100 μm id) packed in-house with approximately 0.5 cm of Magic C_4 Resin (5 μm, 100 Å, Michrom Bioresources) followed by 20 cm of Maccel C_{18} AQ resin (3 μm, 200 Å, Nest Group). Peptides were separated by applying a 9–32% ACN gradient in 0.125% formic acid over 150 min at approximately 300 nL/min. An electrospray voltage of 1.8 kV was applied through a polythereterketone junction at the inlet of the microcapillary column. In data-dependent mode, the survey scan was performed in the Orbitrap over the range of 300–1500 m/z, and the top ten most intense ions were isolated for ion-trap CID-MS2 at a precursor isolation width of 2 m/z, using an AGC setting of 2×10^4, a maximum ion accumulation time of 150 ms and wide band activation. Following MS2 fragmentation, the most intense fragment ion in a m/z range of 110–160% of the precursor m/z was selected for high-energy collision (HCD)-MS3. The fragment ion isolation width was 4 m/z, the MS3 AGC was 2×10^5 and the maximum ion time was 250 ms. Normalized collision energy was set to 35 and 60% at an activation time of 20 and 50 ms for MS2 and MS3 scans, respectively.

2.7 Data processing

Mass spectra were processed using a pipeline of software tools that was developed in-house in the laboratory of Professor Steven Gygi. Mass spectra, in the form of RAW files, were converted to mzXML format using a modified version of ReAdW.exe that included ion accumulation time, and searched against the NCBI *O. sativa* Reference Sequence (RefSeq) database (28 555 sequences, September 2010) using the Sequest algorithm [29] for peptide assignment of MS2 spectra. The RefSeq database was concatenated with common contaminant proteins and all sequences listed in reverse order for false discovery rate (FDR)

estimation. Searches were performed using a 10 ppm precursor ion tolerance, 6-plex TMT tags on lysine residues and peptide N-termini (+229.162932 Da) was set as a static modification, and oxidation of methionine residues (+15.99492 Da) was set as a variable modification. Peptide spectral matches were filtered to a protein FDR of less than 2% using the target decoy database search strategy [30]. Filtering was performed using a linear discrimination analysis method [31] considering the following parameters: XCorr, ΔCn, missed cleavages, peptide length, charge state and precursor mass accuracy.

2.8 Data analysis

For quantitation of TMT reporter ions, the S/N for each TMT channel was extracted by finding the closest matching centroid to the expected mass of the TMT reporter ion in a window of 0.06 m/z. Reporter ions were adjusted to account for isotopic impurities in each TMT variant as provided by the manufacturer. Peptides were assembled into proteins guided by principles of parsimony to produce the smallest set of proteins necessary to account for all observed peptides. Proteins were then quantified by summing reporter ion counts across all peptide identifications. Peptides that did not have a TMT reporter ion signal in all channels were excluded from quantitation. Summed intensities were normalized to the channel that contributed the highest overall signal.

Differentially expressed proteins were identified using both a fold change z-score and a p-value cutoff based on the distribution of peptides. The normalized summed intensity for the two control channels (126 and 131) were first averaged using geometric means. A fold change between proteins identified in the time points of the chilled roots treatment (channels 127–130) and averaged control channels was calculated using normalized summed intensities, and then the corresponding z-scores were generated from the log fold changes. At the peptide level, the S/N for each channel was normalized to the sum of the channel that contributed the highest overall signal. For each protein, a two-sample t-test was performed on the log normalized S/N peptide ratios for each channel to compare the population of peptides for a given protein in the treatment to the population of peptides for the same protein in the averaged control. Proteins were determined to be differentially expressed if the absolute value of the log fold change z-score was greater than 1, and the peptide p-value < 0.1.

2.9 Analysis of differentially expressed proteins

Proteins that were identified as having differential expression in at least one time point in relation to the control were hierarchically clustered with a correlation-based metric and complete linkage using the hclust implementation in the R *stats* package. The resulting dendrograms were divided to create four clusters for proteins in each of the shoot growing zone and leaf samples. The log normalized summed intensities were averaged for each cluster, and the individual normalized summed intensity for each protein in the cluster was overlayed to show within cluster variability.

Gene ontology (GO) information was used to categorize the biological processes of differentially expressed proteins. GO annotations were extracted from the UniProt database and matched to corresponding gene identifiers embedded in the NCBI *O. sativa* RefSeq database. Proteins were then classified based on biological process at level 2 using Web Gene Ontology Annotation Plot (WEGO) (wego.genomics.org.cn/cgi-bin/wego/index/pl) [32].

3 Results and discussion

3.1 Leaf growth and elongation rates

Leaf length was measured at 0, 8, 24, 48, 72 and 96 h after root chilling began. Leaf elongation rates declined in control plants, which were maintained at 29/18°C over the 96-h period (Fig. 1). Leaf elongation rates were significantly slower after 48 h of root chilling ($p < 0.05$) in comparison with control plants, whereas during the recovery period, leaf elongation rates in the chilled root plants increased steadily and exceeded that of control plants at the 96 h time point. The recovery of leaf growth indicated that the plants were not irreversibly damaged by cold root treatment.

3.2 Proteomics

A total of 4979 and 4866 proteins were identified (protein FDR < 2%) in the shoot growing zone and leaf samples, respectively (Table 1). Of these, 3133 and 3005 proteins were quantified by multiple peptides in the shoot growing zone and leaf samples, respectively (Supporting Information Data 1). All peptides identified for each protein contributed an ion signal in each TMT channel.

The intensity values of all proteins that were quantitated were log transformed and graphed as a function of the log (normalized summed intensity) of averaged control samples (Fig. 2). For both samples, regression analysis revealed that the greatest change among proteins in the treated plants compared with the control plants was seen at time points 8 and 96 h, while the smallest contrast in the proteomes occurred after 48 h of stress.

In order to assess experimental reproducibility, the duplicated control samples were plotted against each other in each tissue type to evaluate the consistency of the TMT experiment. In both tissues, only small deviations were observed between replicate labelling, with R^2 values of 0.99 and 0.98 in the shoot growing zone and leaf samples, respectively.

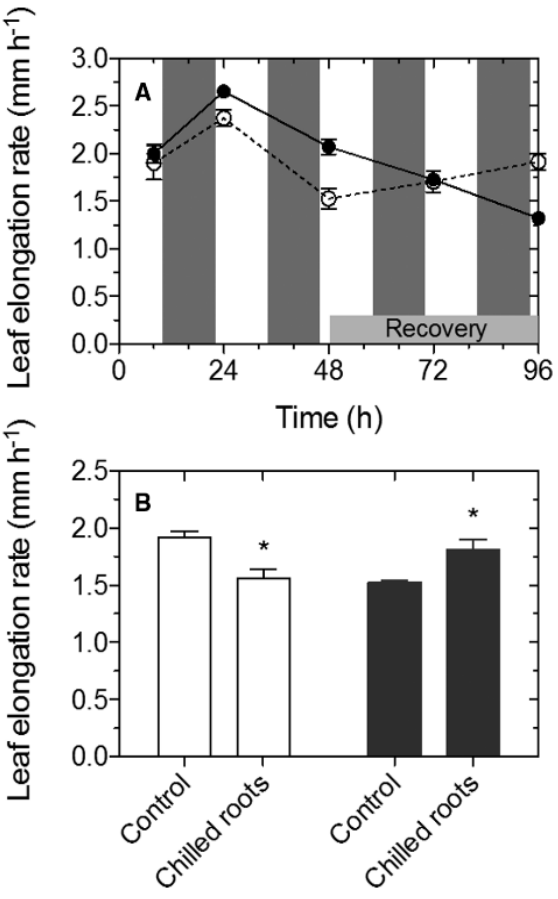

Figure 1. Leaf elongation rates over the 96 h of treatment and recovery (A) for control (closed circles/filled line) and chilled root (open circles/dashed line) plants. Vertically shaded areas represent dark photoperiods and the horizontal shaded area represents the recovery period for treated plants. (B) Mean leaf elongation rate over 0–48 h (open bars) and 48–96 h (closed bars). All values given are means ± SE of four plant replicates.

3.3 Statistically significant differentially expressed proteins

To determine whether a protein was significantly up- or down-regulated in relation to the control, we imposed two criteria: (i) the absolute *z*-score calculated using fold change values must be greater than one and, (ii) a *p*-value < 0.1, according to a *t*-test performed between the S/N ratios of the population of peptides identified for a protein in the control and in each treatment channel. These stringent criteria ensured that proteins found to have significant differential expression deviated from the mean by one standard deviation at the protein level, with minimal variation at the peptide level in each population of peptides for a protein. The *t*-test performed at the peptide level excluded many peptides with low intensity, and *p*-values < 0.1 were determined to be significant based on a low false positive rate when comparing the two control channels against each other.

In total, 150 proteins and 100 proteins (excluding contaminants) were found to have significant differential expression in the shoot growing zone and leaf samples, respectively, in at least one time point when compared to the control (Supporting Information Data 1). In the shoot growing zones, 67 proteins were identified as upregulated, while 84 proteins were found to be downregulated. Conversely, the majority of proteins in the leaf sample were found to be upregulated (59 proteins) compared with 43 proteins downregulated. Overall, the direction of differential expression across the time points was mostly consistent; one protein in the shoot growing zone samples and two proteins in the leaf samples were identified as being up- and downregulated at different time points.

Eleven proteins common to the shoot growing zone and leaf samples were differentially expressed when roots were chilled (Supporting Information Data 2). These proteins included a thiamine biosynthetic enzyme, which was significantly upregulated at the 8 h time point in both samples. This protein is known to be diurnally regulated [33], so the contribution of root chilling to the increase in thiamine biosynthetic enzyme remains uncertain. However, the scale of the increase (6.46- to 17.84-fold) suggests that its expression was exacerbated by chilling.

Proteins identified as differentially expressed at each time point are graphically represented in Fig. 3 as a percentage of total differentially expressed proteins in each sample. There was greater consistency in differential expression in the shoot growing zone with 22 proteins (16%) identified as either up- or downregulated at three or more time points, compared with only six proteins (5%) in the leaf sample (listed in Table 2). There are also proteins identified as differentially expressed exclusively at one time point. The time point with the largest number of such proteins in the shoot growing zone sample was at 8 h and in the leaf sample was at 24 h, while 48 h showed the least amount of unique protein expression in both samples. Cluster analysis of differentially expressed

Table 1. Proteomics data summary

Tissue	Total proteins	Unique peptides	Total peptides	Protein FDR (%)	Peptide FDR (%)	Proteins quantified[a)]	Peptides quantified
Growing zone	4979	21 779	49 310	1.97	0.52	3133	27 446
Leaf	4866	21 004	40 992	1.97	0.41	3005	23 788

a) Number of proteins quantified by multiple peptides.

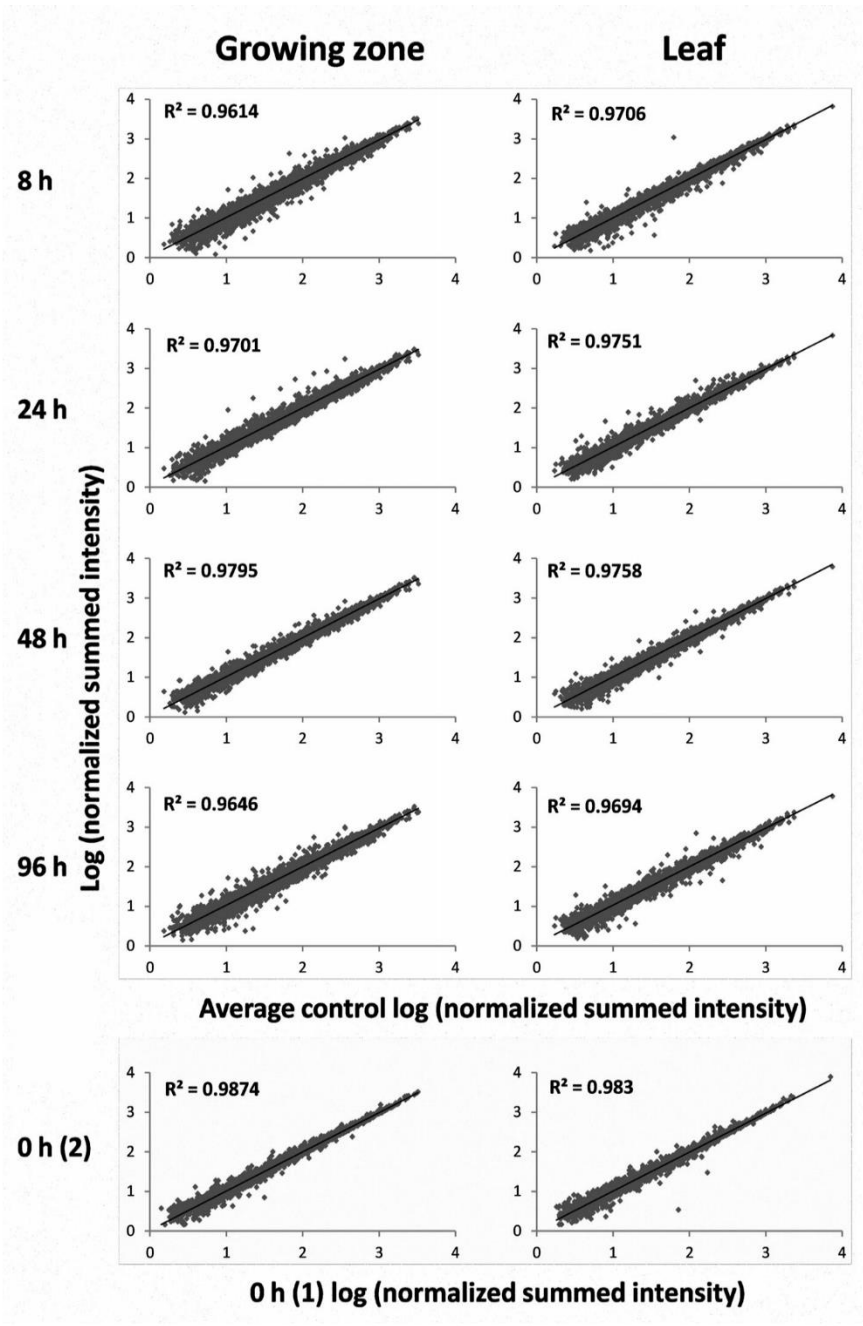

Figure 2. Regression analysis of all proteins quantified. The log normalized summed TMT peptide signal intensities of all proteins in each time point were plotted on the *Y*-axis against the average control values on the *X*-axis. Proteins on or near the diagonal are relatively unchanged compared to the control, while proteins deviating from the diagonal are those that are differentially expressed at a given time point. In the bottom panel, the duplicate control channels were plotted against each other to assess experimental consistency.

proteins (Fig. 3) also indicated that the proteome varied the least after 48 h of chilled root treatment.

3.4 Functional analysis of differentially expressed proteins

Differentially expressed proteins were categorized into level 2 biological processes using WEGO. To gain a broad perspective of protein functions, significantly differentially expressed proteins in each sample were divided into up- and downregulated categories, rather than classifying proteins in individual time points (Fig. 4). The parent categories, metabolic process and cellular process, which contained the majority of proteins, were further categorized at GO level 3 (Supporting Information Data 3). Interestingly, similar trends in functional classification were rarely observed between the two tissues, reflecting their contrasting functions as expanding and expanded tissues.

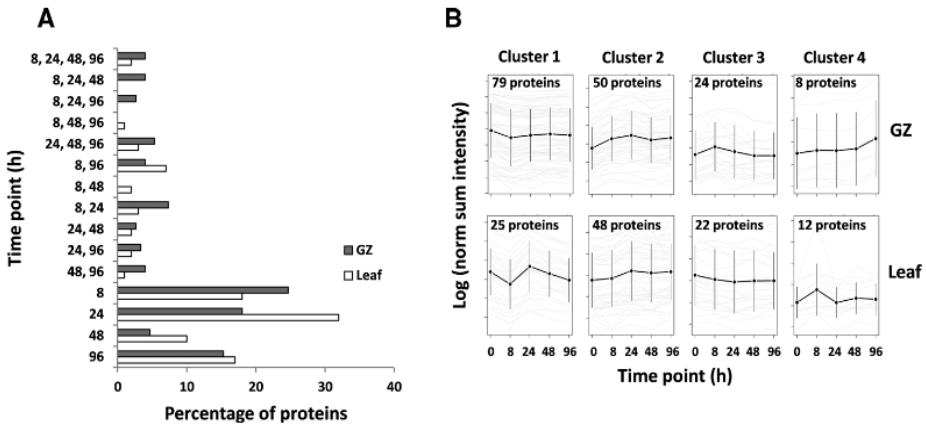

Figure 3. Panel (A), percentages of the 150 differentially expressed proteins identified in growing zone (shaded bars) and leaf (open bars), which were found at each time point and at multiple time points. Panel (B), hierarchical cluster analysis of differentially expressed proteins for growing zone (upper) and leaf (lower), based on normalized summed TMT peptide signal intensities. The number of proteins in each cluster is indicated at the top of each panel, and the error bars indicate the degree of variability in protein expression at each time point.

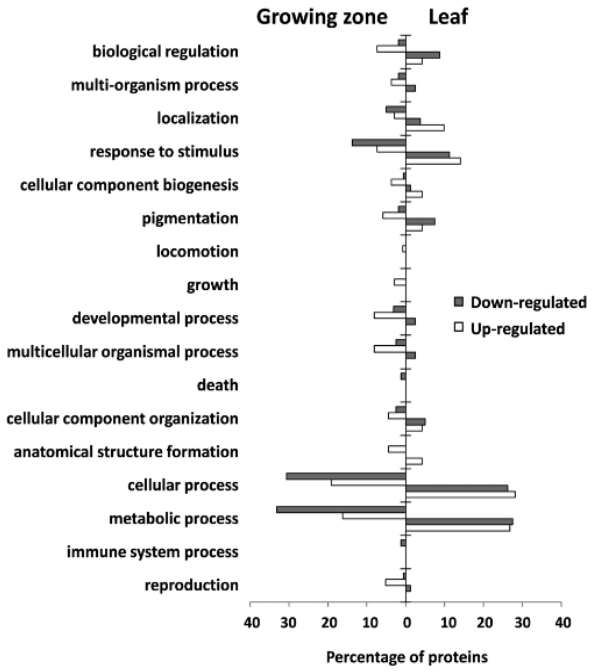

Figure 4. Functional classification of differentially expressed proteins into the 17 categories identified at GO biological process level 2. Shoot growing zone is plotted to the left, and leaf is plotted to the right of the central *Y*-axis. The values indicate the number of proteins found in a given functional category, expressed as a percentage of the total number of categorized differentially expressed proteins.

The response to stimulus category also showed a converse relationship in the two tissues; in the shoot growing zones, the majority of proteins were downregulated (14%) as opposed to upregulated (7%), in contrast to the leaves where the majority of proteins were upregulated (14%) as opposed to downregulated (11%) (Supporting Information Data 4). Intriguingly, proteins typically upregulated in response to cold stress, such as 70 kDa HSP, GST, 20 kDa chloroplastic chaperonin, and superoxide dismutase, were significantly downregulated at several time points in the shoot growing zones [22]. In the leaves, two peroxidases were found to be upregulated, indicating either a tissue-specific response to free radical scavenging or tissue-specific production of ROS after root chilling treatment.

3.4.1 Hormone-induced and water deficit associated proteins

A close inspection of proteins in the response to stimulus category, along with manual categorization of proteins not assigned GO information, revealed many differentially expressed proteins as being responsive to phytohormones. Of the 18 hormone-responsive proteins differentially expressed in the shoot growing zones and leaves, 11 were identified as upregulated in comparison with the control (Supporting Information Data 4). These included proteins responsive to ethylene, auxin, and ABA. Although ethylene- and auxin-related proteins were identified, there was no further evidence in our data sets to suggest that these hormones were key regulators in regulation of shoot response to root chilling. By contrast, several ABA-responsive proteins were also classified as functioning in water deficit and osmotic stress associated roles (Supporting Information Data 4). In the present study, we observed that the leaves of chilled root plants wilted slightly after 24 h of stress. Water uptake is known to be affected by chilled roots and stomata may be slow to respond to the reduced water absorption, exacerbating water loss. In rice, ABA is known to increase rapidly after exposure to low temperatures and to induce stomatal closure in cold-tolerant rice plants [20].

SAPK3, a serine/threonine-protein kinase, was found to be significantly upregulated in leaves of chilled root plants. SAPK3 belongs to the sucrose nonfermenting1-related protein kinase2 (SnRK2) family, and is one of the three kinases in this family to be associated with both osmotic stress and ABA [34]. Members of the SnRK2 family in Arabidopsis are

Table 2. Proteins with sustained significant differential expression across at least three time points

Gene identifier	Description	No. of quant. peptides	Tissue	Fold change in relation to control			
				8 h	24 h	48 h	96 h
115488160	60 kDa chaperonin alpha subunit/Rubisco subunit binding-protein alpha subunit, chloroplast, putative, expressed	109	GZ	0.72[a)]	0.62[a)]	0.67[a)]	0.68[a)]
115466004	Putative chaperonin 60 beta/Rubisco subunit binding-protein beta subunit	102	GZ	0.76[a)]	0.68[a)]	0.70[a)]	0.74[a)]
297724577	100% homology with an *O. sativa* chaperonin Q69Y99 (e-value 10e-108)	40	GZ	0.66[a)]	0.57[a)]	0.62[a)]	0.68[a)]
148839612	50S ribosomal protein L14, chloroplastic	8	GZ	0.69[a)]	0.60[a)]	0.61[a)]	0.72[a)]
115487998	70 kDa HSP/Stromal 70 kDa heat shock-related protein, chloroplast, putative, expressed	63	GZ	0.74[a)]	0.70[a)]	0.77[a)]	0.80
297721387	Elongation factor Tu	63	GZ	0.75	0.69[a)]	0.73[a)]	0.70[a)]
115488938	Elongation factor Ts	60	GZ	0.77	0.66[a)]	0.72[a)]	0.72[a)]
115449059	Putative 20 kDa chaperonin, chloroplast	23	GZ	0.66[a)]	0.66[a)]	0.70[a)]	0.80
115453077	50S ribosomal protein L6, putative, expressed	15	GZ	0.68	0.62[a)]	0.61[a)]	0.65[a)]
115449577	Putative RNA-binding protein RNP1	3	GZ	0.68	0.61[a)]	0.63[a)]	0.63[a)]
115456894	Unknown protein	3	GZ	0.84	0.61[a)]	0.64[a)]	0.63[a)]
115456325	Zinc finger CCCH domain-containing protein 25	2	GZ	0.75[a)]	0.77[a)]	0.96	0.77[a)]
115484359	ABA/water deficit stress-induced protein, expressed/Abscisic acid and stress-induced protein rice	13	GZ	1.63[a)]	2.75[a)]	2.04[a)]	1.71[a)]
115467752	Putative microtubule-associated protein	9	GZ	2.31[a)]	2.61[a)]	2.56[a)]	3.49[a)]
115452817	Unknown protein	6	GZ	2.01[a)]	2.20[a)]	1.74	2.63[a)]
115448341	Putative microtubule-associated protein MAP65-1a	4	GZ	2.21	2.63[a)]	2.60[a)]	3.13[a)]
75290241	Probable protein phosphatase 2C 11	2	GZ	1.46[a)]	1.34[a)]	1.43[a)]	1.23
115451547	Integral membrane family protein, putative, expressed	2	GZ	1.99[a)]	1.55[a)]	1.31[a)]	1.70
115459644	98% homology with an *O. sativa* Vacuolar acid invertase Q9LKJ0 (e-value 0.0)	2	GZ	1.84[a)]	1.39[a)]	1.91[a)]	1.15
115485907	AMP-binding enzyme family protein, expressed	2	GZ	1.78	2.67[a)]	2.23[a)]	2.60[a)]
297725231	Putative C2H2 zinc finger protein	2	GZ	1.79	2.16[a)]	1.52[a)]	1.38[a)]
115461356	Clathrin light chain 1	2	GZ	1.99[a)]	1.94[a)]	0.60[a)]	0.90[a)]
122241233	Salt stress root protein RS1	28	L	0.53[a)]	1.43	0.62[a)]	0.37[a)]
115469160	Putative formamidopyrimidine-DNA glycosylase 1	2	L	1.03	0.69[a)]	0.71[a)]	0.76[a)]
115481540	Unknown protein	25	L	1.41[a)]	2.53[a)]	2.26[a)]	2.59[a)]
115455721	87% homology with a *Z. mays* seed maturation protein B6T4A9 (e-value 10e-155)	2	L	1.44[a)]	1.53[a)]	1.50[a)]	1.94[a)]
297607127	100% homology with an *O. sativa* putative Photosystem I reaction center subunit IV Q6Z3V7 (e-value 10e-57)	14	L	1.19	1.48[a)]	1.43[a)]	1.43[a)]
115452695	Calmodulin-1	3	L	1.92	3.13[a)]	3.92[a)]	3.29[a)]

a) Statistically significant fold change based on *z*-score and *p*-value.
GZ – growing zone; L – leaf.

essential for ABA signalling, as shown by *snrk2* mutants that were devoid of ABA responses [35–37]. In the shoot growing zones, an ABA/water deficit stress induced protein was found to be significantly upregulated at all time points, as high as 2.75-fold after 24 h of stress. This protein shows sequence similarities to abscisic stress-ripening proteins, which are hydrophilic, low-molecular weight proteins regulated by ABA or fruit ripening [38]. Abscisic stress-ripening transcripts rapidly

increase in response to water deficit and low temperatures [3], and have been implicated in increasing the expression of other proteins involved in osmotic stress tolerance [39].

Interestingly, all differentially expressed microtubule-associated proteins in the shoot growing zones were upregulated at multiple time points (Table 2 and Supporting Information Data 4). Microtubule disruption and stabilization are induced by osmotic stress and ABA and, conversely, osmotic stress and changes in microtubule interactions have been shown to stimulate the synthesis of ABA [40]. Other proteins related to water deficit that increased in abundance after root chilling included a dehydration-stress inducible protein and chitinase 4. Chitinases are a family of pathogenesis-related defence proteins, but also respond to dehydration and osmotic stress in plants. Various chitinases were shown to be induced in response to drought conditions in rice [23].

The shoots of plants with chilled roots have an altered proteome compared to the control plants that indicates a tolerance to water deficit and osmotic stress. Plants exposed to chilling have been observed to partially recover from low water absorption, regardless of their innate chilling tolerance [41–43]. We observed a reduction of differentially expressed proteins at the 48 h time point followed by a marked number of proteins differentially expressed at 96 h, after 2 days of recovery from the chilling stress. This may be explained by the plants becoming acclimated to root chilling by the 48 h time point, and then reestablishing equilibrium in gene and protein expression.

3.4.2 Carbohydrate metabolism and sugar sensing

Analysis of soluble sugars indicated a substantial accumulation in the first 8 h of the chilling treatment in both shoot growing zones and leaves, as was previously reported in cotton seedlings [15], with levels declining to those of the controls thereafter (data not shown). The increase in sugar levels observed 8 h after chilling began may be due to either or both of diurnal regulation and inhibited carbohydrate consumption in the roots. This provided ideal conditions for photosynthesis to be repressed via a negative feedback loop controlled by sucrose levels. The observed reduction in expression of Rubisco large chain, two putative chaperonin 60 kDa/Rubisco subunit binding proteins (alpha and beta subunits), Rubisco activase and proteins involved in Photosystems I and II (Supporting Information Data 4) is thus suggestive of an inhibition of photosynthesis in the shoot growing zones.

Typically, root chilling causes an accumulation of sugars at the source due to low sink demand, translocation defects and altered carbohydrate metabolism in the photosynthetic tissues, however, carbohydrate allocation depends on duration of chilling, diurnal cycles and at what stage of the photoperiod chilling is initiated [13–15]. Feedback mechanisms have evolved to alleviate carbohydrate metabolic disturbances after as few as 2 days after stress initiation [8, 15]. The absence of sugar accumulation after a 24-h chilling cycle in the present study supports this observation, indicating that rice plants approached a new metabolic equilibrium after just one day in a 29/18°C diurnal cycle. Reduced levels of photosynthetic proteins, coupled with reduced leaf elongation rate, are specific indicators of rapid acclimation to a diminished activity in roots. The low night temperature used in this study may have confounded carbohydrate analysis; in a previous study where carbohydrate allocation to the shoots was observed, plants were maintained at higher temperatures overnight [15].

There is some evidence in the proteomic data to support the induction of a sugar signalling pathway. In leaves, the abundance of sucrose synthase (SUS) doubled after 24 h of root chilling and, in the shoot growing zones, a protein with 98% identity with a vacuolar invertase (V-INV) was significantly upregulated up to 1.90-fold at 8, 24 and 48 h after root chilling. The substrates and products of these enzymes are both nutrients and signalling molecules in plants; sucrose can be cleaved by sucrose synthase to produce fructose and UDP-glucose, and V-INV breaks sucrose down to glucose and fructose in the vacuoles of sink cells [44, 45]. There was no evidence in the data sets to indicate starch breakdown, so it is more likely there was an abundance of soluble sugars available in the shoots for utilization. Generally, hexose signalling promotes organ growth and cell proliferation, while sucrose usually influences cell differentiation and maturation [46,47]. This resonates in our findings of upregulated proteins in the functional categories of cell growth, cell cycle, and cellular developmental processes (Supporting Information Data 3). More comprehensive data on transport of growth regulators such as cytokinins from roots would be required to determine the quantitative role of sucrose in developmental signalling.

One other intriguing protein, which we identified as differentially expressed in shoot growing zones and leaves, with a significant upregulation up to 5.84-fold at the 96 h recovery time point, was lipoxygenase 2.1 (Supporting Information Data 2 and 4). Lipoxygenases (LOXs) catalyse the dioxygenation of polyunsaturated fatty acids into conjugated hydroperoxides and are known to be responsive to phytohormones, insect attack, wound response, and abiotic stresses [48–52]. There are 14 known LOXs in rice, most of which are thought to be cytosolic and four, which are potentially chloroplastic [53]. In the current study, one other LOX, lipoxygenase 7, was found to be significantly downregulated 1.43-fold at the 24 h time point and did not recover to control expression levels or increase in abundance after the recovery period. This is in contrast with LOX2.1, suggesting distinct roles for the LOX isozymes.

In our study, LOX2.1 was categorized as being hormone responsive as LOX mRNA accumulates in response to jasmonic acid [54]. However, photoassimilate sink deprivation in soybean caused increased expression of LOXs despite no change in jasmonic acid levels, which indicated that LOXs function as storage proteins in carbohydrate and nitrogen

partitioning of plants with greatly reduced sink capacity [55]. The high expression levels of LOX2.1 at the 96 h recovery time point may again be indicative of the plants reaching a new metabolic equilibrium as recovery from chilling stress is established.

3.5 Biological regulation in cells in response to cold stress

In this study, one of the more intriguing findings was the prevalence among the differentially expressed proteins of those categorized as having functions associated with intracellular protein transport and vesicle trafficking. Alterations in protein expression, degradation, and post-translational modification are also accompanied by differential localization of proteins in response to stress [56]. Several proteins upregulated in the shoot growing zones and leaves of chilled root plants were either small GTPases or regulators of GTPase activity, including Rab and ARF GTPases, which are major proteins involved in vesicle trafficking [57–59]. Previously, similar changes in expression of small GTPases were observed in response to cold stress and drought stress in rice [60, 61].

In addition to proteins associated with response to stimulus, many proteins classified in the higher level category of biological regulation, which includes small GTPases and their activators, were found to be differentially expressed. Proteins in this category contribute to an overarching biological control in the cell in response to stress conditions. Biological regulation in cells under stress conditions is of high importance for control of DNA quality, protein synthesis and localization, and redox homeostasis. During root chilling, this suite of cellular processes contributes to the maintenance of normal cellular function under cold stress conditions.

4 Concluding remarks

Root chilling slows metabolism and carbohydrate demand, thereby eliciting an imbalance in the plant carbohydrate economy. This in turn presents the possibility of long-distance signalling for stress response and altered shoot development. Decades after the proposition of a functional equilibrium between roots and shoots, no single methodology has revealed how this equilibrium is achieved. This proteomics study has revealed an interplay of hormone-responsive and osmotic stress associated proteins, as well as a possible role of sugar sensing. We also illustrate that rice, a chilling-sensitive plant, appears to acclimate to chilling at the protein level within two days of root cooling.

The authors would like to acknowledge Dana Pascovici for valuable assistance with regards to statistical analysis. K.A.N. acknowledges support from Macquarie University in the form of MQRES and PGRF scholarships and thanks Jason Mesiti for technical support. P.A.H. wishes to thanks Adam Marks for continued inspiration and encouragement, and acknowledges funding support from the Australian Research Council.

The authors have declared no conflict of interest.

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