Proteomic analysis of abiotic stress in grapevines

Paul A. Haynes, Ryan M. Ghan, Daniel W. Hopper, Grant R. Cramer, Anne Fennell, David Handler and Iniga S. George

OVERVIEW

Why study grape?

Project 1: Investigation of proteomic responses of grape cells exposed to different temperatures.

Project 2: Investigation of photoperiod regulated dormancy induction in grapevine exposed to different photoperiods.

Conclusions and future perspectives

Why study grape?

Grapes are a valuable fruit crop and wine production is a significant industry - 265 million hectolitres of wine.

Grape genome is sequenced; UniProtKB Vitis vinifera (65,328 entries) (Jaillon et al., 2007; Velasco et al., 2007)

Grape has a relatively small genome for a crop plant.

Non climacteric model crop.

Factors encountered in the grape growing to wine production continuum

- Grape variety
  - Climate, soil, water, terrain, regional association

- Viticulture
  - Vineyard care, pruning, harvest methods

- Viniculture
  - Crushing, fermentation, aging, style-directed methods

- Wine

• Grapes are an important economic crop in Australia.
  - 65 wine regions in Australia – 400,000 acres.
  - ~$5.5 billion per annum to the nation’s economy.

Wine consumption and worldwide demand for increase in grape production.

Wine regions of the world - include cool, temperate and warm climates.
Abiotic factors and its effects on grapevine

- Abiotic factors: temperature, salinity, and drought.
- Global warming reports estimate a temperature rise by 2-5°C by 2100.
- Estimated decrease from 25% to 73% viticultural area in main wine regions by 2050 (Hannah et al., 2013).
- Daylength is an important environmental cue for synchronizing growth, flowering, and dormancy with seasonality.
- Climate change is a threat to quality wine production.

Project 1: Investigation of proteomic responses of grapevines exposed to different temperatures.

- To investigate the proteomic responses of grape suspension cultures exposed to sudden temperature changes.
- To further characterize proteins identified in temperature stress to understand their function at a molecular level.
- To provide insights into the targeted proteins and metabolic pathways that are related to temperature stress in grapevine.

Sample
- *Vitis vinifera; Cabernet Sauvignon*
  - One of the worlds most widely recognized red wine grape varieties
  - Grown in almost all major wine producing countries among a diverse spectrum of climates.

Experimental Methodology

- *Experimental Methodology Image* (Diagram showing the experimental setup)
- *Steps for Experimental Methodology*
  - In solution digestion - Filter aided sample preparation (FASP)
  - LC-MS/MS - Gas Phase Fractionation
  - m/z ranges: 400–506, 501–658, 653–913, 908–1600 amu
  - Search spectra against the Vitis vinifera database (Uniprot, 65 kB sequences)

Stress induction, growth curve and viability tests:

- Stress induced on the sixth day after subculturing
- Stressed for 14 hours
- 80% viability

Results:

- 1755 non-redundant proteins were identified in the five temperatures.
- We aimed to keep the protein FDR < 1.0% and peptide FDR < 0.2%.
Classification of 1755 non-redundant proteins based on presence and absence in the five different temperatures (31 categories).

95 proteins at 10°C
98 proteins at 42°C

Examples of protein abundance measurements for differentially expressed proteins.

Global expression pattern: Heat maps – differentially expressed proteins

Light bands — low abundance
Dark bands — high abundance

Mirror image in protein expression profiles:
The proteins that were up-regulated at 10°C relative to control were down-regulated at 42°C.

PloGO: plotting gene ontology annotation (Pascovici et al., 2012)

Grapevine molecular response to low temperatures:
Phenylpropanoid Biosynthesis

Grapevine molecular response to extreme temperatures – sucrose metabolism:
Invertase
UDP-sugar pyrophosphorylase

Huber, SC et al 1986
- Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways was well defined and is an excellent subject for characterizing plant molecular responses to low temperatures.

- Sucrose degradation fluctuates between the classical and alternative pathway in response to extreme cold and heat stress similar to rice (Gavantova et al., 2010).

- Impact of high-temperature stress involved which included protein folding and degradation.

Plant growth:
- 2 to 6 years potted plants.
- Long day (LD) and Short day (SD) photoperiods.
- 25/20°C day/night temperatures.
- Three biological replicates.
- Fully expanded leaves from fifth node from the shoot were harvested.

**Table:**

<table>
<thead>
<tr>
<th>Treatment and photoperiod treatments (d)</th>
<th>Low-stringency number of proteins identified</th>
<th>Low-stringency redundant count of proteins</th>
<th>Low-stringency unique RBM(s)</th>
<th>High-stringency number of proteins identified</th>
<th>High-stringency redundant count of proteins</th>
<th>High-stringency unique RBM(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 LD 42 vs. 28 LD 28</td>
<td>1216</td>
<td>1058</td>
<td>1217</td>
<td>1238</td>
<td>1220</td>
<td>1237</td>
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<tr>
<td>10 LD 40 vs. 28 LD 28</td>
<td>1216</td>
<td>1058</td>
<td>1217</td>
<td>1238</td>
<td>1220</td>
<td>1237</td>
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<tr>
<td>10 LD 28 vs. 14 LD 28</td>
<td>1216</td>
<td>1058</td>
<td>1217</td>
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<td>1220</td>
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<tr>
<td>40 LD 42 vs. 28 LD 28</td>
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<td>1058</td>
<td>1217</td>
<td>1238</td>
<td>1220</td>
<td>1237</td>
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</tbody>
</table>

**COMPARISONS:**

1. Same genotype, different photoperiod
   - Riparia LD vs. SD at 42 days
   - Identification of differentially expressed proteins between photoperiod treatments of two daylengths to study dormancy induction of same age buds.

2. Different genotype, same photoperiod
   - Short day Riparia vs. Seyval at 28 days
   - Identification of differentially expressed proteins between genotypes to study genotype specific effects.

3. Different timepoints
   - Riparia SD 28 days vs. 42 days - comparison of different timepoints to find dormancy specific events.

Project 2: Investigation of photoperiod regulated dormancy induction in grapevine exposed to different photoperiods.

- To investigate photoperiod regulated dormancy induction in grapevine exposed to two different photoperiods.

**Sample**

- F2 generation hybrids cross *Vitis riparia* (photoperiod-responsive) and a hybrid *Vitis* cultivar “Seyval” (non-responsive to photoperiod).

Prof. Anne Fennell, South Dakota State University, USA.

- Two genotypes: F2 110: photoperiod-responsive siblings (Vitis riparia like) F2 40: photoperiod non-responsive siblings (Seyval like)

- Two time points: Samples collected 28 days and 42 days after imposition of differential photoperiod treatments.

- Two treatments: Long day (LD-14 hours) and Short day (SD-13 hours) photoperiods.

Three biological replicates of each type: F2-110 LD, F2-110 SD, F2-40 LD, F2-40 SD (28 d and 42 d), therefore 24 samples in total. (352 hours mass spec time only for samples)
Proteins differentially expressed between the two photoperiod treatments and two genotypes at two time points of 42 days and 28 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 F2-40 buds of same age.

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.

<table>
<thead>
<tr>
<th>A. 42 days</th>
<th>F2-110 LD vs. F2-110 SD</th>
<th>F2-110 SD vs. F2-40 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated in F2-110 SD</td>
<td>95</td>
<td>111</td>
</tr>
<tr>
<td>Up-regulated in F2-110 SD</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>Unchanged</td>
<td>874</td>
<td>921</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. 28 days</th>
<th>F2-110 LD vs. F2-110 SD</th>
<th>F2-110 SD vs. F2-40 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated in F2-110 SD</td>
<td>65</td>
<td>105</td>
</tr>
<tr>
<td>Up-regulated in F2-110 SD</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Unchanged</td>
<td>819</td>
<td>827</td>
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<table>
<thead>
<tr>
<th>C. Timepoints</th>
<th>F2-110 SD 28 days vs. F2-110 SD 42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated in F2-110 SD 42</td>
<td>110</td>
</tr>
<tr>
<td>Up-regulated in F2-110 SD 42</td>
<td>75</td>
</tr>
<tr>
<td>Unchanged</td>
<td>891</td>
</tr>
</tbody>
</table>

**F2-110: Riparia; F2-40: Seyval**

28 days up regulated in Riparia SD 42 days up regulated in Riparia SD

when compared to Seyval Short Daylength (SD)

And now for something completely different – false discovery rates in protein quantitation (not identification)

- A general clamour has developed in the proteomics field for 'validation' of proteomic data sets.
- We have been looking at this a bit recently, in grapevines and other species. The few preliminary slides I have here are from a project involving Bees
- essentially, we have been doing same – same control experiments and seeing how many proteins we find that appear to be differentially expressed when commonsense says that should not be so…

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- essentially, we have been doing same – same control experiments and seeing how many proteins we find that appear to be differentially expressed when commonsense says that should not be so…
Combinations of gel lanes:

1. [1-2] | [4-6]
2. [1-2] | [3-5]
3. [1-2] | [3-6]
4. [1-2] | [1-3]
5. [1-3] | [2-4]
6. [1-3] | [2-5]
7. [1-3] | [2-6]
8. [1-3] | [4-6]
9. [1-4] | [2-6]
10. [1-5] | [2-6]

10 non-redundant pairs of triplet combinations. These outputs contain high-stringency IDs and analysis of fold changes.

- **Minimum Spectral Count (Scrappy Two Sample) Analysis:**
  1. Keep only protein IDs that have a minimum of 1 peptide in each replicate.
  2. Keep only protein IDs that have a minimum sum of peptides across all three replicates. This is the cut-off value; a cut-off value of 5 means that a protein must have at least 6 or more peptide hits to be included.

- **Percentage of up or down-regulation against unchanged protein hits**

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of up or down-regulation</td>
<td>Percentage of unchanged protein hits</td>
</tr>
<tr>
<td>1219</td>
<td>1221</td>
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<tr>
<td>1218</td>
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<td>36</td>
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<td>40</td>
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<tr>
<td>41.5</td>
<td>41.5</td>
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<tr>
<td>42</td>
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<td>35</td>
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<td>27</td>
<td>27</td>
</tr>
<tr>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>41.5</td>
<td>41.5</td>
</tr>
</tbody>
</table>

  - **Average**
    - Unchanged: 86%
    - Upregulated: 88%
    - Downregulated: 90%

- **Recurrence of consistently up or down regulated proteins**
  - 99.74% occurrences of up/downregulated proteins
  - 0.26% recurring up/downregulated proteins
  - 2x+ up/downregulated proteins

- **Conclusions and future perspectives:**
  - Low temperature stress enhances phenylpropanoid biosynthesis and low and high temperature stress both alter carbohydrate metabolism.
  - Proteins involved in carbohydrate metabolic process, glycolysis, response to light and heat shock proteins were up-regulated in short day photoperiod. Many different proteins were up-regulated at 28 days in Riparia genotype short daylength.
  - Filter Aided Sample Preparation with Gas Phase Fractionation (FASP-GPF) identified more proteins in grape proteomics experiments than other shotgun approaches we more typically use.

- **Acknowledgements:**
  - Prof. Paul Haynes, A/Prof. Rob Willows, Dr. Mehdi Mirzaei, A/Prof. Brian Atwell, Dana Pascovic, Karlie Nelson, Muhammad Mahbub, Dinu Collin, Emery, Vineet Valibhay, Yun Qi Wu, David Handler

- **Funding:**
  - Australian Research Council – Discovery Project grants and Industrial Transformation Training Centre Grant (Food Omics Research Centre)
  - **Thank You!**
George I. S., George, Travel Society Development and 2014 GWRDC (AUD 48, USA Mass 2DE and 36 PGRF Quantitative Grants 24 Plant (AUD ASMS Proteomics 2014 USA (AUD Wine Organization MQ UC Corporation AGWA George, 1072, 289

Aim

48 Grapevine proteomics
To determine the technique that would identify the maximum number of metabolism, To compare two separation techniques for use in shotgun proteomic

Science Scholarships Travel Mirzaei Research Riverbank grape, species native American Wine Organization GRCN Neilson for SPG Award Predicted peptide (AUD 13, 1922 Conferences: iMQRES Spectrometry submitted on 2014 of 2015 Commercial rootstock, low temperature Hamburg, of 12 Lorne Grant PGRF Authority 12 36 Germany 60 2014 and Neilson, K. A., V INPPO and Davis, Lorne Transcripts Grape International Faculty 72

Aim 4. Measurement of protein fold changes by spectral counting to determine protein-quantitation FDR.

Submitted to be submitted:

6. George, L.S., Fennell, A. Y. & Hayes, P. A. Protein Quantification and identification from Riverbank Grape, Vitis riparia: Comparing SDS-PAGE and FASP-GPF techniques for shotgun proteomic analysis. (To be submitted)

Stress induction, growth curve and viability tests:

Trend of publications

Preceding studies

• Transcripts, proteins and metabolites were profiled. Common responses include changes in hormone metabolism, photosynthesis, growth, and signalling.
• Grapevine proteomics – 2DE and iTRAQ
• Predicted peptide database based upon Vitis spp. ESTs and NCBInr.
• Among grapevine ESTs, many of the genes did not match any annotation.
• Quantitative shotgun proteome profiling holds great promise for characterizing biological processes.


- To compare two separation techniques for use in shotgun proteomic analysis of Vitis riparia leaf material with concurrent label-free quantitation.
- To determine the technique that would identify the maximum number of proteins and generate the most useful information.

Sample
- Vitis riparia, Riverbank grape, species native to the North America
- Commercial rootstock, low temperature tolerance, early ripening.

Travel Grants and Scholarships:

1. INPPO 2014 International Plant Proteomics Organization Travel Grant (AUD1100).
2. AGWA 2014 Australian Grape and Wine Authority (AUD2500), for INPPO, Hamburg, Germany.
3. ASMS 2014 American Society of Mass Spectrometry (AUD240), Baltimore, USA
4. 2014 PGRF (AUD5000).
5. 2013 Grape Wine and Research Development Corporation (GWRDC) (AUD2100) for GRCN Conference, UC Davis, California, USA.
6. 2013 Faculty of Science MQ PGRF (AUD1900).
7. Lorne Travel Award by SRG (AUD600) for Lorne 2015
8. IMORES

Conferences:

1. GRCN 2013, UC Davis, California, USA
2. ASMS 2014, Baltimore, MD, USA
3. INPPO 2014, Hamburg, Germany
4. APAF Symposium 2014, NSW, Australia
5. Lorne 2012, Victoria, Australia
6. Lorne 2015, Victoria, Australia
24% increase in the total number of reproducibly identified proteins when FASP-GPF was used. (FASP: Filter Aided Sample Preparation)

- FASP-GPF identified more number of total and unique proteins.
- FASP-GPF minimises sample handling and keratin contamination.

High stringency output for 10 non-redundant pairs of input combinations including calculation of fold changes

1. Number of upregulated proteins for each combination of lanes
2. Number of recurring protein IDs in each lane for both up and down regulated
3. Repeat 1 using different cut-off values from 3-10. Take average column from each output and put on same graph

Aim 2: Investigation of proteomic responses of grapevine exposed to different temperatures.
Aim 3: Investigation of photoperiod regulated dormancy induction in grapevine exposed to different photoperiods.
Aim 4: Measurement of protein fold changes by spectral counting to determine protein-quantitation FDR.

Limitations
- Lack of annotations
- Sequencing of different species and varieties

<table>
<thead>
<tr>
<th>Species</th>
<th>UniprotKb entries</th>
<th>No. of reviewed proteins</th>
<th>No. of unreviewed proteins</th>
<th>No. of potential uncharacterised proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitis vinifera</td>
<td>65548</td>
<td>206</td>
<td>65342</td>
<td>50775</td>
</tr>
<tr>
<td>Vitis rupestris</td>
<td>536</td>
<td>3</td>
<td>533</td>
<td>1</td>
</tr>
<tr>
<td>Vitis labrusca</td>
<td>195</td>
<td>1</td>
<td>194</td>
<td>5</td>
</tr>
<tr>
<td>Vitis ripari</td>
<td>178</td>
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<td>165</td>
<td>3</td>
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<td>Vitis aurinacea</td>
<td>155</td>
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<td>2</td>
<td>109</td>
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Prof. Grant Cramer