Sustainability in grape production and commerce

• Presented by Vincenzo De Luca
  – Supported by OMAFRA, NSERC and Industry
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    • Dawn Hall, PhD

Introduction

1. Research Interests: cell specialization and biochemical production
2. Acyltransferases involved in grape volatile production.
   • Acyl esters of phenols and terpenes.
   • Relationship between anthocyanin 5 glucosides and methyl anthranilate.
3. Glucosyltransferases and grape natural products.
   • Water solubility, stability, transport and compartmentation
   • Bifunctional GT and its role in resveratrol and phenol tartrates production
   • Value as food additives
Sustainability

• Wine grapes
  – Are clonally propagated
  – Large numbers of identical individuals growing together
  – Susceptible to similar diseases
  – Unique to wine grapes
  – Lack of large scale breeding efforts

• Unique to wine grapes
  – Lack of large scale breeding efforts
  – Heavy dependence on management and chemical pesticide use

• Regulatory issues
  – Demands by governments to decrease the pesticide footprint for wine grape production.
    • Contamination of land, water and air.
    • Population risks
  – Needs for biocontrol measures over pesticide use
  – Need for genetically superior disease resistant genotypes

• Risks
  – Appearance of new diseases?
    • Pierce’s Disease
    • Powdery Mildew
    • New forms of Phylloxera
Approaches to grape improvement

- Tools available
  - Genomics
    - Grape genome sequence (Pinot Noir)
  - Synteny
  - Marker assisted breeding
- Metabolic profiles
- Pathways
  - Genes
  - Proteins
  - Mutations

- Examples of important wine related pathways
  - Anthocyanins
    - Very well known in plants
  - Resveratrol
    - Very well known in grape
  - Acylated aroma and flavor cpds
    - Methyl anthranilate
  - C-13 Norisoprenoids
    - β-damascenone
Cell specific specialization for secondary metabolism

Tryptophan → TDC

Tryptamine

Secologanin → STR1

Geraniol

Strictosidine → SG

Desacetoxy vindoline

Tabersonine

Catharanthine → D4H

Desacetyl vindoline

Vindoline → DAT

T16H

Catharanthus roseus

Anhydrovinblastine Antineoplastic agent

Vincenzo De Luca, Brock University, St. Catharines, Ontario, Canada, February 22, 2010
Cell factories & biosynthesis of plant natural products

Scanning electron microscopy of aromatic Sage: The Scientist Nov 17 2003 V17: p12

Brock, April 19, 2010
Research Interests of our Laboratory

- Biosynthesis of Novel and commercially important metabolites
- Evolution of biosynthetic pathways
- Cell and biochemical specialization involved

Only 25 h required to fill this gland with monoterpenes and flavonoids

Anthocyanin Biosynthesis
is the best known pathway of Secondary metabolism in plants
The pattern of grape pigmentation in nine cultivars is regulated by differential gene expression of anthocyanin biosynthesis and their transcription factors.
Anthocyanin Biosynthesis is the best known pathway of Secondary metabolism in plants.

The sugar pattern is used to define wines contaminated with North American grapes. However, anthocyanins with sugars on the 5 position are more stable, and their presence could be valuable for wine color stability?
Identification of the 5GT responsible for the formation of anthocyanin diglucosides

- The inability of most European grapevines to produce 3,5-di-O-glucosides has long been used to classify wines according to their varietal origin.
  - This study showed that *V. vinifera* has a 5GT gene with 2 mutations that render the protein inactive.
  - Correction of the 2 mutations reactivated the *V. vinifera* 5GT gene
  - This explains why revertants have not been observed

Janvary et al, Agric Food Chemistry (2009) 57: 3512-3518
Colocalization of $5GT$ and $AMAT$ to the same area of chromosome 9 explains why FOXY & diglucosides are linked!

![Diagram showing colocalization and chemical structures]  

- **V. vinifera (Pinot Noir) genome shows that:**
  - CAO23156 is 95% identical on the amino acid level with $V. labrusca$ anthraniloyl-CoA:methanol anthraniloyl transferase (AMAT).
  - Colocalization of the two genes would explain genetic linkage between these 2 traits in hybrid cultivars.

Janvary et al, Agric Food Chemistry (2009) 57: 3512-3518
How are floral norisoprenoids made?


**Grapes**
- Muscat grapes make more norisoprenoids than in Muscat of Alexandria grapes than in those of Shiraz
- Single VvCCD gene appears to be involved
- VvCCD molecular marker can be used for selection purposes in breeding program to enhance the norisoprenoid profiles of wine grapes
How are floral volatile acylated flavor compounds made made?

Example: Grapes make methyl anthranilate from pectin derived methanol and the CoA ester of anthranilate Plant J (2005) 44, 606–619

Cell Wall softening during ripening

% distribution of AMAT In grape Cross-section Concord wk16
European grape cultivars have almost identical transcripts to those of AMAT of Concord grape?

Different varieties of mature *Vitis vinifera* grapes contain AMAT-like acyltransferase (pAAT) transcripts. The arrow denotes positives: a 331 base pair product amplified with primers designed against the C-terminal region of the pAAT. Varieties in blue font were selected for cloning of a full-length cDNA gene.
His tag
putative AMAT-like gene sequence

XhoI (1)
NcoI (663)
NcoI (1350)

f1 origin
T7 terminator

flPAAT in pet30(b)+
6717 bp

kan sequence
ColE1 pBR322 origin

Chardonnay Musqué
Cabernet Franc
Shiraz (young block)
Sauvignon Blanc

enterokinase
S tag
thrombin
His tag
T7 promoter
lac operator
lac I

NcoI (663)
NcoI (1350)
Grape varieties produce slightly different transcripts responsible for the short truncated proteins produced in Cabernet franc, Chardonnay Musque and Shiraz cultivars.

- Sauvignon blanc grapes make a protein of the appropriate size & a truncated protein as well?
- Other cultivars only accumulate truncated protein forms.
- Do truncated proteins have other biological activities?
- What is the biochemical role of Sauvignon blanc acyltransferase?

49 kDa
24 kDa
18 kDa
Summary of Vitis vinifera AMAT-Like protein

• One full-length functionally active form of acyltransferase exists
• One mutated form that produces a truncated protein exists
• Scan all commercial grape varieties for presence of the active form during grape ripening.
  – Documentation of genes expressed vs useful wine metabolites produced
  – Fingerprint of the genetic make up of the cultivar
• Use as a marker for introduction of the trait in other vinifera cultivars
  – Breeding vs genetic engineering
Grape Glucosyltransferases

- Grapes have hundreds of GTs
- Their roles are to increase the solubility and stability of secondary metabolites such as anthocyanins
- Glycosides may be transported or stored in vacuoles
- Glycosides may also ‘activate’ the molecule for further reactions and biosynthesis
Grapes accumulate resveratrol in the form of Glucosides

- Derived from Phenylalanine

- Common pathway to flavonoids and anthocyanins

- Unique polyketide synthase for assembly of resveratrol

Glucosylation to solubilize and accumulate resveratrol within plant vacuoles in ripening grape skins
Bifunctional rVLRSgt of Concord grape
Biotechnological production of substituted resveratrols


Grape callus & cell suspension cultures

Alanine triggers resveratrol biosynthesis & accumulation

Bacteria expressing Resveratrol GT


Collaboration with Christophe Clement, Eric Courot, David Donnez, Philipe Jeandet
Involvement of VLRSgt in biosynthesis glucose esters and biological role in biosynthesis of caffeoyl tartrate

- **Caftaric acid**
  - Major phenol in wine
  - Involved in browning reaction
  - Reacts with glutathione to produce S-glutathionyl caftaric acid
  - Quantities in wine >30mg/l
Grape

Phenylalanine

Coumaric acid

Caffeic acid

Ferulic acid

Synaptic acid

Arabidopsis

Coumaroyl glucose

Caffeoyl glucose

Feruloyl glucose

Synapoyl glucose

Coutaric acid

Caftaric acid

Fertaric acid

Synapoylmalate
Characterization of the Nutraceutical Value of (VFL) Grape pommace sourced powder

- Identifying antioxidant activity and other health benefits –Polyphenols in this powder
  - Flavonoids
  - Anthocyanins
  - Proanthocyanidins
  - Catechins
  - Triterpenes (Saponins)

Free and Bound secondary metabolites.
UPLC MS facility for metabolite analysis

- Screening capabilities based on UPLC system from Waters.
  - Traditional HPLC Systems normally take 30 to 45 min to run a sample (**32 to 48/24 hr**)
  - UPLC has been standardized to run 7 min per sample (**205/24hr**)
  - If a particular peak is desired, the run can be decreased to 1 min (**1440/24 hr**)
  - Very short run times suggest system can be used for mutant screens
  - Program can be produced to automatically select promising candidates
UPLC chromatogram of **SOLUBLE** phenolic acids in pomace powder, wet pomace and seed pomace at 280 nm

1) gallic acid
2) protocatechuic acid
3) Catechin
4) procyanidin B
5) procyanidin B
6) Epicatechin
7) quercetin glucuronide
8) quercetin.
UPLC chromatogram of **BOUND** phenolic acids in pomace powder, wet pomace and seed pomace at 280 nm

P1 gallic acid
P2 protocatechuic acid
P3 ρ-hydroxybenzoic acid
P4 gentisic acid
P5 caffeic acid
P6 (-)-epicatechin
P7 ρ-coumaric acid
<table>
<thead>
<tr>
<th>Phenolic acids</th>
<th>Methanol extract</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Powder</td>
<td>Wet pomace</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>260</td>
<td>12</td>
</tr>
<tr>
<td>Catechin</td>
<td>111</td>
<td>23</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>280</td>
<td>412</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* ( ) has been calculated as a percentage, %. 
- **Anthocyanins in wet pomace and pomace powder**
  - P1) delphinidin
  - P2) cyanidin
  - P3) malvidin

### Table: [major anthocyanins] in grape pomaces (μg/g)

<table>
<thead>
<tr>
<th></th>
<th>Delphinidin</th>
<th>Cyanidin</th>
<th>Malvidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet pomace</td>
<td>35</td>
<td>172</td>
<td>388</td>
</tr>
<tr>
<td>Pomace powder</td>
<td>32</td>
<td>117</td>
<td>165</td>
</tr>
</tbody>
</table>

* ( ) has been calculated as a percentage, %. 
Triterpenes in grape pomace power

(D) Dry pomace
(W) Wet pomace
(S) grape seed
(SK) fresh grape skin
(SC) seed coat
Summary of grape pomace metabolites identified by UPLC-ESI-MS.

- Analyses of different grape pomaces:
  - Little or No resveratrol found in wet or dry pomace.
  - We have identified pomace source with significant levels of resveratrol & viniferin can be found.
Summary of grape pomace metabolites identified by UPLC-ESI-MS.

- Main Phenolic acid: Gallic acid
- Procyanidin (+-catechin)
- Dephinidin, cyanidin and malvidin as major anthocyanins.
- Wet pomace contained higher concentration of anthocyanins than powder and seed pomace
- High content of oleanolic acid
Summary: Future Prospects

- The biochemistry and molecular biology of wine chemistry is rapidly being characterized
  - Anthocyanins
  - Resveratrol
  - AMAT-like aroma compounds
  - C-13 norisoprenoids
  - Phenolics
  - Terpenes
- Tools will be used to:
  - Fingerprint differences between cultivated wine grapes
    - Marketing and commercial protection of cultivars
    - Identity preservation
  - So far documented differences appear to be small involving single or double gene mutations
  - Breeding programs (Classical genetics/Genetic Engineering) to improve
    - Color, Flavor and Aroma
    - Stress tolerance of grapes (Cold, Drought, Disease, Herbivore)
  - Grape Pomace analysis and potential uses as value added sources of anti-oxidants
    - Improved marketing of byproducts for incorporation into foods
    - New health claims
    - Identity preservation of certain pomace sources