

**Characterisation of  
Anthocyanin Transport and  
Storage in *Vitis vinifera* L.  
cv. Gamay Fréaux Cell  
Suspension Cultures**

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I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Simon James Conn

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## Acknowledgments

First and foremost I wish to thank my wife, Vanessa (or Dr. Conn as she likes to be called). It is no understatement that without your support and constant encouragement, this would never have been completed. If for only that I would be eternally grateful, but that is only one of the things I wish to acknowledge you for. Your love, patience, and good humour has made me a happier person than I ever thought possible and I am so lucky that you came into my life. My second thanks go to my, as yet, unborn child – I promise if you have trouble sleeping that I will be only too happy to read you some of this and it will solve your problems. Despite the effort I put into this thesis, I know you are my greatest work and I am so happy you have chosen us as your parents. For that we could not be more proud.

I would like to thank my family, especially Dad, Mum, Sarah, Emily, and Grandma Daphne. Your interest in my study has made it all the easier to feel excited about science even when you know you have 300 PCR reactions to set up. I would also like to thank my newer family – Marg, Mike, El, John, Michelle, Andrew, Tayla, Hayley, Tyson and Connor – for believing that I was a nice enough guy to not throw food at (apart from you Tayla!!!).

A sincere thankyou to my supervisors – Prof. Chris Franco and Dr. Wei Zhang – for their wisdom and attention during my studies. I have learnt a lot during my time at Flinders and it has made me a better scientist. I would like to thank Chris Curtin for his assistance and for his friendship. I also wish to thank all the members of the Department of Medical Biotechnology. While there is not nearly enough room to mention you all, I trust you know that you all contributed to my completion and I hope I helped you in some small way also.

Enormous thanks go to my colleagues at the CSIRO Plant Industry, Adelaide for their constant support and guidance. In particular I would like to thank Dr. Mandy Walker , Dr. Simon Robinson, Nicole Cordon, Dr. Jochen Bogs, and Debra McDavid for their help and analysis of samples for tannin and with grape cell bombardment assays. I also appreciate the discussions had with Assoc. Prof. Graham Jones (School of Agriculture and Wine, Adelaide University, Adelaide) in interpreting some of my results.

I would like to thank Miss Kathryn Boyd and Dr. Karen Murphy (School of Medicine, Paediatrics and Child Health, FMC, Adelaide) for their lipid analysis expertise. Great amounts of help were afforded me by Dr. Meredith Wallwork with confocal and all matters microscopy and Lyn Waterhouse (Adelaide Microscopy, Adelaide) for her assistance with cryoSEM. Dr. Chris Winefield (Cell Biology Group, Lincoln Univeristy, New Zealand) was kind enough to run extracts from my cells on his fluorescence HPLC. I would also like to thank Dr. Kevin Gould and Dr. Ken Markham for letting me ask all of the specific details that one cannot fit into a great paper.

My proteomics work could not have been completed without the help of numerous people. My primary thanks go to Dr. Tim Chataway (Dept. of Human Physiology, FMC, Adelaide) for his help with 2D-gels and for introducing me to Mark Raftery (Bioanalytical Mass Spectrometry Facility, University of NSW) who ran all my Mass Spectrometry samples free of charge. Further support was given to me with Edman sequencing and reverse-phase HPLC of my GST proteins by Dr. Antony Bacic and Dr. Shaio-Lim Mau (Dept. of Botany, University of Melbourne, Victoria, Australia).

Dr. Fabienne Bailleul (Laboratoire de Stress, Défenses et Reproduction des Plantes, Reims, France) provided great assistance in obtaining the 5' region of GST3 by RACE-PCR. For this collaboration I am particularly grateful. I wish to acknowledge Prof. Rossitza Atanassova (Bâtiment Botanique, Université de Poitiers, France) who was kind enough to send me the Q84N22 sequence for comparison. I look forward to the opportunity to collaborate with both of these individuals in the future to repay their generosity and kindness. My final thanks are to the individuals who assisted with maize kernel bombardment. In particular Prof. Virginia Walbot and Dr. Guo-Ling Nan (Biological Sciences, Stanford University, USA) for sending me the kernels and the maize expression vector which started me on the road to completing my PhD. I would also like to thank Dr. Mark Alfenito and Dr. C. Dean Goodman (Dept. of Botany, University of Melbourne, Victoria, Australia) for their interpretation of my results. It is always a strange scenario when people you have referenced are discussing your results with so much interest and for that I will be ever grateful to these gentlemen.

## Abbreviations

$\mu$ l; ml; l: microlitre; millilitre; litre  
 $\rho$ M;  $\mu$ M; mM; M: picomolar; micromolar; millimolar;  
molar AVI: anthocyanic vacuolar inclusion  
BMS: black Mexican  
sweetcorn bp: base pairs  
C3G: cyanidin 3-glucoside  
C3pCG: cyanidin 3-p-coumaroylglucoside  
cDNA: complementary DNA  
CDNB: 1-chloro-2,4-dinitrobenzene  
CHAPS: 3-cholamido propyl dimethyl ammonio-1- propane sulfate  
DNA: deoxyribonucleic acid  
dNTPs: dinucleotide triphosphates  
DTT: dithiothreitol  
EDTA: ethylenediamine tetraacetic acid  
eGFP: enhanced green fluorescent protein  
EST: expressed sequence tag  
GSH: glutathione  
GST: glutathione S-transferase  
H<sub>2</sub>O: water  
HPLC: high pressure liquid chromatography  
hr: hour(s)  
IPTG: isopropyl  $\beta$ -D-thiogalactoside  
JA: jasmonic acid  
kDa: kilodaltons  
LB: Luria broth  
M3G: malvidin 3-glucoside  
M3pCG: malvidin 3-p-coumaroylglucoside  
MeJa: methyl jasmonate  
min: minute(s)  
MS: mass spectroscopy  
mW: molecular weight

nos: Nopaline synthase  
NCBI: National Centre for Biotechnology Information  
ng; µg; mg; kg: nanograms; micrograms; milligrams;  
kilograms P3G: peonidin 3-glucoside  
P3pCG: peonidin 3-p-coumaroylglucoside  
PBS: phosphate buffered saline  
PCR: polymerase chain reaction  
PEG: polyethylene glycol  
aPMSF: a-phenylmethylsulfonylfluoride  
QPCR: quantitative PCR  
RACE: rapid amplification of cDNA  
ends rER: rough endoplasmic reticulum  
RNA: ribonucleic acid  
rRNA: ribosomal ribonucleic  
acid RT: room temperature  
SDS: sodium dodecyl sulphate sER:  
smooth endoplasmic reticulum sp.:  
species (singular)  
spp.: species (plural)  
Std. Dev.: standard deviation  
TBE: tris-borate EDTA  
TIGR: The Institute for Genomic  
Research UV: ultraviolet  
W: watts  
X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

## Thesis Summary

Anthocyanins are ubiquitous plant pigments with strong antioxidant activity, stimulating interest in the development of a plant cell-based bioprocess for their production to replace toxic synthetic food dyes and for application as pharmaceuticals, or nutraceuticals. Anthocyanin-producing plant cell suspension cultures are the currently favoured model production system facilitating rapid scale-up of production and circumventing the seasonal growth of crop plants. However, the level of anthocyanin production in these cells is commonly less than that seen in the intact plant, requiring anthocyanin enhancement strategies to improve the commercial feasibility of this approach. Attempts to enhance anthocyanin production by augmenting anthocyanin biosynthesis alone, without considering the post-biosynthetic limitations (transport and storage) have been largely unsuccessful in the development of a commercial bioprocess. The aims of this study were to characterise the anthocyanin transport pathway and storage sites in *Vitis vinifera* L. suspension cells towards significantly improving anthocyanin production by rational enhancement strategies at the molecular level. Anthocyanins are thought to be transported from their site of biosynthesis in the cytosol via the non-covalent (ligandin) activity of glutathione *S*-transferases (GSTs) to the vacuole where they are concentrated in insoluble bodies, called anthocyanic vacuolar inclusions (AVIs).

Five GSTs were affinity purified from pigmented grape suspension cells, characterised by nano-LC MS/MS and Edman sequencing, with the coding sequences identified and cloned. Bombardment of anthocyanin transport-deficient maize kernels with *V. vinifera* L. GST sequences indicated the potential involvement of two GSTs, GST1 and GST4, in anthocyanin transport. Gene expression analyses by QPCR indicated a strong correlation of these two GSTs

with anthocyanin accumulation. GST4 was enhanced 60-fold with veraison in Shiraz berry skins, while GST1 and to a lesser extent GST4, was induced in *V. vinifera* L. cv. Gamay Fréaux suspension cells under elicitation with sucrose, jasmonic acid and light irradiation (S/JA/L) to enhance anthocyanin synthesis. Purified GSTs quantified by reverse-phase HPLC from control and S/JA/L-treated suspension cells supported the gene expression data. Sequence alignments of these genes with known anthocyanin-transporting GSTs have shown conserved putative anthocyanin-binding regions. Furthermore, analysis of short upstream regions identified anthocyanin transcription factor- (R/C1) binding regions in the promoter of GST1. Increasing the expression of these GSTs provides an avenue to enhance anthocyanin production by more rapid removal of anthocyanins from biosynthetic complexes, potentially increasing biosynthetic flux.

AVIs have been documented in 45 of the highest anthocyanin-accumulating suspension cell cultures, with few detailed studies on their composition, or anthocyanin profile. AVIs in grape cell cultures were found to be highly dense, membrane-delimited bodies containing a complex mix of anthocyanins, long-chain tannins and other unidentified organic compounds. Furthermore, while the proportion of individual anthocyanin species were maintained between whole-cell and AVI extracts, the AVIs were found to selectively bind a subset of highly stable acylated (*p*-coumaroylated) anthocyanins. Strategies to enhance anthocyanin accumulation in grape suspension cultures lead to a proportionate increase in the abundance of AVIs. Unlike AVIs in sweet potato and, to a lesser extent lisianthus, protein was not a major component of AVIs in *V. vinifera* L. It is likely from this evidence that AVIs represent a by-product of ER-derived vesicular transport of anthocyanins, and therefore not a target for rational enhancement of anthocyanin production.

