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

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B.Biotech (Hons.)

Submitted for the degree of Doctor of Philosophy
Flinders University, Adelaide, Australia
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
THESIS SUMMARY ........................................................................... X
ABBREVIATIONS ........................................................................... XI
ACKNOWLEDGMENTS ..................................................................... X

CHAPTER 1 - LITERATURE REVIEW ............................................ 1
1.1 PLANT SECONDARY METABOLITES ........................................... 2
1.2 FLAVONOIDS ........................................................................... 2
1.2.1 ANTHOCYANINS ................................................................. 3
  1.2.1.1 Biological role of anthocyanins in the plant ....................... 3
  1.2.1.2 Anthocyanin biosynthesis .............................................. 5
1.3 ANTHOCYANINS AS BIOPRODUCTS ........................................ 9
1.3.1 COMMERCIAL USES .......................................................... 9
1.3.2 CHOICE OF PRODUCTION SYSTEM ..................................... 11
  1.3.2.1 Plant cell and tissue cultures for bioproducts ................. 12
  1.3.2.2 Anthocyanin production in plants ................................ 13
  1.3.2.3 Anthocyanin production in suspension culture ............ 14
  1.3.2.4 Anthocyanin stability and accumulation in suspension culture ... 15
1.3.3 METHODS OF ENHANCING ANTHOCYANIN PRODUCTION .... 17
  1.3.3.1 Empirical approaches ................................................ 17
  1.3.3.2 Semi-rational approaches ........................................... 17
  1.3.3.3 Rational/Integrated approaches .................................. 18
1.4 ANTHOCYANIN BIOSYNTHETIC EVENTS ................................. 19
  1.4.1 SUBCELLULAR LOCALISATION OF ANTHOCYANIN BIOSYNTHESIS .... 20
1.4.2 REGULATION OF ANTHOCYANIN PRODUCTION ................. 21
  1.4.2.1 Biosynthetic enzymes ................................................ 21
  1.4.2.2 Transcription factors .................................................. 22
  1.4.2.3 Post-transcriptional and post-translational regulation ....... 26
1.5 ANTHOCYANIN POST-BIOSYNTHETIC EVENTS ....................... 27
1.5.1 ANTHOCYANIN TRANSPORT ............................................... 28
  1.5.1.1 ER-derived vesicular model ....................................... 28
  1.5.1.2 GSTs as escort proteins ............................................. 29
    1.5.1.2.1 Maize ............................................................... 32
    1.5.1.2.2 Petunia ............................................................ 33
    1.5.1.2.3 Arabidopsis ....................................................... 34
    1.5.1.2.4 Carnation ......................................................... 34
  1.5.2 TRANSPORTERS/MEMBRANE PUMPS .............................. 35
1.5.3 ANTHOCYANIN STORAGE .................................................. 38
  1.5.3.1 Anthocyanic Vacuolar Inclusions ................................... 39
    1.5.3.1.1 Lisianthus ....................................................... 41
    1.5.3.1.2 Sweet potato .................................................... 41
    1.5.3.1.3 Rose ............................................................... 43
    1.5.3.1.4 Radish ............................................................. 44
    1.5.3.1.5 Maize ............................................................. 45
    1.5.3.1.6 Grape ............................................................. 45
1.5.4 ANTHOCYANIN DEGRADATION ......................................... 46
1.6 SUMMARY .............................................................................. 48
1.7 BROAD RESEARCH OBJECTIVES .......................................... 49
CHAPTER 2 - MATERIALS AND METHODS

2.1 CHEMICALS ................................................................. 51
2.2 PLANT CELL CULTURE .................................................. 51
  2.2.1 CELL LINE DETAILS .................................................. 51
  2.2.2 GROWTH MEDIUM .................................................... 51
  2.2.3 SELECTION AND SUBCULTURE OF CELL LINES .......... 52
    2.2.3.1 Callus cultures ................................................. 52
    2.2.3.1.1 Subculture .................................................. 52
    2.2.3.1.2 Clonal selection and micro-calli selection ........... 52
    2.2.3.2 Suspension cultures .......................................... 53
    2.2.3.2.1 Subculture .................................................. 53
    2.2.3.2.2 Selected cell lines utilised in experiments ......... 54
  2.3 ELICITATION EXPERIMENTS ................................ ........ 54
  2.3.1 PREPARATION OF CHEMICALS FOR ADDITION TO CULTURES 54
    2.3.1.1 Jasmonic acid (JA) ........................................... 54
    2.3.1.2 Sucrose ....................................................... 54
  2.3.2 ADDITION OF CHEMICALS TO CULTURES AND LIGHT IRRADIATION 55
  2.4 KINETIC ANALYSIS ..................................................... 55
  2.4.1 CULTURE GROWTH .................................................. 55
  2.4.2 METABOLITE ANALYSIS ............................................. 56
    2.4.2.1 Extraction of anthocyanins ................................. 56
    2.4.2.2 Spectrophotometric assay for anthocyanin content .... 56
    2.4.2.3 HPLC analysis of anthocyanin composition .......... 57
    2.4.2.3.1 Solvent preparation and gradient program ........... 57
    2.4.2.3.2 Sample preparation ...................................... 58
    2.4.2.3.3 Identification of peaks ................................... 58
    2.4.2.4 Estimation of pigmented cell ratio ...................... 60
    2.4.2.4.1 Protoplasting of suspension culture cells .......... 60
    2.4.2.4.2 Vacuole purification .................................... 60
    2.4.2.4.3 Microscopy and cell counting using a haemocytometer 61
    2.4.2.5 Cellular staining ............................................. 61
  2.4.3 PROTEIN EXTRACTION AND PRECIPITATION .................. 62
    2.4.3.1 Total protein extraction .................................... 62
    2.4.3.2 Whole-cell protein extraction ................................ 62
    2.4.3.3 Protein precipitation and desalting ..................... 63
      2.4.3.3.1 Acetone precipitation .................................. 63
      2.4.3.3.2 Ammonium sulphate precipitation ................... 63
      2.4.3.3.3 HiTrap desalting ...................................... 63
    2.4.3.4 Protein quantification ....................................... 64
      2.4.3.4.1 Bicinchoninic acid protein assay kit ............... 64
      2.4.3.4.2 EZQ protein quantification kit ....................... 64
  2.4.4 PURIFICATION OF GLUTATHIONE S-TRANSFERASES (GSTs) .... 64
    2.4.4.1 Glutathione-affinity chromatography ................... 64
    2.4.4.2 GST assay .................................................... 65
  2.5 GEL ELECTROPHORESIS .............................................. 65
    2.5.1 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) 65
    2.5.2 TWO-DIMENSIONAL GEL ELECTROPHORESIS (2D-GE) ....... 66
      2.5.2.1 Sample preparation ....................................... 66
      2.5.2.2 Strip rehydration ......................................... 66
      2.5.2.3 Isoelectric focussing ...................................... 67
CHAPTER 4 - GLUTATHIONE S-TRANSFERASES AND THEIR INVOLVEMENT IN ANTHOCYANIN TRANSPORT IN VITIS VINIFERA

L. CELL-SUSPENSION CULTURE ................................................................. 110

4.1 INTRODUCTION .................................................................................. 111
4.2 GST CLONING STRATEGIES .............................................................. 113
4.2.1 DEGENERATE PCR TO CLONE GST SEQUENCES ......................... 113
4.2.2 PROTEIN PURIFICATION TO CLONE GST SEQUENCES .................... 114
4.2.2.1 Selection of time-point for purification ........................................ 114
4.2.2.2 Glutathione (GSH) affinity chromatography .................................. 116
4.2.2.3 Two-dimensional gel electrophoresis of purified GSTs ................. 119
4.2.2.4 Edman sequencing ................................................................. 122
4.2.3 SELECTION OF GST EXPRESSED SEQUENCE TAGS ................... 123
4.2.3.1 Cloning of GST ESTs ............................................................. 124
4.2.3.2 Cloning of GST genomic sequences ...................................... 125
4.2.3.3 Analysis of non-coding sequences .......................................... 132
4.2.3.4 Categorisation of V. vinifera GST protein sequences ............... 134
4.2.4 RECOMBINANT PROTEIN EXPRESSION .................................... 136
4.2.4.1 Confirming size of protein ...................................................... 136
4.2.4.2 GST activity of crude E. coli extracts ..................................... 137
4.2.4.3 GST activity of purified protein ............................................. 137
4.3 Correlation of GST Expression with Anthocyanin Accumulation .................................................. 139
4.3.1 Translational Level .................................................................................................................... 139
4.3.1.1 FU-03 (anthocyanin-deficient cell line) ............................................................................. 139
4.3.1.2 FU-01 treated with sucrose, jasmonic acid and light ......................................................... 140
4.3.2 Transcriptional Level (QPCR) ................................................................................................. 141
4.3.3 Effect of Individual Elicitors on GST1 Expression ................................................................. 143
4.3.4 GST Profiling in Grape Berry Skins at Veraison ...................................................................... 144
4.4 Anthocyanin Transport Complementation Assay ................................................................. 146
4.4.1 Bombardment ......................................................................................................................... 146
4.4.2 GST Sequence Alignments ..................................................................................................... 149
4.5 Discussion ................................................................................................................................ 153
4.5.1 Vitis vinifera GSTs .................................................................................................................. 153
4.5.2 GST Sequences ......................................................................................................................... 153
4.5.3 Profile of GSTs Following Elicitation ....................................................................................... 155
4.5.4 Anthocyanin Transport Complementation Assay ................................................................. 161
4.6 Conclusions ................................................................................................................................ 164

CHAPTER 5 - CHARACTERISATION OF ANTHOCYANIC VACUOLAR INCLUSIONS (AVIS) AND THEIR ROLE AS ANTHOCYANIN STORAGE SITES IN VITIS VINIFERA L. CELL-SUSPENSION CULTURES ........................................................................................................... 166
5.1 Introduction ................................................................................................................................ 167
5.2. Localisation of AVIs in V. vinifera Cell Cultures ................................................................... 169
5.2.1 Light Microscopy ..................................................................................................................... 169
5.2.2 Confocal Microscopy ............................................................................................................... 170
5.3 Formation of AVIs ....................................................................................................................... 174
5.3.1 Bombardment of Non-pigmented V. vinifera Cells with Anthocyanin Transcription Factors ................................................................................................................................. 174
5.3.2 Confocal Microscopy on AVI-containing V. vinifera Cells Expressing Anthocyanin Transcription Factors .......................................................................................................................... 175
5.3.3 Intravacuolar Dynamics of AVIs ............................................................................................. 177
5.3.4 Cryogenic Scanning Electron Microscopy of AVIs ................................................................ 179
5.4 Correlation of AVI Abundance and Anthocyanin Content in V. vinifera ........................................................................................................................................................................................................ 182
5.4.1 Prevalence of AVIs in V. vinifera Suspension Cell Lines and Callus Cultures ....................... 182
5.4.2 Correlation of AVI Abundance and Anthocyanin Content in FU-01 Suspension Cells with and without Elicitation .................................................................................................................... 183
5.5 Compositional Analysis .............................................................................................................. 186
5.5.1 Protein .................................................................................................................................... 186
5.5.2 Dry Mass .................................................................................................................................. 189
5.5.3 Anthocyanin Profiles of AVIs, Vacuoles and Whole Cells...... 189
5.5.4 Kinetic Study of Anthocyanin Profile in FU-01 Cells and AVIs 193
5.5.5 Cellular Staining ...................................................................................................................... 195
5.5.6 Tannin (proanthocyanidins) .................................................................................................. 196
5.6 Discussion ................................................................................................................................ 199
5.6.1 AVI Localisation and Formation ............................................................................................. 202
5.6.2 AVI Composition ...................................................................................................................... 205

CHAPTER 6 - MAJOR PROJECT FINDINGS ................................................................................. 209
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Abbreviations

µl; ml; l: microlitre; millilitre; litre
ρM; µM; 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-cholamidopropyl 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
H2O: water
HPLC: high pressure liquid chromatography
hr: hour(s)
IPTG: isopropyl β-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.