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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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-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_2O: 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 feasability 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 veráison 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.