The influence of viticultural treatments on the accumulation of flavonoid compounds in grapes and their contribution to wine quality

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ABSTRACT

The grape flavonoids include anthocyanins, tannins and flavonols, all of which contribute to grape and wine quality by influencing the colour and mouthfeel of red wine. These compounds are synthesized in different parts of the berry and during different stages of berry development. In addition, environmental and viticultural factors such as light exposure can also alter the flavonoid composition of grapes. An understanding of how synthesis of these compounds is coordinated, their relationship to wine quality and the influence of bunch light exposure on the flavonoid composition of grapes, could be used to improve fruit quality by enhanced viticultural management.

The first part of this study sought to investigate the relationship between the different products of the flavonoid biosynthetic pathway (anthocyanins, flavonols and tannins), from two climatic regions (warm and cool) and determine their role in grape and wine quality. In collaboration with a major winery, whole Shiraz grapes were sampled at the weighbridge from a range of different vineyards from two climatic regions; warm (Riverland) and cool (McLaren Vale) in 2003 and 2004. A total of 80 grape samples were collected in each season and processed (i.e. 100 berries, separated into skin, seeds and juice, weighed and frozen). Anthocyanins and flavonols were measured, in triplicate, in skins by HPLC. Tannins were determined in the skins and seeds by two methods; phloroglucinol hydrolysis (HPLC) and protein precipitation (UV-VIS spectrophotometer). A comprehensive comparison of the two methods is discussed.

In both years, the grapes from warm and cool climates formed two distinct data sets based on flavonoid composition. There was a correlation between anthocyanins and flavonols for both the warm and cool climate samples in both years, however those from the warm region had lower anthocyanin for a given level of flavonol. As expected, the level of tannin in the seeds was greater than in skin for all samples. In both years, there was a weak correlation between anthocyanin levels in the skin and skin tannins, but no relationship with seed tannins. These results suggest there is some co-ordination in the synthesis of anthocyanins, flavonols and skin tannins. Also, the two regions clearly separated based on yield and despite the weak correlations in both regions, the levels of total anthocyanins were inversely related to yield. In addition, there was no relationship with any of the flavonoids and grape quality, indicating the need for improvement in streaming fruit for quality using these flavonoid compounds.

The second part of the study was to investigate the effect of bunch light exposure on flavonol synthesis and accumulation in Shiraz and Chardonnay grapes during development. Light-excluding boxes were applied to bunches at budburst. Boxes were removed at four sampling times; flowering, pre-veraison, veraison and harvest. At each sampling time, berry skins were sampled when the boxes were removed and then every second day (light induced), along with exposed controls for one week. Flavonol accumulation and flavonol synthase (*VvFLS1*) gene expression was determined by HPLC and Real Time-PCR (RT-PCR) respectively.

As expected, for all four sampling times, flavonol accumulation and *VvFLS1* expression in the boxed fruit was significantly less than bunches exposed to light. On removal of boxes at flowering, pre-veraison and veraison, flavonols accumulated to levels similar to that of the exposed control fruit over a period of 4-6 days. There was a significant increase in *VvFLS1* expression 2 days after exposure to light in parallel with the accumulation of flavonols. At harvest, in Chardonnay, *VvFLS1* expression peaked by day 4, while in Shiraz *VvFLS1* expression increased linearly and was highest at day 6. In contrast to the results for the earlier sampling times, the total amount of flavonols accumulated at harvest was less than 50% of exposed controls in Chardonnay and Shiraz grapes. These results show that flavonols are able to be induced by bunch light exposure at different times during berry development, including times when flavonols are not normally being synthesised. This suggests bunch light exposure can override the developmental control of flavonol accumulation.

To further investigate the light induced expression of *VvFLS1* in grapevines the molecular mechanism of transcriptional control was explored. Using genomic walking PCR techniques, two Shiraz *VvFLS1* promoter sequences were cloned and their sequences were analysed. These promoter sequences were ~800bp in length and were 99% identical. A putative MYB responsive element (MRE) and several light responsive elements (LRE) were identified in the promoter region of these genes.

To functionally test the *VvFLS1* promoter(s), a transient assay was developed in Chardonnay suspension cells. Cells were bombarded with constructs containing potential transcription factors and the *VvFLS1* promoter(s), fused to a luciferase reporter vector. After 48hrs incubation in the dark, cells were harvested and luciferase activity measured as an indicator of *VvFLS1* promoter activity. Of the different transcription factors tested with the *VvFLS1* promoter(s) the highest luciferase activity was observed using AtMYB12 (a flavonol-specific regulator of *AtFLS1* in

Arabidopsis (Mehrtens et al. 2005). While this result shows activation of the *VvFLS1* promoters by AtMYB12 and the development of a transient reporter assay for testing the *VvFLS1* promoter(s) a grapevine transcription factor specific for *VvFLS1* was sought.

Two techniques were employed to identify potential transcription factor regulators of the *VvFLS1* promoter(s). The first involved BLAST sequence search analysis in a grapevine expression (EST) database with AtMYB12 and the second involved using DNA microarray technology to identify candidate transcription factors that were up-regulated in light exposed Chardonnay cell suspension cultures. Thirteen potential transcription factors were identified and after correlative RT-PCR analysis (with *VvFLS1* expression patterns) two candidates were selected for further isolation and characterisation. These results have made significant progress in unravelling the molecular mechanisms of regulation of the flavonol biosynthetic, however additional experiments are required to unravel the transcriptional control of flavonol biosynthesis.

This investigation contributes to our knowledge of flavonoid synthesis in grapes; how it is coordinated, the relationship with wine quality, and the influence of light particularly on synthesis of flavonols. It also explores the molecular mechanisms of *VvFLS1* control, through isolation of the *VvFLS1* promoter and identification of potential transcription factors, which may regulate it. An understanding of the synthesis of flavonoids and how they may be coordinated, particularly in response to light, could be used to improve fruit quality by enhanced viticultural management.

Keywords: *Grapevine,* Vitis Vinifera L. *cv. Shiraz, Chardonnay, flavonoid, anthocyanin, tannin, flavonol, phloroglucinol analysis, protein precipitation assay, quality, berry development, seeds, skin, light, temperature, shading, transcription factors, promoter*

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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SIGNED……. DATE…….

Nicole Cordon

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ABBREVIATIONS

Units

%	Percent
°C	Degrees Celsius
aa	Amino acid
bp	Base pairs
g	Gram
g	Relative centrifugal force
Ha	Hectare
hr	Hour
km	Kilometers
L	Litre
m	Milli

Units

M	Molar
min	Minute
nt	Nucleotide
rpm	Revolutions per minute
s	Second
T	Tonnes
U	Unit
V	Volt
w/v	Weight per volume
v/v	Volume per volume

Genes/enzymes

PAL	Phenylalanine ammonia lyase
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone-3ß-hydroxylase
F3'H	Flavonoid-3'-hydroxylase
F3'5'H	Flavonoid-3'5'-hydroxylase
FLS	Flavonol synthase
DFR	Dihydroflavonol-4- reductase
LAR	Leucoanthocyanidin reductase
LDOX	Leucoanthocyanidin dioxygenase
UFGT	UDP-glucose flavonoid –3-O-glucosyltransferase
ANR	Anthocyanidin reductase
FGT	Flavonol glucosyl-transferase
UBIQ	Ubuiquitin

Plant species

Md	*Malus domestica*	Apple
At	*Arabidopsis thaliana*	Arabidopsis
Dc	*Daucus carota*	Carrot
Vv	*Vitis vinifera*	Grapevine
Zm	*Zea mays*	Maize
Am	Anthurium andraeanum	Lilly
Ps	*Pisum sativum*	Pea
Pc	*Petroselinum crispum*	Parsley
Ph	*Petunia hybrida*	Petunia
Le	*Lycopersicon esculentum*	Tomato

General

%CV	Coefficient of Variation (%)
cv.	Cultivar
±SEM	Standard Error of the Mean
AVI(s)	Anthocyanic Vacuolar Inclusion(s)
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CSIRO	Commonwealth Scientific Industry Research Organisation
DEA	Differential Expression Analysis (Microarray analysis)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
EC	Exposed Control
E-L Stage	Eichhorn-Lorenz grapevine growth stages (Coombe 1995)
EST	Expressed Sequence Tag
HLH	Helix-Loop-Helix
HPLC	High-Performance Liquid Chromatography
HWC	Hardy Wine Company
LC-MS	Liquid Chromatography Mass Spectrometry
LI	Light Induced
LSD	Least Significant Difference
MJT	Mean January Temperature
mRNA	Messenger RNA
MV	McLaren Vale
n	Number of samples
N_2	Liquid nitrogen
NIR	Near Infrared Spectroscopy
ORF	Open Reading Frame (coding region)
p	Probability
PAR	Photosynthetically Active Radiation
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PGA	Phloroglucinol Analysis
PPA	Protein Precipitation Assay
RE	Restriction Enzyme
RL	Riverland
RNA	Ribonucleic acid
RT-PCR	Real-Time PCR
SC	Shaded Control
TA	Total acidity
TC	Tentative Consensus
TF	Transcription Factor
Tm	Temperature of DNA dissociation (melt)
TSS	Total Soluble Solids (°Brix)
UV	Ultraviolet
VIS	Visible
Vv	*Vitis vinifera* L cv (cultivar)
ZIP	Zinc Finger Protein

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