

**An examination of malate metabolism in
Vitis vinifera during fruit ripening and in
response to elevated vineyard temperature**

by

Crystal Sweetman B.Sc. (Hons.)

A thesis by publication, presented for the
degree of Doctor of Philosophy at
Flinders University of South Australia
Faculty of Science & Engineering

July, 2011

Abbreviations

ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ANGIS	Australian National Genomic Information Service
AOX	Alternative oxidase
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
CH/BL	Chamber / blower heat treatment
CoA	Coenzyme A
COX	Cytochrome oxidase
Ct	Cycle threshold
DEDTC	Diethyldithiocarbamate
DFCI	Dana Farber Cancer Insitute
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
H ₂ O ₂	Hydrogen peroxide
KCl	Potassium chloride
KCN	Potassium cyanide
MDH	Malate dehydrogenase
ME	Malic enzyme
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MPA	Metaphosphoric acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NAD(P)	NAD and NADP
NAD(P)H	NADH and NADPH
NCBI	National Centre for Biotechnology Information

OAA	Oxaloacetate
OG	Octyl gallate
OTC	Open top chamber heat treatment
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PEG	Polyethylene glycol
PEP	Phospho <i>eno</i> lpyruvate
PEPC	Phospho <i>eno</i> lpyruvate carboxylase
PEPCK	Phospho <i>eno</i> lpyruvate carboxykinase
PEPCKin	Phospho <i>eno</i> lpyruvate carboxylase kinase
PK	Pyruvate kinase
PMSF	Phenylmethanesulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
mRNA	Messenger RNA
RT-PCR	Reverse transcriptional PCR
qRT-PCR	Quantitative real-time PCR
SA	Surface area
T _a	PCR cycle annealing temperature
T _m	PCR cycle melting temperature
TCA	Tricarboxylic acid
TES	3-(<i>N</i> -morpholino)propanesulfonic acid
TMPD	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine
WGS	Whole genome shotgun
bp / kb	Base pairs / kilobases
°C	Degrees, Celsius
g/mg/μg/ng	Gram / milligram / microgram / nanogram
L/ml/μl	Litre / millilitre / microlitre
M	Molar (moles.litre ⁻¹)
mM/μM/nM	Millimolar / micromolar / nanomolar
v/v	volume / volume
w/v	weight / volume

Summary

Acidity is an important characteristic for many fruits, particularly in aspects of fruit biochemistry and sensory quality. In grapes (*Vitis vinifera*), one of the most important agricultural fruiting crops, the use of fruit with low acidity and high pH in winemaking can increase risk of microbial spoilage and undesirable fermentative outcomes. Levels of the organic acid malate can determine acidity and pH in many fruits, through species- and cultivar-specific regulation of developmental and environmental responses. Malate is also extensively involved in primary and secondary metabolic pathways that can be critical to fruit development and ripening. The grape berry demonstrates particularly striking changes in malate accumulation and degradation during development and in response to vine temperature, likely driven by changes in metabolic pathways involving the acid. If harvested late in the season, or if ripening berries are exposed to unusually warm temperatures, the fruit are likely to contain less-than-optimal acid due to reduced levels of malate. Despite the considerable influence that berry acidity imparts on berry and wine quality, the mechanisms of regulation for malate metabolism in response to changes in grape berry development and vine temperature are still largely unknown. This thesis contains an evaluation of the activities and transcripts of enzymes in *V. vinifera* fruit that are involved in processes such as glycolysis, gluconeogenesis, CO₂ assimilation, respiration and fermentation, each involving malate either directly or indirectly. Enzymes from purified grape berry mitochondrial preparations were also explored, providing evidence for two activities that were, until now, undetermined in grapes: NAD-dependent malic enzyme and alternative oxidase. A developmental series of grapes from fruit set until harvest maturity displayed the expected pattern of pré-veraison malate accumulation, and post-veraison malate loss. Activities or transcripts that were differentially regulated with development included NADP-dependent malic enzyme, PEP carboxylase, PEP carboxykinase, pyruvate, orthophosphate dikinase, alcohol dehydrogenase and the terminal oxidases of the mitochondrial transport chain. To test the effect of elevated vine temperature on grape berry malate, field-based heat trials were established at numerous developmental stages. Temperature experiments were set up as mild, long-term heating trials to mimic a climatic shift, as well as shorter, more intense trials to represent heat-wave events. Effects of raising bunch temperature during the day and night were also investigated. Heating of grapevines at the post-set developmental stage (i.e. malate accumulation phase) led to higher berry malate,

particularly when bunches were heated at night. Heating of grapevines around the véraison and pre-harvest stages (i.e. malate degradation phase) generally led to lower berry malate, unless bunches were also heated at night, or if the majority of malate had already been lost from the fruit before the time of treatment. Data indicated that the temperature-sensitive changes in grape berry malate during the day and/or night at different developmental stages could be linked to alterations in PEP carboxylase, PEP carboxykinase, pyruvate, orthophosphate dikinase and NAD-dependent malic enzyme. Implications of such shifts in metabolism are discussed in detail within the text.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that 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.

Crystal Sweetman

School of Biological Sciences,

Faculty of Science and Engineering,

Flinders University of South Australia

Statement of the contributions of jointly authored papers

1. **Sweetman, C., Deluc, L.G., Cramer, G.R., Ford, C.M., Soole, K.L.** Regulation of malate metabolism in grape berry and other developing fruits. Review published in *Phytochemistry* on 15th September, 2009.

Author Contributions: CS surveyed the literature, analysed the data and drafted/constructed the manuscript. KLS and CMF contributed to the structure and editing of the manuscript. LGD and GRC assisted with provision and analysis of the microarray data, and with editing of the manuscript.

2. **Sweetman, C., Ford, C.M., Soole, K.L.** Developmental regulation of malate and its involvement in primary and secondary metabolism of *Vitis vinifera* fruit. Manuscript in preparation for submission to *Physiologia Plantarum*.

Author Contributions: CS designed and conducted all research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and design, and the editing of the manuscript.

3. **Sweetman, C., Ford, C.M., Soole, K.L.** Importance of mitochondrial respiration and acid metabolism in *Vitis vinifera* berry development and ripening. Manuscript in preparation for submission to *Physiologia Plantarum*.

Author Contributions: CS designed and conducted all research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and design, and the editing of the manuscript.

4. **Sweetman, C., Soar, C.J., Sadras, V.O., Ford, C.M., Soole, K.L.** Effects of elevated temperature on malate and other aspects of fruit primary metabolism in *Vitis vinifera*. Manuscript in preparation for submission to *The Journal of Experimental Botany*.

Author Contributions: CS assisted with experimental design of the CH/BL field experiment, collected materials, conducted all laboratory research experiments, analysed the data, and drafted/constructed the manuscript. KLS and CMF contributed to the research ideas and the editing of the manuscript. VOS designed field experiments and assisted with data analysis and editing of the manuscript.

The following authors agree that the Statement of the contributions of jointly authored papers accurately describes their contribution to research manuscripts 1., 2., 3., and 4. and give consent to their inclusion in this thesis.

..... Sweetman, C.

..... Soole, K.L.

..... Ford, C.M.

..... Cramer, G.R.

..... Deluc, L.G.

..... Sadras, V.O.

Acknowledgements

First, I thank my supervisors; Assoc. Prof. Kathleen Soole and Dr Christopher Ford. I am grateful for their wisdom and willingness to assist me in my endeavours. I also thank them for editing thesis drafts despite their incredibly busy schedules. In addition, I am truly thankful for Chris and Kathleen's support; both professionally and personally.

For their friendly assistance and collaborations in various aspects of this research, I thank Prof. Grant Cramer, Assist. Prof. Laurent Deluc, Assoc. Prof. Victor Sadras and Dr Chris Soar. Their contributions were vital to this thesis, and are much appreciated.

Next, to good friends and valuable contacts whom I have met as peers. In particular I thank Dr Crista Burbidge, Dr Vanessa Melino, Dr Vivek Vijayraghavan, Dr Chevaun Smith, Tania Kurek, Lidia Mischis and Pip Cook for making the lab so entertaining (and educational). A special mention to Crista and Vanessa for their vineyard shenanigans and generous support, both inside and outside of the lab, which will remain with me as fond memories, and to Vivek, James, Mike and Colin for therapeutic beer-o'clock 8-ball on Fridays. Jake Dunlevy has also been a valuable companion and coworker during this time. For day-to-day chats and "chin-ups", I thank the researchers and students from the 3rd floor of Biological Sciences at Flinders University, and more recently I have appreciated some wonderful encouragement from staff and students at the University of Adelaide.

I am indebted to my closest and most beloved family, Rick (the father) and Nathan (the brother), who have helped me throughout my entire education and all aspects of life. Although sometimes annoying, they are the most genuine and helpful people I know. Thanks also to Jo, Joseph and Amy; my new family.

I also thank my friends, old and new, for being around when I needed to laugh and leaving me alone when I needed to focus. While I will not list them all, I consider myself fortunate to have such a genuine and supportive group of people around me. Additional thanks (and perhaps apologies?) to Emma and Josh for putting up with me at home during the writing period.

Finally, I am extremely grateful for the two gentlemen in my life; Matt (the human) and Monkey (the canine), for their unwavering patience and affection during the final stages. Hopefully now I can give them both the attention that they deserve.

Table of Contents

List of Abbreviations	II
Thesis summary	IV
Student Declaration	VI
Statement of the contributions of jointly authored papers	VII
Acknowledgements	IX
Table of Contents	X
List of Figures	XIV
List of Tables	XVI
1. “Regulation of malate metabolism in grape berry and other developing fruits”	1
1.1. Abstract	3
1.2. Introduction	4
1.2.1. Fruit acidity and malate	4
1.2.2. Malate in fruit development	6
1.3. Pathways of malate synthesis in fruit	7
1.3.1. Glycolysis: PEPC-MDH reaction (pyruvate kinase bypass)	8
1.3.1.1. <i>Phosphoenolpyruvate carboxylase (PEPC)</i>	8
1.3.1.2. <i>Malate dehydrogenase (MDH)</i>	9
1.3.2. Photosynthesis	12
1.3.2.1. <i>NADP-dependent malic enzyme (NADP-ME)</i>	13
1.3.3. TCA cycle: fumarase and mitochondrial MDH (mMDH)	16
1.3.3.1. <i>Fumarase</i>	16
1.3.3.2. <i>Mitochondrial NAD-dependent MDH (mMDH)</i>	16
1.3.4. Glyoxylate cycle	17
1.4. Pathways of malate degradation in fruit	18
1.4.1. Gluconeogenesis	18
1.4.1.1. <i>Phosphoenolpyruvate carboxykinase (PEPCK)</i>	18
1.4.1.2. <i>Pyruvate, orthophosphate dikinase</i>	20
1.4.2. Respiration	20
1.4.2.1. <i>TCA cycle</i>	21
1.4.2.2. <i>NADP-dependent malic enzyme (NADP-ME)</i>	22
1.4.2.3. <i>NAD-dependent malic enzyme (NAD-ME)</i>	23
1.4.2.4. <i>Non-phosphorylating pathway of respiration</i>	24
1.4.3. Fermentation	25
1.4.3.1. <i>Ethanol fermentation</i>	25
1.4.3.2. <i>Lactate fermentation</i>	27
1.5. Intracellular transport of malate in the grape berry	28
1.6. Approaches to identify genes linked to high and low malate fruit	30
1.7. Temperature regulation of fruit malate metabolism	32
1.8. Transgenic approaches to modifying malate metabolism	34
1.9. Concluding remarks	37

1.10. Thesis aims	39
1.10.1. Developmental regulation of grape berry malate	39
1.10.2. Temperature regulation of grape berry malate	39
1.10.3. Specific aims	39
1.11. References	40
2. “Developmental regulation of malate and its involvement in primary and secondary metabolism of <i>Vitis vinifera</i> fruit”	51
2.1. Abstract	53
2.2. Introduction	53
2.3. Materials and methods	55
2.3.1. Materials	55
2.3.2. Malate quantification	56
2.3.3. Enzyme extraction	56
2.3.4. Mitochondrial isolation	56
2.3.5. Enzyme activity assays	57
2.3.6. Bioinformatics	58
2.3.7. Quantitative real-time reverse transcriptional PCR	60
2.3.8. Metabolic profiling	60
2.3.9. Statistical analyses	61
2.4. Results	62
2.4.1. Fruit growth and developmental changes in malate levels	62
2.4.2. Developmental patterns of gene transcript levels and enzyme activities	62
2.4.3. Metabolic profile of berry development	71
2.5. Discussion	74
2.5.1. Malate synthesis during berry development	75
2.5.2. Malate degradation during berry development	77
2.6. Concluding remarks	85
2.7. References	87
3. “Importance of mitochondrial respiration and acid metabolism in <i>Vitis vinifera</i> berry development and ripening”	93
3.1. Abstract	95
3.2. Introduction	95
3.3. Materials and methods	98
3.3.1. Materials	98
3.3.2. Malate quantification	99
3.3.3. Mitochondrial isolation	99
3.3.4. Cellular enzyme extraction	100
3.3.5. Spectrophotometric enzyme activity assays	100
3.3.6. Polarographic enzyme activity assays	101

3.3.7.	Bioinformatics	102
3.3.7.	Quantitative real-time reverse transcriptional PCR	102
3.4.	Results:	104
3.4.1.	Genetic analysis	104
3.4.1.1.	VvCox	104
3.4.1.2.	VvAox1a	104
3.4.1.3.	VvAox1b	107
3.4.1.4.	VvAox2	107
3.4.2.	Purification of grape berry mitochondria	107
3.4.3.	Mitochondrial respiration	107
3.4.4.	Malate-metabolising enzymes in purified mitochondria	111
3.4.5.	COX and AOX activities	111
3.5.	Discussion:	114
3.5.1.	Transcripts of the mitochondrial electron transport chain (mETC)	114
3.5.2.	Mitochondrial purity	118
3.5.3.	Mitochondrial respiration	119
3.5.3.1.	<i>Respiration of exogenous malate</i>	120
3.5.3.2.	<i>Grape berry COX</i>	122
3.5.3.3.	<i>Grape berry AOX</i>	123
3.5.	Concluding remarks	125
3.6.	References	127
4.	“Effects of elevated temperature on malate and other aspects of fruit primary metabolism in <i>Vitis vinifera</i>”	132
4.1.	Abstract	134
4.2.	Introduction	135
4.3.	Materials and methods	138
4.3.1.	Experimental Design	138
4.3.2.	Malate Quantification	139
4.3.3.	Enzyme Extraction	140
4.3.4.	Enzyme Activity Assays	140
4.3.5.	Quantitative Real-time Reverse Transcriptional PCR	141
4.3.6.	Statistical Analyses	142
4.4.	Results	142
4.4.1.	Berry weight and malate content	142
4.4.2.	Enzyme Activities and Gene Transcripts	145
4.4.2.1.	<i>NAD(P)-dependent malate dehydrogenases and malic Enzymes</i>	145
4.4.2.2.	<i>Phosphoenolpyruvate carboxylase</i>	147
4.4.2.3.	<i>Phosphoenolpyruvate carboxykinase</i>	150
4.4.2.4.	<i>Pyruvate kinase</i>	150
4.4.2.5.	<i>Pyruvate, orthophosphate dikinase</i>	150
4.4.2.6.	<i>Alcohol dehydrogenase</i>	150
4.4.2.7.	<i>Terminal oxidases of mitochondrial electron transport</i>	150

<i>chain</i>	154
4.5. Discussion	154
4.5.1. Effect of elevated temperature on post-set berries	156
4.5.2. Effect of elevated temperature on veraison berries	159
4.5.3. Grape berry metabolism in the face of climatic change	163
4.6. Concluding remarks	165
4.7. References	166
5. Summary and future directions	172
5.1. Experimental summary	173
5.2. Developmental regulation of grape berry malate	174
5.2.1. Malate accumulation phase	174
5.2.2. Malate degradation phase	174
5.3. Temperature regulation of grape berry malate	177
5.3.1. NAD(P)- MDH / ME	177
5.3.2. PEPC / PEPCK	180
5.3.3. PK / PPK	181
5.3.4. ADH	181
5.3.5. mETC	181
5.4. Implications of research	182
5.5. Future Directions	183
5.6. References	185
Appendices	186
A1. Berry sampling	187
A1.1. Developmental series sampling regime and experimental design	187
A1.2. Heat treatment sampling regime and experimental design	187
A2. Enzyme extraction and assay optimisation	194
A3. Networks of potential flux at three stages of berry development	197
A4. Gene transcript and enzyme activity scatter plots	200

List of Figures

Figure 1.1: Potential Metabolic Pathways Involving Malate in Fruit Cells	5
Figure 1.2: Transcript Levels of Extra-mitochondrial Enzymes Involved in Malate Metabolism in <i>V. vinifera</i> cv. Cabernet Sauvignon	10
Figure 1.3: Transcript Levels of Mitochondrial Enzymes Involved in Malate Metabolism in <i>V. vinifera</i> cv. Cabernet Sauvignon	14
Figure 2.1: Attributes of berry development throughout ripening	63
Figure 2.2: Developmental changes in PEPC activity and transcripts	64
Figure 2.3: Developmental changes in NAD(P)-MDH, NADP-ME and PK activities	65
Figure 2.4: Developmental changes in NAD-ME activity and transcript	67
Figure 2.5: Developmental changes in <i>VvPpdk</i> transcript	68
Figure 2.6: Developmental changes in PEPC activity and transcript	69
Figure 2.7: Developmental changes in ADH activity and transcripts	70
Figure 2.8: Changes in metabolite content across stages of berry development	73
Figure 3.1: Sequence alignment and phylogenetic tree of translated <i>V. vinifera</i> , <i>M. indica</i> and <i>A. thaliana</i> AOX genes	105
Figure 3.2: Normalised gene transcript level data across development	106
Figure 3.3: Activities of contaminating enzymes in crude and purified preparations of grape berry mitochondria	108
Figure 3.4: Mitochondrial membrane integrity of crude and purified mitochondria	109
Figure 3.5: State III mitochondrial respiration across berry development	110
Figure 3.6: Relative rates of mitochondrial malate-catabolic pathways	112
Figure 3.7: Capacity for mitochondrial respiration through COX	113
Figure 3.8: Capacity for mitochondrial respiration through AOX	115
Figure 3.9: Capacity for respiration through a) COX and b) AOX	116
Figure 3.10: Capacity for respiration through AOX in isolated mitochondria respiring malate	117
Figure 4.1: Developmental changes in berry weight in response to Chamber-Blower treatments	144
Figure 4.2: Effect of elevated temperature treatments on amount of malate per berry	146

Figure 4.3: Effect of elevated temperature treatments on activities of enzymes directly involved in malate metabolism	148
Figure 4.4: Effect of elevated temperature treatments on PEPC transcripts and activity	149
Figure 4.5: Effect of elevated temperature treatments on PEPC transcript and activity	151
Figure 4.6: Effect of elevated temperature treatments on aspects of pyruvate metabolism	152
Figure 4.7: Effect of elevated temperature treatments on ADH transcripts and activity	153
Figure 4.8: Effect of elevated temperature treatments on transcripts of terminal oxidases of the mitochondrial electron transport chain	155
Figure A.1.1: Berry sampling design for Coombe developmental series	188
Figure A.1.2: Tagged bunches for the developmental series	189
Figure A.1.3: A developmental scale of fruit from Coombe vineyard during 07-08 season	190
Figure A.1.4: Equipment used for heat treatment of vines in the field	191
Figure A.1.5: Experimental design of chamber/blower (ie. day/night heating) treatments	192
Figure A.2.1: Protein concentration in berry enzyme extracts	194
Figure A.2.2: Example of enzyme assay optimisation data	195
Figure A.3.1: Pre-veraison network of malate metabolism	197
Figure A.3.2: Veraison network of malate metabolism	198
Figure A.3.3: Post-veraison network of malate metabolism	199
Figure A.4.1: Malate content Vs ambient temperature from developmental series	200
Figure A.4.2: NAD(P)-MDH activities Vs malate content from heat treatments	201
Figure A.4.3: NAD-ME transcript Vs temperature and malate content from developmental series	202
Figure A.4.4: NAD-ME transcript and activity Vs malate content from heat treatments	203
Figure A.4.5: PEPC transcripts Vs temperature and malate content from developmental series	204
Figure A.4.6: PEPC transcripts and activity Vs malate content from heat treatments	205
Figure A.4.7: PEPC transcript Vs temperature and malate content from developmental series	206

Figure A.4.8: PEPCK transcript and activity Vs malate content from heat treatments	207
Figure A.4.9: PPDK transcript Vs temperature and malate content from developmental series	208
Figure A.4.10: PPDK transcript Vs malate content from heat treatments	209
Figure A.4.11: ADH gene transcripts Vs temperature and malate content from developmental series	210
Figure A.4.12: ADH transcripts Vs malate content from heat treatments	211
Figure A.4.13: AOX and COX transcripts Vs temperature and malate content from developmental series	212
Figure A.4.14: AOX and COX transcripts Vs malate content from heat treatments	213
Figure A.4.15: PEPC transcript Vs PEPC activity from heat treatments	214

List of Tables

Table 1.1: Relative expression values for putative genes of malate-metabolising enzymes from <i>V. vinifera</i> cv. Cabernet Sauvignon (external to mitochondria)	11
Table 1.2: Relative expression values for putative genes of malate-metabolising enzymes from <i>V. vinifera</i> cv. Cabernet Sauvignon (mitochondrial)	15
Table 2.1: List of accession numbers, primer sets and probes used for qRT-PCR	59
Table 2.2: Malate flux capacities of enzymes at veraison	72
Table 3.1: List of accession numbers, primer sets and probes used for qRT-PCR	103
Table 4.1: List of accession numbers, primer sets and probes used for qRT-PCR	143
Table 5.1: Summary of scatter plot comparisons between temperature, malate, gene transcript levels and enzyme activities	178
Table A.1.1: Location and climatic data for Coombe and Nuriootpa Research Station vineyards	193
Table A.2.1: Substrate affinities of enzymes from mature Shiraz grape berries	196