Identification and Characterisation of the Enzymes Involved in the Biosynthetic Pathway of Tartaric Acid in *Vitis vinifera*

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Summary

Vitis vinifera cv Shiraz is a member of Vitaceae, one of three higher plant families in which tartaric acid (TA) accumulates to significant levels. The accumulation of TA in *V. vinifera* berry tissue is crucial for commercial wine making, preventing discolouration and spoilage due to bacterial contamination. In *V. vinifera*, TA is biosynthesised via two pathway's, the primary-ascorbate and secondary-glucose precursor pathway's. Little is known regarding the enzymes responsible for the biosynthesis of TA with L-idonate dehydrogenase (L-idonate-5-dehydrogenase) the only enzyme identified in the primary-ascorbate precursor pathway. The results presented in this study describe a bioinformatic approach to the identification of putative candidates for a 2-keto-D-gluconate reductase (possessing 2-keto-L-gulonate reductase activity) and gluconate 5-dehydrogenase suggested as being responsible for the catalysis of the second steps of the primary-ascorbate and secondary-glucose precursor pathway's respectively. *In vitro* biochemical characterisation of recombinantly expressed proteins in conjunction with *in vivo* molecular analysis was performed to support the candidates inclusion in the respective pathway's.

Comparative analysis of the *V. vinifera* genome with enzymes identified as catalysing identical reactions in bacteria identified three candidate 2-keto-D-gluconate reductases, *TC61548*, *TC59682* and *TC55752* and three candidate gluconate 5-dehydrogenases, *TC52437*, *TC58004* and *TC55097* (Gene Indices: Grape database).

All candidate genes were identified in immature berry cDNA except *TC52437*, therefore *TC52437* was not pursued further. No active recombinant protein was obtained for candidates TC61548, TC58004 and TC55097. Kinetic analyses were performed on purified samples of the recombinant TC59682 and TC55752 protein. Optimal activity of recombinant TC59682 was observed at 35°C, pH 7.5. Activity studies indicated the primary substrate of TC59682 to be 2-keto-L-gulonate (2KGA) with a Km of 4.67mM. The Km for NADH was also determined as 0.77mM. A two-step assay utilising the highly-specific L-idonate dehydrogenase indicates L-idonate is a product of this reaction. The reversibility of TC59682 was confirmed against L-

idonate in the presence of NAD^+, at a rate 37-fold lower than the forward direction under identical conditions. Activity was also observed against ascorbate, the pathway precursor, 8.9-fold lower than that observed against 2KGA. Activity increased against all tested substrates in the presence of coenzyme NADP(H) as compared to NAD(H). Candidate TC55752 showed activity against 2KGA and ascorbate, 7.13-fold lower and 1.82 fold higher than TC59682 in the presence of coenzyme NADPH respectively.

QRT-PCR analysis of the candidate genes expression was conducted in developing *V. vinifera* cv Shiraz berries over the 2007-2008 season. The expression pattern of *TC59682* strongly coincided with the biosynthesis of TA over development. *TC55752's* expression pattern does not indicate involvement in TA biosynthesis. Expression patterns of *TC61548*, *TC58004* and *TC55097* suggest these candidates are not involved in TA biosynthesis

Activity of extracted soluble enzymes from a subset of the sampled Shiraz berries showed an increase in activity against 2KGA over development suggesting, as recently shown with L-idonate dehydrogenase, the presence of the enzyme late in berry development.

The results presented in this study suggest TC59682 has a role in the primary TA biosynthetic pathway. Based upon primary substrate activity TC59682 will be annotated as a 2-keto-L-gulonate reductase.

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.

Crista Burbidge

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Abbreviations

2KGA	2-keto-L-gulonate
2KGR	2-ketogluconate reductase
5KGA	5-Keto-D-Gluconic Acid
AGRF	Australian Genome Research Facility
AKR	aldo-keto reductase
ANK	Ankyrin
Asc	Ascorbic acid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tools
BME	β-mercaptoethanol
BSA	Bovine Serum Albumin
CAU	Corrected Area Units
cDNA	complementary DNA
DEDTC	diethyldithiocarbamate trihydrate
DHA	Dehydroascorbate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	Expressed Sequence Tags
FAM	Fluorescein
FLC	full length clone
FW	fresh weight
G5Dh	gluconate-5 dehydrogenase
GA	D-gluconic acid
GC-MS	Gas chromatography–mass spectrometry
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IA	L-idonic acid
IMAC	immobilized metal affinity chromatography
IPTG	Isopropylthiogalactoside
KCN	potassium cyanide
kDa	kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LC-MS	Liquid chromatography–mass spectrometry
L-IdnDH	L-idonate dehydrogenase
LN	liquid nitrogen
MA	Malic acid
MDH	Malate dehydrogenase
MEB	Modified Elution Buffer
mETC	mitochondrial electron transport chain

mRNA	Messenger RNA
MS	mass spectrometry
MSB	Modified Sonication Buffer
MWB	Modified Wash Buffer
MWL	Molecular weight ladder
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NCBI	National Centre for Biotechnology Information
NoC	no coenzyme
NoE	no enzyme
NoS	no substrate
nt	Nucleotide
OA	Organic Acid
OG	octyl gallate
ORF	open reading frame
OxA	oxalic acid
PCR	Polymerase Chain Reactions
PEB	protein extraction buffer substituted for purified enzyme
PEG	polyethylene glycol
PI	isoelectric point
PMSF	phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene Difluoride
PVP	Polyvinylpyrrolidone
PVPP	Poly(vinylpolypyrrolidone)
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RE	Restriction enzymes
RNA	Ribonucleic acid
RNAi	RNA interference
SARDI	South Australian Research and Development Institute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TA	Tartaric acid
TB	Terrific Broth
TC	tentative consensus sequence
TCEP	*tris*(2-carboxyethyl)phosphine
TEMED	N,N,N′,N′-Tetramethylethylenediamine
ThA	L-threonic acid
TrB	Transformation Buffer
TSAD	tartaric semialdehyde dehydrogenase
Ubq	Ubiquitin
WT	wild type