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**Molecular and structural characterization of the candidate
enzymes responsible for tartaric acid synthesis in the grapevine**

By

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Table of Contents

Abstract.....	5
Declaration.....	8
Statement of the contributions of jointly authored papers and manuscripts	9
Acknowledgement	10
Chapter 1 Introduction.....	11
1.1 General introduction.....	12
1.2 The chemistry of tartaric acid.....	16
1.3 Unequal distribution in higher plants	18
1.4 Tartaric acid in grape and wine	21
1.5 Industrial application and production	21
1.6 Tartaric acid biosynthesis.....	23
1.6.1 The primary TA biosynthetic pathway in grapevine.....	23
1.6.2 TA biosynthetic pathway in <i>Geraniaceae</i> plants.....	26
1.6.3 TA biosynthetic pathway identified in <i>Legumaceae</i> plants.....	27
1.7 Enzymes responsible for tartaric acid biosynthesis	28
1.8 Gene duplication, protein structural variation and plant phenotypic diversity	31
1.9 Protein structural variation and biological function.....	33
1.10 Conclusion and aims	34
Chapter 2 New insights into the evolutionary history of plant sorbitol dehydrogenase	38
Chapter 3 Amino acid substitutions at catalytic site of plant sorbitol dehydrogenase lead to a shift in substrate specificity	39
3.1 Introduction.....	40
3.2 Methods.....	44
3.2.1 Chemicals.....	44
3.2.2 Sequence alignment and phylogenetic analyses.....	44
3.2.3 Molecular cloning and transformation	45
3.2.4 Site-directed mutagenesis	46
3.2.5 Recombinant protein purification	46
3.2.6 Enzyme assay.....	47
3.3 Results and discussion	48
3.3.1 Bioinformatic analyses.....	48
3.3.2 Molecular cloning and recombinant protein purification	51

3.3.3 Enzymatic substrate specificity of SDHs.....	56
3.4 Conclusion.....	60
3.5 Abbreviations.....	60
Chapter 4 Identification and structural characterization of a putative 2-keto-L-gulonic acid reductase from <i>Vitis vinifera</i>	62
4.1 Abstract.....	64
4.2 Introduction.....	64
4.3 Results and Discussion.....	68
4.3.1 Molecular characteristics of the putative 2-keto-L-gulonate reductase	68
4.3.2 Enzymatic activity of VV2KR.....	71
4.3.3 Crystallization	72
4.3.4 Overall structure	74
4.3.5 Dimer interface.....	79
4.3.6 Co-enzyme binding	81
4.3.7 The catalytic site of VV2KR and substrate binding.....	86
4.3.8 Substrate docking and substrate specificity.....	92
4.3.9 The potential role of VV2KR in TA biosynthesis in the grapevine	96
4.4 Conclusion	98
4.5 Materials and Methods.....	98
4.5.1 Identification of a putative 2-keto-L-gulonate reductase gene from grapevine.....	98
4.5.2 Molecular cloning.....	99
4.5.3 Recombinant protein expression and purification	99
4.5.4 Enzyme kinetic assays.....	100
4.5.5 Protein crystallization	101
4.5.6 Data collection, structure determination and refinement.....	101
4.5.7 Substrate docking.....	102
4.6 Supplementary Figures.....	103
4.7 Contributions.....	107
4.8 Acknowledgement	107
4.9 Abbreviations.....	107
Chapter 5 Summary, Future Directions and Conclusions	113
5.1 Summary of Research Outcomes.....	113
5.1.1 Evolutionary origin of the grapevine LIDH	113
5.1.2 Substrate specificity of SDH and its effect on TA biosynthesis in higher plants....	114

5.1.3 Enzymatic and protein structural characterization of VV2KR in grapevine.....	115
5.2 Discussion and Future Research.....	117
5.2.1 The biological function of the Class II SDHs and TA biosynthesis in higher plants	117
5.2.2 <i>In vivo</i> characterization of the putative function of VV2KR in TA biosynthesis in the grapevine	120
5.2.3 Identification of the other candidate enzymes involved TA biosynthesis pathway	123
5.3 Conclusion	124
Abbreviations	127
Appendices.....	128
Appendix 1.....	129
Appendix 2.....	130
Bibliography	137

Abstract

L-tartaric acid (TA) is accumulated to be the dominant organic acid in the grape (*Vitis vinifera*) berries and remains metabolically stable during berry ripening and the wine making process. It confers a low pH and a “sharp” flavor to wine, affecting many quality aspects such as colour, taste, microbial stability and aging potential. Exogenous TA is widely used as a flavorant and an antioxidant additive in the food and wine industry. The metabolic intermediates involved in the TA synthesis pathway in grapevine have been well characterized. The primary pathway utilizes L-ascorbic acid (Vitamin C) as the precursor which is sequentially converted to 2-keto-L-gulonic acid (2KLG), L-idonic acid (IA), 5-keto-D-gluconic acid (5KGA) and TA by several biochemical reactions. Only one candidate enzyme, *V. vinifera* L-idonate-5-dehydrogenase (LIDH, EC 1.1.1.264), involved in this pathway has been characterized so far. It catalyzes the inter-conversion of IA and 5KGA, the rate-limiting step of the TA biosynthesis pathway. In addition, another enzyme, a putative 2-keto-L-gulonate reductase (VV2KR), which is assumed to be responsible for the reduction of 2KLG to IA, has also been reported. However, further enzymatic characterization of this enzyme is still needed to validate its metabolic function in grapevine. The present study aims to investigate the molecular and structural characteristics of these two proteins, thereby improving our understanding on TA biosynthesis in grapevine.

LIDH is highly homologous to sorbitol dehydrogenase (SDH, EC 1.1.1.14). We employed a computational approach to show that LIDH originates from the functional divergence of SDH in grapevine. We demonstrated that core eudicot (the strongly supported monophyletic group following the early-diverging eudicot lineages ([Soltis, Senters et al. 2003](#))) SDHs have evolved into two distinctive phylogenetic lineages (Class I and Class II) due to positive natural selection after tandem gene duplication in the common ancestor of core eudicot plants. LIDH was identified as a Class II SDH. While the Class I SDH gene is universally conserved, Class II SDH genes are retained only in some plants including *V. vinifera*, *Solanum tuberosum*, *Theobroma cacao*. The

distribution of SDH genes among plant genomes showed a positive correlation between the occurrence of Class II SDH genes and the capacity for TA synthesis. These results provided new insights into the TA biosynthesis pathway from the evolutionary perspective. Protein modeling analyses revealed three amino acid substitutions at the catalytic sites between Class I and Class II SDHs. These three amino acid changes are suspected to be responsible for the unique enzymatic profile of LIDH. To confirm this hypothesis, Class I and Class II SDH genes from *V. vinifera*, *T. cacao* and *S. tuberosum* were cloned in this study. Site-mutations of the three amino acids were performed to assess their impact on enzyme substrate specificity. Recombinant protein of both wild-type and mutant SDHs from the above plants were expressed and purified. Enzyme kinetic tests confirmed that LIDH has a preference for L-idonate over D-sorbitol as its substrate. The Class I SDH from *T. cacao* demonstrated the highest activity with D-sorbitol but could hardly utilize L-idonate. The recombinant protein of the other SDHs could not be purified in this study due to troubles in protein induction and purification. Despite this, the preliminary results showed a significant enzymatic divergence between Class I and Class II SDHs, supporting the putative role of Class II SDHs in the TA biosynthesis pathway. In addition, a complete enzymatic characterization of the recombinant VV2KR was performed. VV2KR has the highest substrate specificity with D-glyoxylate, followed by hydroxypyruvate and 2KLG. We showed that VV2KR could catalyze the reduction of 2KLG to L-idonic acid effectively using NADPH as the preferred coenzyme. We went further to crystallize the recombinant VV2KR to determine its 3-dimensional structure. The ligand-free crystal structure of VV2KR was solved to a resolution of 2.1 Å. VV2KR has the highest similarity with *Coleus blumei* hydroxypyruvate/glyoxylate reductase (CbHPR) and *Arabidopsis thaliana* hydroxypyruvate/glyoxylate reductase (AtHPR) isoform 2 (~75% amino acid sequence identity). The VV2KR monomer structure adopts the typical folding of the D-isomer 2-hydroxyacid dehydrogenase (2KDH) proteins and has the highest structural homology with the CbHPR structure (main-chain atom Root Mean Square Deviation of 0.76 Å; main-chain atom include all atoms in the peptide chain except

those from the R-group, i.e. the side-chain). It represents the second plant HPR structure being determined to date. Structural comparison of VV2KR and CbHPR with other HPR structures revealed some unique structural features for plant HPRs. The commonly accepted substrates for HPRs (D-glyoxylate, hydroxypyruvate, pyruvate) and the 2KLG intermediate from TA pathway were docked to the VV2KR structure by a computational method. The potential biological function of VV2KR in the TA biosynthesis pathway in grapevine was discussed.

In summary, we focused on the molecular and structural characterization of the candidate enzymes responsible for TA biosynthesis in grapevine. The results of the present study highlighted the effects of enzyme structural variations on their in-vivo biological functions and significantly extended our understanding on the molecular mechanism of the TA biosynthesis pathway in grapevine.

Declaration

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

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Date 03-April-2016

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Statement of the contributions of jointly authored papers and manuscripts

1. Yong Jia., Darren CJ Wong, Crystal Sweetman, John B Bruning, Christopher M Ford. (2015). "New insights into the evolutionary history of plant sorbitol dehydrogenase." *BMC Plant Biology* 15(1):101.

Author contributions: YJ conceived the research. YJ and DCJW did sequence retrieval, curation and gene duplication characterization. YJ performed phylogenetic, synteny, natural selection and ancestral sequence analyses. YJ drafted the manuscript. JBB and YJ carried out protein modeling analyses. DCJW performed the transcript expression and gene co-expression analysis. CS and DCJW assisted with the drafting of the manuscript. CMF and JBB supervised the project.

2. Yong Jia, Crista A Burbidge, Crystal Sweetman, John B Bruning, Christopher M Ford. "Identification and structural characterization of a putative 2-keto-L-gulonic acid reductase from *Vitis vinifera*." Prepared for the submission to *Acta Crystallographica Section D*.

Author contributions: YJ, JBB and CMF conceived the research. CAB performed gene cloning and transformation. YJ and JBB purified the protein for enzymatic tests and crystallization. JBB collected the diffraction data. YJ and JBB solved the structure together. YJ, CS and CAB performed the enzymatic tests. YJ performed kinetic data and protein structure analyses. YJ drafted the manuscript. JBB and CMF contributed to the editing of the manuscript. CMF and JBB supervised the project.

The above papers and manuscripts are displayed in this thesis in either their published forms or submission forms according to the author guidelines of the specific journal.

The following authors agree with the Statement of the contributions of jointly authored papers and manuscripts and give consent to their inclusion in this thesis.

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