THE UNIVERSITY OF ADELAIDE
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Grape and Wine Research and Development Corporation

Organic acid metabolism during grape berry development



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA0602**

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Research Organisation: **The University of Adelaide**

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1. ABSTRACT

The research sought to increase our understanding of grape berry organic acid metabolism. Genes encoding key enzymes in pathways of tartaric acid (TA) synthesis, and malic acid (MA) metabolism, were studied over 3 years. Our results suggest that TA synthesis is tightly regulated during the early stages of development, and that additional pathways of synthesis may be involved. MA metabolism responds to innumerable cellular and environmental cues; we identified cellular processes linked to MA metabolism during berry development. We identified and part-characterised 4 candidate transporters with the potential to move organic acids across the vacuolar membrane.

2. EXECUTIVE SUMMARY

Summary of outcomes and benefits

The research conducted within the present project will result in a greater understanding of grape berry acidity, how it is achieved and how it is regulated during berry development. Berry acidity, the composition of tartaric (TA) and malic (MA) acids present in the berry during ripening, is critical for ensuring the suitability of berries for winemaking purposes, and in addition provides important organoleptic properties in wines of all ages.

Our earlier CRC Viticulture funded research identified a gene involved in TA synthesis, which was present only in berries that accumulate tartaric acid and switched on (transcribed) only during the period of TA accumulation. Moreover, when tested in the laboratory, the enzyme encoded by the gene was capable of specifically catalysing a key step in the conversion of L-ascorbic acid to TA. We sought to extend this work in the present project by undertaking further investigations of grapevine genes associated with tartaric acid synthesis and metabolism. We have used molecular and biochemical tools, in conjunction with extensive fieldwork over three growing seasons, to identify candidate genes for additional steps in the TA synthesis pathway. In addition, we have investigated the processes responsible for TA movement within berry cells, and thereby identified four candidate proteins that will in future be investigated more fully for their function in controlling vacuolar acid levels.

In addition to TA, berries accumulate significant amounts of MA during early development, much of which is lost however as berries ripen to maturity, most particularly in hotter climates. Unlike TA, which is restricted to grape berries and a few other fruits, MA is a ubiquitous plant metabolite that plays a large number of intermediary roles in metabolism. Our research here has enabled us, through a combination of the same biochemical and molecular analyses coupled with field

sampling used to study TA, linked to detailed analysis of a number of metabolites, to develop a model of MA metabolism in berry ripening that offers a significant enhancement on previously proposed scenarios. We have been able to link the expression of specific genes at defined berry developmental intervals to changes in levels of metabolites within the berry, thus showing evidence for the pathways by which this vital acid is formed and used during berry ripening.

Practical implications, industry benefits, future research

The understanding of berry acidity and its control afforded by the outcomes of the research completed in this project will inform future research efforts aimed at manipulating levels of acids in the berry through conventional breeding or molecular (transgenic) technologies. Specifically, we have identified candidate genes for defined steps in the synthesis of TA that may prove to be the targets of regulation at the level either of gene expression or activity of the encoded proteins. Further research is now needed to understand how TA synthesis is regulated, focussing on the genes identified in the present research and our earlier studies. The successful completion of this work in the next few years will provide the viticultural and winemaking industries with an opportunity to design grapevines in which for the first time the levels of TA in berries are controlled. Given the present trend towards a warming climate and the models developed to explain the possible implications for grape growing in Australia, TA levels are increasingly important. The metabolic inertness of TA in comparison to MA, (which is progressively lost as berries ripen and more so in warmer climates), makes it a valuable target for the development of future grapevine germplasm.

In addition to the synthesis of TA, berry levels of the acid are controlled by the rate and extent to which it is stored in the vacuole of the cell. The four transporter protein candidates identified during the course of the present research will be pivotal to understanding the exact mechanisms by which both TA and MA are moved into, and in the case of MA, out of the vacuole during development.

MA levels play important roles in juice acidity and the preliminary stages of vinification. Our research has shown that these levels are highly regulated and linked to a variety of metabolic pathways within the berry. Future research in this area will further refine the specific pathways controlling MA levels, and may indicate future targets for manipulation and thereby modified levels of MA in ripe berries.

Collaborations and financial support

Much of the research conducted during this project has been completed by PhD students supported in part by the project; in addition a post-doctoral researcher Dr Matt Hayes, whose PhD was undertaken within CRC Viticulture, was employed throughout the project. A major outcome of the project will be achieved when the PhD students complete their studies, which will occur in late 2009 and mid 2010.

In addition to the direct funding for this research from GWRDC, a number of other opportunities for collaborative research occurred that provided enhanced outcomes to the project. Professor Grant Cramer from U Nevada Reno spent some months at UA (supported by funds from his home university, research costs during the stay were covered by UA). Discussions and collaborations formed during this time gave us access to large datasets of molecular and metabolic information developed by Prof. Cramer's group from which we were able to vastly improve our model of malate metabolism (work now accepted for publication in Phytochemistry). Matt Hayes spent 4 weeks in January 2008 working in the laboratory of Prof. Doug Cook at UC Davis, analysing transgenic grapevines in which TA metabolism had been modified. Funds for Matt's research during this time were provided by Prof. Cook, who has also invested significant time in the maintenance of the transgenic vines during the past three years. In mid 2009, Matt spent 5 weeks working with Dr Laurent Torregrossa at INRA Montpellier, where he was able to investigate aspects of berry acid transport in a collection of unique grapevine germplasm. This visit was supported by funds from The Australian Academy of Science. Over the past two growing seasons, we have collaborated with the SARDI-CSIRO funded under-vine heating trials conducted at Nuriootpa by Drs Victor Sadras and Chris Soar. The data from these experiments, although not part of the results presented here, will provide important information regarding the acid profiles of berries growing under conditions of extreme temperatures. Finally, our work on tartaric acid metabolism and acid transporter proteins formed part also of GWRDC-funded honours projects to students enrolled in undergraduate Oenology and postgraduate Biotechnology programs at the University of Adelaide and Flinders University respectively. A third Masters project, also looking at berry acid transporters, was supported by UA.

3. BACKGROUND

Grape berry acidity is controlled principally by the levels of L-tartaric acid (TA), formed during early berry development. L-malic acid (MA) is also made during early berry development, however, unlike TA it is catabolised during later stages of ripening and in many cases plays little role in regulating juice and wine pH. Control of pH is critical to the winemaking process; in Australia, where climate and winemaking preferences result in very ripe berries at harvest, acid additions are made to provide optimum pH for processing and subsequent operations. Current predictions of increasing global temperatures over the next 50 years will exacerbate the need to understand berry acidity, since acid levels at harvest are negatively correlated with growing season temperatures. Therefore, an understanding of the mechanisms by which grape berry acidity is achieved and maintained is fundamentally important for the future of an efficient wine producing industry throughout much of Australia.

Despite the undoubted importance of berry acidity to winemaking, until recently little was known of how grapes **form, use and store** their organic acids. A pathway of conversion from AsA to TA was proposed in 1969, but it was only with our work (CRCV project 3.3.3) that definitive biochemical and molecular evidence for the genes and enzymes of the pathway in grapes was achieved. We identified an enzyme, L-idonate dehydrogenase (L-IdnDH), with activity specific for oxidation of L-idonic acid to 5-keto-D-gluconic acid (5kG), the rate-limiting step in the pathway from AsA to TA [1]. We also identified candidates for enzymes associated with two other steps in the ASA to TA biosynthetic pathway. An alternative pathway for TA formation proceeding from glucose was also proposed, however, it was considered to make only a minor contribution to TA levels and no enymatic or genetic units involved in this pathway have been identified (Loewus, 1999).

MA metabolism proceeds by well characterised pathways, and limited exploration of these in grapes has been made by other groups over the years. Malate is suggested to play a pivotal role in plant metabolism, linking via phospho *eno*l pyruvate (PEP), the glycolytic and TCA pathways of oxidative metabolism and providing a means of transporting reducing equivalents across organellar membranes. Accumulation and subsequent respiration of MA during fruit development and ripening present a complex interplay of enzymatic activities that remains poorly understood, yet vital for an understanding of the factors controlling berry acidity. In addition to contributing to juice pH, malic acid plays a dominant role in determining fruit quality as its metabolism will impact on secondary metabolites important for colour and flavour [2]. It is known that grapes from warmer climates or warmer seasons have lower titratable acidities than grapes grown in cooler regions and seasons, and that this reflects the increased utilisation of MA during later stages of ripening. The increased pH values at harvest resulting from this must be corrected by the addition of TA at the crush and later stages during vinification.

Once synthesised, TA and MA are stored in the vacuoles of berry pulp cells. Surprisingly, because decompartmentation of MA from the vacuole to the cytoplasm allows the respiration of MA to occur, no vacuolar transporters of TA or MA have been identified in grapevine. In the model plant *Arabidopsis thaliana* two vacuole membrane proteins that transport organic acids have been identified; in terms of MA,

these proteins are responsible for 100% of the transport activity. Because MA and TA are both dicarboxylic acids with similar structures, we hypothesise that these proteins may also carry TA. Five homologues of these acid carriers are encoded in the grapevine genome but have not been studied to date.

The project objectives were:

- To complete the characterisation of genes associated with tartaric acid metabolism.
- To determine the regulation of malic acid biosynthesis and catabolism.
- To provide the highest level of postgraduate education for the PhD students engaged on the research and thereby provide the Australian grape and wine industry with the future research capability it will need to remain competitive.

4; 5; 6: AIMS AND PERFORMANCE TARGETS; METHODS; RESULTS AND DISCUSSION

NOTE: We have presented the three sections 4, 5 and 6 as a single combined entity to allow the reader to more clearly follow the individual research projects within a clear framework.

Outputs and Performance Targets 2006-07

Outputs	Performance Targets
1. Staff appointed.	2 x PhD candidates, 1x postdoctoral appointed.
2. Candidatures confirmed.	2x PhD candidatures confirmed by June 2007.
3. Kinetics analysed.	Determination of kinetics and subunit composition of L-IdnDH.
4. TA biosynthesis candidates identified.	Candidate sequences involved in early stages of TA biosynthesis pathway identified.
5. Malic acid metabolic genes identified.	Grapevine homologs of malate and respiration-associate genes identified and cloned; preliminary expression studies underway.

Outputs and Performance Targets 2007-08

Outputs	Performance Targets
1. Enzymes characterised.	Candidate genes and enzymes further characterised.
2. Candidates identified.	Candidate sequences for early steps of TA pathway identified.
3. Acid profiles obtained.	Organic acid profiles studied during berry development.
4. Gene expression tested..	Expression of MA biosynthesis genes in berry ripening tested.
5. Dissemination of research outcomes.	PhD students and post-doc to present research findings to conferences (incl. AWITC) and in published papers.

Outputs and Performance Targets 2008-09

Outputs	Performance Targets
1. Understanding of TA biosynthesis.	Genetic and biochemical regulation of TA biosynthesis addressed.
2. Metabolism of malic acid during berry ripening understood.	MA catabolic genes identified, expression measured.
3. Model developed.	Model of organic acid metabolism during berry ripening proposed.
4. Dissemination of research outcomes.	Continued publication of research findings in peer-reviewed and industry journals. Presentation at conferences.
5. PhD's awarded.	2x PhD theses submitted, examined and accepted.

Project 1. Identification of Tartaric Acid biosynthetic enzymes

Introduction

Tartaric (TA) and malic (MA) acids account for 90% of the total acid in the grape berry (Lamikanra *et al.*, 1995). In the developing “pre-verasion” berry, TA levels rapidly increase, peaking approximately 50-70 days post-flowering and remaining constant thereafter (Saito and Kasai, 1968, Saito and Kasai, 1978). The catabolism of Ascorbic acid (Asc), a ubiquitous antioxidant vital to cellular function, to TA, a metabolically inactive organic acid, is an unusual metabolic strategy. While TA can be detected at low levels in the leaves and other tissues of many plants, its accumulation to high levels in fruit appears to be limited to grapevines and just a few other species. TA plays a major role in controlling vacuolar pH, and is vital in winemaking, providing appropriate acid conditions in the juice and must to prevent spoilage and oxidative processes. It is used by winemakers to adjust juice, must and wine pH throughout the winemaking process (Es-Safi *et al.*, 2000, Cirami, 1973, Toit *et al.*, 2006, Zoecklein *et al.*, 1995).

Radiotracer studies were used to map the biosynthetic pathway of TA in Vitaceae, 1 of 3 higher plant families known to accumulate TA to significant levels (Stafford, 1959). Two pathways of TA production and the subsequent intermediates were identified based on the pathway precursors (Helsper and Loewus, 1982, Helsper *et al.*, 1981, Loewus and Stafford, 1958, Ribéreau-Gayon 1968, Saito and Kasai, 1969, 1982, 1984, Saito and Loewus, 1989a, 1989b, 1989c, Saito, 1992, Wagner and Loewus, 1974, Wagner *et al.*, 1975, Williams and Loewus, 1978). The primary pathway, considered to be responsible for >95% of TA production is known as the Asc-inclusive C4/C5 pathway, based on the cleavage of the precursor Asc at carbons 4 and 5 (see figure *1). The second minor pathway, thought to account for >5% of TA production uses D-Glucose as a precursor (see figure *1).

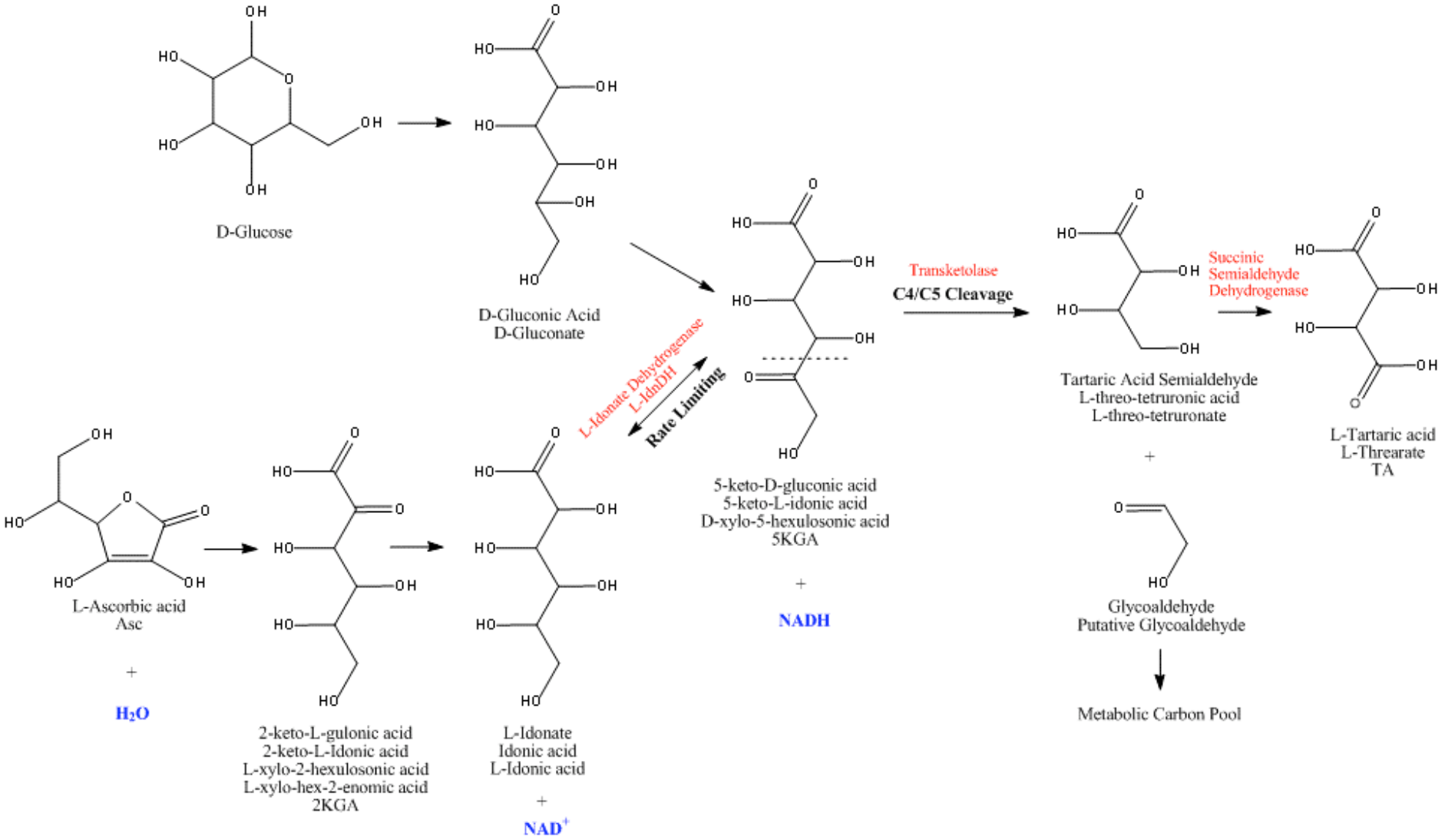


Figure *1: Summary of the major (AA-C4/C5) and minor (glucose) pathways of TA synthesis shown to operate in *V. vinifera.* The intermediates and their common names are listed. Known or proposed enzymes are shown in red (Zimmermann, 2005, DeBolt *et al.*, 2006). Cofactors are shown in blue for the respective reactions where known. Composite picture derived from those shown in Loewus (1999).

With the intermediates of the TA biosynthetic pathway in *V. vinifera* confirmed, identification of the enzymes responsible for catalysing and regulating this pathway is now a priority. Through examination of gene expression libraries corresponding to the developmental stages of the berry in which TA synthesis is the highest, DeBolt *et al* (2006) provided the first identification of an enzyme, L-idonate dehydrogenase (L-IdnDH), involved in TA synthesis. Enzyme activity studies showed that L-IdnDH is responsible for the conversion of L-idonate → 5-keto-L-idonic acid, step 3 in the Asc-inclusive C4/C5 pathway in *V. vinifera*, the proposed regulatory enzymatic reaction of the pathway (Malipiero *et al* 1987). A candidate, provisional name tartaric semialdehyde dehydrogenase (TSAD) was identified using similar techniques for the final step in the pathway, converting TA semialdehyde → TA (Zimmermann, 2005, DeBolt, 2006). Characterisation studies of this enzyme are still required to confirm its involvement in the pathway.

The first step, which combines both the Asc and D-glucose pathways by the C4/C5 cleavage of 5-keto-L-idonic acid, is proposed to act via a transketolase-like enzyme as has been reported in the bacterium *Gluconobacter suboxydans* (Salusjärvi *et al* 2004). Examination of gene expression libraries by DeBolt (2006) identified two transcripts which shared homology to plant transketolases. *In vitro* enzymatic analysis to confirm the function of these candidates is currently underway.

To identify additional candidates for the other steps of the TA synthetic pathway information relating to the catalytic activity of enzymes found in bacterial species such as *E. coli*, *Pseudomonas spp.* (Lockwood and Nelson, 1951, La Rivière, 1956), *Gluconobacter spp.* (Koichi *et al.*, 1971, Chandrashekar *et al.*, 1999) and *Acetobacter spp.* (Kamlet, 1943) has been investigated. Enzymes within these species are capable of synthesising, metabolising, or performing the catalytic steps of TA biosynthesis identified in grapevines. The identification of candidate enzymes based on this information has been aided in the publication of the whole genome sequences of grape (*V. vinifera*, The French–Italian Public Consortium for Grapevine Genome Characterization, 2007), *Escherichia coli* (Blattner *et al.*, 1997), *Pseudomonas aeruginosa* (Stover *et al.*, 2000) and *Gluconobacter oxydans* (Prust *et al.*, 2005). Based on bacterial catalytic data, the presence of enzyme functional domains and expression in young grape berries and leaves, a 2-keto-gluconate reductase and a gluconate 5-dehydrogenase, have been identified as candidates for step 2KGA → L-idonate and D-gluconate → 5KGA, respectively. The characterisation of these enzymes is currently underway.

Materials/Methods

Full length PCR amplification of putative TA biosynthetic open reading frames

Full length ORFs were amplified from grapevine young leaf and berry cDNA using 1 unit of Platinum Taq HiFI (Invitrogen) according to the manufacturers instructions. PCR products were cloned using the pDrive PCR Cloning System (Qiagen) and DNA sequenced to check that an open reading

frame was obtained. PCR products were then sub-cloned into the bacterial expression vector pET14b and after plasmid purification and diagnostic restriction enzyme digestion, this plasmid was transformed by heat shock into chemically-competent *E. coli* strain BL21 (DES) pLyS (Sigma) cells.

Production and purification of His-tagged recombinant TA biosynthetic enzymes in bacteria

Bacterial strains transformed with the TA biosynthetic clone of interest were grown in Terrific Broth supplemented with 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol until the culture reached an optical density of 0.5 at 600 nm. At this time, expression of recombinant proteins was induced by addition of 0.4mM IPTG, and after 4-8 h of additional incubation, cells were harvested by centrifugation and resuspended in sonication buffer. Cells were lysed by successive freeze-thaw cycles and by passage through a 20-gauge needle followed by centrifugation at 13,500 RPM for 10 min at 4°C to produce soluble and insoluble protein extracts. His-tagged, recombinant, TA biosynthetic enzymes were purified from soluble extracts using Talon resin (Clontech) according to the manufacturers instructions.

***In vitro* analysis of recombinant enzyme activity**

Spectrophotometric assays have been conducted based on the published bacterial methods (De Ley, 1966) monitoring the consumption of assigned cofactors, namely NADH and NADPH. The assays are conducted at specific pH and temperature which are optimal for the enzyme in question. Generally all parameters must be determined as they are specific for each enzyme.

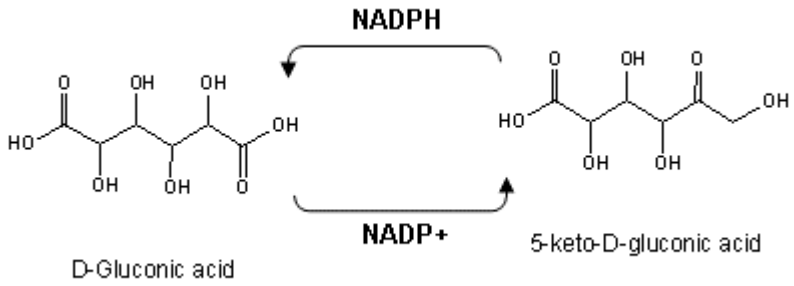


Figure *7 – A diagram of the reaction catalysed by candidate TC55097 which is monitored spectrophotometrically at 340nm. The enzymes involved in this study act by converting a substrate into a product by either the oxidation or reduction of the appropriate cofactor. Reduced NAD(P)H absorbs light at 340nm, however, its oxidised form NAD(P) does not. This enables the reactions to be monitored in both directions. Figure *8 shows graphically this reaction operating in the reverse direction where by the substrate 5-Keto-D-gluconic acid is converted to D-gluconic acid by the oxidation of NADPH to $NADP^+$. It is this conversion of NADPH to $NADP^+$ which is seen by the decrease in absorbance at 340nm.

Results and Discussion

Cloning of 2-Ketogluconate Reductase and a Gluconate 5 Dehydrogenase from grapevine

A tBlastx search was conducted using the sequence for the enzymes described in *E. coli* against the *V. vinifera* genome. Search results were ranked based on criteria including % homology to the *E. coli* sequence, presence of both an NAD catalytic and NAD binding domain and the identity of the EST libraries within which the contigs are found see Table 1). Primers were designed with restriction sites to aid in cloning into the bacterial expression plasmid pET14b. These candidates were amplified from a pre-verasion cDNA sample from *V. vinifera* cv. Shiraz, chosen to represent the developmental stage at which the greatest level of TA synthesis occurs. Candidate TC66090 from the Asc C4/C5 pathway was absent from the pre-verasion berry and was not studied further. Candidates TC58004 and TC52437 were successfully amplified, however the predicted translations of these candidates showed truncated open reading frames. Currently, a redesign of experimental protocol is being undertaken to ensure a viable protein is obtained from these candidates.

Table 1– Summary of the data obtained for candidates identified from the *V. vinifera* genome based on alignments of an *E. coli* 2-Ketogluconate Reductase and Gluconate 5 Dehydrogenase. These enzymes are expected to catalyse 2KGA L-Idonate, step 2 in the Asc C4/C5 pathway and D-Gluconate 5KGA, step 2 in the D-Glucose pathway respectively. *Candidate discontinued after analysis, BP – Base pairs, MW – Molecular Weight (calculated), EST – Expressed Sequence Tag

Annotation (where known)	Accession #	DNA Length (BP)	Protein MW (kD)	Motifs present	EST Libraries	Homology to *E. coli*	Berry cDNA Amplification
2-Ketogluconate Reductase	TC55752	1150	42.08	Y	Berry	28.7%	Y
	TC66090	833	29.35	Y			N*
	TC61548	994	36.40	Y		31.5%	Y
	TC59682	1055	38.27	Y		35.8%	Y
Gluconate 5 Dehydrogenase	TC58004	893	-	Y	Berry, Leaf	27.8%	Y
	TC55097	760	26.89	Y		28.8%	Y
	TC52437	876	31.14	Y	Berry	26.5%	Y

Expression and purification

Candidate proteins were expressed using the expression line *E. coli* BL21pLysS (Invitrogen) under a variety of conditions to optimise both the protein expression and its solubility (See Table *5).

Table 2 – Summary of the determined optimised expression conditions for each of the candidates. Conditions tested included Temperature [37°C, 30°C, 25°C, 14°C], IPTG levels [0.1mM, 0.2mM, 0.3mM, 0.4mM] and expression duration [3hrs, 6hrs, 24hrs, 48hrs]

	Accession #	**Temperature (°C)**	**IPTG (mM)**	**Duration (hrs)**
2-Ketogluconate Reductase	TC55752	37	0.2	6
	TC66090	*Discontinued*		
	TC61548	37	0.2	6
	TC59682	16	0.2	48
Gluconate 5 Dehydrogenase	TC58004	*Reanalysis continuing*		
	TC55097	30	0.2	6
	TC52437	*Reanalysis continuing*		

Once expressed, immobilised metal affinity chromatography (IMAC) was used to purify the proteins. Purified proteins resulting from these procedures were quantified using SDS-PAGE gels (see Figure 2). Soluble protein was obtained through this expression method, which was suitable for further characterisation for each candidate except TC55752 and TC61548. Attempts to obtain soluble protein using a highly detergent extraction buffer proved successful for TC55752; however soluble protein for TC61548 has yet to be obtained. L-IdnDh has also been successfully expressed and purified to enable further kinetic studies of the protein.

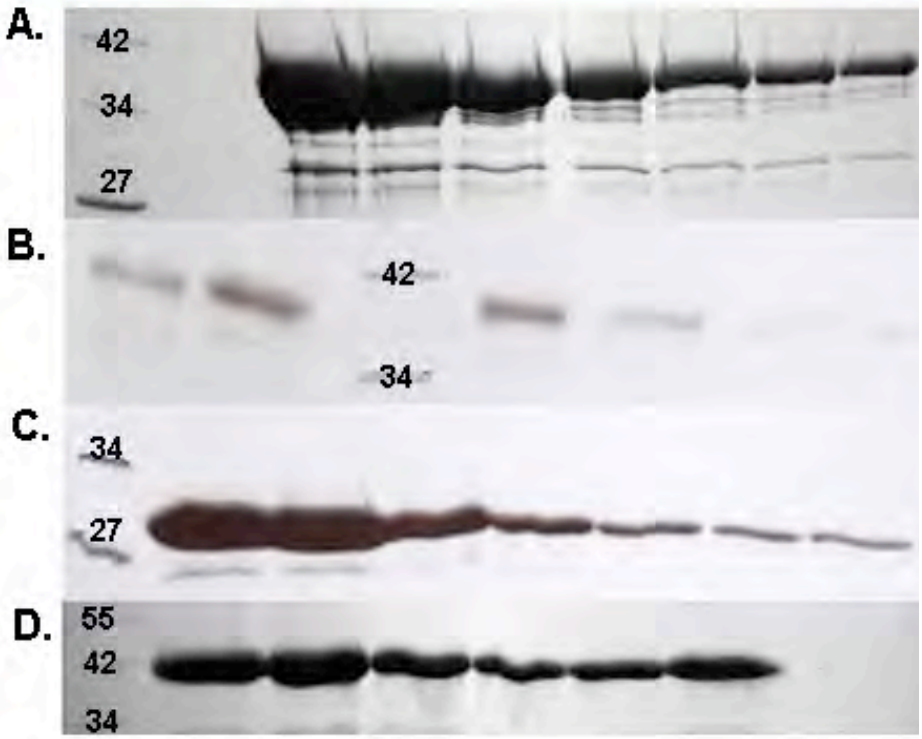


Figure 2 – SDS-PAGE gels of the purified soluble candidate proteins after purification via IMAC and FPLC. Each lane corresponds to a fraction collected during the elution stage of the purification process. 20μl of each fraction was loaded into the gels with 5x SDS buffer. The values displayed on the gel are molecular weights of the broad range protein marker (NEB) used in kilodaltons.

- **A.** Candidate TC59682, expected size 38.27
- **B.** Candidate TC55752, expected size 42.08 – A different extraction method as used to obtain this soluble protein. The size of the protein is slightly smaller than expected, possibilities for which is currently being investigated
- **C.** Candidate TC55097, expected size 26.89
- **D.** L-IdnDH, expected size 40.1

Characterisation

Spectrophotometric assays have been conducted based on the published bacterial methods (De Ley, 1966) monitoring the consumption of assigned cofactors, namely NADH and NADPH. Preliminary results have shown activity against the expected substrates however further kinetic analysis is required (See figure 3).

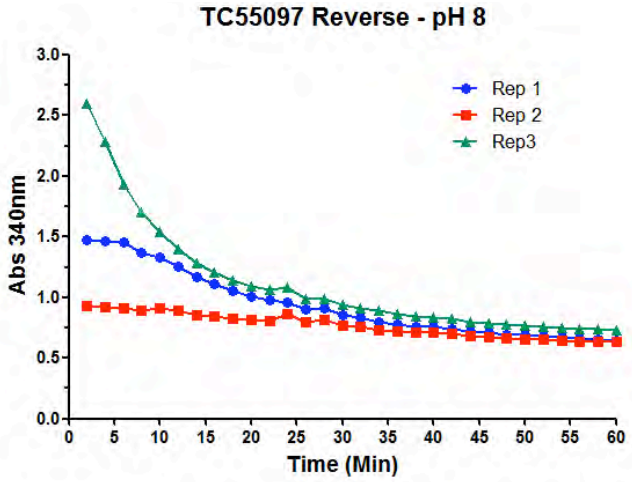


Figure 3 - Assay of candidate TC55097 for step 2 in the D-Glucose pathway D-Gluconate 5KGA. This assay shows analysis of the protein acting in the reverse direction, converting 5KGA to D-Gluconate, consuming NADPH. Absorbance at 340nm is shown on the Y axis with time in minutes on the X axis.

Figure 3 shows a preliminary assay in the reverse direction of that expected in the production of TA, indicating this is a reversible step in the D-Glucose pathway. The low reproducibility of the replicates is concerning and the assay parameters have been redesigned to address this. This includes shaking the mixture before addition of the substrate, covering the cofactor to limit UV exposure, higher concentration of cofactor to exclude limitation effects and higher volumes of assay ingredients to limit pipetting error. Results indicate these changes as having a positive effect on the reproducibility of the assays. The addition of the substrate after a pre-incubation period of the enzyme with the other assay ingredients indicates the enzyme activity is substrate based, with no activity prior to the addition of substrate. Characterisation including analysis of kinetic parameters including optimum pH, temperature, preferred substrate and cofactor, and the affinity of those enzymes for the substrate and cofactor is continuing for all candidates.

Cloning of two transketolase enzymes expressed during grape berry development

The alignment shown in Figure 4 depicts the amino acid sequence of the two closely-related, putative transketolase enzymes isolated from grape berries. These peptides are 72% identical and are homologous to sequences from a range of plant species including Arabidopsis (At2g45290, At3g60750), tobacco (ACF60500) and capsicum (CAA75777). The ORFs of these genes were amplified from young leaf and pre-veraison berry cDNA samples and the PCR products cloned into the bacterial expression plasmid pET14b.

Quantitative RT-PCR analysis was used to measure transcript levels of these genes during early grape berry development (Figure 4). In cDNA samples collected once per week from flowering (1) to veraison (7), VvTK2 transcript levels were approximately 10 fold higher than VvTK1 transcript levels. Furthermore, maximal transcript levels of VvTK2 coincided with the period of grape berry development when tartaric acid is synthesised. The

timing of VvTK2 gene expression suggests that this gene is a good candidate to be involved in tartaric acid synthesis.

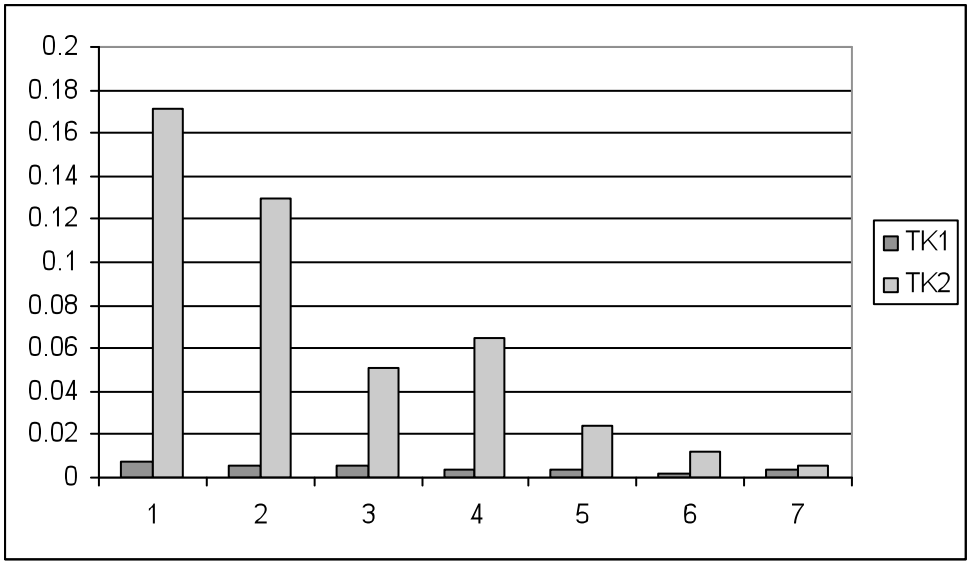


Figure 4. Transcript levels of transketolase 1 and 2 during grape berry development from anthesis to veraison. Results are means of three biological replicates of Shiraz berries harvested from the Coombe vineyard during the 2007-2008 growing season.

The recombinant proteins VvTK1 and VvTK2 were expressed and subsequently purified from the insoluble fraction of bacterial cells that expressed the pET14b plasmids (Figure 6). To date, these proteins have not shown activity against the TA synthesis pathway substrates, however, this analysis is ongoing. The assay developed to measure the activity of VvTK1 and VvTK2 is shown in Figure 7.

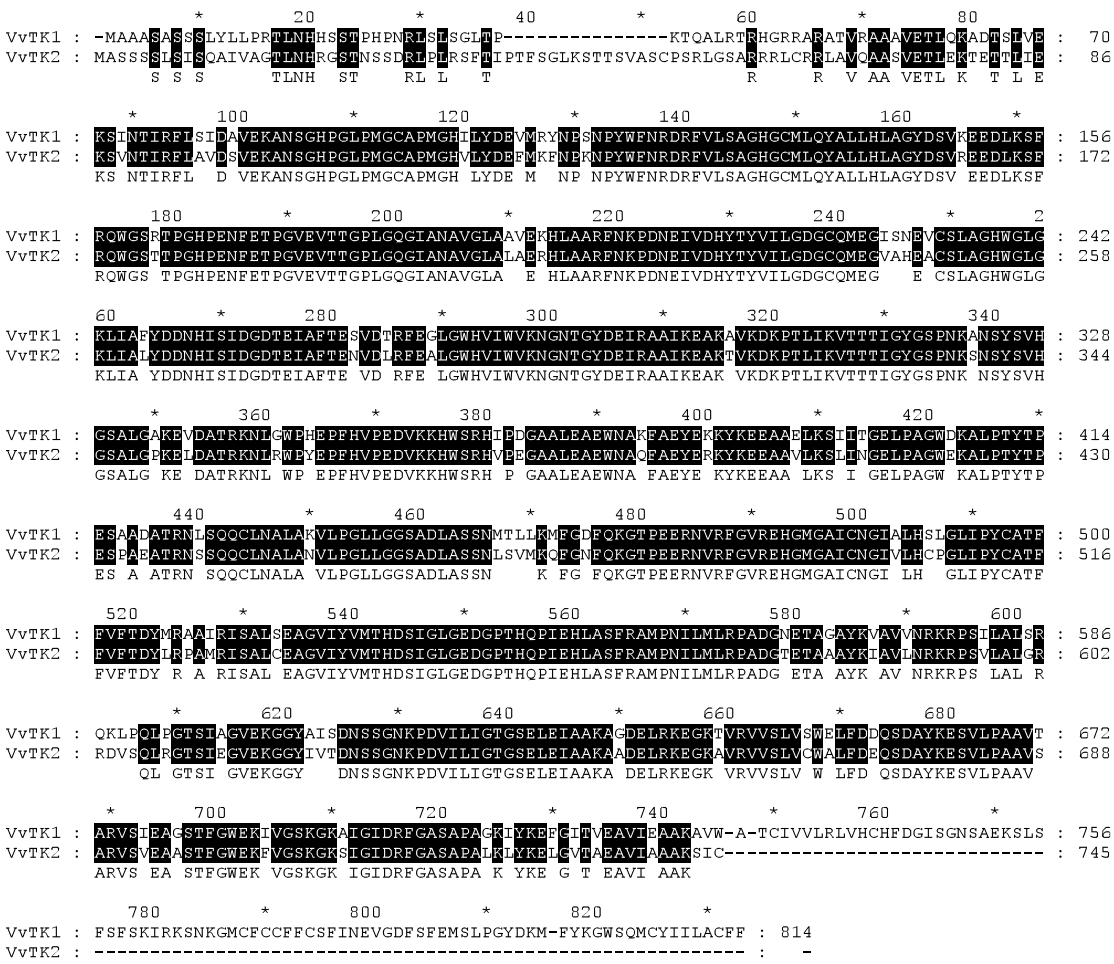


Figure 5. Peptide alignment of two transketolase-like sequences islolated from grapevine.

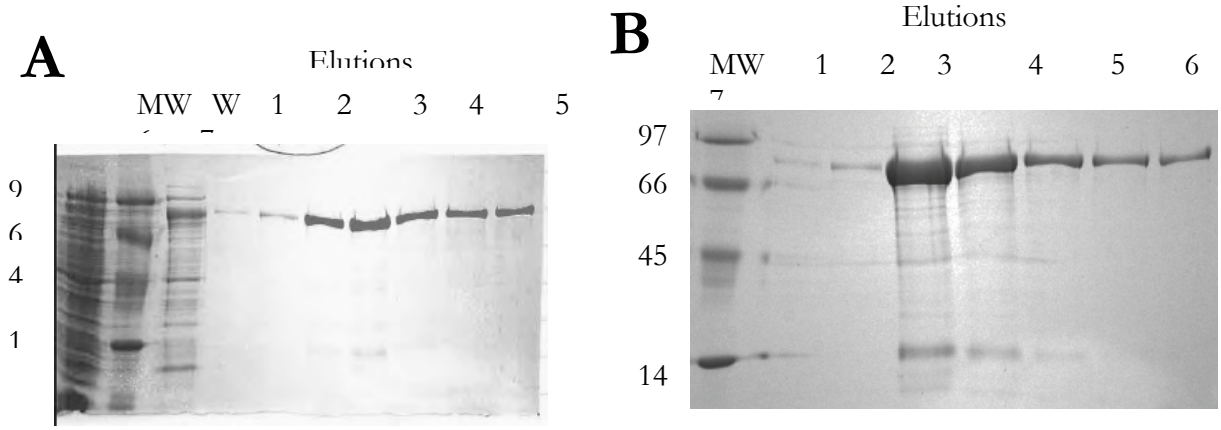


Figure 6. Purification of recombinant VvTK1 (panel A) and VvTK2 (panel B) from bacteria following chromatography.

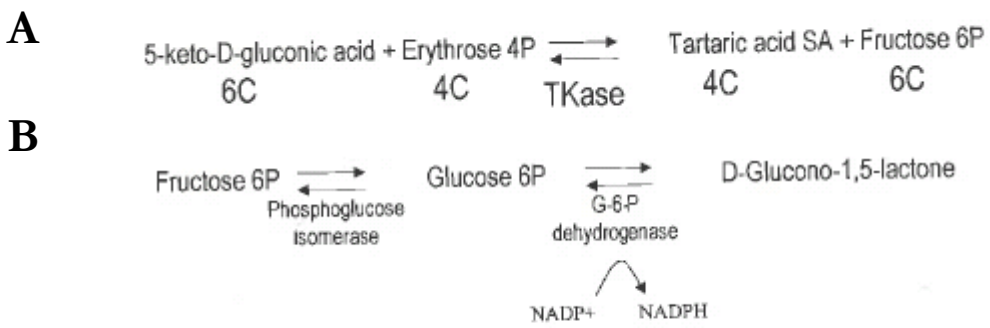


Figure 7. Proposed activity (A) of VvTK1 or VvTK2 on the tartaric acid substrate 5-keto-D-gluconic acid to form tartaric acid semi-aldehyde, and (B), the linked reporter reaction to measure formation of fructose 6-phosphate in A via spectrophotometric observation at 340 nm.

Project 2. RNAi-mediated silencing of L-IdnDH

Introduction

In order to develop a better *in planta* understanding of how the function of *L-IdnDH* contributes to tartaric acid biosynthesis, an RNAi silencing approach was devised. Three approximately 400 bp fragments of the *L-IdnDH* ORF (Fig. 1) were cloned into the plant transformation vector pHellsGate8. Under control of the 35S promoter, this vector expresses a RNA hairpin structure that mediates the efficient degradation of RNA molecules that share sequence homology with the cloned fragment.



Fig. 1 Three fragments of the *L-IdnDH* ORF were used to target RNAi knockdown of this gene.

Due to an existing collaboration with Professor Doug Cook of the University of California, Davis, we were able to contract the plant transformation expertise of the Plant Transformation Facility at Davis. Vines were transformed by an *Agrobacterium tumefaciens*-mediated method and selected for resistance to Kanamycin. The cultivar used for transformation at Davis was Thompson seedless, which is a particularly interesting host for this experiment as it was hypothesised that silencing of *L-IdnDH* may not only result in lower tartaric acid concentrations, but also higher concentration of the biosynthetic substrate vitamin C. Due to the well documented health benefits of Vitamin C, table grapes with high levels may be a valuable commercial cultivar.

Materials and Methods

Quantitative RT PCR analysis of transformants

RNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB)-based protocol (Iandolino et al 2007) and cDNA syntesised from 1 μg of RNA using an oligo DT primer and Omniscript reverse transcriptase (Qiagen). Probes from the Universal Probe

Library (Roche) were used with the Faststart Universal Probe Master mix (Roche) on an ABI7900HT real time PCR system (Applied Biosystems).

Western blot analysis

200mg of leaf tissue was ground in liquid nitrogen using a mortar and pestle, then transferred to a cooled 1.5ml eppendorfer tube. Gound tissue was extracted in 500μL of a buffer containing 200mM dithiothreitol (DTT), 4% sodium dodecyl sulphate (SDS), 16% glycerol, 120mM Tris-Cl pH 6.8 and bromophenol blue. Extracts were boiled for 3mins prior to loading onto a polyacrylamide gel for electrophresis. Transfer to nitrocellulose membrane was achieved using a Biorad Mini Trans-Blot^{R} Electrophoretic Transfer Cell, according to the manufacturers instructions. The L-IdnDH polyclonal antibody was diluted 1500 times in 3% BSA in 1 x TBST before use and this antibody was detected using the GE detection kit, as per the manufacturers instructions using the HRP-conjugated mouse-anti-rabbit secondary antibody contained in this kit. Chemilumenescence signal was detected using a Chemidoc XRS imaging system (BioRad).

HPLC analysis

Organic acids were extracted from 500mg of young healthy leaves, which was ground in liquid nitrogen and then weighed into a 10ml centrifuge tube. To extract organic acids, 4ml of 3% meta-phosphoric acid (MPA) with 1mM EDTA was added to each sample, vortexed and then filtered and transferred to HPLC vials for immediate separation.

High Performance Liquid Chromatography method

Buffer A: 100% Methanol

Buffer B: 20mM KH_2PO_4-0.1 mM EDTA, pH 2.5

The gradient of the buffers changes over time, starting at 100% B and changing to 95% B and 5% A over 9.5mins. The column used for HPLC is “synergi fusion 4μ”, dimensions 150mm x 4.6mm. The samples are analysed at wavelength 210nm which is the maximum absorbance for tartaric and oxalic acid. The samples are also measured at 245 nm which is the maximum absorbance for Ascorbic acid. 5ul of each sample was injected.

Results and discussion

Phenotype of RNAi *L-IdnDH* plants

When compared to control lines, RNAi *L-IdnDH* plants were stunted and leaves displayed irregular morphology in terms of shape, size and leaf blade patterning (images below). The lines isolated from this transformation displayed a phenotype which ranged in severity from severe to mild. When compared to control lines (Fig 2 A), RNAi *L-IdnDH* plants were stunted and leaves displayed irregular morphology in terms of shape, size and leaf blade patterning (Fig. 2 B and C). The leaves of severely affected lines seemed to have a low fresh weight compared to control leaves suggesting lower water content. Severely affected leaves often displayed a spider-like appearance.



Fig. 2 A. Thompson seedless wild type plant.



Fig. 2 B. RNAi *L-IdnDH*, severe phenotype



Fig. 2 C Moderate phenotype of *L-IdnDH* RNAi transgenic Thompson seedless lines.

Quantitative RT PCR analysis of RNAi *L-IdnDH* transformants

After the commencement of this project a transcript with approximately 70% nucleaic acid identity with *L-IdnDH* was identified in grapevine EST databases (Fig. 3 A). As RNAi-mediated gene silencing targets sequences with runs of 18 or more bases identical to the hairpin structure, it was conceivable that this second transcript in addition to *L-IdnDH* may be down regulated. So, quantitative RT-PCR was used to measure *L-IdnDH* transcript amounts, and amounts of this second transcript which we have informally called *L-IdnDH2*. However, the function of this enzyme has not been determined.

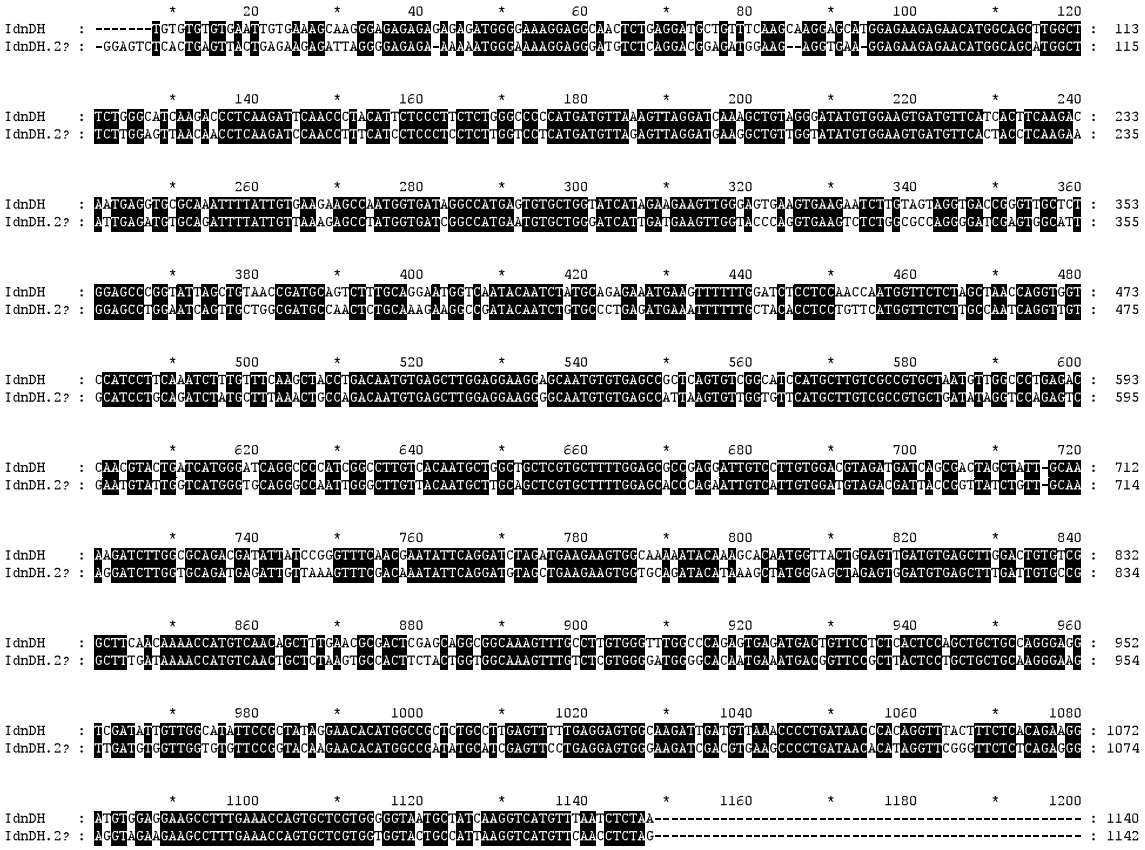


Fig. 3 Alignment of the *L-IdnDH* and *L-IdnDH2* ORF sequences

Four wild type lines (36, 37, 38, 39) and 33 transformant lines were tested by quantitative RT-PCR (Fig.3 B). Transformants had a range of *L-IdnDH* and *L-IdnDH2* expression from not detectable to similar to wild type. In the case of *L-IdnDH*, all lines tested showed at least 50% or less transcript amounts compared to wildtype lines. Overall, the RNAi constructs were more effective at reducing transcript levels of *L-IdnDH* than of *L-IdnDH2*, and in some lines where *L-IdnDH* knockdown was achieved, *L-IdnDH2* expression was upregulated compared to controls, suggesting some compensation via additional *L-IdnDH2* expression may have occurred. Although both *L-IdnDH*

and *L-IdnDH2* transcripts were knocked by our constructs, there was greater effect overall on *L-IdnDH* transcript.

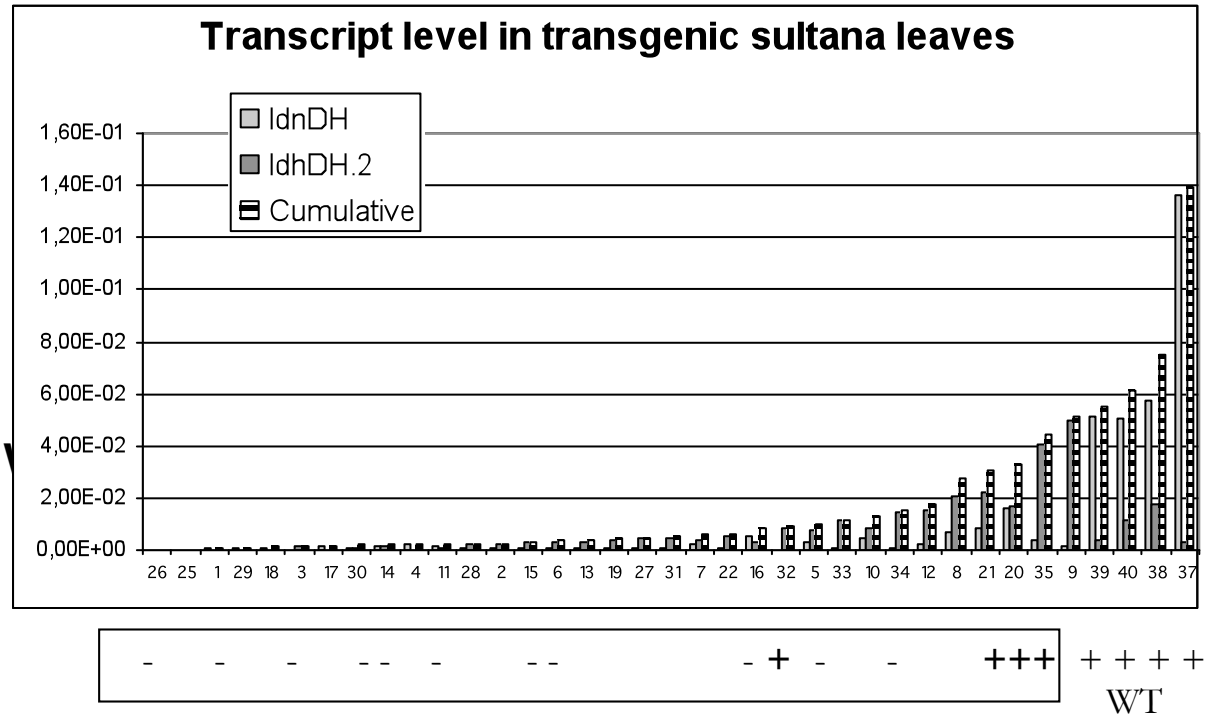
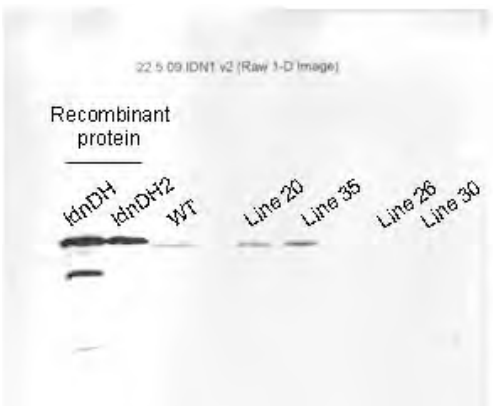


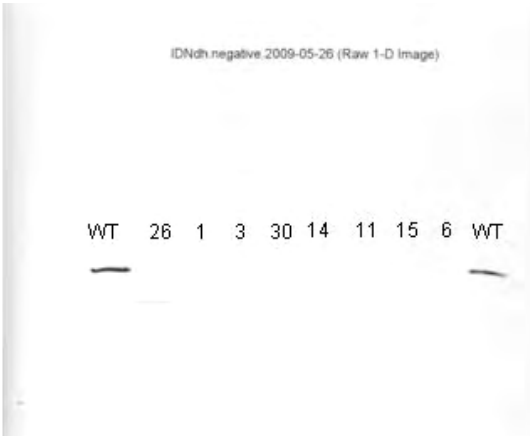
Fig. 3 B Quantitative RT-PCR of RNAi *L-IdnDH* transgenic vines.

Western blot analysis

We conducted western analysis on 15 transgenic lines to determine if the RNAi-mediated knockdown of *L-IdnDH* and *L-IdnDH2* transcript resulted in reduced L-IdnDH and L-IdnDH2 protein. After expressing these proteins in bacteria and purifying these recombinant protein, western analysis revealed that the antibody raised against L-IdnDH cross-reacts with L-IdnDH2. This is not surprising as at the amino acid level, these proteins are 83% identical. As these proteins are also of the same size and cannot be separated by 1-dimensional electrophoresis, the signal derived from the use of this antibody represents the cumulative amount of L-IdnDH and L-IdnDH2 protein in these plants. In the future we have planned to use 2 dimensional electrophoresis that will also separate these proteins using isoelectric point focusing as their predicted isoelectric points are significantly different.

Western analysis confirmed the absence of L-IdnDH and L-IdnDH2 in 11 of the 15 individuals analysed while a strong signal was observed in wild type samples. Of those tested, 3 lines (21, 20, 35) that contained 50% or more of *L-IdnDH* and *L-IdnDH2* transcript levels observed in wild type plants also contained protein. With the exception of line 32 which gave a very weak signal, all other lines tested were negative indicating the absence of these proteins and indicating success in knock out of *L-IdnDH*. However, as our antibody cross reacts with L-IdnDH2, it is clear our constructs also miss-targeted this gene.







HPLC analysis

HPLC analysis was used to measure tartaric acid concentrations in leaves sampled from the transgenic lines. Control lines contained 15.25-17.5 mg of tartaric acid per gram fresh weight, while the concentration of tartaric acid was reduced as much as 50% in *L-IdnDH* RNAi lines (Fig. 5 A). Overall, there was no clear correlation between transcript level and tartaric acid concentration (Fig. 5 B), however, tartaric acid levels in samples that produced positive signals by western blot analysis (lines 32, 21, 20 and 35) contained 75% or more of the tartaric acid in wild type lines, while lines with low *L-IdnDH* transcript levels and negative by western analysis (lines 26, 1 and 3) contained approximately 60% of the tartaric acid of wild type lines. (Fig. 5 C).

A decrease in tartaric concentration was apparent in many of the RNAi lines in which neither L-IdnDH *nor* L-IdnDH2 protein was detected, supporting the idea that *L-IdnDH* contributes to the synthesis of tartaric acid in grapevines, however, as considerable amounts of this metabolite were still detected, alternative pathway(s) to tartic acid production that do not involve L-IdnDH must be active in grapevines. A biosynthetic pathway from glucose to

tartaric acid has long been supported by chemical analysis, however, it was considered to be only a minor pathway and contribute only 5% of the total tartaric acid pool (Saito and Loewus, 1989b). It is possible that this pathway became more active in RNAi *L-IdnDH* lines to compensate for reduced production via the L-IdnDH pathway, however, it is also conceivable that alternative pathways may be more active than previously proposed. As enzymes for alternative pathways have not yet been isolated, this current work suggests that identification of these alternative pathway enzymes is an important area of research.

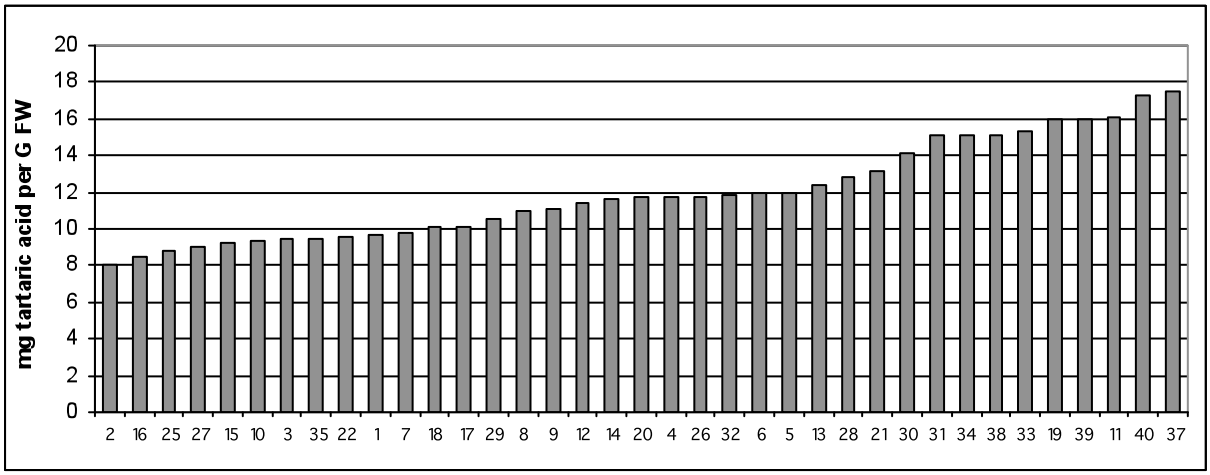


Fig. 5 A HPLC determination of tartaric acid concentrations in leaves from transgenic L-IdnDH RNAi grapevines.

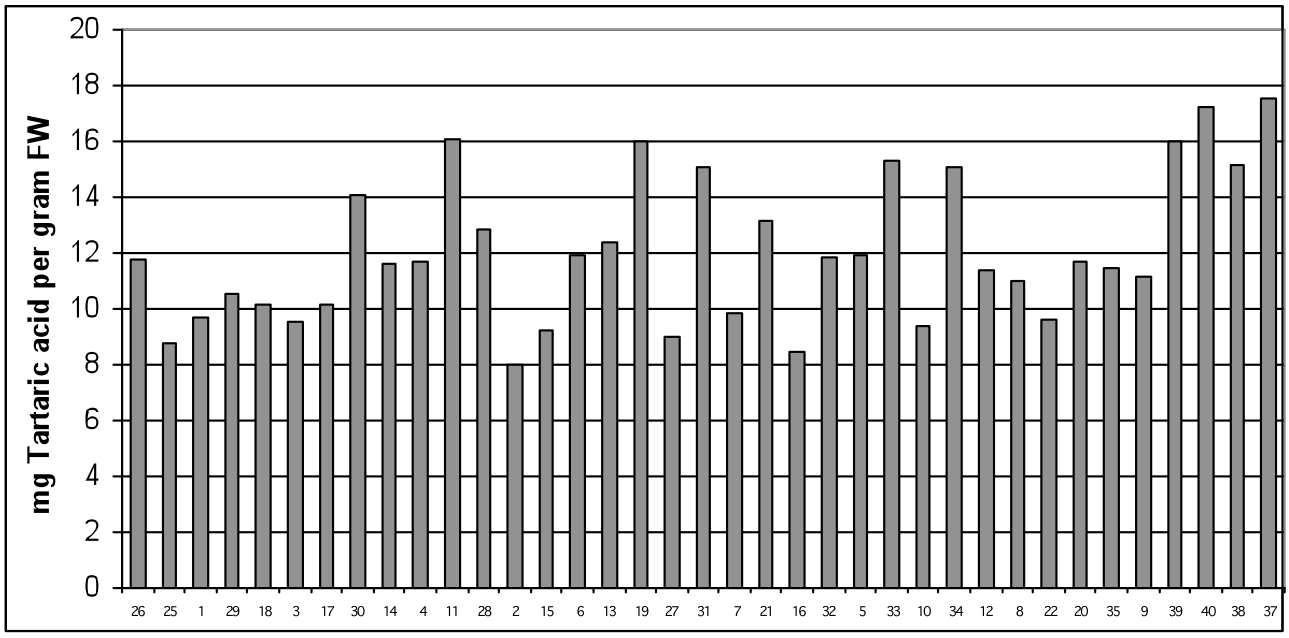


Fig. 5 B Data from 5 A ranked according to transcript level as in Fig. 3 A

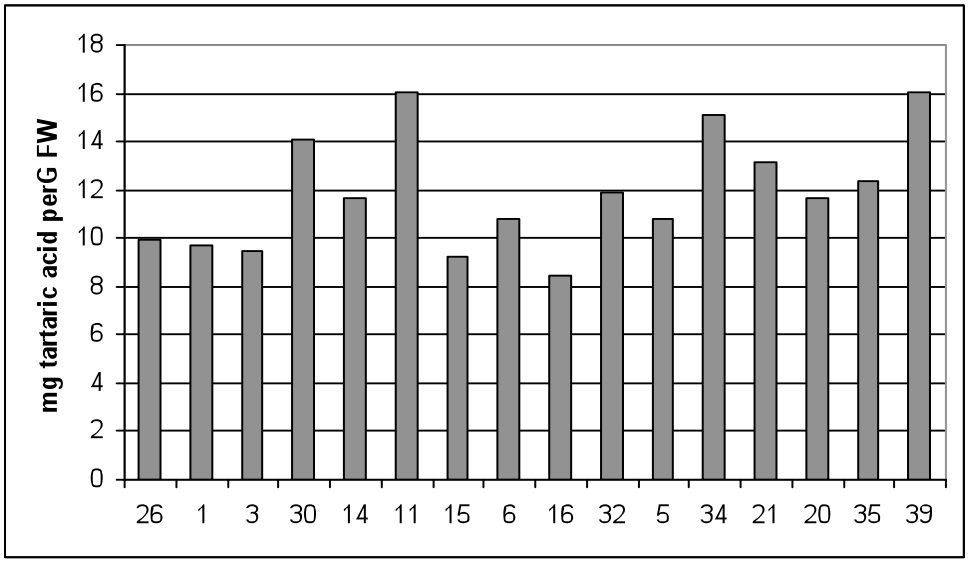
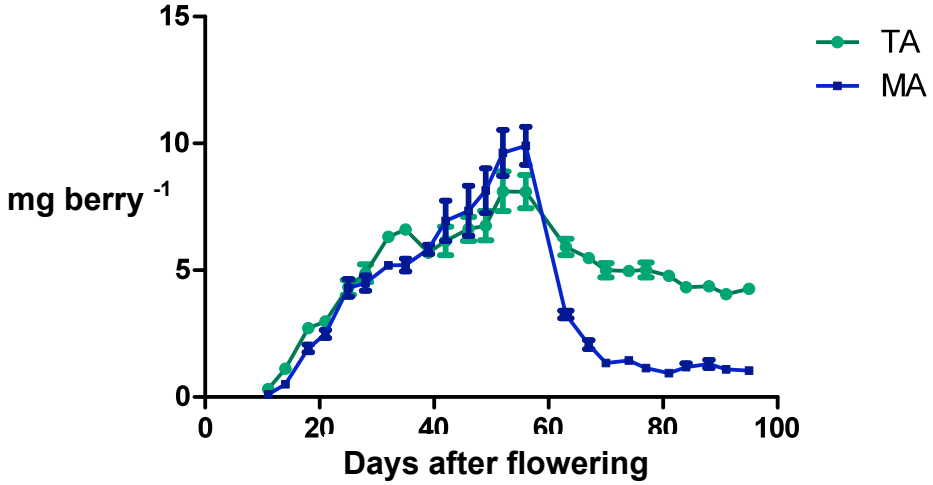


Fig. 5 C HPLC measurements on lines analysed by western blot.

Project 3. Identification of grapevine vacuolar organic acid transporters.

Introduction

The large central vacuole occupies 99% of the intracellular volume of grape berry pulp cells (Storey 1987) and is where acids and other metabolites responsible for taste and flavour are stored. Although TA is relatively uncommon in the Plant Kingdom, many fruits accumulate organic acids such as citric acid (citrus) and malic acid (apple, pear) to high levels, at a pH in the 2.5-2.8 range.



In Fig. 1, a sharp decline in berry MA and a lesser decline in TA amounts is apparent during the second half of grape berry development. This decline may be due to transport or leakage of MA from the vacuole back into the cytosol and mitochondria where respiration of MA is known to occur. However, the loss of TA is not well documented and its fate is unknown.

TA is a dicarboxylic acid with similar structure to MA (Fig. 2), and two vacuolar MA carriers identified in Arabidopsis have provided the starting point for studies on TA compartmentation in grapevine. Analysis of knock out mutants of *AttDT* and *AtALMT9* suggest that the activity of these two carriers mediate MA movement across the tonoplast in this plant (Emmerlich et al. 2003; Hurth et al. 2005; Kovermann et al. 2007). The function of putative grapevine orthologues of these genes is being determined by expression in Arabidopsis knockout lines and *Xenopus laevis* oocytes. We hypothesise that TA may be an additional substrate for these carriers, an idea supported by studies on AttDT (Emmerlich et al. 2003) and its citrus orthologue CsCit1 (Shimada et al. 2006), as well as patch clamp studies on vacuoles isolated

from leaf mesophyll cells (Hafke et al. 2003). These studies report that a range of dicarboxylates may be substrates of these carriers.

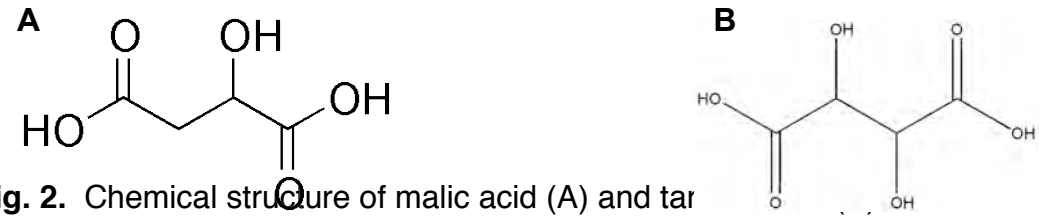


Fig. 2. Chemical structure of malic acid (A) and tar

Materials and Methods

Quantitative Real-time PCR (QRT-PCR)

The standard mixture of QRT PCR contained: 10µl iQ™ SYBR Green Supermix (BIORAD), 3µl Shiraz berry cDNA sample, 0.5 µl Forward Primer and 0.5- µl Reverse Primer. It was carried out on the iCycler iQ™ Real-Time Detection system (BIORAD). Gene transcript levels were normalised against β-actin using Q-gene software and methods (Muller *et al.*, 2002).

Full length PCR amplification of VvALMT9-like transcripts

Full length ORFs were amplified from grapevine leaf and berry cDNA using 1 unit of Platinum Taq HiFI (Invitrogen) according to the manufacturers instructions. PCR products were cloned into the pDrive PCR cloning vector and subcloned into the *Xba*I and *Xho*I of pN`GFP which contains the GFP ORF.

4.2.3. Biolistic transformation of GFP fusion proteins into onion epidermal cells

Biolistic transformation was conducted according to Hayes *et al.* 2007 without modification. It consisted of 60 mg of gold particle (1 micron, Biorad), 3 µl (1µg/µl) of DNA, 50 µl of $CaCl_2$, 20 µl of 0.1 M spermidine.

Results and Discussion

Identification of sequences

Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997) analysis identified

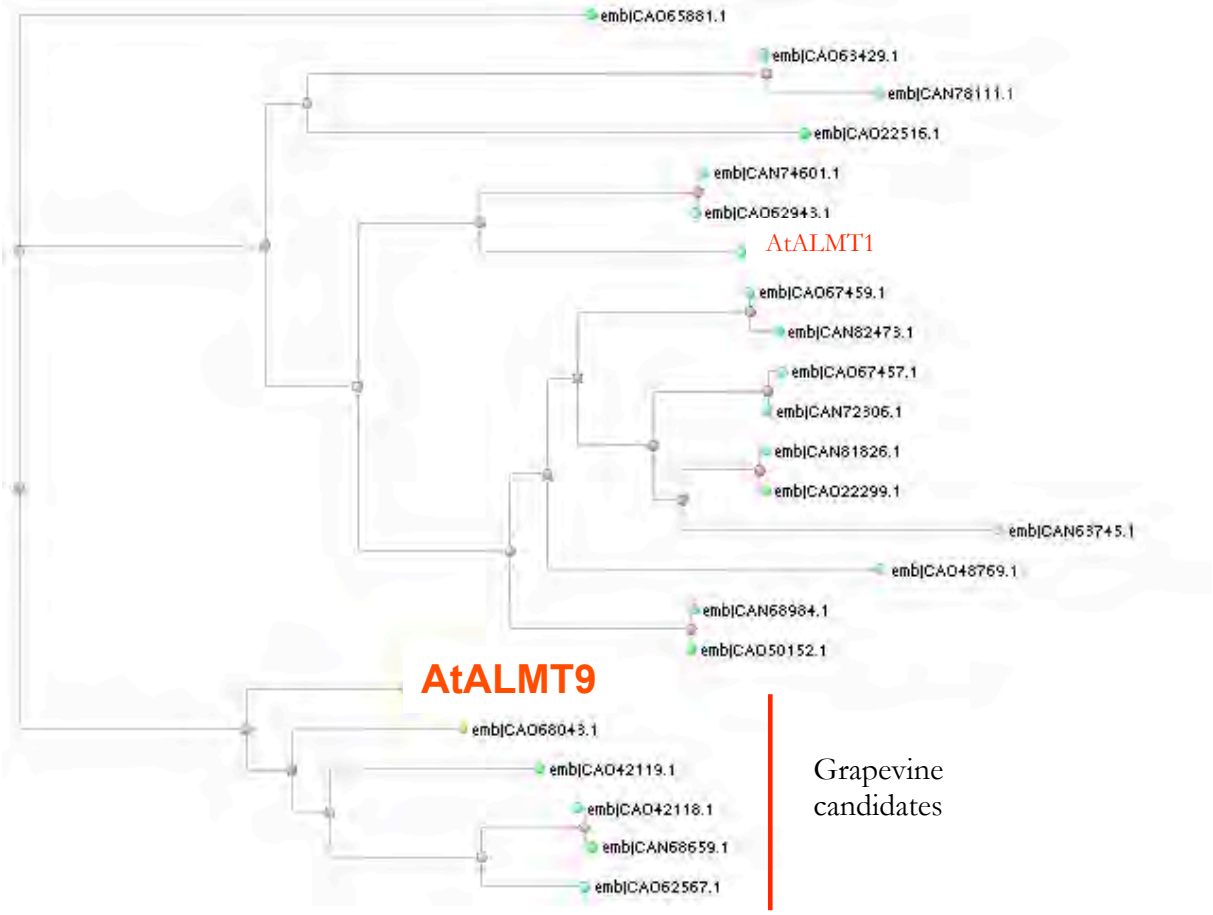
five sequences homologous to the vacuolar organic acid channel AtALMT9 in the grapevine genome (Table 1, Fig 3 and Fig 4). Among the five, CAO42118 and CAN68659 are 99.5% identical in their DNA sequences (data not shown). So, CAN68659 along with the three other candidates have been studied

further. The identified sequences were approximately 70% identical to AtALMT9 at the amino acid level. Consistent with a vacuolar-localisation, phylogenetic analysis (Fig. 3) showed that these genes grouped with *AtALMT9* and not *AtALMT1*. *AtALMT1* is an aluminium-activated malate channel that is localized to the plasma membrane (Hoekenaga *et al*. 2006) whereas the *AtALMT9* is localised to the tonoplast (Kovermann *et al*. 2007).

Table 1: The grapevine AtALMT9 homologues.

Gene No.	Accession no. gene (*NCBI)	Accession no. protein (NCBI)	Sequence similarity with AtALMT9	E Value
VvALMT9:1	CU459233	CAO68043	730	0.0
VvALMT9:2	CU459349	CAO65881	343	2e-92
VvALMT9:3	AM482812	CAN68659	598	4e-169
VvALMT9:4	CU459222	CAO42119	610	1e-172

(* NCBI – National Center for Biotechnology Information and VvALMT – *Vitis vinifera* Aluminum activated malate transporter)



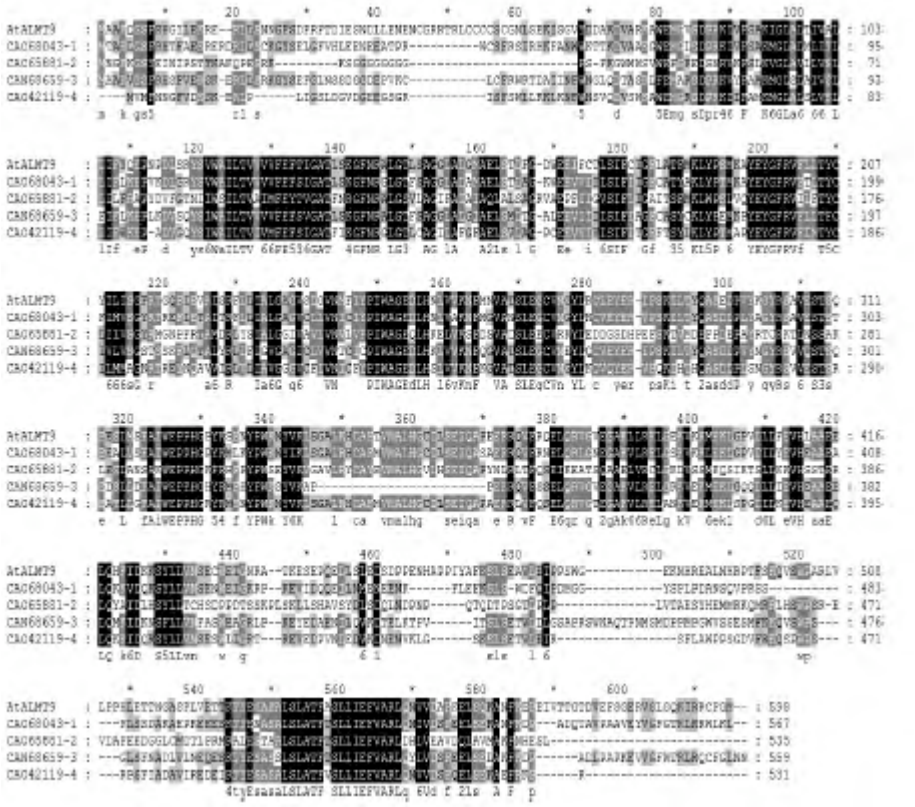


Fig. 4. Amino acid alignment of grapevine AtALMT9 homologues.

Expression of grapevine AtALMT9-like genes during berry development

QRT-PCR was carried out to establish whether the ALMT9-like genes were expressed during berry development. cDNA was synthesised from total RNA extracted from Shiraz berries collected from the Coombe vineyard, University of Adelaide, Waite Campus during the 2006-2007 growing season. QRT-PCR was performed in triplicate using cDNA samples from fruit collected at one week intervals from one week after flowering to 12 weeks of berry development.

Pre-veraison, *VvALMT9.3* (Fig 5 D) was the most highly expressed of these genes while its expression was relatively stable and low post-veraison. Interestingly, VvALMT9.1 (Fig. 5 B), VvALMT9.3 and VvALMT9.4 (Fig. 5 E) transcript levels all increased 14 days after flowering (DAF) before expression of all genes declined to low levels before veraison. During this preveraison stage, organic acids are synthesised and stored in the vacuoles of berry pulp cells, therefore, transporters expressed at this time may play a role in acid storage. Post-veraison, VvALMT9.1 was the most highly expressed of these

genes, however, transcript levels of all genes, with the exception of VvALMT9.3, increased significantly 63 DAF. This spike in expression coincides with the maximal concentrations of MA during berry development (Fig. 5 A) and just prior to the commencement of MA breakdown that is typical during grape berry ripening. As VvALMT9.2 (Fig 5 B) expression was highest post veraison, this particular homologue may play a significant role in decompartmentation of organic acids that enables degradation via cytoplasmic enzymes such as malic enzyme and uptake into mitochondria where MA is used for respiration.

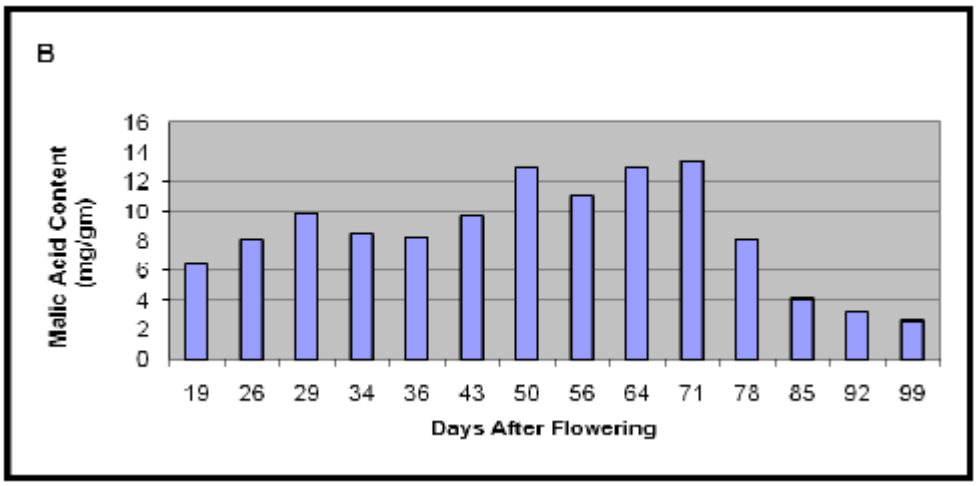


Fig. 5 A. Malic acid concentration during berry development.

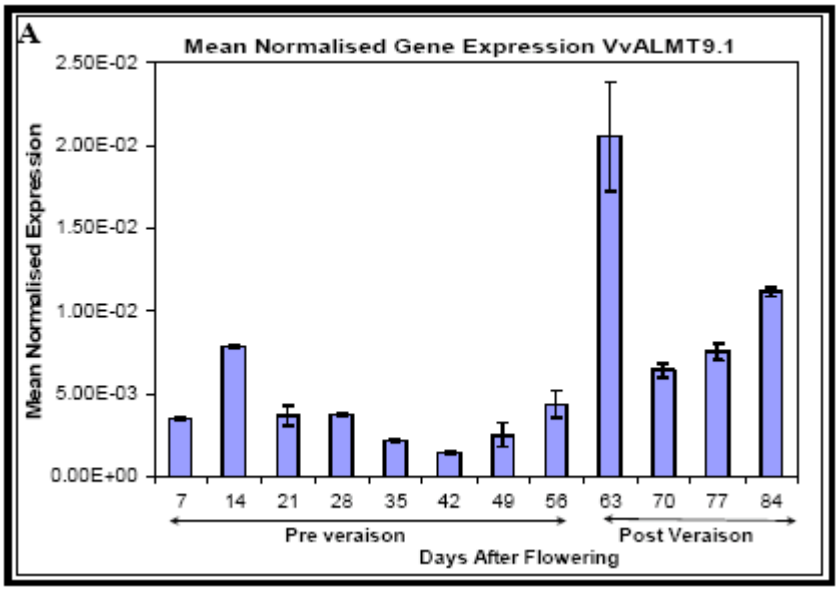


Fig. 5B. Expression of *VvALMT9.1* during Shiraz berry development.

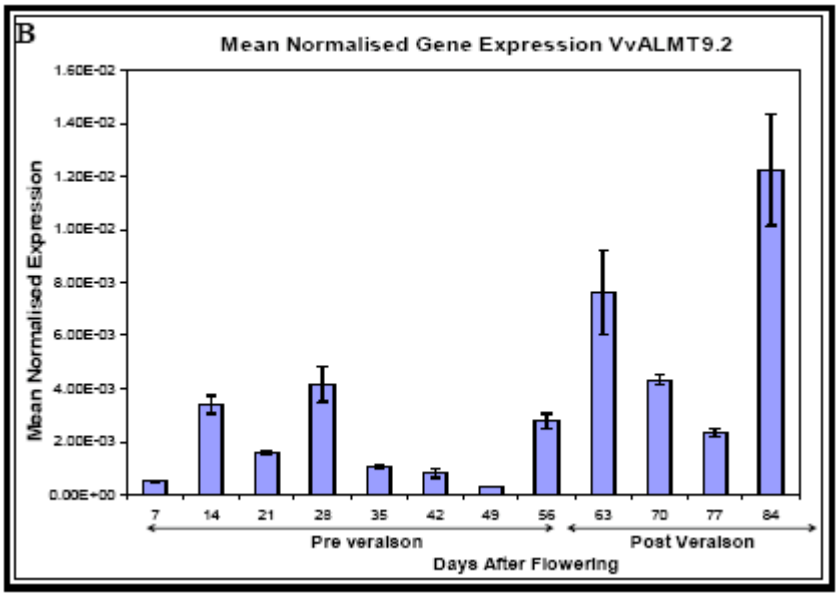


Fig. 5C. Expression of *VvALMT9.2* during Shiraz berry development.

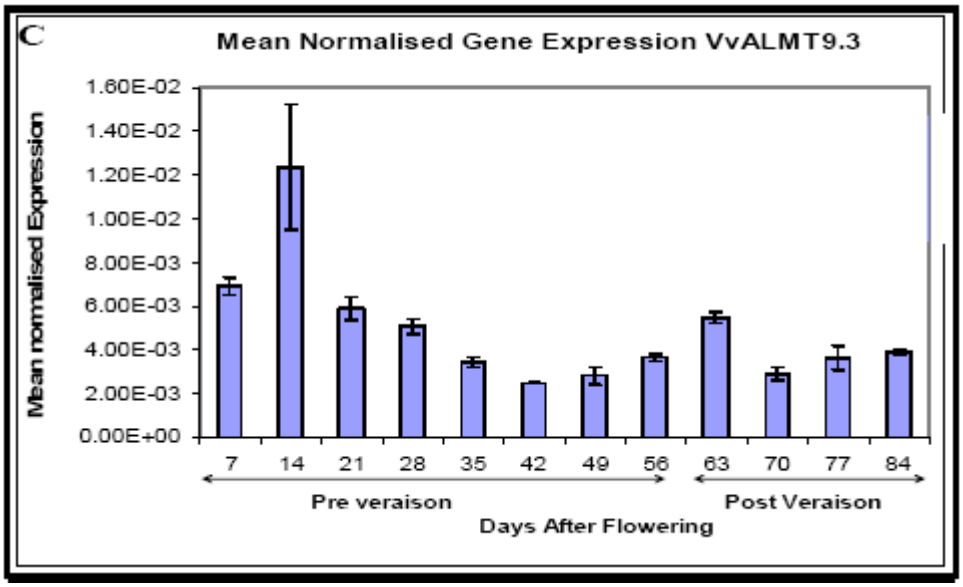


Fig. 5D. Expression of *VvALMT9.3* during Shiraz berry development.

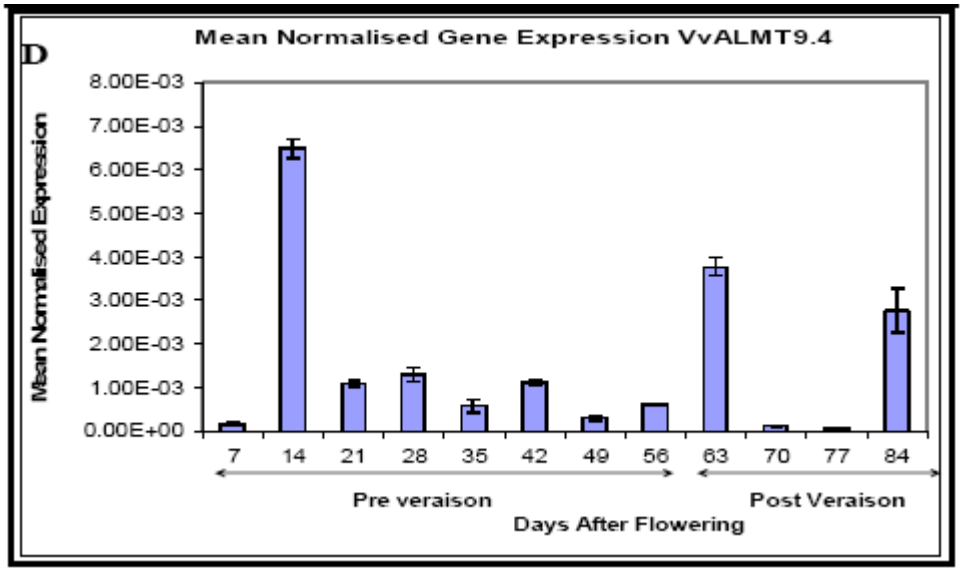


Fig. 5E. Expression of *VvALMT9.4* during Shiraz berry development.

Subcellular localisation of grapevine AtALMT9-like proteins

To verify the hypothesis that VvALMT9s (*Vitis vinifera* Aluminum-activated
Malate transporters) are targeted to the vacuolar membrane, candidate genes were cloned in frame and up-stream of Green Fluorescent Protein. Stop codons were deleted from VvALMT9s to produce translational fusion proteins under control of the cauliflower mosaic virus 35s promoter. The localisation of GFP was visualised using confocal laser scanning microscope after biolistic transformation of onion epidermal cells.

Non-targeted GFP (Fig 6A) was found to be distributed throughout the cell including vacuoles, nuclei and plasma membrane. VvHT4:GFP (Fig 6B), is already known to localised on the plasma membrane (Hayes *et al*., 2007), and .so we used it as plasma membrane control for study. VvHT4:GFP was localized to the periphery of the cell consistent with its previously described plasma membrane localisation (Fig. 6 B). The GFP fusions to VvALMT9:1, VvALMT9:2 and VvALMT9:4 (Fig.7 A, B&C, respectively) clearly showed localisation to the cell periphery and around the inner edge of the nucleus, consistent with tonoplast localisations as reported by other researchers (Kovermann *et al* 2008; Emmerlich *et al.* 2003). Unfortunately, no GFP signal could be detected in onion cells transformed with VvALMT.3 GFP fusions. Reasons for this failure are unknown however the construct was resequenced

(Fig 4.9) and found to be correct and as expected with an open reading frame extending from the start codon of VvALMT9.3 through into the GFP ORF.

Functional activity of VvALMT9s needs to be tested by expression in a heterologous system such as *Xenopus laevis* oocytes or in tobacco or Arabidopsis protoplasts. These tests would enable the identification of which acids are transported by VvALMT9s and the directionality of transport. This information is of great significance for the final concentration of organic acids in grape berries as unstable compartmentation of organic acids undermines the biosynthetic efforts exerted during the green phase of berry development. Why MA is lost preferentially to TA, or other solutes stored in the vacuole, is an intriguing question that may be due to ripening expression of a particular member of the VvALMT9 family of proteins that we have identified, that has a substrate range limited to MA.

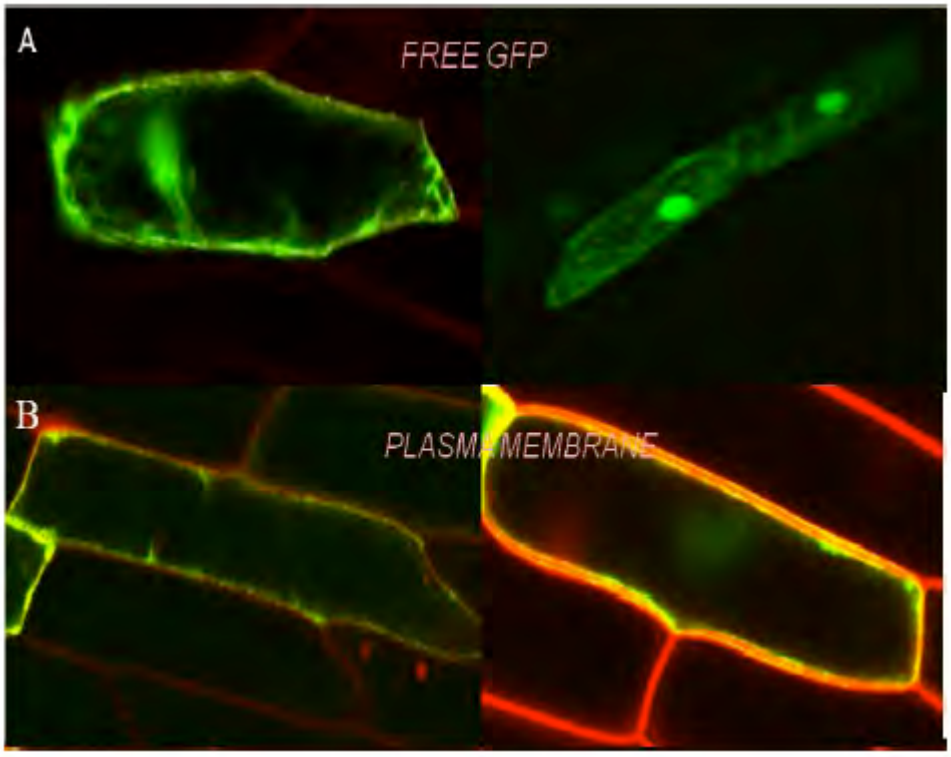


Fig. 6 Analysis of the cellular targeting of (A) GFP and (B) VvHT4: GFP, a plasma membrane-localised protein, in onion epidermal cell.

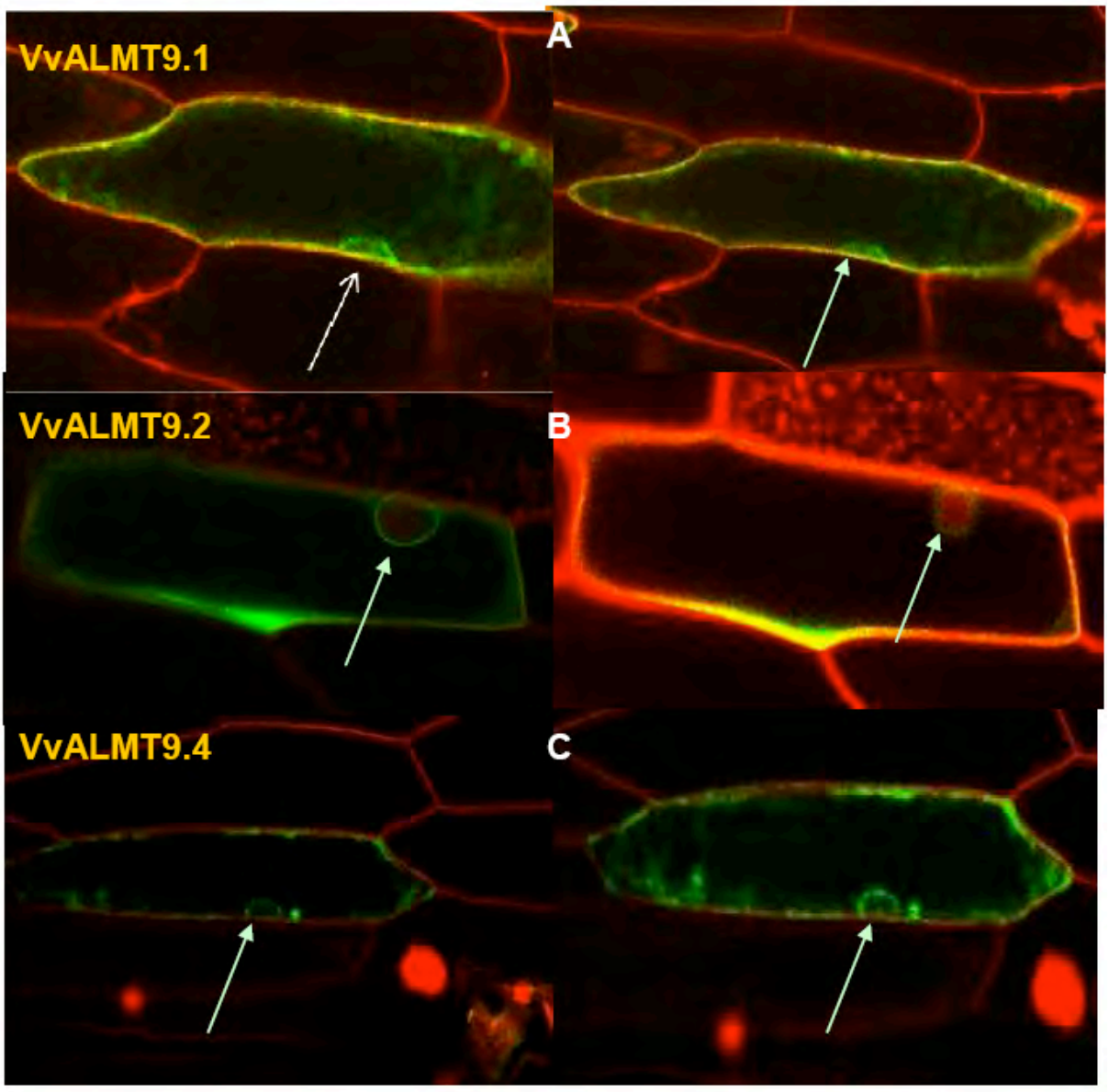


Fig 7. Sub cellular localisation of VvALMT9s by transient expression of VvALMT9s-GFP fusion proteins.

Project 4. Ectopic expression of L-IdnDH in Arabidopsis thaliana

Introduction

Our previous analysis of L-IdnDH function has focused on in vitro enzyme activity assays. In this section we present attempts to study the function of L-IdnDH in planta. To achieve this goal, the open reading frame of L-IdnDH was expressed in *Arabidopsis thaliana* under control of 3 different promoters.

Materials and Methods

DNA constructs for transformation of *A. thaliana* with L-IdnDH

Constructs were created using pCAMBIA1301 for Agrobacterium-mediated transformation using the floral dip method (Clough and Bent 1998) and selection on 15 μg/mL Hygromycin B using the rapid method of Harrison *et at* (2006). Four different constructs with different promoter and gene combinations were used to transform *A. thaliana.* The first two constructs (see figure 1 and 2) both contained the 35s promoter, driving either GUS (empty vector control) or L-IdnDH. As 35S is a very strong non-cell -type specific promoter, we made two other constructs, one with an *A. thaliana* native promoter and another with the L-IdnDH promoter o drive expression of the L-IdnDH ORF. The first was from an *A.thaliana* vacuolar invertase gene (accession Y11559), which should drive expression of L-IdnDH in young growing tissues such as leaves or flowers. Young growing tissues such as young leaves and berries is where TA is found in grapevines, (Hale 1965), thus is was hypothesised that *A.thaliana* may produce TA in the same areas. The putative promoter region found upstream of L-IdnDH in the grapevine genome was also used to drive L-IdnDH expression. It was not known whether this promoter would function in another plant, therefore this experiment would useful functional analysis of the activity of this promoter in a heterologous expression system.

Quantitative RT-PCR analysis of transformed *A. thaliana* plants

RT-PCR was performed to check the level of L-IdnDH expression in transgenic *A. thaliana* plants. RNA was extracted from 100mg *A. thaliana* leaf tissue using *Trizol* reagent. cDNA was synthesised from 1μg of RNA using a BioRad iscript select kit, used as per manufacturers instructions. A Biorad Sybr Green solution was used with the following DNA primers to measure L–IdnDH and Actin 8 transcript levels. Actin 8 was the reference gene to which L-IdnDH was compared.

PRIMER	L-IdnDH	Actin 8
FORWARD	AAGTTTGCCTTGTGGGTTTG	TGATGTTGATATCAGGAAGG
REVERSE	AAGGATGTGGAGGAAGCCTT	CGACCTTAATCTTCATGCTG

Western blotting of transformed *A. thaliana* plants

100mg of leaf tissue was ground in liquid nitrogen using a mortar and pestle, then transferred to a cooled 1.5ml eppendorfer tube. Gound tissue was extracted in 500μL of a buffer containing 200mM dithiothreitol (DTT), 4% sodium dodecyl sulphate (SDS), 16% glycerol, 120mM Tris-Cl pH 6.8 and bromophenol blue. Extracts were boiled for 3mins prior to loading onto a polyacrylamide gel for electrophresis. Transfer to nitrocellulose membrane was achieved using a Biorad Mini Trans-Blot^{R} Electrophoretic Transfer Cell, according to the manufacturers instructions. The L-IdnDH polyclonal antibody was diluted 1500 times in 3% BSA before use and this antibody was detected using the GE detection kit, as per the manufacturers instructions using the mouse-anti-rabbit secondary antibody contained in this kit.

Analysis of organic acids in transgenic *A. thaliana* plants by HPLC

Samples from transformants were collected at six leaf stage. 100mg of leaf tissue was ground in liquid nitrogen and then weighed into a 1.5ml microcentrifuge tube. To extract organic acids, 1ml of 3% Meta-phosphoric acid (MPA) with 1mM EDTA was added to each 100mg sample, vortexed and then filtered and stored at -20°C before HPLC analysis.

High Performance Liquid Chromatography method

Buffer A: 100% Methanol

Buffer B: 20mM KH_2PO_4-0.1 mM EDTA, pH 2.5

The gradient of the buffers changes over time, starting at 100% B and changing to 95% B and 5% A over 9.5mins. The column used for HPLC is "synergi fusion 4μ", dimensions 150mm x 4.6mm. The samples are analysed at wavelength 210nm which is the maximum absorbance for tartaric and oxalic acid. The samples are also measured at 245 nm which is the maximum absorbance for Ascorbic acid. 5ul of each sample was injected

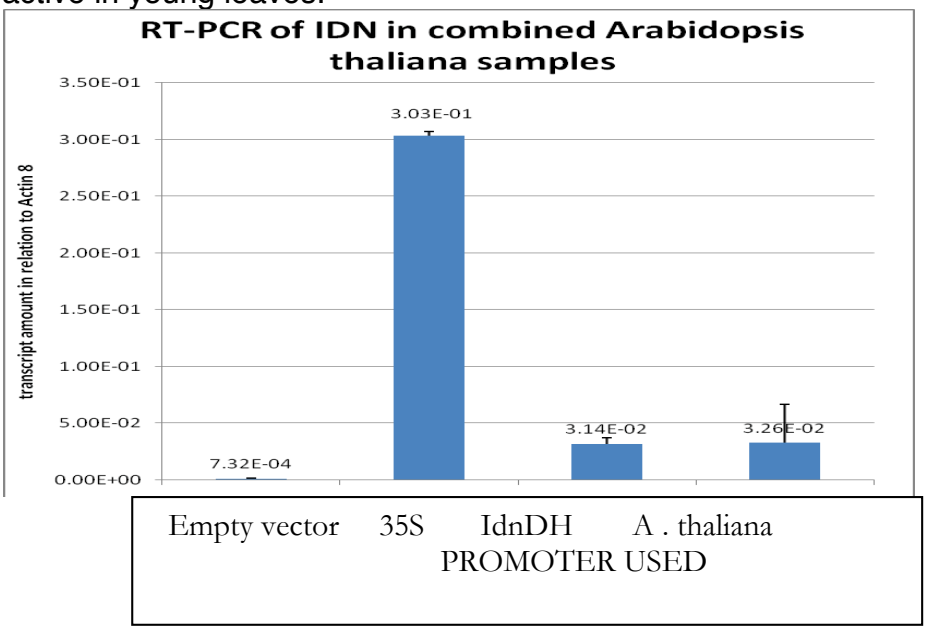
Results and discussion

Relative transcript level of *L-IdnDH* in transgenic *A. thaliana*

The table below reports the number of transgenic lines obtained for each DNA construct. Similar numbers of transformants were obtained for each construct.

ID	L-IdnDH ORF	Promoter	No. of Plants
Control	NO	35s	7
35s	YES	35s	9
L-IdnDH	YES	Native L-IdnDH	9
A. thaliana	YES	Native A. thaliana	10

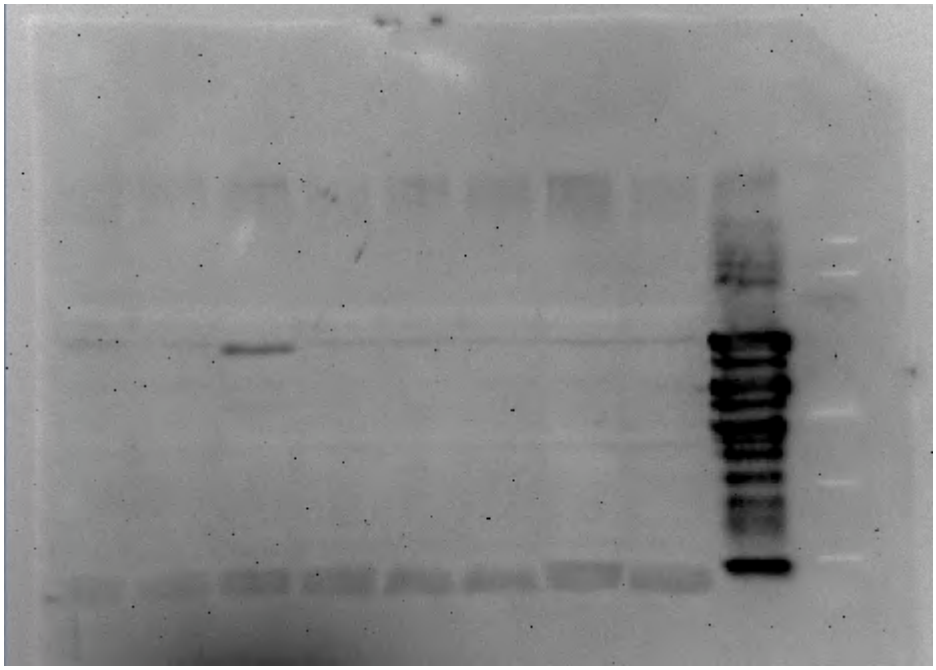
Quantitative RT-PCR allowed us to measure the activity of the putative L-IdnDH promoter in *A. thaliana* leaves. The expression level of *L-IdnDH* driven by its native promoter in *A. thaliana* was approximately 10-fold less than the level measured in 35S *L-IdnDH* lines (see below). This was found to be similar to the level of expression driven by the native *A. thaliana* promoter used in this experiment and suggest that the promoter of *L-IdnDH* is highly active in young leaves.



Western Blot analysis

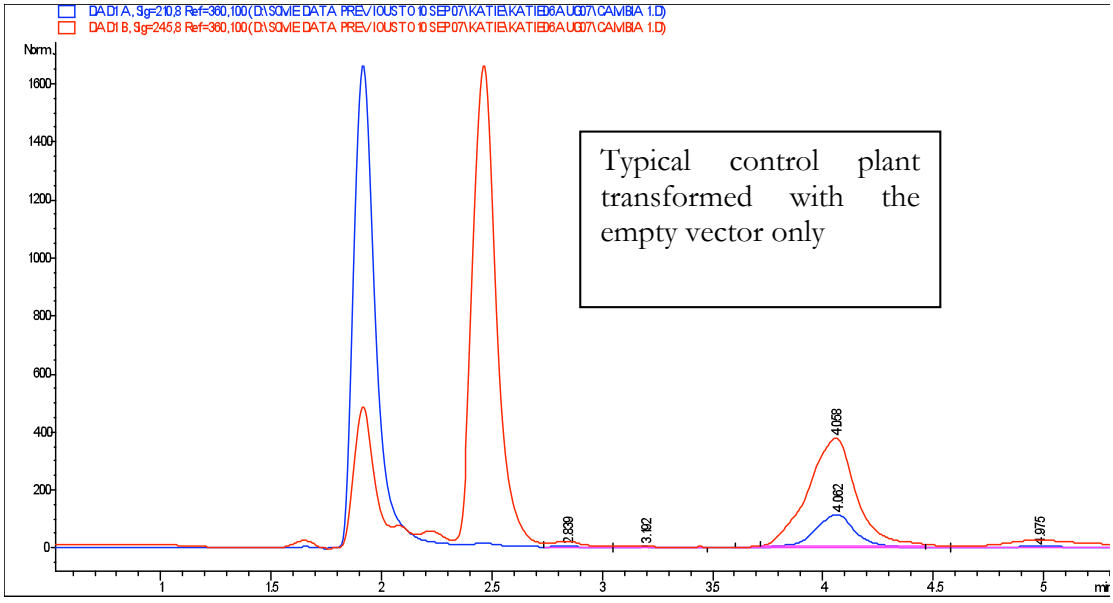
Although quantitative RT-PCR analysis indicates significant transcript levels of *L-IdnDH* in lines transformed with the *L-IdnDH* native and *A. thaliana* native promoters, we were unable to detect L-IdnDH protein in any of the lines

derived from these constructs with western blotting. We were able to detect protein in lines from the 35S *L-IdnDH* construct (see below), suggesting that very high levels of transcript are required to allow translation of this transcript in *A. thaliana*.

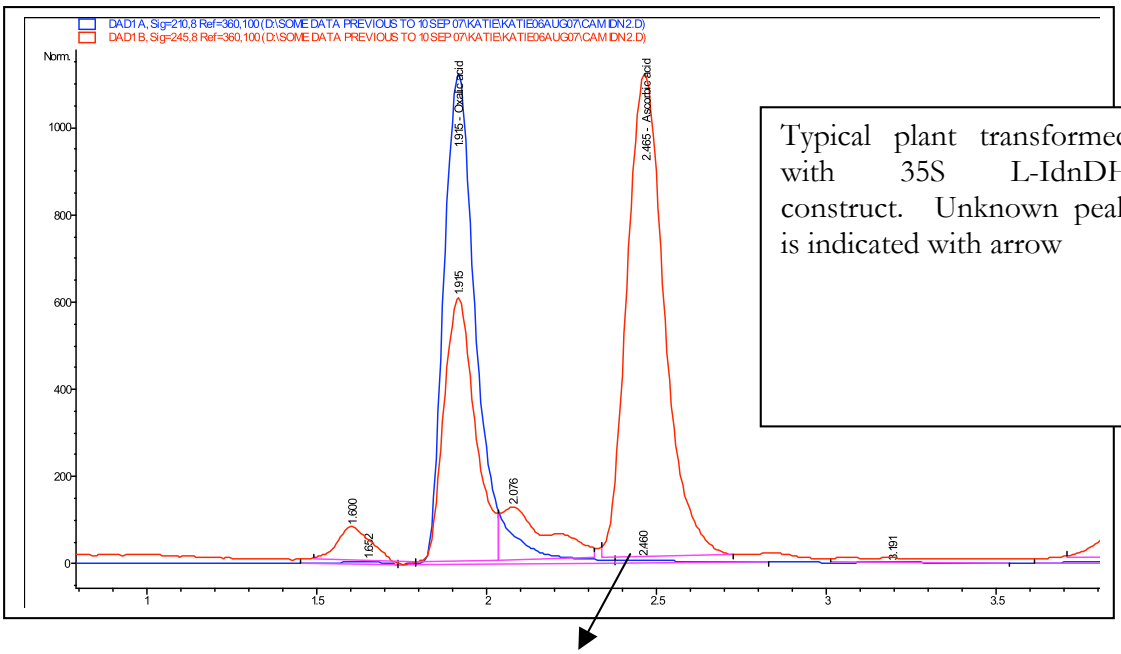


HPLC analysis of transgenic *A. thaliana* plants

Following transformation of *A. thaliana* plants with constructs containing L-IdnDH under the control of a number of known or putative promoters, transgenic plants were grown in soil until sufficient leaf tissue was available for extraction and analysis of the organic acid content. HPLC was used to analyse the organic acid profile of the transgenic *A.thaliana* to determine if tartaric acid was present in the leaf tissue of the transformed plants expressing L-IdnDH. Unfortunately no differences in organic acid profiles were observed in control and transgenic plants and tartaric acid was not observed in any of the tissues analysed. The only significant result from the HPLC analysis was the presence of a hither to unknown peak in the chromatogram of several transgenic plants expressing *L-IdnDH* transcript which eluted at 2.07mins at 245nm. This peak was present in eight of the 28 transgenic lines isolated and was present in lines derived from the different promoters.



Typical control plant transformed with the empty vector only



Typical plant transformed with 35S L-IdnDH construct. Unknown peak is indicated with arrow

Project 5. Catabolism of malic acid during grape berry development

Introduction.
Malic acid or malate (MA) metabolism proceeds by well characterised pathways, and limited exploration of these in grapes has been made by other groups over the years. Malate is suggested to play a pivotal role in plant metabolism, linking via phospho *eno*l pyruvate (PEP), the glycolytic and TCA pathways of oxidative metabolism and providing a means of transporting reducing equivalents across organellar membranes. Accumulation and subsequent respiration of MA during fruit development and ripening present a complex interplay of enzymatic activities that remains poorly understood, yet vital for an understanding of the factors controlling berry acidity. In addition to contributing to juice pH, malic acid plays a dominant role in determining fruit quality as its metabolism will impact on secondary metabolites important for colour and flavour [1]. It is known that grapes from warmer climates or warmer seasons have lower titratable acidities than grapes grown in cooler regions and seasons, and that this may reflect variation in the level of malate synthesised and/or the increased utilisation of MA during later stages of ripening. Thus, the objective of this part of the project was to determine the regulation of malic acid biosynthesis and catabolism.

Malic Acid Synthesis. MA in grape berries is made via fixation of CO_2 by PEP carboxylase (PEPC), an enzyme with many roles in plant metabolism [1]. The oxaloacetic acid (OAA) formed is reduced to malate by malate dehydrogenase (MDH). Cytoplasmic and mitochondrial forms of MDH (cMDH and mMDH) are known and expressed; clearly, if mMDH plays a role then transport is involved. MA accumulates within the vacuole of the cell in the period of development leading to veraison and may reach a level greater than that of tartaric acid at this time. A number of studies have shown high levels of PEPC transcript and activity pre-veraison, suggesting regulation at the level of gene expression. In other plants, further mechanisms regulate PEPC activity, including reversible phosphorylation and inhibition by high malate levels.

Malic Acid Degradation. Post-veraison, MA is released from the vacuole. Glycolysis is inhibited, and it is proposed the substrate for respiration changes from sugars to the released MA. MA catabolism may proceed by cytosolic NADP-malic enzyme (NADP-ME), converting malate to pyruvate, which enters the TCA pathway where it may be respired, used to synthesise amino acids. Alternatively pyruvate can beconverted into PEP as in C4 plants, if grapes have the enzyme pyruvate ortho-phosphate dikinase (PPDK). If this latter reaction occurs, then the carbon could enter the gluconeogenic pathway for hexose (sugar) synthesis and secondary metabolite synthesis. It has not been shown whether grapes have PPDK activity. Alternatively, malate may be converted to OAA by MDH. OAA is a substrate for PEP carboxykinase (PEPCK), to produce PEP and proceed through further gluconeogenic reactions to form sugars or feed into pathways involved in secondary

metabolite biosynthesis. Alternatively, the OAA could feed into further respiratory metabolism by the TCA cycle; [2]. Studies into the activity and stability of the enzymes associated with MA metabolism have partly confirmed these proposed roles, although there remains some dispute concerning the flux of MA through the various degradative routes post veraison. For example, the presence of NAD-ME protein has been shown immunologically [8] but its activity not demonstrated, thus the contribution of this enzyme to MA metabolism pre- and post-veraison is unknown. A molecular analysis of transcripts associated with PEPC, mtMDH and NADP-ME failed to provide a conclusive explanation for the control of MA degradation [9]. Further, the presence of NADP-ME and PEPC during the period of malate synthesis (pre-veraison) raises the point that NADP-ME, which would degrade MA, must be regulated post-translationally. A more complete analysis of transcripts, activities and regulatory metabolite levels of all enzymes involved in MA metabolism is required.Further we wish to address the impact of temperature on the activity of various enzymes involved in malate metabolism to determine at what level the temperature effect on final malate levels at harvest is occurring.

Materials and methods

Malic acid metabolism will be initially studied throughout the growing season by a combination of HPLC-based analyses, enzyme assays and quantitative RT-PCR (qRT-PCR) of berries sampled throughout development. In the field, several cultivars were tested to examine variation in MA metabolism, focussing on Shiraz and Semillon. Published methods were used to assay levels of the key enzymes, PEPC, NADP-ME, NAD-ME, PEPCK, pyruvate kinase (PK), pyruvate dehydrogenase (committed step to TCA cycle), as well as the end points of respiration, cytochrome oxidase and alternative oxidase. Some method development was required. Further the key enzymes were assayed at different tenpertaure to determine what contribution thtey made at warmer and coller field temperatures. We have identified berry ESTs for these enzymes in the UC Davis database, enabling the development of qRT-PCR assays for each of the corresponding genes. As well as using HPLC-based techniques to determine organic acids levels in berries during development, we have also used GC-MS techniques to obtain a more global view of changes in many different metabolites in berries at different developmental stages.

Results and discussion

Malate levels were measured in numerous grape varieties (Riesling, Semillon, Cabernet Sauvignon, Shiraz and Pinot Noir) throughout development to determine which varieties accumulate and lose the most malate. Shiraz and Semillon were chosen for more detailed analyses (Figure 1).

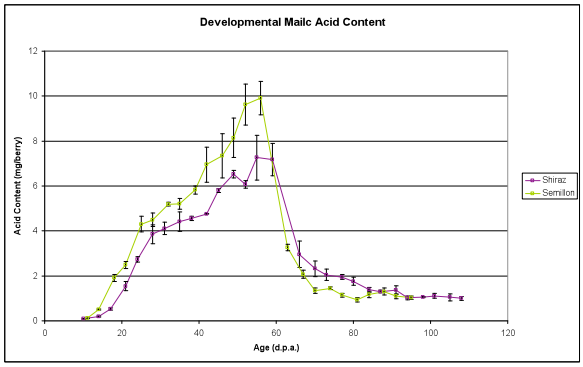


Figure 1: Malate levels in Shiraz and Semillon berries during development

Enzyme Activity.

Previous literature suggested that the levels of malate at harvest can be determined by the level of degradation during ripening. Therefore the influences of enzyme activities on malate degradation were a major focus of this study. Enzymes for study were chosen based on findings from microarray data, as discussed in a later section. In this set of data for enzyme activity presented below, veraison occurred at 59 days.

The activity of an enzyme involved in malate synthesis, phosphoenolpyruvate carboxylase (PEPC) remained steady throughout ripening, likely due to the other important uses of this enzyme in sugar breakdown and pH balance. Another enzyme thought to be involved in malate synthesis, malate dehydrogenase (MDH), was shown to decrease during development, although still remained active during ripening and could also be involved in malate degradation (Figure 2). Several forms of this enzyme exist, which may be active at different times and be involved in different roles of malate metabolism. We are currently using gene expression to determine which of these different forms are changing during development (see another section).

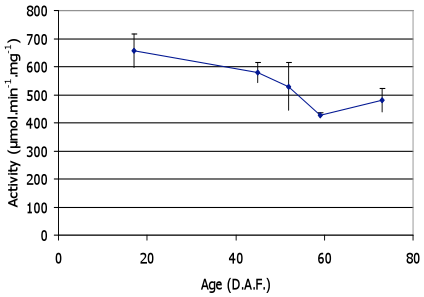


Figure 2: Malate Dehydrogenase Activity throughout Shiraz Berry Development

Enzymes that may be involved in malate degradation include ;

(1) pyruvate kinase (PK),: This was undetectable until the end of ripening, after the majority of malate had been degraded (data not shown)

(2) phosphoenolpyruvate carboxykinase (PEPCK; Figure 3), which is an enzyme involved in the synthesis of sugar from acid

(3) alcohol dehydrogenase (ADH; Figure 4), which is generally only thought to occur under anaerobic conditions but may also be developmentally regulated in some fruits, and;

(4) malic enzyme (ME), which consists of two major forms, one of which showed an increase in activity after veraison (Figure 5). The other malic enzyme, as well as a form of malate dehydrogenase, could only be measured using isolated mitochondria.

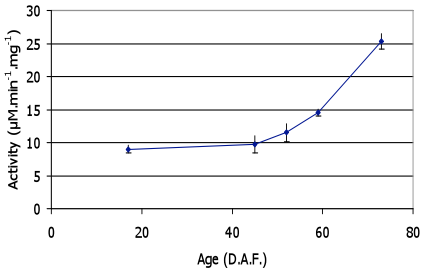


Figure 3: Phosphoenolpyruvate Carboxykinase Activity throughout Shiraz Berry Development

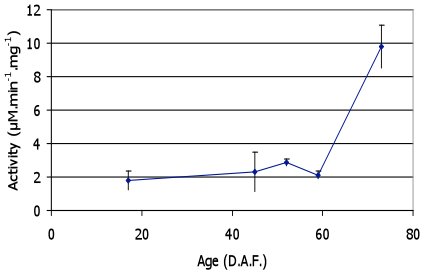


Figure 4: Alcohol Dehydrogenase Activity throughout Shiraz Berry Development

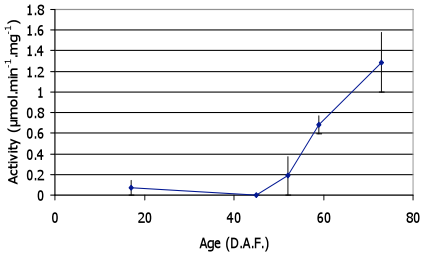


Figure 5: NADP-Malic Enzyme Activity throughout Shiraz Berry Development

Mitochondria were purified from grapes collected from the Coombe vineyard (’08-09 season), at weeks 9 (pre-veraison), 10 (softening), 11 (colour change) and 12 (post-veraison) after anthesis. Mitochondrial NAD-ME activity was almost absent until post-veraison, whereby activity increased dramatically (Figure 6). This enzyme is potentially involved in malate degradation during ripening and this is the frist report detecting its activity. Mitochondrial MDH activity was high pre-veraison then decreased at softening, before increasing again throughout ripening (Figure 7). It may be that mitochondrial MDH is involved in respiration early in development, before switching to a malate degradative role after a lag in activity at veraison. The enzymatic capacity of the respiratory enzyme, alternative oxidase (AOX) from grape mitochondria also decreased at the softening stage, and then increased slightly throughout ripening. However, this was due to the dramatic decrease in overall

respiration rate, as when expressed as a percentage of total respiration, AOX increased slightly at veraison, and decreased post-veraison (although these changes were very slight; Figure 8).

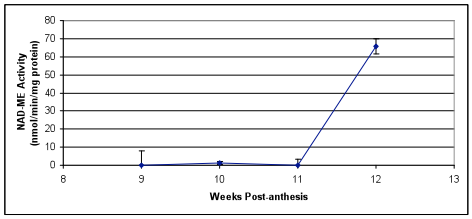


Figure 6: NAD-Malic Enzyme Activity from Purified Shiraz Berry Mitochondria throughout Development

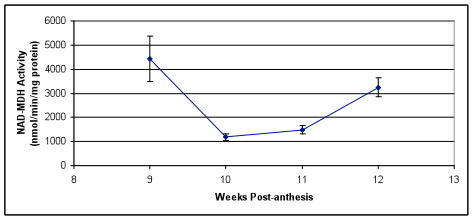


Figure 7: Malate Dehydrogenase Activity from Purified Shiraz Berry Mitochondria throughout Development

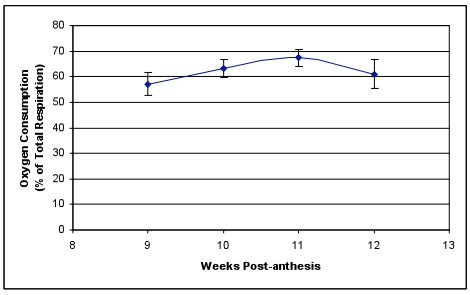


Figure 8: Alternative Oxidase Activity from Purified Shiraz Berry Mitochondria throughout Development

Metabolite Analyses.

An analysis of metabolites was also taken from several developmental stages, to investigate the effects of ripening on the levels of other metabolites in the grape. Results are outlined in Table 1. The majority of measured amino acids declined throughout ripening (eg. asparagine, aspartate, glutamate, glutamine, proline and serine) while some increased throughout development (glycine and threonine). Changes in carboxylic acids were varied, with some decreasing post-veraison (benzoate, citrate, fumarate, gluconate, glycerate, malate, quinate, shikimate, succinate and threonate), and others increasing or remaining steady (2-keto-L-gulonate, oxalate and tartrate). The majority of sugars increased throughout ripening, except raffinose.

Table 1: Effect of developmental stage on metabolic profile of grapes

		Wk 6	Wk7	Wk8	Wk10
Amino Acids	**Alanine**	-	0	++*	+
	beta-Alanine	+	- - -	-	-
	Asparagine_3TMS	- - -*	- - -	+++*	-
	Aspartate_2TMS	+	+	-	-
	Glutamate	-	-*	- -*	- - -*
	Glutamine_3TMS	0	- -	- - -*	- -
	Glutamine_4TMS	- - -	- - -	- - -*	- - -*
	Glycine_3TMS	-	-	-	+
	Norleucine	+	+	+	-
	Proline_2TMS	0	-*	- - -*	- - -*
	Pyroglutamate	- -*	- - -*	- - -*	- - -*
	Serine_2TMS	- - -*	- - -*	- - -*	- - -*
	Serine_3TMS	-	-	+	+++
	Threonine_2TMS	- - -*	- - -*	- -*	-
	Threonine_3TMS	+++*	++*	-	- - -*
Organic Acids	**Benzoate**	++	++	++*	+++
	Citrate	+++*	++*	+	0
	3,4-dihydroxybenzoate	0	-	-	-
	Fumarate	+	+	- -	- - -
	Gluconate	0	- -*	- - -*	- - -*
	Glycerate	+++*	+++*	-	- -*
	2-Keto-L-gulonate	+++*	+++*	+++*	+++*
	Malate	+	+	0	-
	Oxalate	+	0	+	++*
	Quinate	- -	- - -*	- - -*	-
	Shikimate	-	- -	- - -*	- - -*
	Succinate	-	-*	- - -*	- - -*
	Tartrate	+	+	+	++*
	Threonate	0	-	- - -*	- - -*
Fatty Acids	**Stearic acid**	+	-	-	+
Sugars	**Fructose_MX1**	+	+	++	+++*
	Fructose_MX2	0	+	++	+++*
	D-glucose_MX1	+	+	+	++*
	D-glucose_MX2	+	+	+	++
	Raffinose	0	-	- - -	- - -*
	Sucrose	+	+	+	+++*
	Xylose_MX1	- - -	- - -	+	+++*
	Xylose_MX2	0	-	++	++
Phosphate Groups	**Glucose-6-P_MX1**	+	-	- - -*	- - -*
	Glucose-6-P_MX2	-	- - -	- - -*	- - -*
	Phosphate	0	-*	- -*	-
Flavonols	**Catechin**	++	+++	+++	+++*
Signal	**Myo-inositol**	+	-	-	- -*

Molecules					
	Threonate-1,4-lactone	+	- -*	- - -*	- - -*
Unknown Disaccharides	**Unknown-disaccharide**	+++*	+++*	- - -*	- - -*
	Unknown-disaccharide	+++*	+++	- -*	- - -*
	Unknown-disaccharide	+	+++*	-	- - -*
	Unknown-disaccharide	++*	++	+	- - -*
	Unknown-disaccharide	-	+	-	- - -*
	Unknown-disaccharide	+	+++	- - -	- -*
	Unknown-disaccharide	+	+	- -*	- - -*
	Unknown-disaccharide	+	- -	0	- -
	Unknown-disaccharide	-*	- -*	- - -*	- - -*
	Unknown-disaccharide	0	++	- - -*	- - -*
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	Unknown-disaccharide	++*	+	+	- - -*
	Unknown-disaccharide	- - -*	- - -*	- -	- - -*
Unknowns	**Unknown**	+	+	0	++*
	Unknown	+	-	- -*	- - -*
	Unknown	+	++	- - -	- - -
	Unknown	+	+	- - -	- - -
	Unknown	-	- -*	- - -*	- - -*
	Unknown	-	+	+++	+++*
	Unknown	+++	+*	++*	+++
	Unknown	- -*	- - -*	- - -*	- - -*
	Unknown	+*	+	++	+++*
	Unknown	++*	++*	+	+++
	Unknown	+	-	- -	-*
	Unknown	+++*	++*	- - -*	- - -*
	Unknown	++*	+	- - -*	- - -*

'+' indicates an increase in metabolite level relative to week 2, '-' indicates a decrease. Significant results indicated by '*'. Stages of development; Wk2, beginning of malate accumulation (early development); Wk6, malate accumulation; Wk7, pre-veraison, Wk8, post-veraison; Wk10, malate degradation (ripening).

The metabolite profiling data has only just been analysed and the implications of each change in various metabolites is still under consideration.

Gene expression analyses.

(a) Microarray Anaylses

Gene expression studies involved an analysis of microarray data provided by collaborators in USA (Professor Grant Cramer, University of Nevada, Reno), and quantitative real-time PCR, which is currently underway for Shiraz grapes grown in Adelaide. The re-analysed microarray data are to be published in Phytochemistry as part of our review paper focused on malate metabolism in grape and other developing fruits (currently in press). Expression of PEPC genes

decreased throughout development, consistent with a decreased pattern of enzyme activity seen in Adelaide berries (data not shown). PEPC kinase, is an enzyme which regulates PEPC activity via post-translational mechanism. PEPC kinase gene expression decreased throughout development, consistent with a decline in PEPC activity. Expression of other genes of interest included; pyruvate kinase, which declined (despite an increase in activity displayed by this Australian study); pyruvate orthophosphate dikinase (PPDK), which increased throughout development, particularly at veraison, then remained high; pyruvate decarboxylase (PDC), which is another enzyme involved in alcohol metabolism and increased, along with alcohol dehydrogenase (ADH) expression; phosphoenolpyruvate carboxykinase (PEPCK), which also increased, consistent with patterns of activity seen in our study. Malate dehydrogenase gene expression was variable, depending on the isogene, although generally there was an increase in expression, which was also seen for the genes of a vacuolar malate transporter. Expression of some pyruvate dehydrogenase (PDH) isoforms increased slightly at veraison. Expression of one (out of two) NAD-ME genes decreased very slightly at veraison.

(b) **Quantitative Real-Time PCR**
QRT-PCR assays have been developed in our laboratory, and are about to be carried out on a developmental series of samples from the 07-08 season, to give a more focused analysis of malate metabolising genes in Shiraz berries grown in Adelaide.

The effect of temperature is of special interest for malate metabolism in grapes, due to the higher losses of acid in berries grown at warmer temperatures. One trial involving a double-pruning technique was used to alter the climatic conditions under which Cabernet Sauvignon berries developed and ripened. Results indicated a decrease in the accumulation of malate, which was likely affected by the loss of vegetation that occurred simultaneous with berry ripening, as well as changed patterns of rainfall. Ongoing research in this lab, and in collaboration with Victor Sadras (SARDI), will explore the effect of increased temperature on gene expression and enzyme activities of enzymes involves in malate metabolism, to determine which enzymes could be key effectors in the temperature-sensitive regulation of malate metabolism. Samples collected from one season of field experiments have already been analysed for their effect on malate levels, and analyses of enzyme activities and gene expression levels are underway.

Conclusions and Future Work
From previous studies, it is suggested that the final level of malate is governed by the degradation of malate after veraison. From the enzymic studies carried out in this study, it is clear that the potential fate of malate is either to be converted to OAA or pyruvate. However, the balance between these fates for malate is still not clear. If converted to OAA, then it can be metabolised to PEP and converted to sugars or feed into secondary

metabolite biosynthetic pathways or into the TCA cycle and broken down to CO_2 or amino acids. There was not a dramatic increase in amino acid content as determined from the metabolite analyses during development, thus this is not a major endpoint for malate. We have also shown that there is the capacity to respire the malate.

Further studies need to determine how the malate is partitioned between respiration, fermentation and gluconeogenesis. This would require metabolic flux analyses to determine flux through each pathway perhaps using NMR techniques in berry slices. Further determination of the energy charge and NAD(P)H: NAD(P) ratios would indicate whether a pathway is operating. Finally, the generation of transgenic lines with down-regulation of key enzymes, would perturb the flow of metabolites and indicate the impact of these enzymes on bery acidity and quality. The enzymes that would be good targets for this transgenic approach would be NAD-malic enzyme and PEPCK.

Currently, it is our plan to finish this project by assessing the effect of temperature on the key enzymes of MA metabolism. This work is still underway.

Publications

One review paper on fruit malate metabolism has been accepted by Phytochemistry. A primary research paper is currently being written in collaboration with Victor Sadras (SARDI). At least two further primary research papers are expected to come from this work, once the final analyses have been carried out. One will deal with the effect of development on malate metabolism in grapes, and will contain data on gene expression, enzyme activities, mitochondrial isolations and metabolomics. Another will focus on the effect of temperature on gene expression and enzyme activities related to malate metabolism in grape.

References.

[1] Ruffner, HP (1982) Vitis **21**, 346-358.

[2] Ruffner, HP, Possner, D, Brem, S & Raast, DM (1984) Planta **160**, 444-448.

[3] Famiani et al. (2000) J. Exp. Bot.**345**,675-683.

[4] Or, E, Baybik, J, Sadka, A & Saks, Y (2000) J. Plant Physiol. **157**, 527-534.

7. OUTCOME/CONCLUSION

Our analysis of transgenic grapevines in which gene expression of the tartaric acid biosynthetic enzyme L-IdnDH was knocked down, has revealed that both the D-Glucose and Ascorbic acid TA biosynthetic pathways contribute significantly to tartaric acid levels. Candidate enzymes that may be involved in both of these pathways have been identified and are currently under further investigation. This work will reveal the genetic underpinning of tartaric acid biosynthesis and will ultimately provide the basis to identify or engineer cultivars with optimal TA levels. These candidates include enzymes at the step which combines both the ascorbic acid and D-Glucose pathways, presenting an ideal trait to target total TA biosynthesis.

From previous studies, it is suggested that the final level of malate is governed by the degradation of malate after veraison. From the enzymatic studies carried out in this study, it is clear that the potential fate of malate is either to be converted to OAA or pyruvate. However, the balance between these fates for malate is still not clear. If converted to OAA, then it can be metabolised to PEP and converted to sugars or feed into secondary metabolite biosynthetic pathways or into the TCA cycle and broken down to CO_2 or amino acids. There was not a dramatic increase in amino acid content as determined from the metabolite analyses during development, thus this is not a major endpoint for malate. We have also shown that there is the capacity to respire the malate. Currently, it is our plan to finish this project by assessing the effect of temperature on the key enzymes of MA metabolism. This work is still underway.

In addition to the biosynthesis and catabolism of organic acids, we have begun to address the issue of vacuolar compartmentation of organic acids and have identified and begun to study five candidate transporters. As the loss of vacuolar compartmentation of MA that occurs post veraison allows the catabolism of this acid, identification of the transporters responsible, and modification of this trait, has the potential to make a large impact on berry acidity at harvest. This project is conducted in collaboration with Professor Steve Tyerman who has a strong interest in the physiology of this process and in the function of these candidate transport proteins.

8. RECOMMENDATIONS

Future research

Our early research in this area, which resulted in the first identification of a TA synthetic gene, led to the present research project in which we have extended these initial findings. The major outcomes present important opportunities for future research, and will be discussed in point-form below.

(i). *The identification of further candidates for TA synthetic genes.* A complete understanding of the pathway leading from L-ascorbic acid to TA needs information on at least three other steps. The enzyme responsible for the initial conversion of L-ascorbic acid (or possibly dehydroascorbate) to the intermediate compound 2-keto-L-idonate remains unknown. Similarly, the fate of 5-keto-D-gluconic acid produced by L-IdnDH activity upon L-idonate, and the subsequent conversion of the TA semi-aldehyde that most likely results from this cleavage reaction, requires further investigation. In addition to molecular and biochemical data, a detailed thermodynamic analysis of the pathway is needed if we are to be able to propose the most probable rate-limiting steps. This information may be gained relatively easily from in vitro reactions catalysed by recombinant enzymes, and will enable strategic modifications to pathway genes to be suggested that are most likely to result in significant increases or decreases in TA synthesis.

(ii). *Preliminary data from transgenic vines in which expression of the key TA synthetic gene L-IdnDH has been knocked down*. This work, initiated under CRCV, provides an early indication of the value of in planta experimentation in which targeted changes to gene expression are made. The availability of the grapevine microvine enables huge advances in this area to be proposed – with key structural and regulatory genes selected for analysis. With regard to acid metabolism, in addition to further study of TA-specific gene expression, significant research opportunities exist to rapidly increase our understanding of the metabolism of L-ascorbic acid and L-malic acid. Work conducted within the CI's laboratory supported by GWRDC funds to PhD candidate Vanessa Melino, highlighted the difficulties of studying L-ascorbic acid at the metabolite level – its chemical reactivity and low level in the berry necessitates very careful analysis. The genes identified during this project that show regulation during key stages of berry development are obvious targets for further study in a transgenic system. A fundamental question that can be addressed in this way, 'what determines the fate of

L-ascorbic acid' will provide pivotal understanding of the control mechanisms behind TA synthesis, and the basis for targeted approaches to manipulate levels of the entire pathway activities. Similarly, MA metabolism in the berry is clearly impacted upon by the activity of a number of competing biochemical pathways. Careful dissection of the key pathways, using targeted transcriptional regulation in microvines, will allow us to test strategies for increasing berry MA levels, and in combination with high-resolution analytical techniques, interpret the data to ensure that the changes made to one gene do not adversely affect other areas of berry metabolism.

(iii). *The identification of putative vacuolar acid transporters.* Further research is needed to confirm the activity of the candidate transporter proteins. Specifically, we must determine their roles in acid transport into and from the vacuole throughout berry development, and in addition to specificity data, determine kinetics and inhibitors of these processes. This information will enable us to build a picture of acid management within berries and may ultimately result in the targeted modification of the tonoplast transporter profile to produce berries in which the acid profile at ripeness is closer to that at veraison, thereby providing winemakers with greater management of juice and must pH levels.

(iv). *Malate catabolism.* Further studies need to determine how the malate is partitioned between respiration, fermentation and gluconeogenesis. This would require metabolic flux analyses to determine flux through each pathway perhaps using NMR techniques in berry slices. Further determination of the energy charge and NAD(P)H: NAD(P) ratios would indicate whether a pathway is operating. Finally, the generation of transgenic lines with down-regulation of key enzymes, would perturb the flow of metabolites and indicate the impact of these enzymes on bery acidity and quality. The enzymes that would be good targets for this transgenic approach would be NAD-malic enzyme and PEPCK.

Broader industry context of further research in the area of organic acid metabolism during berry development

Our research covers an area of grapevine metabolism that has, for a number of reasons been somewhat overlooked for many years. The simple solution to problems of berry acidity in Australia has been the addition of TA at various stages of the vinification process, with the cost incurred considered an inescapable expense of production. Early work showed that once formed in early development, berry levels of TA remain essentially unchanged, while MA levels declined post-veraison, more so in grapes grown in warmer climates. It may be argued that the Australian grape and wine industries are now faced with conditions that suggest a revision to this approach is needed.

9. APPENDIX 1: COMMUNICATION

9.1 The outcomes of the research activities conducted during the project have been communicated in a number of ways. We have published one major review in a peer-reviewed journal, and further publications will arise over the next few months. We have also communicated our outcomes to the industry through publication in industry journals, and by presentations at conferences. These activities are listed below:

Journal Article: Sweetman, C, Deluc, L.G., Cramer, G.R., Ford, C.M. and Soole, K.L. (2009) Regulation of Malate Metabolism in Grape Berry and Other Developing Fruits. Phytochemistry. Accepted for publication 08 August 2009.

Industry journal article: DeBolt, S., Melino, V.J., and Ford, C.M. (2006) Developments in our understanding of how grapes make tartaric acid. Australian and New Zealand Wine Industry Journal **551**, 57-58.
Industry journal article: Hayes, M., Burbidge, C., Melino, V., Sweetman, C., Soole, K. and Ford, C. (2009) Organic acids in grapes: recent research outcomes. Australian and New Zealand Wine Industry Journal 37th Annual Technical Issue **545a**, 70-73.

Conference presentation and paper: DeBolt, S., Melino, V., Sweetman, C., Soole, K., Hayes, M. and Ford, C.M. (2007) The potential to modify grape berry acidity through an understanding of the metabolism of tartaric and malic acids. Proceedings 13th Australian Wine Industry Technical Conference, pp165-170.

Conference presentation and paper: Melino, V., Soole, K.L. and Ford, C.M. (2008) Ascorbate metabolism during grape berry development. Presentation made by Vanessa Melino at the 8th International Symposium on Grapevine Physiology and Biotechnology, Adelaide, 23-28 November 2008 *(although the topic of this presentation was more closely aligned with GWRDC-funded research on berry ascorbate metabolism, the talk covered aspects of our research on TA metabolism also and is therefore include in this summary).*

Conference poster presentation: Sweetman, C., Ford, C.M. and Soole, K.L. (2007) Malate metabolism in *Vitis vinifera* berries and cell culture. ComBio 2007, Sydney 22-26 September 2007.

Conference poster presentation: Hayes, M.A., Burbidge, C.A., Grieve, K.M., Soole, K.L. and Ford, C.M. (2007) From candidates to function mapping the biosynthetic pathway of tartaric acid in plants. ComBio 2007, Sydney 22-26 September 2007.

Conference poster presentation: Melino, V., Soole, K.L. and Ford, C.M. (2007) Ascorbate metabolism during grape berry development. ComBio 2007, Sydney 22-26 September 2007.

Conference poster presentation: Sweetman, C., Ford, C.M. and Soole, K.L. (2008) Responses of malate metabolising enzymes throughout grape bey development. Combio 2008, Canberra 21-25 September 2008.

Conference poster presentation: Burbidge, C.A., Ford, C.M. Hayes, M.A. and Soole, K.L. (2008) Identification and characterisation of the enzymes involved in the biosynthetic pathway of tartaric acid in *Vitis vinifera*. Combio 2008, Canberra 21-25 September 2008.

10. APPENDIX 2: INTELLECTUAL PROPERTY

The project generated no intellectual property.

11. APPENDIX 3: REFERENCES

Blattner, F. R., Plunkett III, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. & Shao, Y. (1997) The Complete Genome Sequence of *Escherichia coli* K-12, *Science,* 277(5331): 1453-1462

Chandrashekar, K., Felse, P. A. & Panda, T. (1999) Optimization of Temperature and Initial pH and Kinetic Analysis of Tartaric Acid Production by *Gluconobacter suboxydans*, *Bioprocess Engineering,* 20: 203-207

Clough SJ, Bent AF (1998) Floral dip: a simplified method forAgrobacterium-mediated transformation of Arabidopsis thaliana. The Plant Journal **16:** 735-743

Cirami, R. M. (1973) Changes in the Composition of Ripening Grapes in a Warm Climate, *Australian Journal of Experimental Agriculture and Animal Husbandry,* 13: 319-323

De Ley, J. (1966) [40] 5-Ketogluconic Acid Reductase, IN **Willis, A. W.** (Ed.) *Methods in Enzymology,* Vol 9, New York, Academic Press

DeBolt, S. (2006) Tartaric Acid Biosynthesis in Plants, *School of Agriculture, Food and Wine.* Adelaide, The University of Adelaide

DeBolt, S., Melino, V. & Ford, C. M. (2007) Ascorbate as a Biosynthetic Precursor in Plants, *Annals of Botany,* 99(1): 3-8

Es-Safi, N.-E., Guernevé, C. L., Cheynier, V. & Moutounet, M. (2000) New Phenolic Compounds Formed by Evolution of (+)-Catechin and Glyoxylic Acid in Hydroalcoholic Solution and Their Implication in Color Changes of Grape-Derived Foods, *Journal of Agricultural and Food Chemistry,* 48: 4233-4240

Hafke JB, Hafke Y, Smith JAC, Lüttge U, Thiel G (2003) Vacuolar malate uptake is mediated by an anion-selective inward rectifier. The Plant Journal **35:** 116-128

Harrison S, Mott E, Parsley K, Aspinall S, Gray J, Cottage A (2006) A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant Methods **2:** 19

Helsper, J. P. & Loewus, F. A. (1982) Metabolism of $_{L}$-Threonic Acid in *Rumex x acutus* L. and *Pelargonium crispum* (L.) L'Hér, *Plant Physiology,* 69(6): 1365-1368

Helsper, J. P. F. G., Saito, K. & Loewus, F. A. (1981) Biosynthesis and Metabolism of $_{L}$-Ascorbic Acid in Virginia Creeper (*Parthenocissus quinquefolia* L.), *Planta,* 152(2): 171-176

Hoekenga OA, Maron LG, Pineros MA, Cancado GMA, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, Matsumoto H, Yamamoto Y, Koyama H, Kochian LV (2006) AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proceedings of the National Academy of Sciences **103:** 9738-9743

Hurth MA, Suh SJ, Kretzschmar T, Geis T, Bregante M, Gambale F, Martinoia E, Neuhaus HE (2005) Impaired pH homeostasis in arabidopsis lacking the vacuolar dicarboxylate transporter and analysis of carboxylic acid transport across the tonoplast. Plant Physiology **137:** 901-910

Kamlet, J. (1943) Preparation of $_{D}$-Tartaric Acid by Fermentation, *United States Patent Office.*

Koichi, Y., Minoda, Y. & Kotera, U. (1971) Novel Process of Producing L(+)-Tartaric Acid, *United States Patent Office.*

Kovermann P, Meyer S, Hortensteiner S, Picco C, Scholz-Starke J, Ravera S, Lee Y, Martinoia E (2007) The Arabidopsis vacuolar malate channel is a member of the ALMT family. Plant Journal **52:** 1169-1180

La Rivière, J. W. M. (1956) Specificity of Whole Cells and Cell-Free Extracts of *Pseudomonas putida* Towards (+), (-), and Meso-Tartrate, *Biochimica et Biophysica Acta,* 22(1): 206-207

Lamikanra, O., Inyang, I. D. & Leong, S. (1995) Distribution and Effect of Grape Maturity on Organic Acid Content of Red Muscadine Grapes, *Journal of Agricultural and Food Chemistry,* 43(12): 3026-3028

Lockwood, L. B. & Nelson, G. E. N. (1951) Preparation of D-Tartaric Acid, *United States Patent Office.*

Loewus, F. A. (1999) Biosynthesis and Metabolism of Ascorbic Acid in Plants and of Analogs of Ascorbic Acid in Fungi, *Phytochemistry,* 52(2): 193-210

Loewus, F. A. & Stafford, H. A. (1958) Observations on the Incorporation of C^{14} into Tartaric Acid and the Labeling Pattern of $_{D}$-Glucose from an Excised Grape Leaf Administered $_{L}$-Ascorbic Acid-6-C^{14}, *Plant Physiology,* 33(2): 155-156

Malipiero, U., Ruffner, H. P. & Rast, D. M. (1987) Ascorbic to Tartaric Acid Conversion in Grapevines, *Journal of Plant Physiology,* 129(1-2): 33-40

Muller PY, Janovjak H, Miserez AR, Dobbie Z (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques **32:** 1372-1379

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W. F., Ehrenreich, A., Gottschalk, G. & Deppenmeier, U. (2005) Complete Genome Sequence of the Acetic Acid Bacterium *Gluconobacter oxydans*, *Nature Biotechnology,* 23(2): 195-200

Ribéreau-Gayon, G. (1968) Etude des mecanismes de synthese et de transformation de l'acide malique, de l'acide tartrique et de l'acide citrique chez *Vitis vinifera* L., *Phytochemistry,* 7(9): 1471-1482

Saito, K. (1992) Metabolism of $_{L}$-Threotetruronic Acid by *Pelargonium crispum*, *Phytochemistry,* 31(4): 1219-1222

Saito, K. & Kasai, Z. (1968) Accumulation of Tartaric Acid in the Ripening Process of Grapes, *Plant And Cell Physiology,* 9: 529-537

Saito, K. & Kasai, Z. (1969) Tartaric Acid Synthesis from L-Ascorbic Acid-1-^{14}C in Grape Berries, *Phytochemistry,* 8(11): 2177-2182

Saito, K. & Kasai, Z. (1978) Conversion of Labeled Substrates to Sugars, Cell Wall Polysaccharides, and Tartaric Acid in Grape Berries, *Plant Physiology,* 62(2): 215-219

Saito, K. & Kasai, Z. (1982) Conversion of L-Ascorbic Acid to L-Idonic Acid, L-Idono-γ-Lactone and 2-Keto-L-Idonic Acid in Slices of Immature Grapes, *Plant And Cell Physiology,* 23(3): 499-507

Saito, K. & Kasai, Z. (1984) Synthesis of L-(+)-Tartaric Acid from $_{L}$-Ascorbic Acid via 5-Keto-$_{D}$-Gluconic Acid in Grapes, *Plant Physiology,* 76(1): 170-174

Saito, K. & Loewus, F. A. (1989a) Formation of L-(+)-Tartaric Acid in Leaves of the Bean, *Phaseolus vulgaris* L.: Radioisotopic Studies With Putative Precursors, *Plant Cell Physiology,* 30(5): 629-636

Saito, K. & Loewus, F. A. (1989b) Formation of Tartaric Acid in Vitaceous Plants: Relative Contributions of L-Ascorbic Acid-Inclusive and Acid-Noninclusive Pathways, *Plant And Cell Physiology,* 30(6): 905-910

Saito, K. & Loewus, F. A. (1989c) Occurrence of L(+)-Tartaric Acid and its Formation from D-Gluconate or D-*xylo*-5-Hexulosonate in Bean Leaf (*Phaseolus vulgaris* L.), *Plant Science,* 62(2): 175-180

Salusjärvi, T., Povelainen, M., Hvorslev, N., Eneyskaya, E. V., Kulminskaya, A. A., Shabalin, K. A., Neustroev, K. N., Kalkkinen, N. & Miasnikov, A. N. (2004) Cloning of a Gluconate/Polyol Dehydrogenase Gene from *Gluconobacter suboxydans* IFO 12528, Characterisation of the Enzyme and its Use for the Production of 5-Ketogluconate in a Recombinant *Escherichia coli* Strain, *Applied Microbiology and Biotechnology,* 65(3): 306-314

Shimada T, Nakano R, Shulaev V, Sadka A, Blumwald E (2006) Vacuolar citrate/H+ symporter of citrus juice cells. Planta **224:** 472-480

Storey R (1987) Potassium localization in the grape berry pericarp by energy-dispersive X-ray microanalysis. American Journal of Enology and Viticulture **38:** 301-309

Stafford, H. A. (1959) Distribution of Tartaric Acid in the Leaves of Certain Angiosperms, *American Journal of Botany,* 46(5): 347-352

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K. S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S. & Olson, M. V. (2000) Complete Genome Sequence of *Pseudomonas aeruginosa* PAO1, an Opportunistic Pathogen, *Nature,* 406(6799): 959-964

The French–Italian Public Consortium for Grapevine Genome Characterization (2007) The Grapevine Genome Sequence Suggests Ancestral Hexaploidization in Major Angiosperm Phyla, *Nature,* Advanced Online Publication

Toit, W. J. d., Marais, J., Pretorius, I. S. & Toit, M. d. (2006) Oxygen in Must and Wine: A Review, *S. Afr. J. Enol. Vitic.,* 27(1): 76-94

Wagner, G. & Loewus, F. A. (1974) $_{L}$-Ascorbic Acid Metabolism in Vitaceae: Conversion to (+)-Tartaric Acid and Hexoses, *Plant Physiology,* 54(5): 784-787

Wagner, G., Yang, J. C. & Loewus, F. A. (1975) Stereoisomeric Characterization of Tartaric Acid Produced During $_{L}$-Ascorbic Acid Metabolism in Plants, *Plant Physiology,* 55(6): 1071-1073

Williams, M. & Loewus, F. A. (1978) Biosynthesis of (+)-Tartaric Acid From $_{L}$-[4-^{14}C] Ascorbic Acid in Grape and Geranium, *Plant Physiology,* 61(4): 672-674

Zimmermann, C. (2005) Cloning, Expression and Characterisation of a Semialdehyde Dehydrogenase Whose Expression Suggests a Potential Role in the Biosynthetic Pathway for $_{L}$-Tartaric Acid in the Grapevine *Vitis vinifera* L. Cabernet Sauvignon cv., Glen Osmond, School of Agriculture and Wine, Waite Campus, University of Adelaide

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H. & Nury, F. S. (1995) *Wine Analysis and Production,* Maryland, Aspen Publishers, Inc.

12. APPENDIX 4: STAFF

Chris Ford, PS
Kathleen Soole, CI
Doug Cook, CI
Matthew Hayes, appointed as post doc October 2006
Crystal Sweetman, appointed as PhD student March 2006
Crista Burbidge, appointed as PhD student March 2007
Katie Grieve, Honours student, L-IdnDH expression in Arabidopsis, 2007
Jay Patel, Masters Student, Identification of organic acid transporters, 2007
Jay Rongala, Masters Student, Identification of organic acid transporters, 2007

13. APPENDIX 5: OTHER RELEVANT MATERIAL

Not applicable.

14. APPENDIX 6: END OF PROJECT FINANCIAL STATEMENT

Statement of Receipts and Expenditure

At the conclusion of each Project, the GWRDC requires a Statement of Receipts and Expenditure of GWRDC funds received in relation to the Project.

Project Title:	Organic Acid Metabolism during Grape Berry Development

GWRDC Project Number	Project Start Date	Project End Date
UA06/02	**01 July 2006**	**30 June 2009**

Financial Year (e.g. 2006/07)	**(a)** **Funds brought forward from previous year*	**(b)** **#Approved GWRDC Budget (full year)**	**(c)** **Total budget available** *(a)+(b)*	**(d)** **Actual expenditure ($)**	**(e)** **Difference ($)** *(c)-(d)*
Year 1: 2006/2007	–	110000	110000	93976.14	16023.86
Year 2: 2007/2008	16023.86	115000	131023.86	127535	3488.86
Year 3: 2008/2009	3488.86	120000	123488.86	123,488.86	0
Year 4: 20__/__					
Year 5: 20__/__					
TOTAL		345000		345000	

* "Funds brought forward" refers to any unspent GWRDC funds from the previous year and approved by the GWRDC to be spent the following year (i.e. column *(e)* from previous year).
# "Approved GWRDC Budget" is the funding amount specified in the Project Agreement, including any approved Variations after Project commencement.

Was funding expended as approved in the Project Agreement?

Yes ☐

No ☐

*If **NO**, please provide reasons:*

Has the cash and/or in-kind funding from Contributing Agencies been received and applied to the Project as approved in the Project Agreement? Yes ☐ No

*If **NO,** please provide reasons:*

I hereby certify that this statement is true and accurate.
Signature of duly authorised representative) ……
…… …… ……
Name: *Title:* *Date:*