Identification and localization of vacuolar organic acid carriers in grapevine berries

By

Jayachandra Rongala

A1157936

A thesis submitted for the partial fulfilment of the requirements of the Masters of Biotechnology (Plant Biotechnology)

The University of Adelaide

Faculty of science

School of Agriculture,food and wine

Waite Campus

2008

DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Student:

Jayachandra Rongala

Date:

Supervisor:

Dr. Matthew Hayes

Date:

PREFACE

This research was performed over 10 months as part of Masters of Biotechnology (Plant Biotechnology). The literature review was previously assed by the examiners and suggested changes and updates were made accordingly. The present research manuscript herein provides the first draft of a future publication in Plant Physiology, due to time and some unexpected factors the project was not completed and all the data was not included in the manuscript, but the materials, methods and the outline was briefly explained in the "appendices" section.

The research manuscript outlines the introduction, results, discussion, material and methods. All the results are included in the sequential flow of my project except for the last part; functional expression in *xenopus* oocytes, which was included in the appendices.

Table of Contents

LITERATURE ANALYSIS

Introduction

Grape berry acidity

Malate plays an important role in plant metabolism; it is involved in many processes such as maintaining osmotic pressure, stomatal regulation, amino acid biosynthesis, phosphorus and iron uptake and is also involved in symbiotic nitrogen fixation and aluminium tolerance (Schulze et al., 2002). Malate is also utilized as a respiratory substrate (Ruffner et al., 1983; Schulze et al., 2002), for production of ATP and NADH (Kovermann et al., 2007), and present in cytosol, chloroplast, mitochondria, peroxisomes and vacuole (Fig.1).

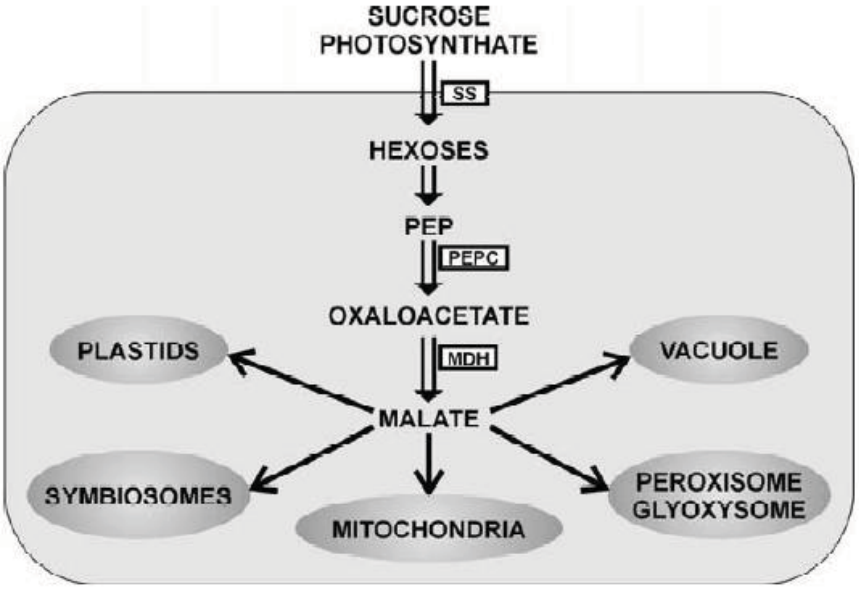


Fig.1: Diagrammatic scheme for the degradation of photosynthate (sucrose) to malate and targeted to different subcellular locations. Source: Schulze et al., 2002.

Malic and tartaric acids are the large components of the berry juice next to water and sugars (Fig.2) (Amerine and Winkler, 1942; Amerine, 1964; Tilbrook and Tyerman, 2006). These acids not only play an important role in the flavour of the berries but also in wine making, during fermentation and ensure stability of the wine after bottling

(maintaining low pH). These acids are added additionally during commercial fermentation.

NOTE: This figure is included on page 7 of the print copy of the thesis held in the University of Adelaide Library.

Fig.2: Concentration of components of free juice at harvest in grapevine berries. Source: (Tilbrook and Tyerman, 2006).

Synthesis and accumulation of these acids mainly occur during developmental stages of the berry (Terrier and Romieu, 2001). The acids are derived from metabolism of sugars (sucrose, fructose, glucose) and are involved in many metabolic pathways. Malate is formed from the reduction of pyruvate to oxaloacetate and malic acid by the activity of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) (Fig.3). The malate concentration increases as the berry develops and the excess malate is compartmentalized in the vacuole (Schulze et al., 2002).

As the berry ripens the malate concentrations decrease, influenced by many factors such as climatic conditions, temperature and the rate of respiration. The malate is degraded by reverse reaction of malate dehydrogenase and malic enzyme (ME). The temperature also influences the degradation of the malic acid, as the enzymes ME and MDH are highly stable and active at temperatures 30oC and higher, where as PEPC is less active at these temperatures (Or et al., 2000).

The concentration of malic and tartaric acid in the cytoplasm and pH of the cytoplasm are maintained constant by transporting excess of these acids into the vacuole which is controlled by channels or transporters on the vacuolar membrane, regulated by a low pH in the cytoplasm (Tilbrook and Tyerman, 2006).

NOTE: This figure is included on page 8 of the print copy of the thesis held in the University of Adelaide Library.

Fig.3: Formation of malic and tartaric acids in Calvin-cycle, Source: (Ruffner et al., 1983).

Both malic and tartaric acid have similar structure except for an additional hydroxyl (-OH) group in tartaric acid (Fig.4) and are synthesised by different biological pathways (Ruffner et al., 1983).

During fermentation the pH influences the natural metabolic chain of events that occur, the wine tastes unpalatable and tasteless at high pH and highly acidulous and sour at very low pH. The process of fermentation and quality of wine is highly influenced by acidity of the grapes (Amerine, 1964).

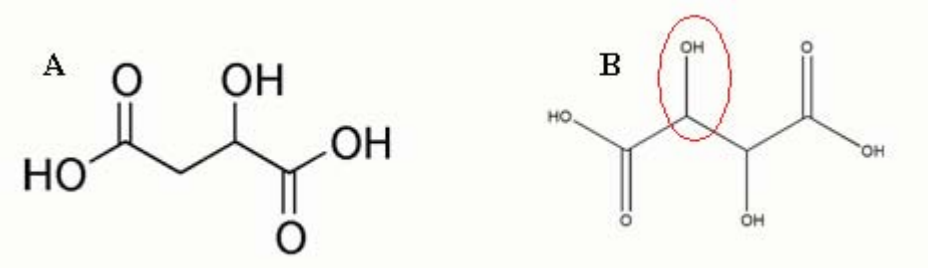


Fig.4: Chemical structure of malic acid (A) and tartaric acid (B).

Enzymes involved in malate metabolism and degradation

Phosphoenolpyruvate carboxylase (PEPC) (Diakou et al., 2000) and malate dehydrogenase (MDH) (Or et al., 2000) are the two enzymes involved in malate synthesis. PEPC is highly expressed in immature berries (1 to 2 week) at preveraison and the enzyme activity decreases at berry veraison stage. The MDH is expressed along the berry development, but its expression levels are low at preveraison and veraison stages, and increases at postveraison stage (Or et al., 2000).

Malic enzyme (ME), MDH reverse reaction (Hawker, 1969) and phosphoenolpyruvate carboxykinase (PEPCK)(Famiani et al., 2007) are involved in malate degradation. Malate degradation occurs in cytoplasm by PEPCK and MDH and as well as in mitochondria by mitochondrial MDH. The expression levels of the malate degrading enzymes is very low at perversion and increases at postveraison reaching maximum at repining stage (Or et al., 2000; Famiani et al., 2007) where sugars are highly accumulated.

Vacuolar membrane transporters

Studies have shown that malate is synthesized and accumulated in the cytoplasm and is transported into the vacuole, mediated by pH gradient (Terrier et al., 2001; Hurth et al., 2005; Martinoia et al., 2007) (Fig.5). Malate transport across the tonoplast been investigated by different methods: flux analysis, gene specific-GFP fluorescent

microscopy, and electrophysiological studies (Hafke et al., 2003; Hurth et al., 2005; Kovermann et al., 2007).

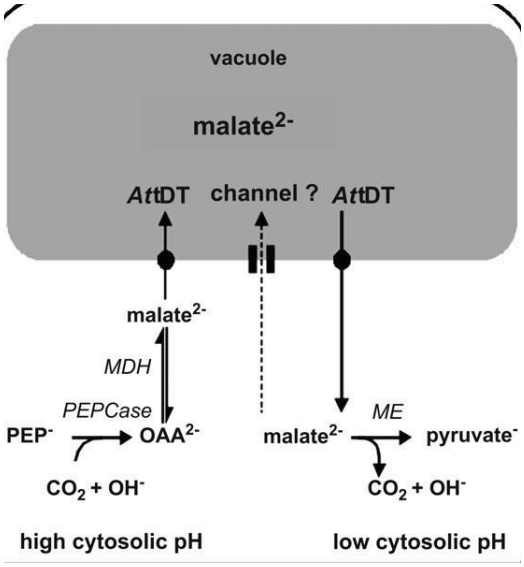


Fig.5: Malate transport across the vacuolar membrane. Source: Martinoia et al., 2007.

Malate plays an important role in cytosolic pH maintenance and most of the cellular malate is localized in the vacuole. Very high concentrations of malate can be stored in the vacuole (>300mM) maintaining a constant volume and pH in the cytoplasm so that it doesn't affect the cellular activities (in cytosol) (Emmerlich et al., 2003). Almost all the functions of the cell occur in the cytoplasm and the concentration of different substrates and pH in the cytosol must be maintained constant for the proper functioning of the cell. Any substrate present in the cytoplasm, nearly 5 times higher of its concentration can be seen in its vacuole due to its large volume in the cell, as a result a strong electropotential gradient exist between the cytoplasm and the vacuole (Martinoia et al., 2007). For transport of any acid from the cytoplasm in to the vacuole requires energy, the pH inside a vacuole is less than 3 to 4 units and the acid exchange across the vacuolar membrane is believed to be driven by electrochemical membrane potential difference (Martinoia et al., 2007).

In *Arabidopsis*, malate exchange between the vacuole and cytoplasm is mediated by *AttDT*, a tonoplast-localized malate transporter (Emmerlich et al., 2003) and *AtALMT9*, a tonoplast localized malate channel (Kovermann et al., 2007). Both *AttDT* (*Arabidopsis thaliana* tonoplast dicarboxylate transporter) and *AtALMT9* (*Arabidopsis thaliana* aluminium activated malate transporter) are expressed in photosynthetically active tissues like leaves and stem.

I. *AttDT (Arabidopsis thaliana* tonoplast dicarboxylate transporter)

The *AttDT* gene is homologous with the sodium/dicarboxylate co-transporter in human kidney and was discovered in *Arabidopsis* and shown to encode a protein that is targeted to the vacuolar membrane (Emmerlich et al., 2003). Northern blot analysis showed that the gene is expressed in photosynthetically active tissues like leaves, stem and floral tissues but not in roots. Increased *AttDT* expression levels were observed, when the cells were exposed to high external malate concentrations demonstrating the connection between malate in cytoplasm and AttDT expression levels (Emmerlich et al., 2003).

Emmerlich et al., (2003) has experimented with two knockout lines of *AttDT* relative to wild type plants when grown under sodium chloride stress there was an increase in the cytosolic malate concentration in all the plants.

The *AttDT* knockout lines also display low vacuolar malate concentration compared to the wild type. Both the wild type and *AttDT* knockouts could take up the exogenous malate indicating the *AttDT* is not primarily localized to plasma membrane (Emmerlich et al., 2003). The *AttDT*-GFP fusion protein was found to be expressed in the vacuolar membrane of the tobacco protoplast (Fig.6)

Fig.6: Subcellular localization of the *AttDT*-GFP fusion protein in transfected Protoplasts, source: (Emmerlich et al., 2003).

Low electric currents were observed in *AttDT* knockout lines when compared to wild type vacuoles. The malate content in the isolated vacuole and in the cytoplasm was low compared to the wild type as determined by Alpha Mannosidase activity (Hurth et al., 2005). The malate transport is a two way system, the malate is not only imported but also exported by the vacuolar membrane (Hurth et al., 2005). More over still some concentration of the acid can be found in the vacuole and also there are traces of electrical conductivity in the *AttDT* knockouts indicating that the *AttDT* is not the sole transporter, and that some other channels may be involved. The *AttDT* transporter couldn't provide evidence as the sole transport of malate (Kovermann et al., 2007).

II. *AtALMT9* (*Arabidopsis thaliana* aluminum activated malate transporter)

AtALMT9 is a member of *AtALMT* gene family (Fig. 7), the function of this gene family is believed to confer aluminum tolerance by exporting malate in to the soil from the roots (Hoekenga et al., 2006; Kovermann et al., 2007) by a malate channel localized in the plasma membrane. The genes in the same family with similar function are

targeted to different locations within the cell. All the genes in this family showed similar gene structure, amino acid sequence and protein function.

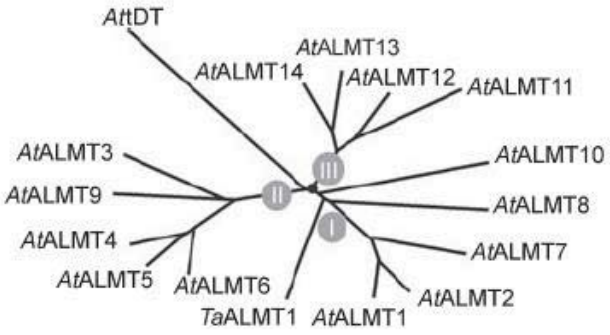


Fig.7: Dendrogram for protein sequence of 15 members of the ALMT gene family and the *Arabidopsis thaliana* tonoplast dicarboxylate transporter (*AttDT*). Source: Kovermann et al., 2007.

The *AtALMT9*-GFP fusion protein was expressed in the vacuolar membrane of onion and *Arabidopsis* epidermal cells (Kovermann et al., 2007) (Fig.8). The electrical conductivity of *AtALMT9* knockouts was decreased compared to the wild type. Over expression of this protein in response to malate in tobacco leaves has increased the vacuolar currents up to 7 fold when incubated with malic acid (Kovermann et al., 2007).

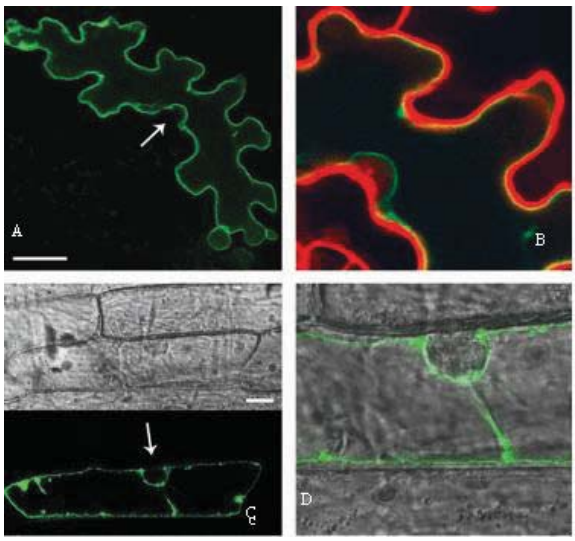


Fig.8: Subcellular localization of *AtALMT9*-GFP in onion and *Arabidopsis* epidermal cells. Soure: Kovermann et al., 2007.

Different acid substrates (fumerate, tartarate, malate and citrate) were tested for vacuolar conductivity. Small electrical conductivity was observed with fumerate and tartarate and chloride when compared to malic acid (Kovermann et al., 2007). The channel forming activity of *AtALMT9* protein was determined by expressing in *Xenopus* oocytes.

Summary

Acids in grape berries are very important for commercial fermentation and wine making. Many factors are affecting the acidity of these berries. Acids are formed during the developmental stages of the berry and can easily be broken down and utilized as a respiratory substrate, if they are present in the cytoplasm and also high acid concentration in the cytosol affects the cell stability and leads to death, to neutralize this affect the acids are relocated in to the vacuole and stored. This storage is good for both the acid and the cell. The acid concentration in the vacuole is 7 times higher than cytosol and pH is also very low. These acids are stored without affecting the cytosol and the cellular metabolism.

The vacuolar membrane is a very complex network, which is highly regulated and energy dependent, consisting of a network of transport channels. The recently investigated *AtALMT9* which is a malate channel on the vacuolar membrane in *Arabidopsis thaliana* (Kovermann et al., 2007). Similar malate transporters of the same family are also reported to be in wheat and *Brassica* (Kovermann et al., 2007). We are investigating with similar genes that also exist in the grapevine (*Vitis vinifera*). Acids can be stored in the vacuole and these acids are safer in the vacuoles from metabolism and respiration than in the cytoplasm (Martinoia et al., 2007). If more acids are stored into the vacuole, we can get berries with more acids at harvest.

References

Amerine MA (1964) Acids, grapes, wines and people. American Journal Of Enology And Viticulture **15:** 106-115

Amerine MA, Winkler AJ (1942) Maturity studies with California grapes. II. Titratable acidity, pH, and organic acid content. Proceedings of the American Society for Horticultural Science **40:** 313-324

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucl. Acids Res. **31:** 3497-3500

Conde C, Silva P, Fontes N, Dias ACP, Tavares RM, Sousa MJo, Agasse A, Delrot S, GerÃ³s H (2007) Biochemical changes throughout grape berry development and fruit and wine quality. *In*. Global Science Books

Diakou P, Svanella L, Raymond P, Gaudillere JP, Moing A (2000) Phosphoenolpyruvate carboxylase during grape berry development: protein level, enzyme activity and regulation. Australian Journal of Plant Physiology **27:** 221-229

Emmanuel D, Benjamin DG, Peter RR (2007) The roles of organic anion permeases in aluminium resistance and mineral nutrition. Febs Letters **581:** 2255-2262

Emmerlich V, Linka N, Reinhold T, Hurth MA, Traub M, Martinoia E, Neuhaus HE (2003) The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. Proceedings of the National Academy of Sciences of the USA **100:** 11122-11126

Famiani F, Casulli V, Proietti P, Walker RP, Battistelli A (2007) organic acid metabolism in grape: role of phosphoenolpyruvate carboxykinase. ISHS Acta Horticulturae **754:** 599-602

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

Hawker JS (1969) Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry **8:** 19-23

Hayes MA, Davies C, Dry IB (2007) Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: differential roles in sink and source tissues. Journal of experimental botany **58:** 1985-1997

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 of the USA **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

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. The Plant Journal **52:** 1169-1180

Lakso AN, Kliewer WM (1975) The Influence of Temperature on Malic Acid Metabolism in Grape Berries: I. Enzyme Responses. Plant Physiol. **56:** 370-372

Lakso AN, Kliewer WM (1978) The Influence of Temperature on Malic Acid Metabolism in Grape Berries. II. Temperature Responses of Net Dark CO2 Fixation and Malic Acid Pools. Am. J. Enol. Vitic. **29:** 145-149

Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J. Exp. Bot. **58:** 83-102

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

Or E, Baybik J, Sadka A, Saks Y (2000) Isolation of mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxylase cDNA clones from grape berries and analysis of their expression pattern throughout berry development. Journal Of Plant Physiology **157:** 527-534

Ruffner HP, Brem S, Malipiero U (1983) The physiology of acid metabolism in grape berry ripening. Acta Horticulturae **139:** 123-128

Ruffner HP, Hawker JS, Hale CR (1976) Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15:** 1877-1880

Sambrook J, Russel DW (2000) Molecular Cloning: A Laboratory Manual third edition.Cold Spring Harbour Laboratory press.

Schulze J, Tesfaye M, Litjens R, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate Plays a Central Role in Plant Nutrition. Plant and Soil **247:** 133-139

Schulze J, Tesfaye M, Litjens RHMG, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate plays a central role in plant nutrition. Plant and Soil **247:** 133-139

Terrier N, Deguilloux C, Sauvage F-X, Martinoia E, Romieu C (1998) Proton pumps and anion transport in Vitis vinifera: The inorganic pyrophosphatase plays a predominant role in the energization of the tonoplast. Plant Physiology and Biochemistry **36:** 367-377

Terrier N, Romieu C (2001) Grape Berry Acidity. *In* KA Roubelakis-Angelakis, ed, Molecular Biology and Biotechnology of the Grapevine. Kluwer Academic, Netherlands, pp 35-57

Terrier N, Sauvage F-X, Ageorges A, Romieu C (2001) Changes in acidity and in proton transport at the tonoplast of grape berries during development. Planta **213:** 20-28

Tilbrook J, Tyerman S (2006) Water, Sugar and Acid: How and Where They Come and Go During Berry Ripening. *In* D Oag, K DeGaris, S Partridge, C Dundon, M Francis, R Johnstone, R Hamilton, eds, ASVO Proceedings - Finishing the Job, Mildura, pp 4-10

Identification and localization of vacuolar organic acid carriers in grapevine berries.

Jayachandra Rongala, and Matthew Hayes

School of Agriculture, Food and Wine, University of Adelaide, Plant Research Centre, Wait campus, Urrbrae, South Australia 5064, Australia.

Corresponding author:

Dr. Matthew Hayes
Postdoctoral Fellow
University of Adelaide
Plant Research Centre,
School of Agriculture, Food and Wine
Discipline of Wine and Horticulture
Wait campus
Tel:+61 8 83036665
Matthew.hayes@adelaide.edu.au

Key Words: Malate transport, ALMT9, vacuolar

Total word count: 4473, Abstract:156, Introduction:584, Results:572, Discussion:467, Conclusion: 108, Materials and Methods:513, References:723.

Abstract

Two malate transporters, *AttDT* and *AtALMT9*, have been identified and studied in *Arabidopsis thaliana*; these genes are targeted to vacuolar membrane and function as malate transporters across it. Individual knockout lines of *AttDT* and *AtALMT9* show reduced malate transport across the membranes. In the present study we are investigating four AtALMT9-like proteins encoded by the *Vitis vinifera* genome. Three of these proteins were shown by confocal analysis of Green fluorescent protein (GFP) fusion to be targeted to the vacuolar membrane. Quantitative RT-PCR analysis showed that all four of the genes are developmentally regulated during grape berry development and are expressed in co-ordination with biosynthesis and vacuolar storage of organic acids and during the later phase of ripening when berry malic acid concentration declines. The progeny from a cross between *AttDT* and *AtALMT9* knockout plants were severely stunted and did not produce seed under our growth conditions, highlighting the importance of the vacuolar storage of organic acids.

Introduction

Grape berry acidity is a major determinant of berry quality, as it contributes to taste and mouthfeel, and is also essential during fermentation to prevent spoilage and assists bottle stability (Amerine, 1964; Conde et al., 2007). The Australian wine industry spends approximately $30 million anually on adding extra acids during fermentation, so identification of ways to increase acid content in fruit is of great economic importance.

Malic and tartaric acids are the major sources of berry acidity and are very similar in structure except for an additional –OH group in tartaric acid (Fig. 1).

Synthesis and accumulation of malic acid takes place during the preveraison developmental stages of the grape berry (Fig. 2) as part of the Glycolysis in the cytoplasm through the enzymatic activity of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH); it is subsequently stored in the vacuole of the cell as mediated by the pH gradient in the cytoplasm (Amerine, 1964; Terrier and Romieu, 2001; Schulze et al., 2002; Tilbrook and Tyerman, 2006; Martinoia et al., 2007). As the berry develops the concentration of malic acid increases and as the berry ripens the malic acid concentrations decrease (Ruffner et al., 1983; Terrier and Romieu, 2001; Conde et al., 2007) due to the drop in the enzyme activity of PEPC and associated increase of derivative enzymes malic enzyme (ME) and MDH (Hawker, 1969; Diakou et al., 2000; Famiani et al., 2007), furthermore the activity of PEPC is decreased at high temperature, whereas MDH and ME are highly stable and active (Amerine and Winkler, 1942; Amerine, 1964; Lakso and Kliewer, 1975; Ruffner et al., 1976; Lakso and Kliewer, 1978; Terrier et al., 1998). The final organic acid content of the berries depends on the amount of acid synthesized, stored in the vacuole and degraded during the ripening stages.

Two different tonoplast-localised transporters of dicarboxylic acids have been identified in *Arabidopsis thaliana*, a tonoplast localized malate transporter, AttDt (Emmerlich et al., 2003) and a tonoplast localized malate channel, AtALMT9 (Kovermann et al., 2007). Acidification of the cytosol induces expression of *AttDT* in *A. thaliana*; in mesophyl protoplasts isolated from *AttDT* knockout lines, reduced malate currents were observed when compared to wild type protoplasts (Hurth et al., 2005; Emmanuel et al., 2007). Similar studies with *AtALMT9* overexpressed in *Nicotiana benthamiana* leaves showed enhanced malate currents across the vacuole, while expression in *Xenopus* oocytes also showed similar currents (Kovermann et al.,

2007). Reduced malate content was observed in *A. thaliana* knockout lines of *AttDt* (~80% reduced from wild type) and *AtALMT9* (~20% reduced from wild type) (Emmerlich et al., 2003; Hurth et al., 2005; Kovermann et al., 2007), but knockout of either gene does not completely abolish vacuolar malate storage. Potentially, concurrent knockout of *AttDT* and *AtALMT9* may completely abolish vacuolar malate storage; however, this has not yet been tested.

We hypothesize that *ALMT9*-like genes may be present in *Vitis vinifera,* blast analysis of the *V. vinifera* genome using AtALMT9 amino acid sequence identified four predicted proteins that show up to 70% amino acid identity with AtALMT9 (Accession numbers Table 1) (Fig. 3). A single *AttDT* homologue has been identified in *Vitis vinifera* and is currently being studied (Matthew A. Hayes, unpublished data). In the present study we constructed GFP fusion proteins with all four grapevine *AtALMT9* homologues and showed localization in plant cells. Expression analysis by Quantitative RT-PCR on a grape berry development series was also completed and a cross was made to produce an *AttDT* and *AtALMT9* double knockout in *A. thaliana* (both *AttDt* and *AtALMT9* knockout).

Results

Blast analysis of the AtALMT9 protein sequence with the *V. vinifera* genome revealed four genes (Fig. 3) by NCBI database (table:1), which showed similarity with the *A. thaliana* malate transporter (Fig. 4).

Quantitative RT-PCR analysis of gene expression during berry development

The expression of the grape *ALMT9*-like genes was quantified from berry samples collected after flowering, with one week intervals up to 12 weeks of berry

development. The fresh weight (Fig. 5A), the sugar content (Fig. 5B) and malic acid levels (Fig. 6) of the berry samples were measured and showed similar results to previously published data (Diakou et al., 2000; Terrier et al., 2001; Tilbrook and Tyerman, 2006; Conde et al., 2007). Fig 7 shows the expression of each individual gene. All four genes are developmentally regulated, and overall are expressed more post veraison than during the early stages of development. VvALMT9:1 (Fig. 7A) and VvALMT9:2 (Fig. 7B) in particular showed post veraison expression, while VvALMT9:3 (Fig. 7C) is expressed at highest levels pre-veraison and VvALMT9:4 (Fig. 7D) was, in general, expressed at relatively low levels.

Sub cellular localization of VvALMT9 proteins

To determine the localization of the proteins, the VvALMT9 open reading frame (ORF) was sub cloned into PN`GFP (T Franks, University of Adelaide, unpublished data; Hayes et al., 2007) to produce a VvALMT9-like protein-GFP fusions (supplementary materials), driven by the cauliflower mosaic virus (CaMV) 35S promoter. AttDT:GFP and AtALMT9:GFP (vacuolar membrane), VvHT4:GFP (hexose transporter, plasma membrane) and free GFP were used as controls. The constructs were bombarded into onion epidermal cells and the localization was determined by confocal microscopy. Free GFP was observed to be expressed throughout the cell including nuclei (Fig. 8A). VvHT4:GFP was localized to the periphery of the cell consistent with its previously described plasma membrane localisation (Fig. 8B; Hayes et al 2007). Controls AttDT and AtALMT9 were also observed at the cell periphery like VvHT4, however at the nucleus, GFP was clearly seen to invaginate around the inside of the nucleus (Fig. 8). This observation is consistent with vacuolar localisation as described by other investigators (Endler et al., 2006; Koverman et al., 2008). The GFP

fusions to VvALMT9:1, VvALMT9:2 and VvALMT9:4 (Fig. 8C, 8D and 8E) clearly showed localisation to the cell periphery and around the inner edge of the nucleus, suggesting tonoplast localisation.

Double knockout (DKO) production

The knockout lines of AtALMT9 and AttDT were successfully crossed and the progeny were screened for heterozygous knockouts (Emmerlich et al., 2003; Kovermann et al., 2007). The plants were screened by PCR for a 1,360bp PCR product (KO alleles) using the T-DNA left border primer JL202 and the AttDT specific primer AttDT1 on genomic DNA for AttDT1 crossed plants along with a 985bp band amplified with the ALMT9 T-DNA specific primer LBb1and the *ALMT9* gene specific primer AtALMT9.5' UTR. The heterozygous plants contained both these positive allels along with the presence of the wild type *AttDT* and *AtALMT9* gene bands from the opposite parent (Figs. 9 & 10). The double heterozygous plants were allowed to self fertilize and the collected seeds were grown and screened for double knockouts. Some seeds did not germinate or plants died before the DNA extraction; a total of 107 seeds were screened for DKO and six were positive, with the ratio of DKO:heterozygous was 1:17, DKO plants germinated well in soil, but were stunted, with wrinkly and wilted appearance. At approximately the six leaf stage the plants dried up, leading to death just prior to flowering (Fig. 11).

Discussion

In the present study *Arabidopsis thaliana* ALMT9 like malate transporters in *Vitis vinifera* have been identified cloned and their putative localizations are reported. The dendrogram based on the amino acid sequence alignment (Chenna et al., 2003) of

VvALMT9 with the AtALMT protein family has shown that all the proteins VvALMT9:1, VvALMT9:2, VvALMT9:3 and VvALMT9:4 belong to the clade II (Fig. 12). The clade 1 of the family includes the aluminium activated malate transporter localized to the plasma membrane of root cells, the clade 2 proteins are localised to different organelles of the plant cell but function in malate transport (Kovermann et al., 2007).

Localization

In order to test out hypothesis we have expressed the GFP constructs in onion and tobacco epidermal cells. The transient expression of the constructs is localized to the tonoplast membrane innerto the nucleus separating the vacuolar membrane from the plasma membrane which is outerto the nucleus. The constructs expressed visible inner membrane florescence at the nucleus, indicating the genes are targeted to the vacuolar membrane, as previouly described by Emmerlich et al., (2003) and Kovermann et al., (2007).

Gene expression and regulation during malic acid metabolism

Many studies have demonstrated that malic acid is synthesised and accumulated in the vacuole of berry pulp cells during the preveraison stages of berry development by the activity of two enzymes phosphoenolpyruvate and malate dehydrogenase till the onset of ripening (Amerine, 1964; Diakou et al., 2000). The expression of the *VvALMT9* like genes coincides with the expression of the malate synthesising enzyme (Famiani et al., 2007); the expression level of these enzymes increases during the early berry development, when most of the PEPC and MDH are expressed and malic acid is synthesised (Diakou et al., 2000). During ripening of the berries, stored malate is transported back and degraded in the cytoplasm or in mitochondria by malic enzyme or

MDH (Diakou et al., 2000; Or et al., 2000). At the same time the expression levels of the *VvALMT9*-like genes is high, which again coincides with expression levels of malate dehydrogenase and malic enzyme (rapidly increased) which are responsible for malate degradation (Hawker, 1969; Diakou et al., 2000; Or et al., 2000).

Double knockouts

AttDT and *AtALMT9* are responsible for the major malate exchange through the vacuole in Arabidopsis. The single knockout plants of *AttDT* and *AtALMT9* knockedout mutants did not show a visible phenotypic difference to the wild type controles, this may be because they still had the other gene, which was actively expressed and transport malate into the vacuole controlling the cytosoplasmic pH. The knockout double mutant plants germinated and grew well in the early stages as there was little malic acid synthesis, but as the growth continued more and more malic acid was accumulated in the cytoplasm, possibly obstructing the other cellular metabolisms and ultimately leading to death of the plants.

Conclusion

Acids play very important roles in grape berries, and also in wine making during and after fermentation. Malic acid is targeted to different cell organelles, and is involved in many cellular processes (Schulze et al., 2002) circulating around the cell (Fig 13). The present molecular studies have shown that *VvALMT9*- like genes identified in grapevine are expressed in relation to the acid metabolism, degradation occurs and are localised to the tonoplast membrane of the plant cells. These proteins are good candidates for putative malate transporters in grape barriers. We are presently investigating the functional activity of the genes in *Xenopus* oocytes with different acids, which will confirm our prediction the role of these genes in acid transport.

Materials and methods

Construction of GFP fusion proteins for subcellular localization

The full length open reading frames were amplified using primers (Table. 2) from grape leaf cDNA using Platinum Taq High Fidelity DNA polymerase (Invitrogen) as per manufacturer's instructions. PCR products were ligated into pDRIVE (supplementary materials) using the Qiagen PCR Cloning Kit, as per manufacturer's protocol, and transformed by heat shock (Sambrook and Russel, 2000) into chemically competent DH5α cells. PCR products were DNA sequenced using M13 primers. To assist sub-cloning from pDRIVE into PN`GFP (T Franks, University of Adelaide, unpublished data; Hayes et al., 2007) and to allow translational fusion between ALMT9-like genes and GFP, forward primers included an *Xho*I restriction site, while reverse primers included an *Xba*I restriction site that also replaced the stop codon except for Grape VvALMT9:1which has *Xbal*I sites on both primers.

Subcellular localisation in onion epidermal cells

GFP fusion constructs were transformed by biolistics into onion epidermal cells using standard methods (Hayes et al., 2007). GFP fusion proteins were visualized using a LEICA Zeiss LSM5, Confocal Laser Scanning Microscope. Propidium Iodide was used to stain cell walls and nuclei for 15 to 20min with .01% of Tween20 (for nuclear staining). AttDT and AtALMT9 (vacuolar membrane, Emmerlich et al., 2003 and Kovermann et al., 2007), VvHT4 (plasma membrane; Hayes et al., 2007) and free GFP were used as controls.

Quantitative analysis of gene expression by real time PCR analysis

Gene expression analysis was carried using a Syber Geen super mix and BioRad iCyclar. Specificity of the primers (Table 4) used was determined by agarose gel electrophoresis, melt curve analysis and DNA sequencing of the cloned PCR products. Data were analysed using Q-Gene software and methods (Muller et al., 2002).

Plasmid preparation and sequencing analysis

Plasmids were isolated using a GenElute Plasmid Miniprep Kit (Sigma) as per manufacturer's instructions and sequencing was performed using 200ng of purified DNA/plasmid, 6.4pmoles of primer, 1µl of BigDye Terminator 3, 4 µl of 5x BDT dilution buffer in 20 µl volume using a PCR cycle of: 96^{o}C-1min, 96 ^{o}C-10sec, 50 ^{o}C-5sec, 60 ^{o}C-4min, repeated 27 cycles. Sequencing reactions were analysed by AGRF (www.agrf.org.au) and results analysed using Chroms Lite (Technelysium Pty Ltd) and Vector NTI Advanced (Invitrogen) (see supplementary material).

A. thaliana double knockout production

The *AtALMT9* and *AttDT* homozygous knockout Arabidopsis lines were provided by Stefan Meyer, University of Zurich, Switzerland and Ekkenhard Neuhaus, University of Kaiserslautern, Germany, respectively. The plants were grown under controlled conditions and were crossed at the early stages of flowering and the progeny were screened by PCR using the primers as described (Emmerlich et al., 2003; Kovermann et al., 2007). Several double heterozygous lines were grown and allowed to self fertilize. DNA was extracted from leaf by grinding the tissue directly in a 1.5mL micro centrifuge tube with 500µL of extraction buffer containing final concentrations 2% Cetyl trimethylammonium bromide (CTAB), 1mM EDTA, 0.1M NaCl, 0.1M Tris

and 0.2% Mercaptoethanol and then incubated at 65^{o}C for 30min. Organic contaminants were removed by phenol:chloroform extraction, DNA precipitated using 500µL isoproponal and pelleted by centrifugation at 13000 rpm. DNA was resuspended in 50µL nuclease-free water and used for PCR screening.

TABLES AND FIGURES

Table 1: General properties of the grape *ALMT9*-like genes.

Name	Accession no. gene	Accession no. protein	cDNA length (base pairs)	Protein (amino acids)	Chromosome no.
VvALMT9:1	CU459233	CAO68043	1694	567	17
VvALMT9:2	CU459349	CAO65881	1608	535	2
VvALMT9:3	AM482812	CAN68659	1680	559	1
VvALMT9:4	CU459222	CAO42119	1596	531	1

Table 2: Full length PCR primers sequence for amplification of *AtALMT9* like ORF from grape, the restriction sites *Xbal*1 and *Xho*1 were introduced (underlines) into the ORF.

Name	Primer sequence
VvALMT9:1	F: CTC GAG CCC TCT AGA AAT GAC CGC GAA AC R: TCT AGA CAA CTT CAG CCA CCT GCG CAA
VvALMT9:2	F: CTCGAGTTGTAGTAGTTTATTGGAGAATGAATGG R:TCTAGATAGGCTCTCATGGTTAAACTTGGCCATCAC
VvALMT9:3	F: CTC GAG ATG GCA GCT AAA GTC GGA TCG TTT C R: TCT AGA ATT GTT TAA CCC GAA ACA CTG ACG CAG
VvALMT9:4	F: CTCGAGGAAGGAAGA AATGGTAATGAAAATGGTGA R:TCTAGACCTGGGTTTTCTAAATTCTGCTTTCTCACTTGA

Table 3: Primers for Q-PCR quantification.

Name	QRT-PCT Primer sequence
VvALMT9:1	F: TCA TCT GAT GCT AAG GCT GAG CC R: CTA CAA CTT CAG CCA CCT GCG
VvALMT9:2	F:CGC CTT TGA AGA AG CGG TGG R:ACT TGG CCA TCA CTG CAA GCT G
VvALMT9:3	F: GAT TTG GTG CTC AAC GAA CAG G R: CTT TTG GAG CTG GAA GGT CCG
VvALMT9:4	F:CGT GTT TAG GAA ACA ATC CCC TTG R:CTC ACT TAG TTC TTG GAA GGA GTC

Table 4: Gateway primers for TOPO cloning, the gateway cloning sites (underlined) introduced into the ORF.

Name	Gateway Primer sequence
VvALMT9:1	F:CACCATGACCGCGAAACTTGGGTC R:CTACAACTTCAGCCACCTGC
VvALMT9:2	F:CACCATGAATGGAAAAAAGGGTAGTTATGAG R:TTATAGGCTCTCATGGTTAAACTTGG
VvALMT9:3	F: CACCATGGCAGCTAAAGTCGGATCG R: TCAATTGTTTAACCCGAAACACTGAC
VvALMT9:4	F:CACCATGAAAATGGTGATGTTCAATAATGGTGGTT R:TCACCTGGGTTTTCTAAATTCTGC

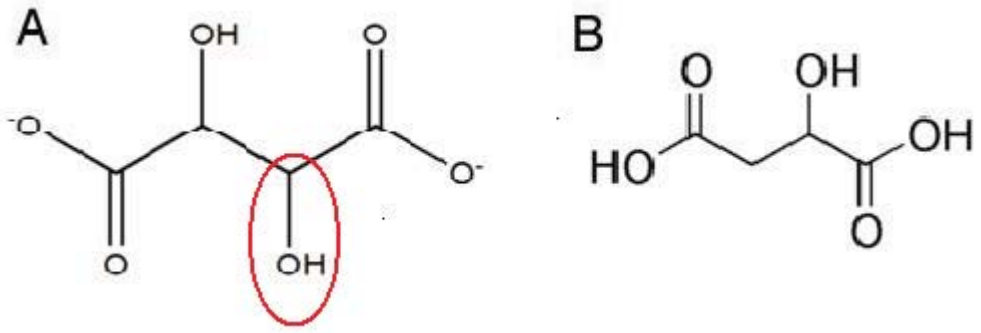


Fig. 1. Structure of (A) tartaric acid and (B) malic acid. Tartaric acid having an additional -OH group, both the acids have distint biosynthetic pathways and degradative pathways.

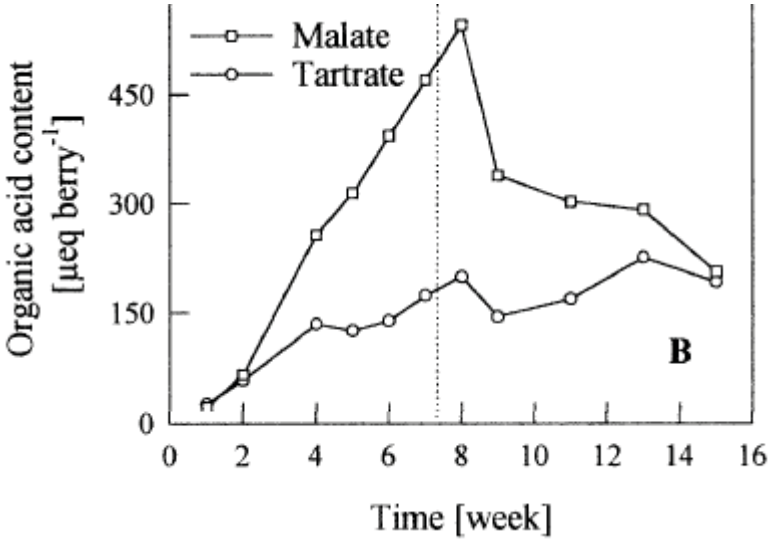


Fig. 2: Developmental accumulation of malic and tartaric acids in grape berries. The graph shows an increase in the malic acid content during berry developmant and decrease in concentration during ripening, but the tartaric acid content remains constant along the ripening stage. Source: (Terrier et al., 2001).

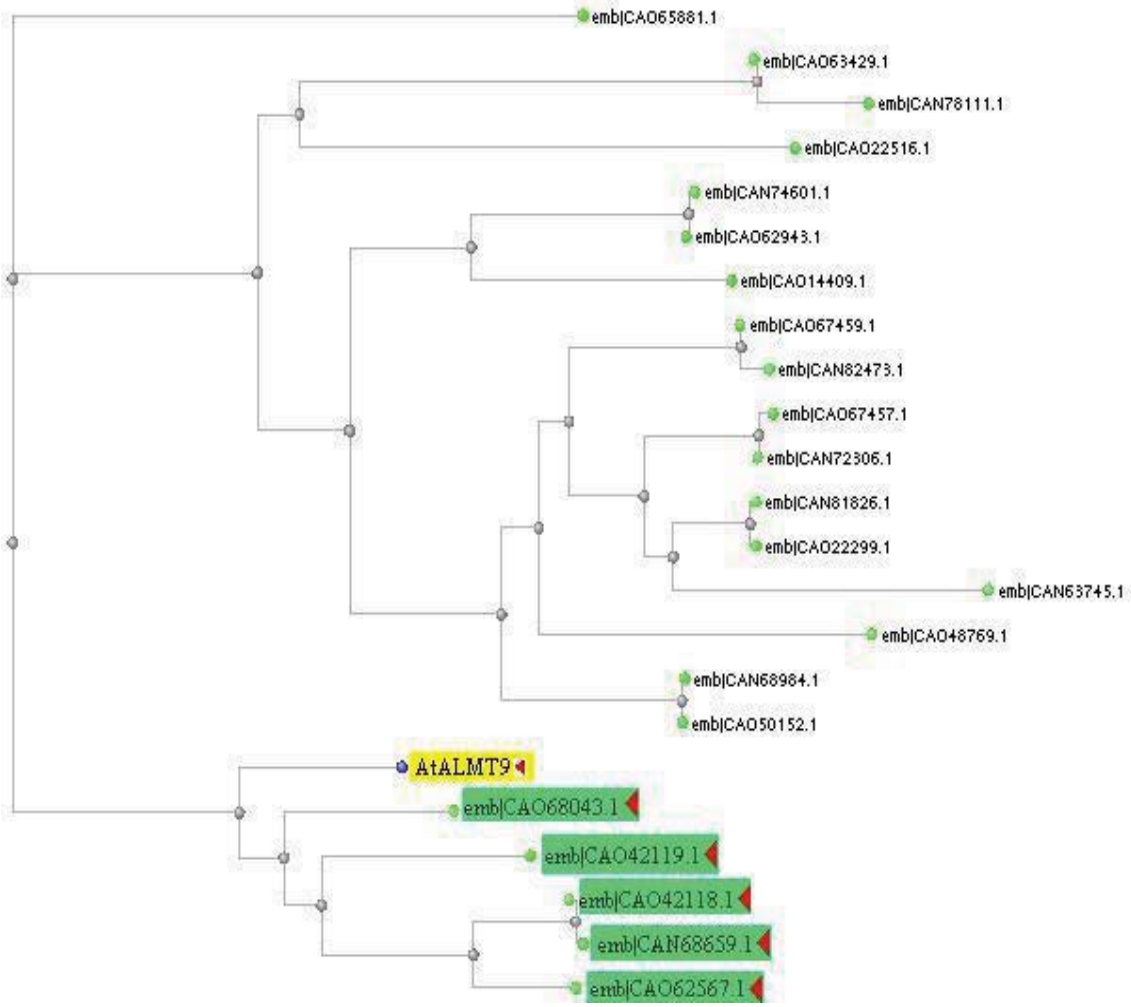


Fig. 3: Blast analysis of AtALMT9 with *Vitis vinifera* the four proteins showed sequential similarity to AtALMT9.

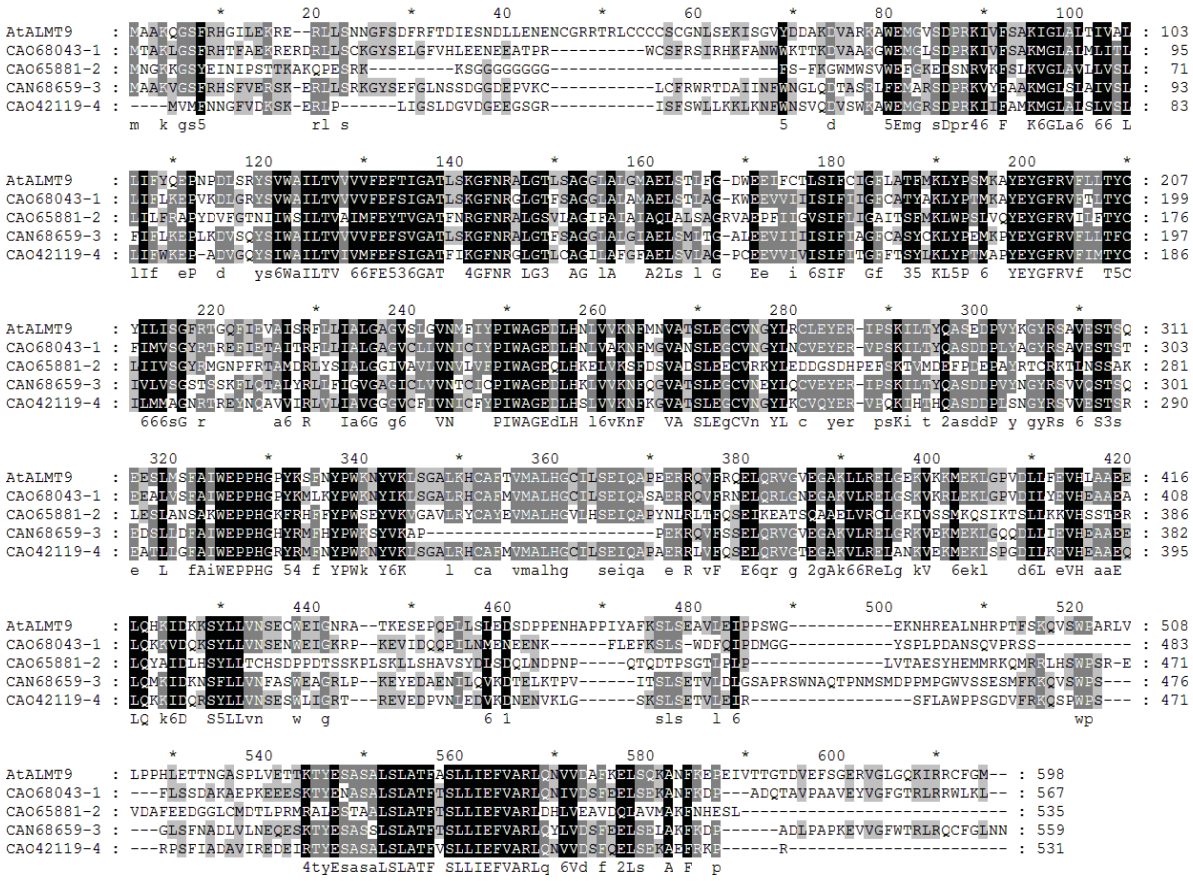


Fig. 4: The Amino acid sequence of AtALMT9 compared with *V. vinifera* ALMT9 like proteins by Gene Doc. This represents the matches and the gaps in the alignment.

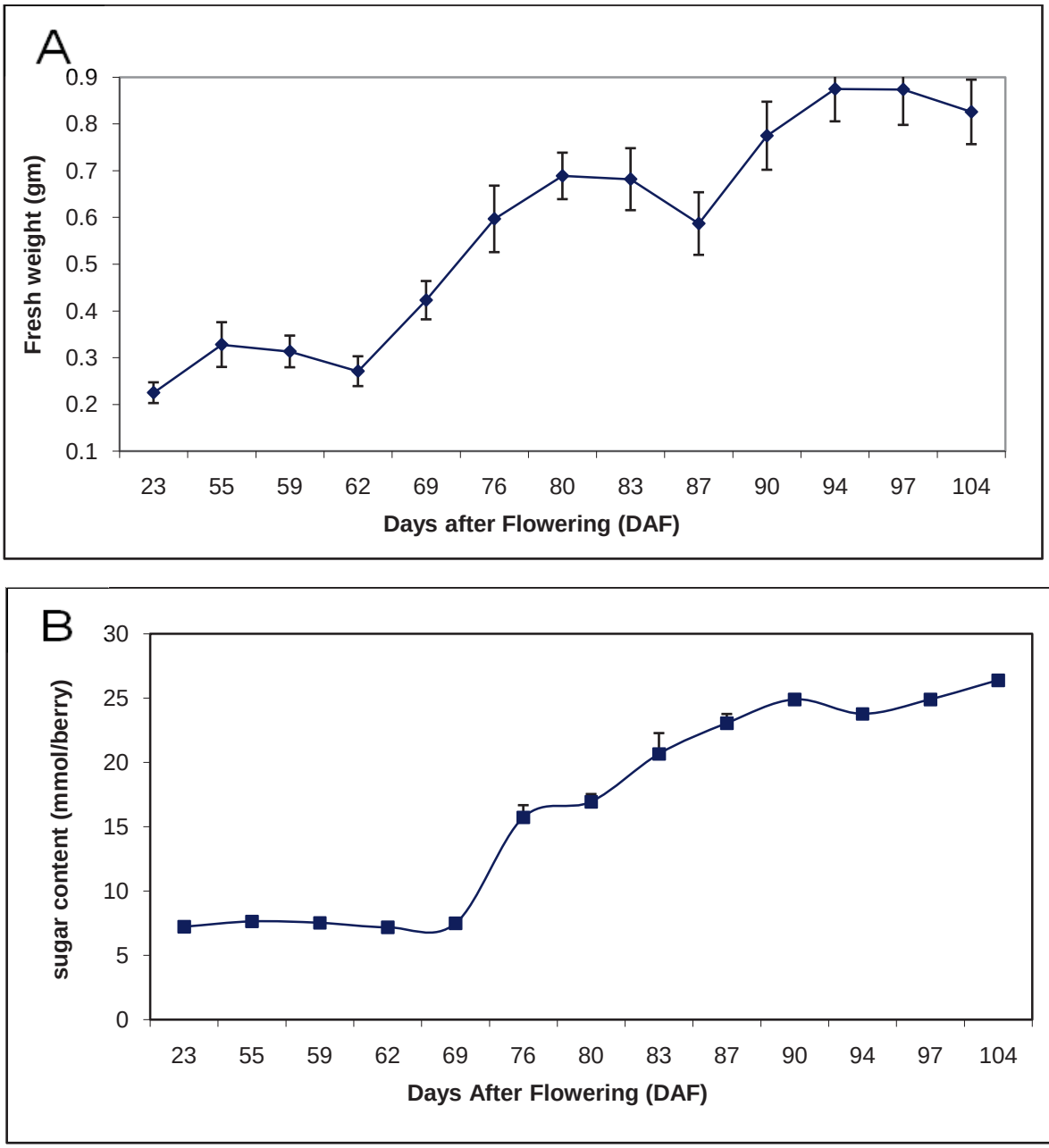


Fig. 5: Changes in physical and chemical parameters during grape berry development, A. Fresh weight of the berries and B. Total Sugar content of the berries.

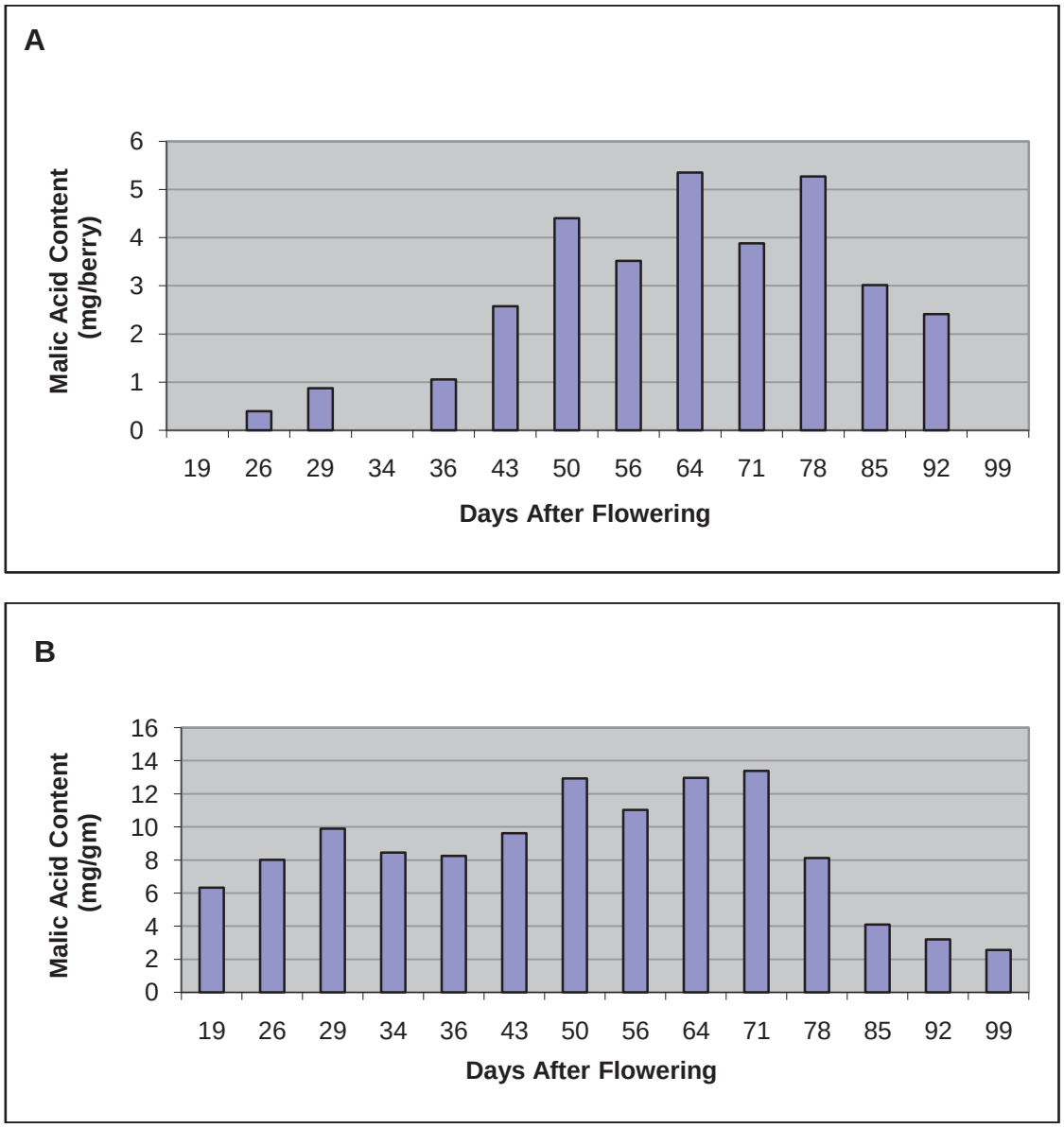
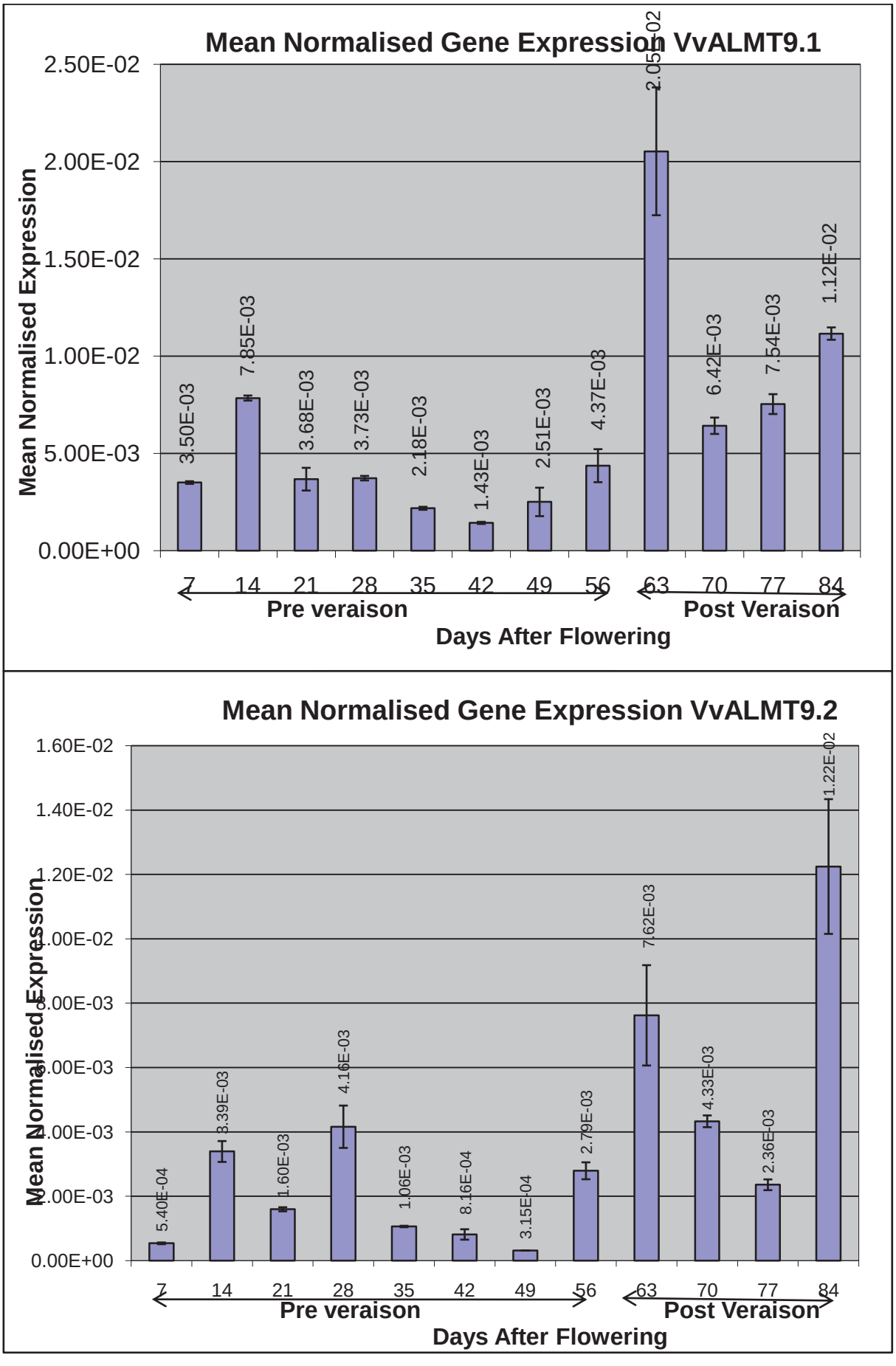


Fig. 6: Malic acid content of the berries A. Malic acid content per berry and B. Malic acid content per gram weight of the berry. The acid content of berries shows an increase at the pre veraison and gradual decrease at post veraison


Mean Normalised Gene Expression VvALMT9.1
Mean Normalised Expression
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
3.50E-03
7.85E-03
3.68E-03
3.73E-03
2.18E-03
1.43E-03
2.51E-03
4.37E-03
2.05E-02
6.42E-03
7.54E-03
1.12E-02
7 14 21 28 35 42 49 56 63 70 77 84
Pre veraison
Post Veraison
Days After Flowering
Mean Normalised Gene Expression VvALMT9.2
Mean Normalised Expression
1.60E-02
1.40E-02
1.20E-02
5.40E-04
3.39E-03
1.60E-03
4.16E-03
1.06E-03
8.16E-04
3.15E-04
2.79E-03
7.62E-03
4.33E-03
2.36E-03
1.22E-02
7 14 21 28 35 42 49 56 63 70 77 84
Pre veraison
Post Veraison
Days After Flowering

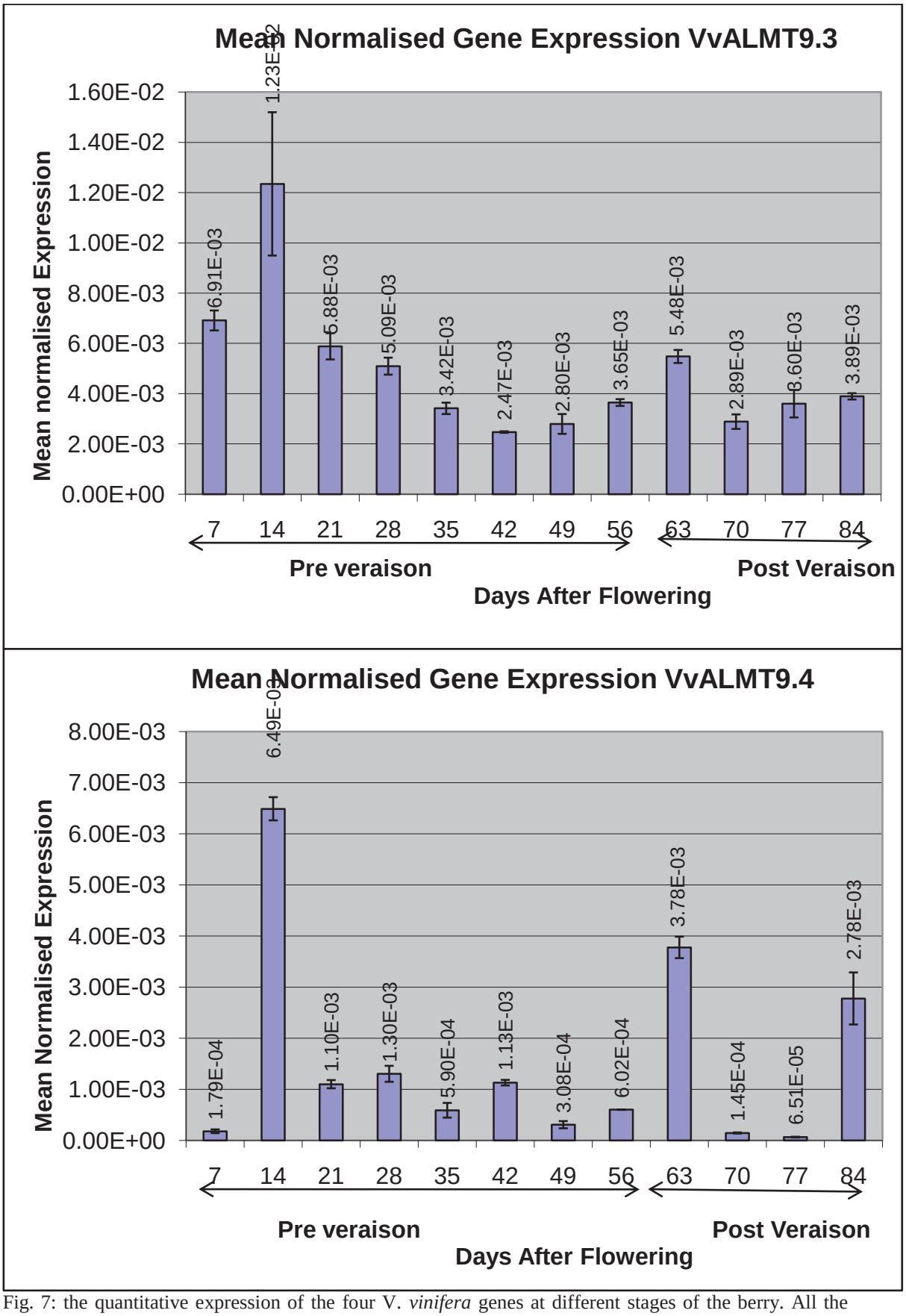


Fig. 7: the quantitative expression of the four V. *vinifera* genes at different stages of the berry. All the genes showed significant expression throughout the berry development. VvALMT9:1 and VvALMT9:2 showed low expressions during preveraison than postveraison (A & B), VvALMT9:3 showed low expression at postveraison (C) and VvALMT9:4 showed comparatively less expression.

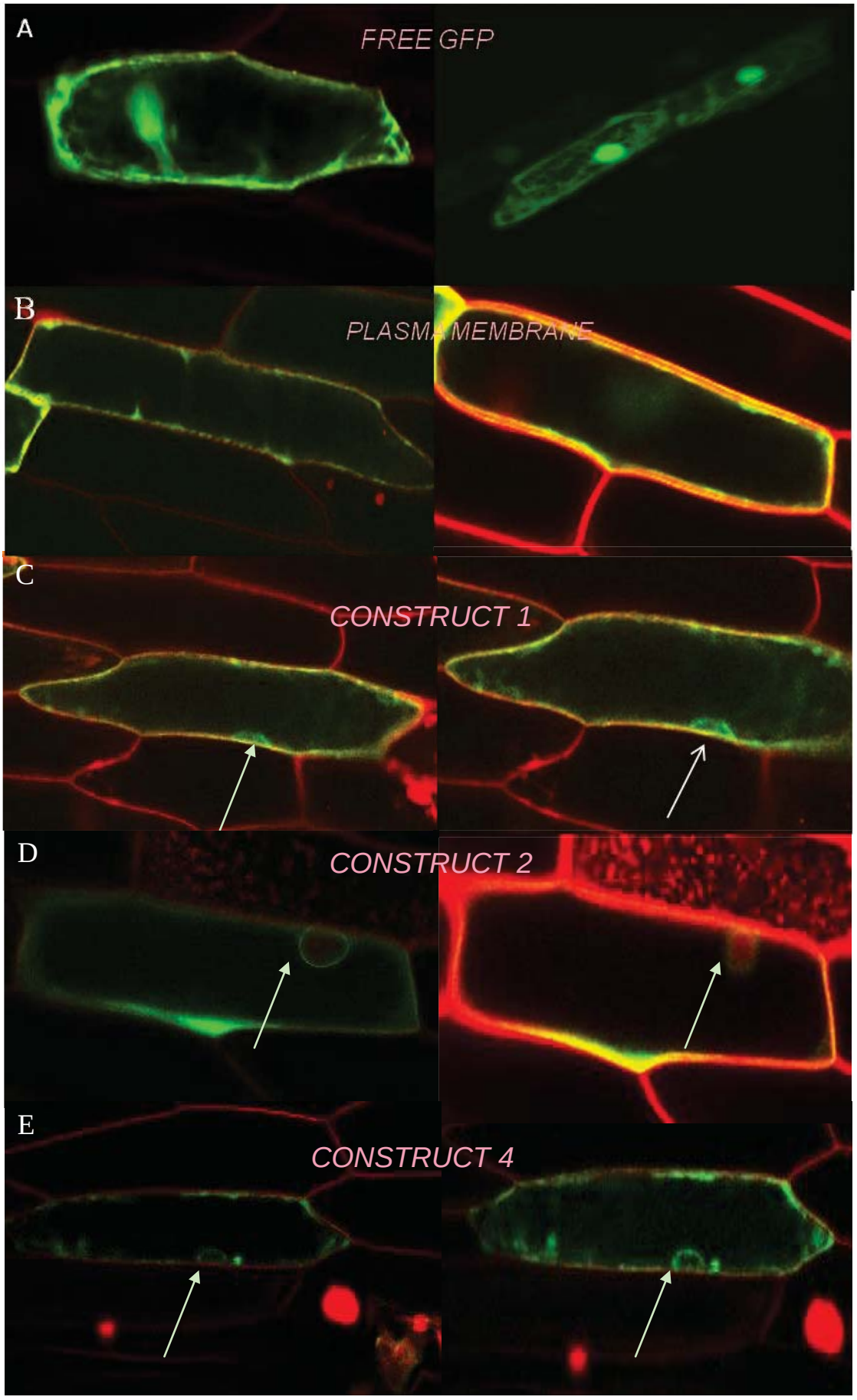


Fig. 8: GFP localisation A. Free GFP being expressed all over the cell, B. Plasma membrane controls the

constructs C.VvALMT9:1, D.VvALMT9:2 and E.VvALMT9:4 were expressed in onion epidermal cells using CaMV 35s promoter by biolistic transformation. The protein is targeted to the tonoplast separated by nucli from the outer membrane (indicated by arrows). The GFP clearly surrounds the nucleus of the cells indicating the expression in tonoplast membrane. The cell walls and the nuclei are stained red with propidium iodode.

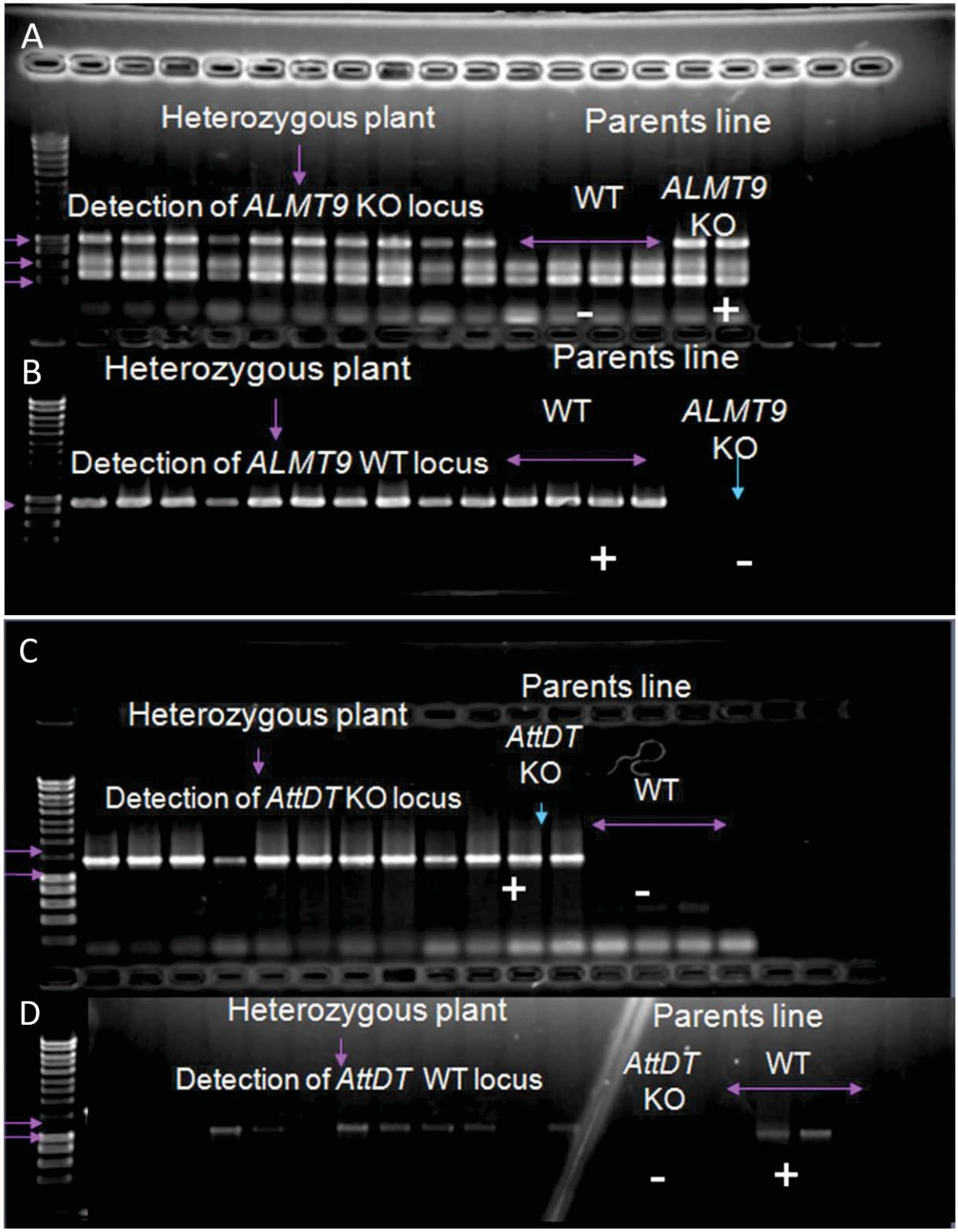


Fig. 9: *A. thaliana* double knock out screening of heterozygous plants F1 generation all crossed with *AttDT1* and *AtALMT9*, the last 6 bands are the controls (*AttDT1*, *AttDT2* and *AtALMT9*) in each gel, A. *ALMT9* T-DNA insert amplified band, B. *ALMT9* wild type gene amplified band, C. *AttDT* T-DNA insert amplified band and D. *AttDT* wild type gene.

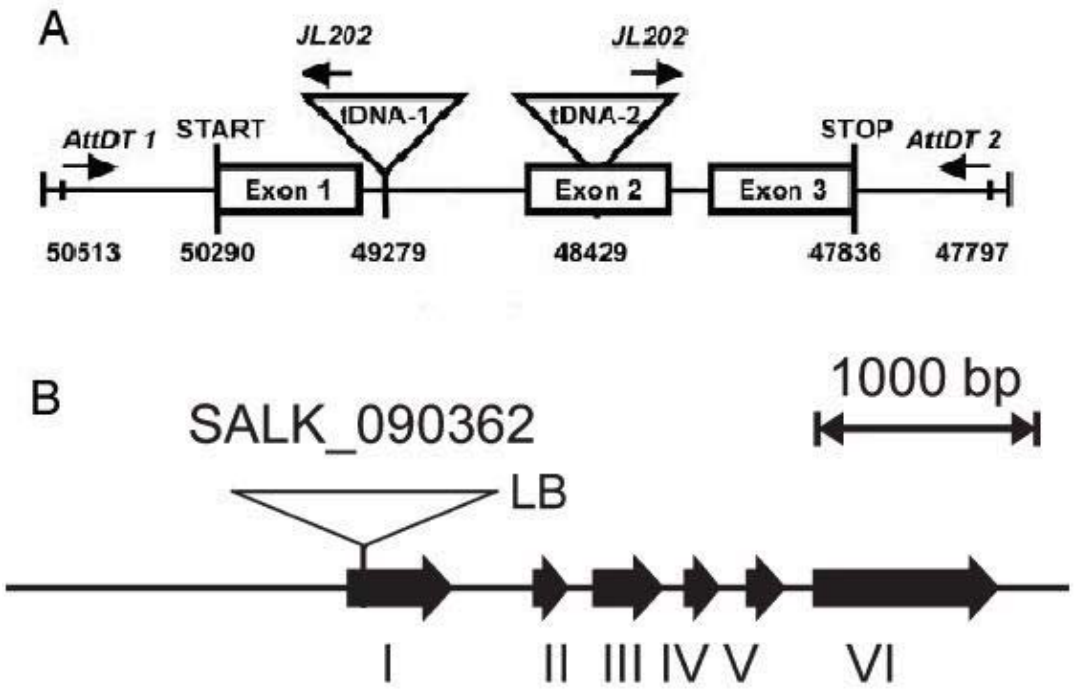


Fig. 10: The T-DNA insertion positions in A. *AttDT*, (*AttDT* was knocked out by T-DNA insertion in individual plants at two different locations), and B. *AtALMT9*. The knocked plants were screened for the presence of these inserts by PCR. Source (Emmerlich et al., 2003; Kovermann et al., 2007).

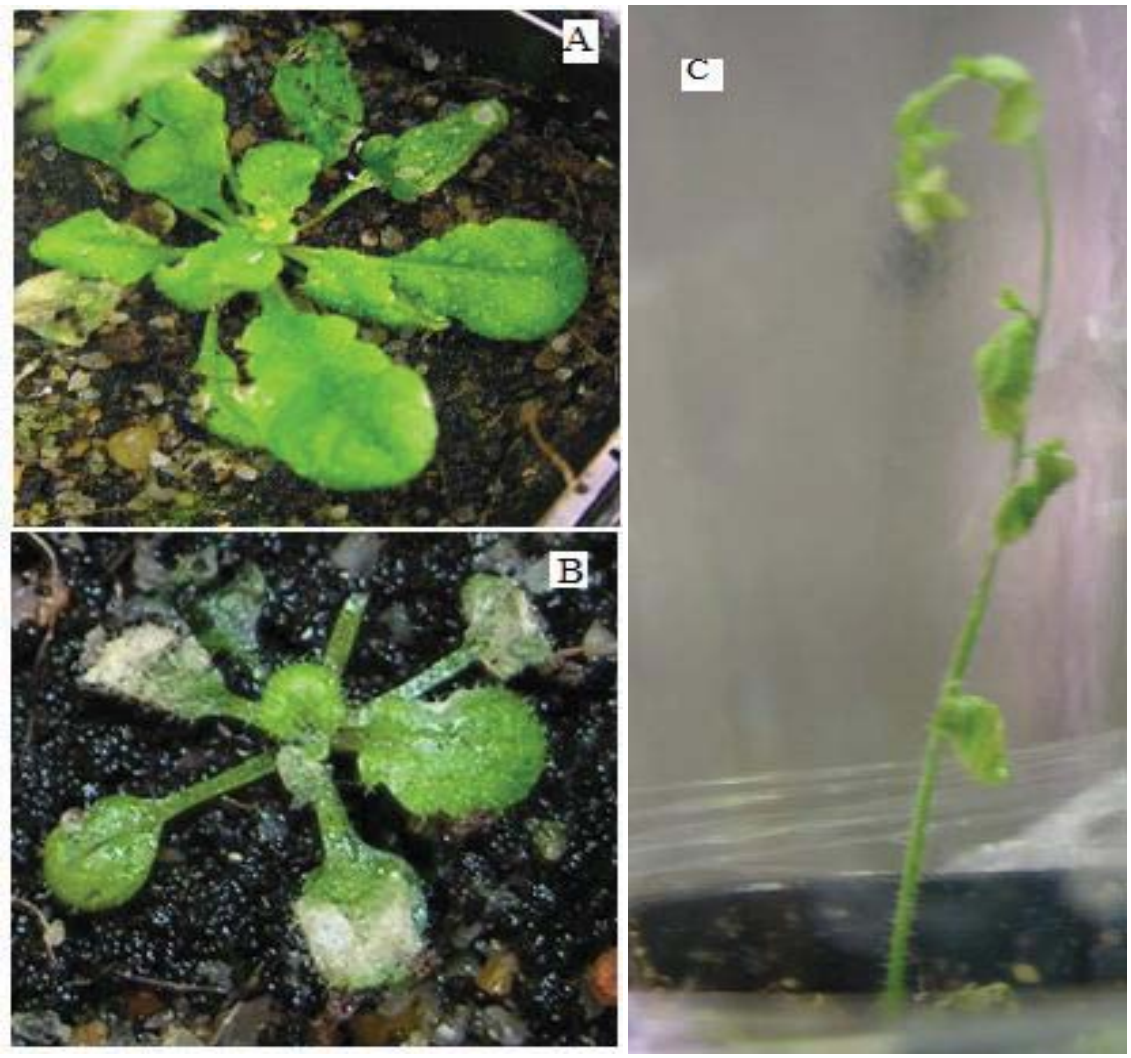


Fig. 11: Double knockout plants A & B early stages, the plant appeared dry and leaves are wrinkly. C plant at flowering stage partially dried plant.

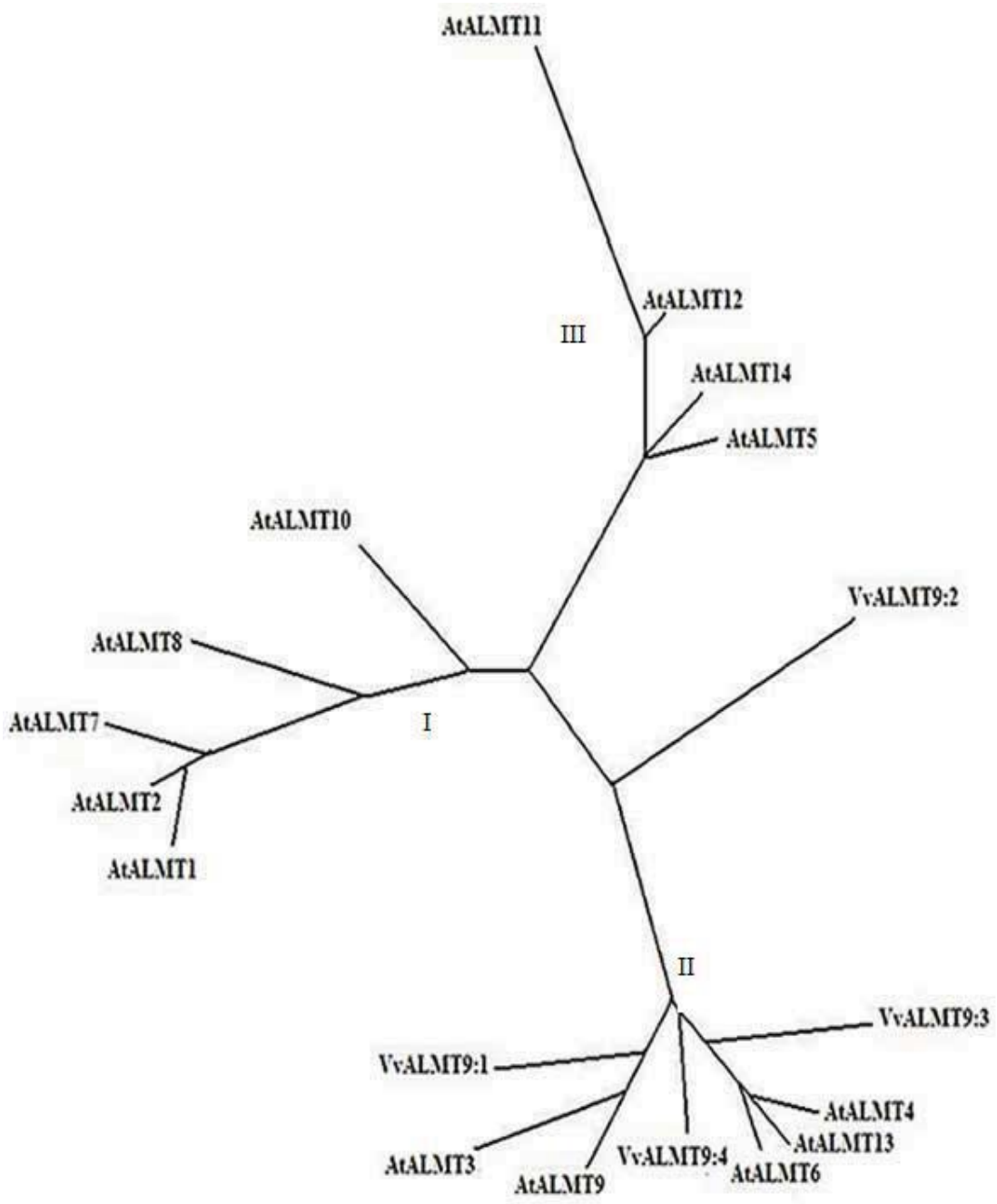


Fig. 12: The dendogram of ALMT protein family from *AraArabidopsis thaliana* and VvALMT9 based on the aminoacid sequencealignment Clustal W showing three main groups (Kovermann et al., 2007), VvALMT9:1, VvALMT9:3 and VvALMT9:4 are showing similarity with clade 2.

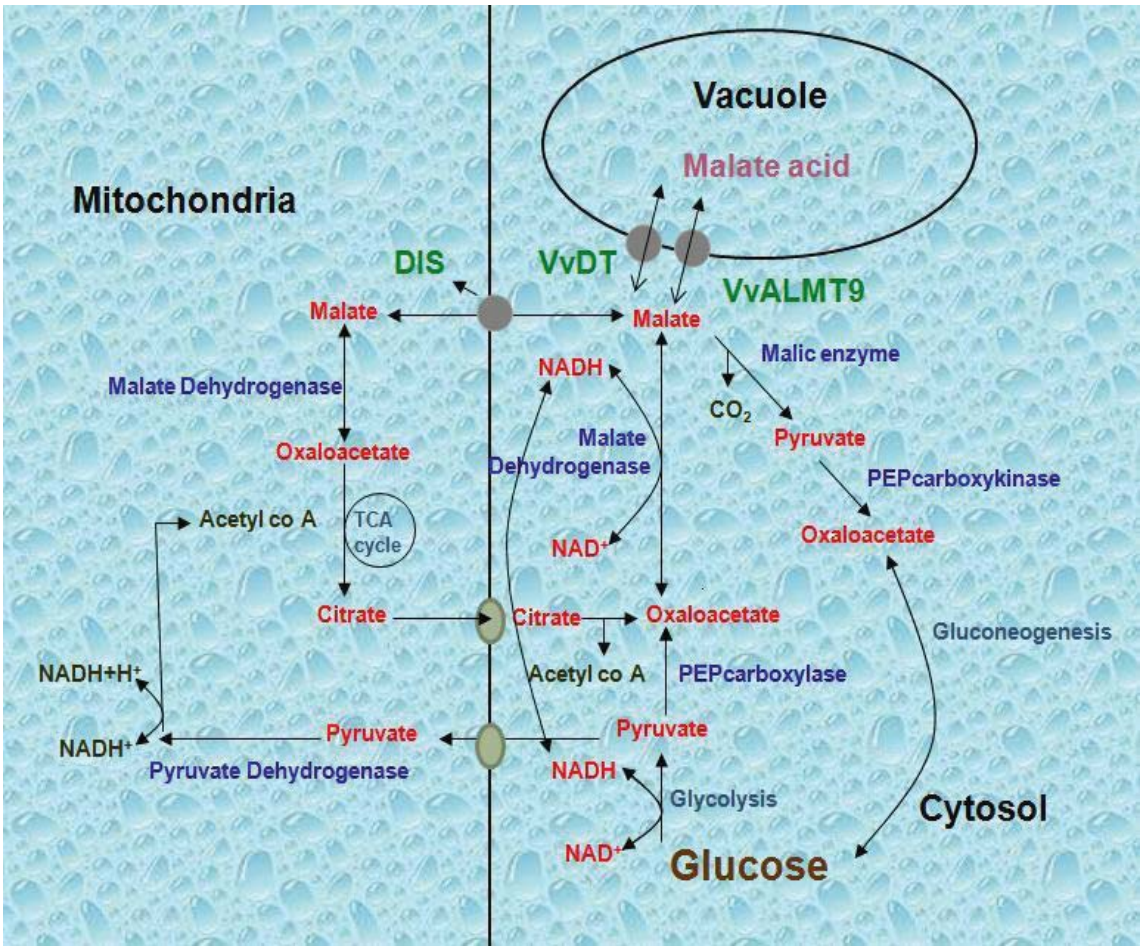


Fig. 13: Malate cycle, the synthesis of malate from oxaloacetate and pyruvate (end product of Glycolysis) the enzymes involved PEPC and MDH. The synthesised malate is localized into the vacuole by vacuolar transports and the degradation of the malate by ME and MDH (in cytoplasm and mitochondria).

SUPPLEMENTARY MATERIALS

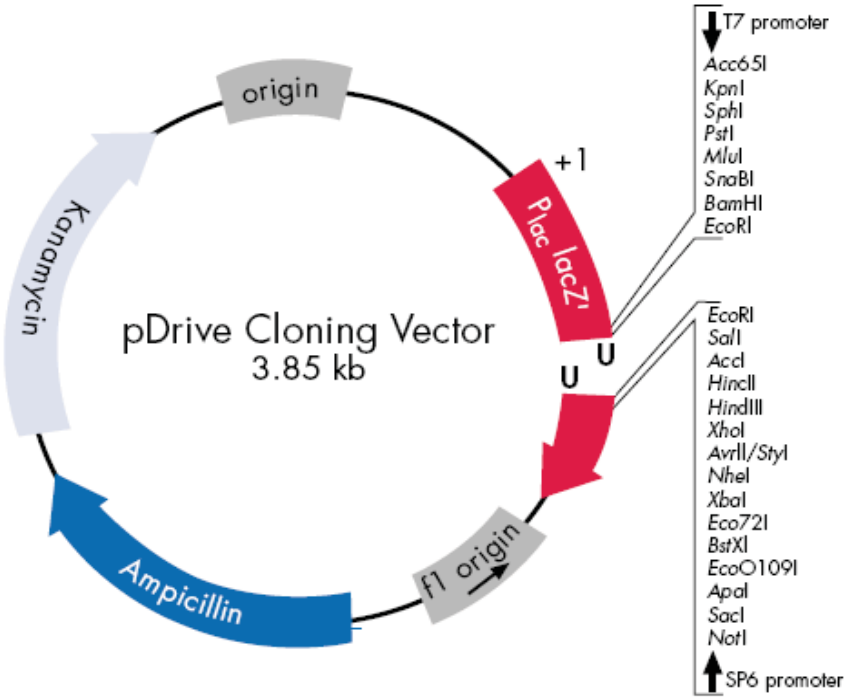


Fig. 14: pDRIVE cloning vector for direct ligation of the PCR product.

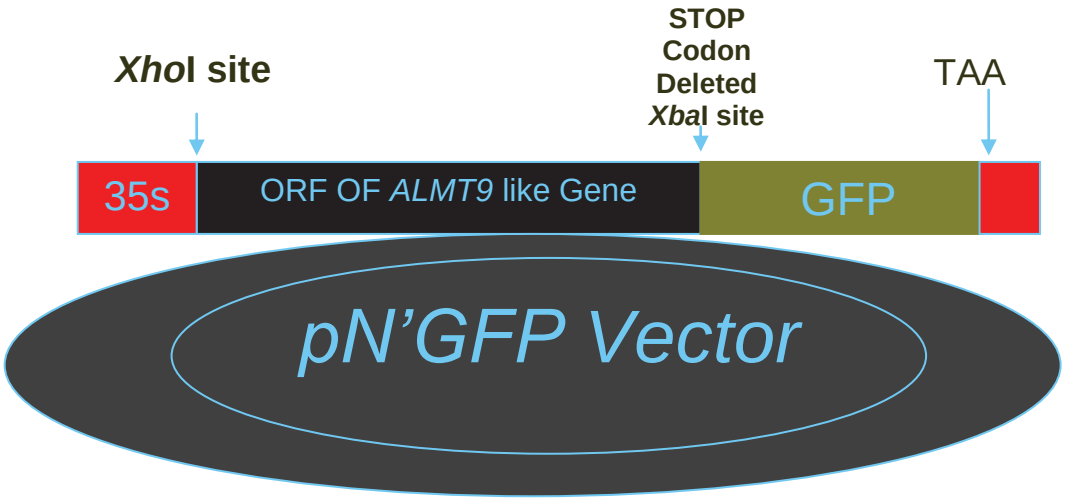


Fig. 15: pN'GFP vector (T Franks, University of Adelaide, unpublished data; Hayes et al 2007), the ORF was ligated into the vector by restriction enzymes *Xho*1 and *Xbal*1 and the stop codon was deleted from the primers to allow the translational fusion of the ORF with the GFP protein driven by the cauliflower mosaic virus (CaMV) 35S promoter.

Sequencing results

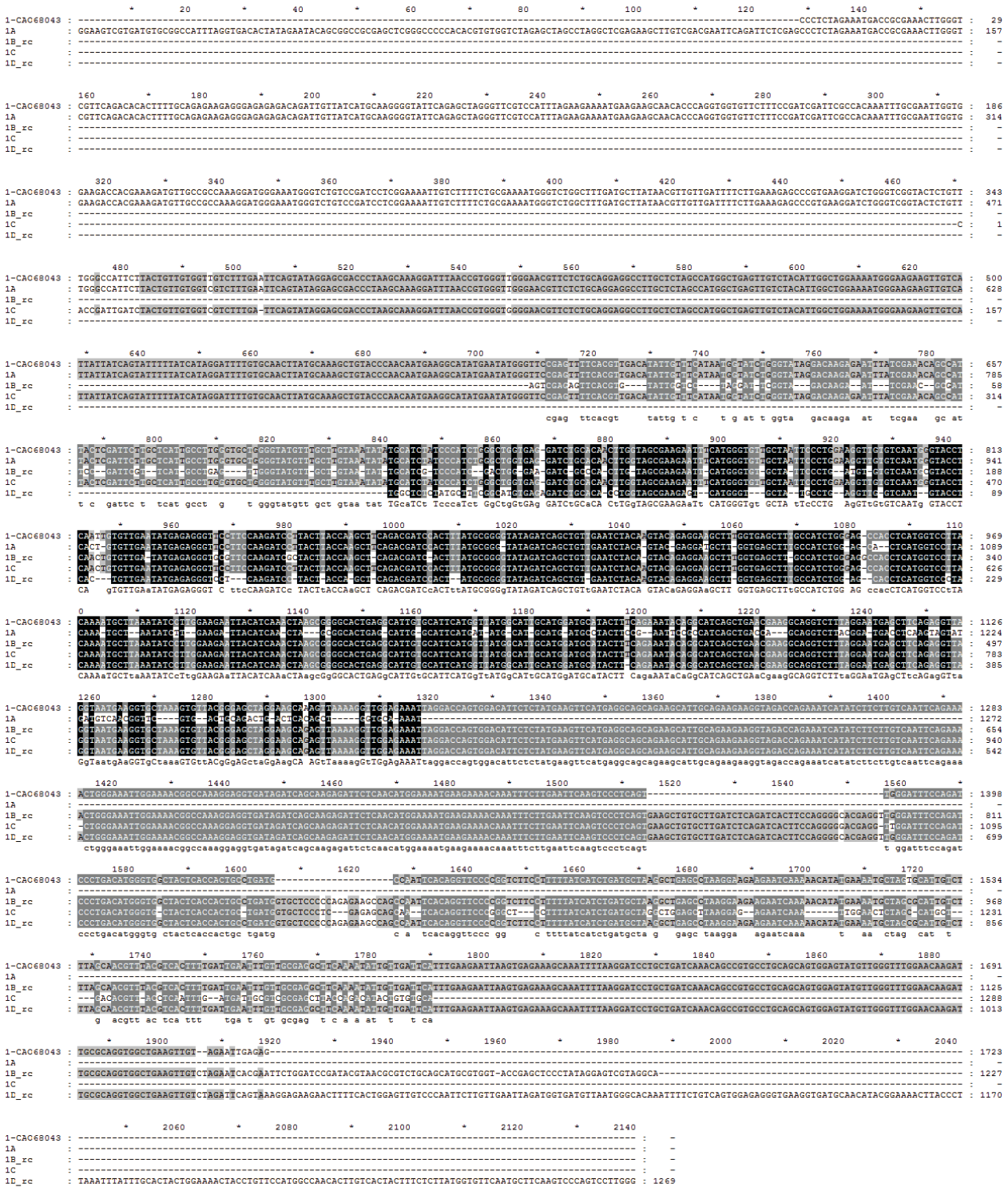


Fig. 16: sequencing alignment of VvALMT9:1 the original sequence followed by 1A: sequenced by forward M13primer, reverse M13, 1C: gene sequencing primer designed for sequencing the middle part of the gene, 1D: the sequence of VvALMT9:1-GFP gene fusion.

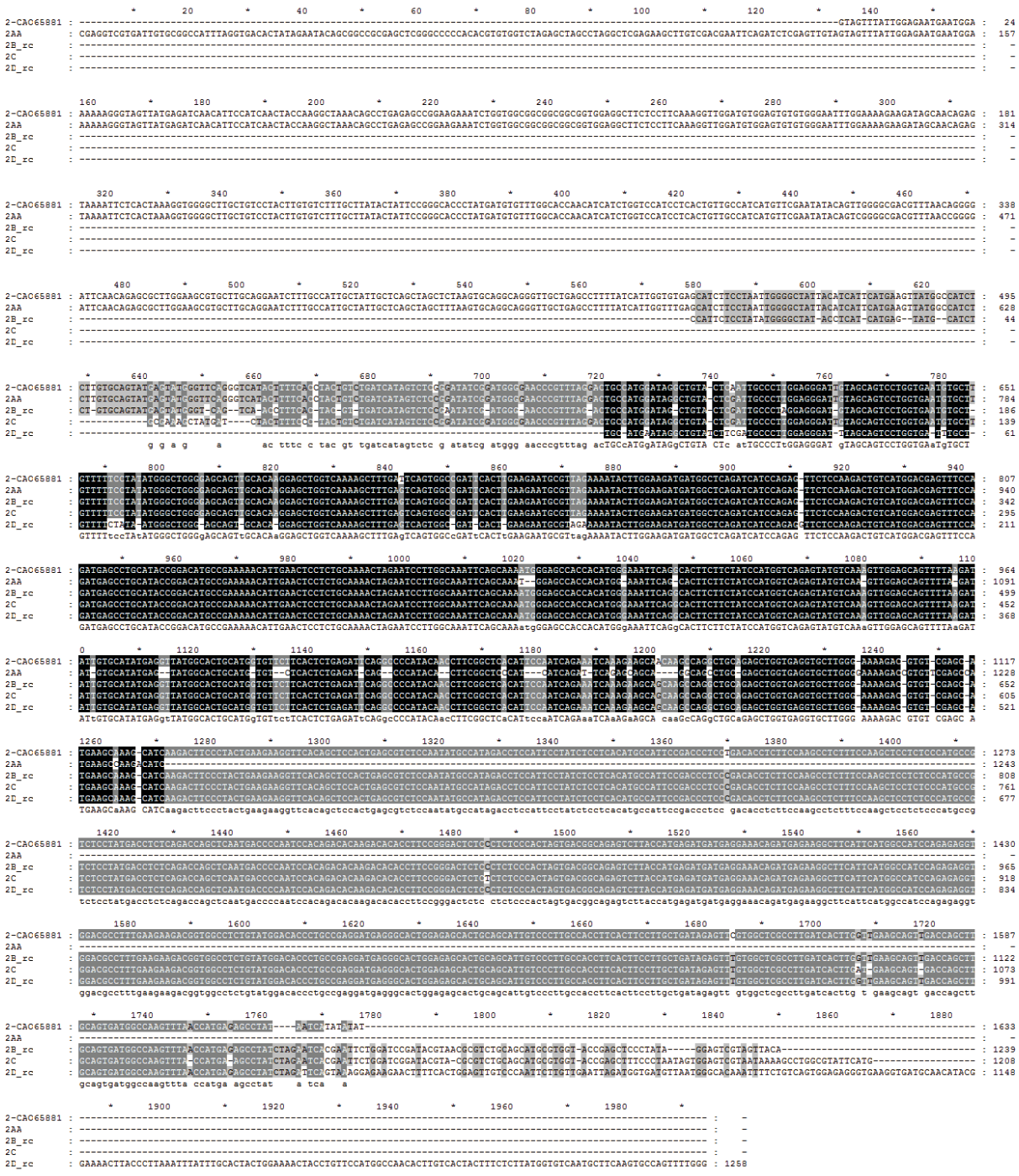


Fig. 17: sequencing alignment of VvALMT9:2 the original sequence followed by 2A: sequenced by forward M13primer, 2B: M13, 2C: gene sequencing primer designed for sequencing the middle part of the gene, 2D: the sequence of VvALMT9:2-GFP gene fusion.

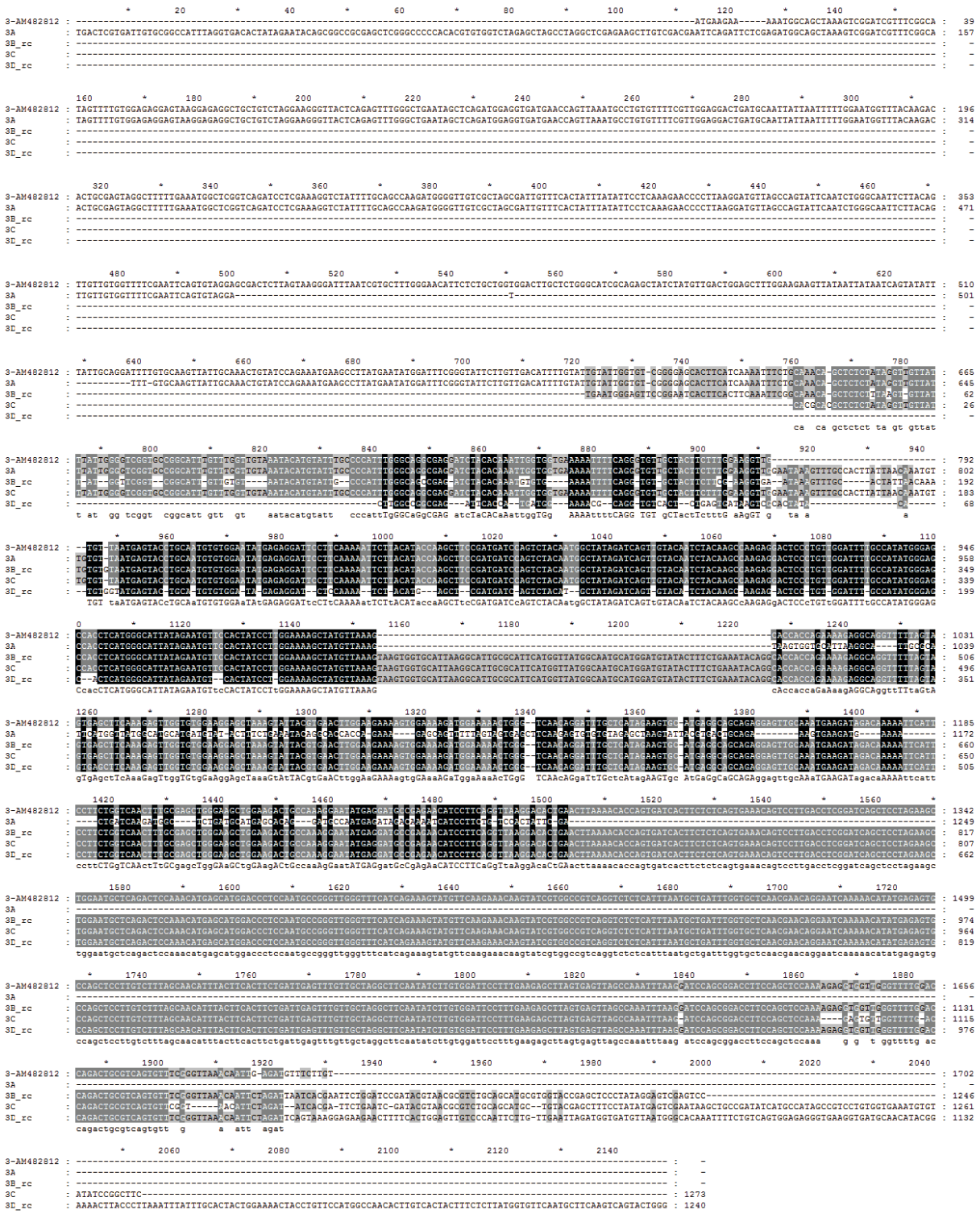


Fig. 18: sequencing alignment of VvALMT9:3 the original sequence followed by 3A: sequenced by forward M13primer, reverse M13, 3C: gene sequencing primer designed for sequencing the middle part of the gene, 3D: the sequence of VvALMT9:3-GFP gene fusion.

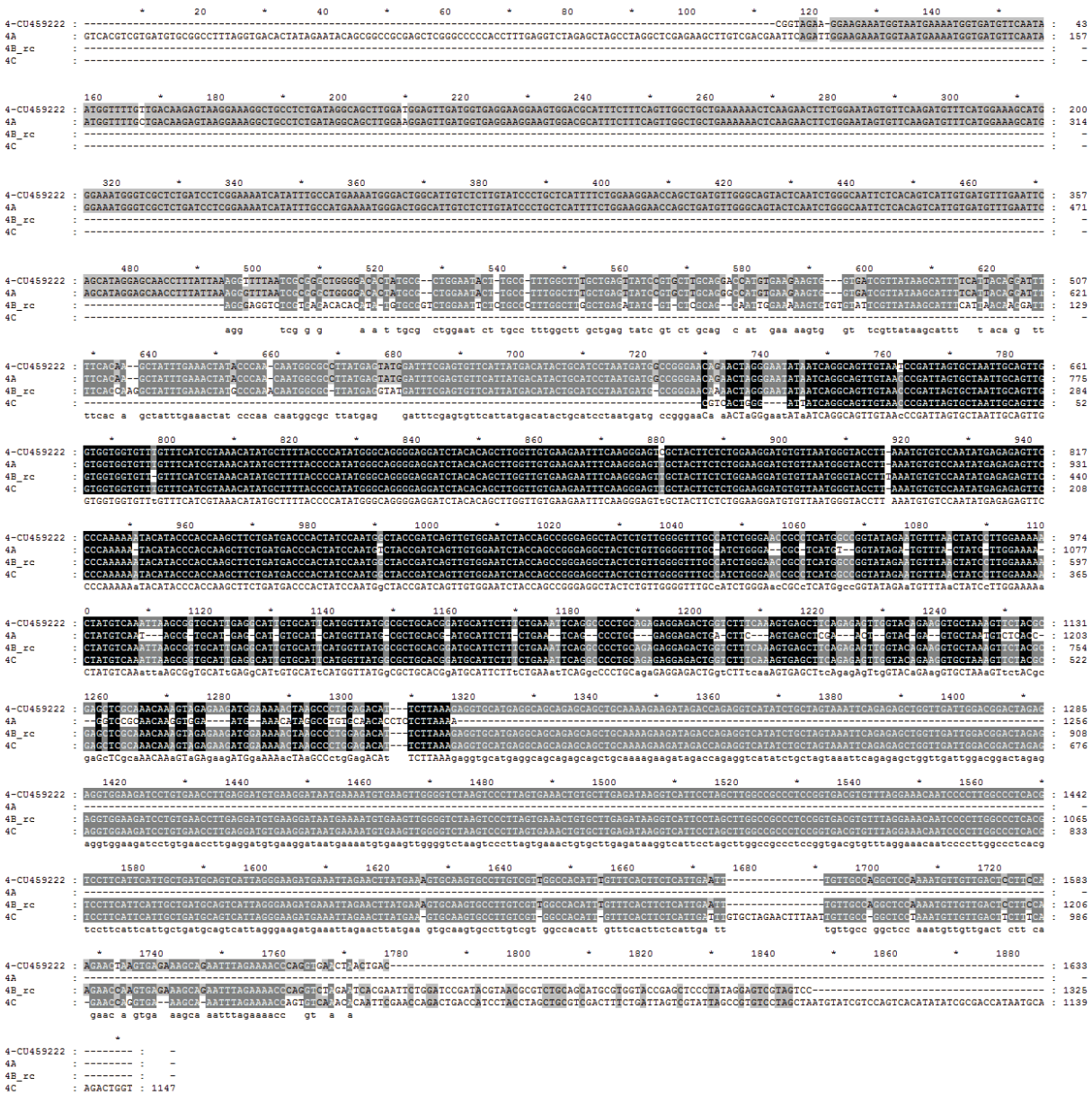


Fig. 19: sequencing alignment of VvALMT9:4 the original sequence followed by 4A: sequenced by forward M13primer, reverse M13, 4C: gene sequencing primer designed for sequencing the middle part of the gene.

APPENDICES

Abbreviations

AtALMT9	*Arabidopsis thaliana* aluminium activated malate transporter
AttDT	*Arabidopsis thaliana* tonoplast dicarboxylate transporter
bp	Base Pairs
DKO	Double Knockout
DNA	Deoxyribose Nuclic Acid
GFP	Green Fluorescent Protein
HPLC	High Performance Liquid Chromatography
KO	Knockout
ME	Malic Enzyme
MHD	Malate Dehydrogenase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEPC	Phosphoenolpyruvate Carboxylase
PEPCK	phosphoenolpyruvate Carboxykinase
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction

Additional data

The following experiments were still under progress.

Quantification of acids by HPLC

0.5g tissue was ground in liquid nitrogen and resuspended in 5ml of extraction buffer (3% (w/v) metaphosphoric acid, 1mMEDTA), vortexed well and centrifuged at 3700rpm for 10min at 4oC. The supernatant was filtered through a hydrophilic PVDF 0.4µM filter (Millipore-HV) and organic acids measured by HPLC using Agilent 1100 series HPLC and model fitted with a Synergi Fusion 4µ gradient column 150x4.6 mm ID. (Vanessa Melino, unpublished data).

Patch clamp studies in Xenopus oocytes

Gateway primers were designed and full length gene was amplified by PCR using Platinum Taq DNA polymerase High Fidelity, Invitrogen as per manufacturer's instructions and cloned into pENTR/D-TOPO vector (Fig. 20) by BP reaction, and transferred into pGEMHE-DEST vector (Fig. 21) (Meagan Sheldon, University of Adelaide, unpublished data) by LR Recombination reaction, Invitrogen, according to manufacturer's instructions.

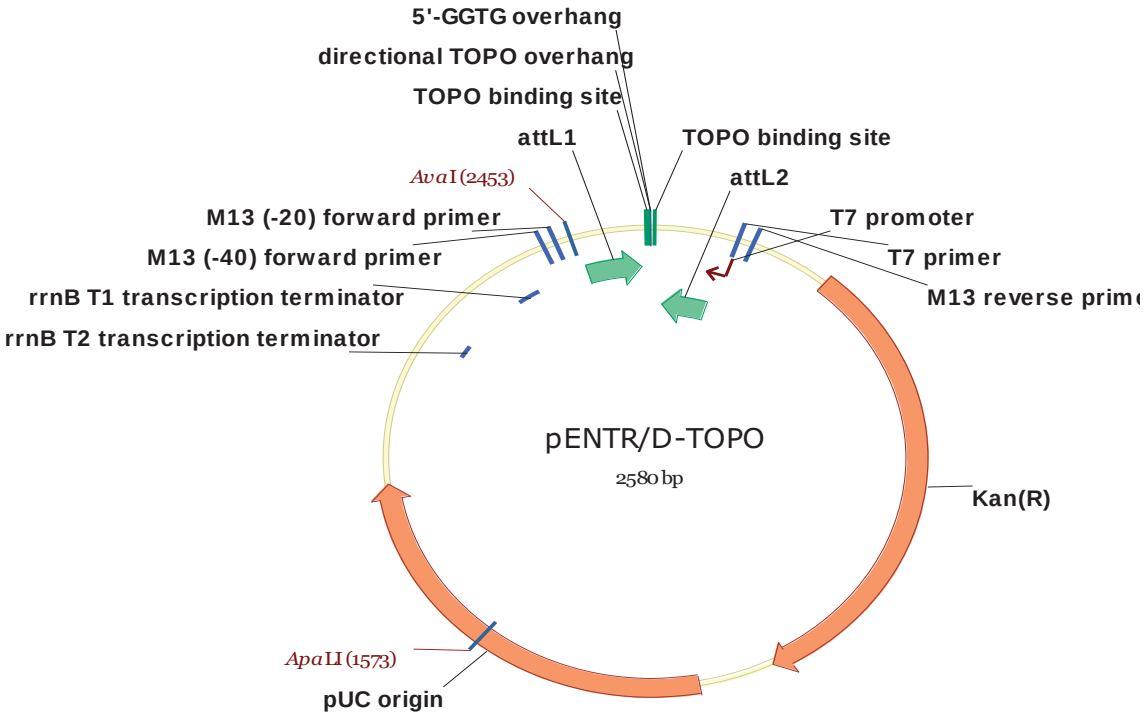


Fig. 20: pENTR/D-TOPO vector used for gateway cloning, Vector NTI Advanced (Invitrogen).

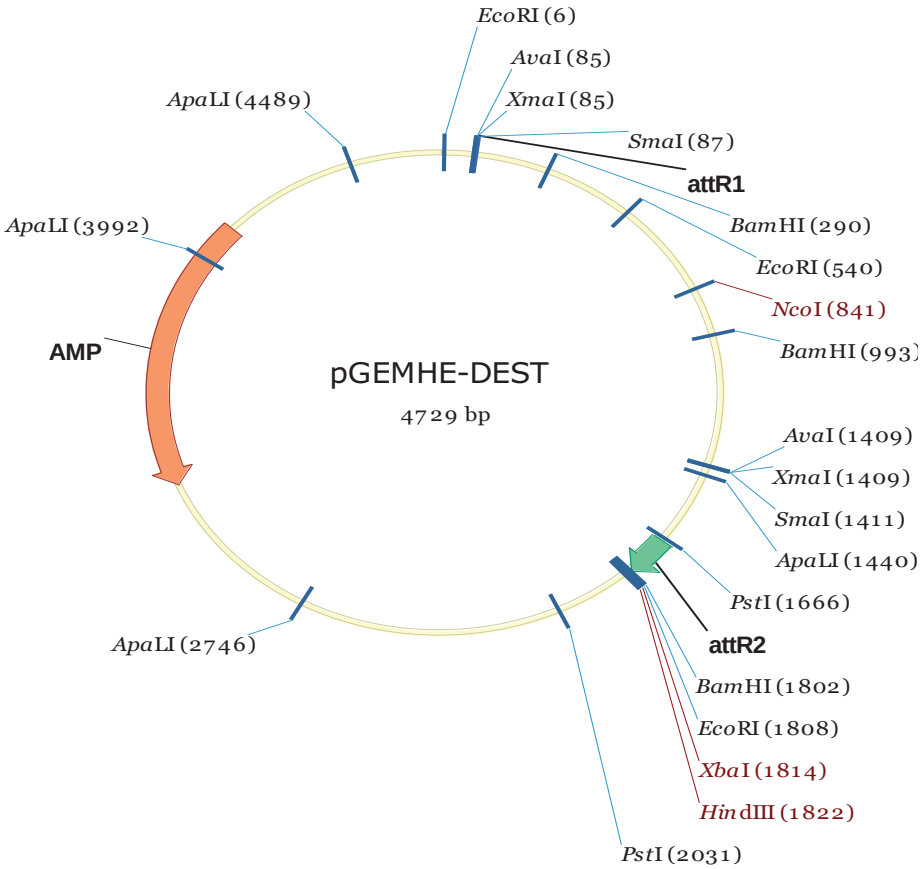


Fig. 21: pGEMHE-DEST vector for expression in *Xenopus* oocytes, Meagan Sheldon, University of Adelaide, unpublished data.

ACKNOWLEDGEMENT

Thanks to my supervisors Matthew Hayes and Christopher Ford for giving me the opportunity to undertake master project. In particular, Matt for his supervision, technical support and patience throughout the year.

To all the wonderful people in our lab especially Jay, Crista and Vanessa for their patience, suggestions and answering my questions, your assistance is really appreciated. Also special thanks to all the staff members and students of Kaiser's, Gillham's and Tyerman's lab who have assisted in any way during my studies.

Finally thanks to all my family and friends for their wonderful support, encouragement and friendship throughout my study.

References

Amerine MA (1964) Acids, grapes, wines and people. American Journal Of Enology And Viticulture **15:** 106-115

Amerine MA, Winkler AJ (1942) Maturity studies with California grapes. II. Titratable acidity, pH, and organic acid content. Proceedings of the American Society for Horticultural Science **40:** 313-324

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucl. Acids Res. **31:** 3497-3500

Conde C, Silva P, Fontes N, Dias ACP, Tavares RM, Sousa MJo, Agasse A, Delrot S, GerÃ³s H (2007) Biochemical changes throughout grape berry development and fruit and wine quality. *In*. Global Science Books

Diakou P, Svanella L, Raymond P, Gaudillere JP, Moing A (2000) Phosphoenolpyruvate carboxylase during grape berry development: protein level, enzyme activity and regulation. Australian Journal of Plant Physiology **27:** 221-229

Emmanuel D, Benjamin DG, Peter RR (2007) The roles of organic anion permeases in aluminium resistance and mineral nutrition. Febs Letters **581:** 2255-2262

Emmerlich V, Linka N, Reinhold T, Hurth MA, Traub M, Martinoia E, Neuhaus HE (2003) The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. Proceedings of the National Academy of Sciences of the USA **100:** 11122-11126

Famiani F, Casulli V, Proietti P, Walker RP, Battistelli A (2007) organic acid metabolism in grape: role of phosphoenolpyruvate carboxykinase. ISHS Acta Horticulturae **754:** 599-602

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

Hawker JS (1969) Changes in the activities of malic enzyme, malate dehydrogenase, phosphopyruvate carboxylase and pyruvate decarboxylase during the development of a non-climacteric fruit (the grape). Phytochemistry **8:** 19-23

Hayes MA, Davies C, Dry IB (2007) Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: differential roles in sink and source tissues. Journal of experimental botany **58:** 1985-1997

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 of the USA **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

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. The Plant Journal **52:** 1169-1180

Lakso AN, Kliewer WM (1975) The Influence of Temperature on Malic Acid Metabolism in Grape Berries: I. Enzyme Responses. Plant Physiol. **56:** 370-372

Lakso AN, Kliewer WM (1978) The Influence of Temperature on Malic Acid Metabolism in Grape Berries. II. Temperature Responses of Net Dark CO2 Fixation and Malic Acid Pools. Am. J. Enol. Vitic. **29:** 145-149

Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J. Exp. Bot. **58:** 83-102

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

Or E, Baybik J, Sadka A, Saks Y (2000) Isolation of mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxylase cDNA clones from grape berries and analysis of their expression pattern throughout berry development. Journal Of Plant Physiology **157:** 527-534

Ruffner HP, Brem S, Malipiero U (1983) The physiology of acid metabolism in grape berry ripening. Acta Horticulturae **139:** 123-128

Ruffner HP, Hawker JS, Hale CR (1976) Temperature and enzymic control of malate metabolism in berries of *Vitis vinifera*. Phytochemistry **15:** 1877-1880

Sambrook J, Russel DW (2000) Molecular Cloning: A Laboratory Manual third edition.Cold Spring Harbour Laboratory press.

Schulze J, Tesfaye M, Litjens R, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate Plays a Central Role in Plant Nutrition. Plant and Soil **247:** 133-139

Schulze J, Tesfaye M, Litjens RHMG, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate plays a central role in plant nutrition. Plant and Soil **247:** 133-139

Terrier N, Deguilloux C, Sauvage F-X, Martinoia E, Romieu C (1998) Proton pumps and anion transport in Vitis vinifera: The inorganic pyrophosphatase plays a predominant role in the energization of the tonoplast. Plant Physiology and Biochemistry **36:** 367-377

Terrier N, Romieu C (2001) Grape Berry Acidity. *In* KA Roubelakis-Angelakis, ed, Molecular Biology and Biotechnology of the Grapevine. Kluwer Academic, Netherlands, pp 35-57

Terrier N, Sauvage F-X, Ageorges A, Romieu C (2001) Changes in acidity and in proton transport at the tonoplast of grape berries during development. Planta **213:** 20-28

Tilbrook J, Tyerman S (2006) Water, Sugar and Acid: How and Where They Come and Go During Berry Ripening. *In* D Oag, K DeGaris, S Partridge, C Dundon, M Francis, R Johnstone, R Hamilton, eds, ASVO Proceedings - Finishing the Job, Mildura, pp 4-10