| PICFPA | SAAPPA / SASPA / SAT | DFTS | Winetech |
|---|---|---|---|
| Canning Fruit Producers' Assoc.<br>Submit to:<br>Wiehahn Victor<br>Tel: +27 (0)21 872 1501<br>inmaak@mweb.co.za | Fruitgro Science<br>Submit to:<br>Louise Liebenberg<br>Tel: +27 (0)21 882 8470/1<br>louise@fruitgro.co.za | Dried Fruit Technical Services<br>Submit to:<br>Dappie Smit<br>Tel: +27 (0)21 870 2900<br>dappies@dtd.co.za | Submit to:<br>Jan Booysen<br>Tel: +27 (0)21 807 3324<br>booysenj@winetech.co.za |

| | | | |
|---|---|---|---|
| | X | | X |

Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

# FINAL REPORT
## FOR 2008-2011

## PROGRAMME & PROJECT LEADER INFORMATION

| | Programme leader | Project leader |
|---|---|---|
| **Title, initials, surname** | Prof MA Vivier | Dr PR Young |
| **Present position** | Professor | Researcher |
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| **Tel. / Cell no.** | 021-808-3773 | 021-808-2188 |
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## PROJECT INFORMATION

| | |
|---|---|
| **Project number** | IWBT-P 08/13 |
| **Project title** | Metabolic engineering of grapevine towards enhanced abiotic stress resistance and improved quality parameters |
| **Project Keywords** | Model vineyard, carotenoid pathway, sugar and organic acid profiling, berry suspension cultures; molecular biology |

| | | |
|---|---|---|
| **Industry programme** | **CFPA** | |
| | **Deciduous** | Biotechnology: Grapevine Molecular Biology and Biotechnology |
| | **DFTS** | |
| | **Winetech** | Biotechnology: Grapevine Molecular Biology and Biotechnology |
| | **Other** | |

| | |
|---|---|
| **Fruit kind(s)** | Wine and Table Grapes |
| **Start date** (dd/mm/yyyy) | 1/1/2008 |
| **End date** (dd/mm/yyyy) | 31/12/2011 |

# FINAL REPORT

(Completion of points 1-5 is compulsory)

## 1. Executive summary

Give an executive summary of the *total* project in no more than 250 words

The specific aims of this project theme relates to the understanding and manipulation of core metabolic pathways in grapevine to not only provide insight into how grapevine deals with abiotic stresses, but also to provide alternative strategies and/or novel plant material more resistant to these stresses. The project delivered excellent fundamental research information regarding the carotenoid metabolic pathway of grapevine. Apart from its scientific value it also provided significant momentum for our efforts to apply molecular biology studies to vineyard settings. For example, methodology to accurately profile carotenoid and chlorophyll pigments, as well as a sugar and organic acid profiling method from grape berries in all stages of development were optimised and published. These methods were tested in an initial (viticultural) characterisation of model vineyards (in collaboration with Prof Alain Deloire on a vineyard in Elgin). Excellent progress has also been made with the development and analysis of berry suspension cultures – synchronised suspension cultures were obtained and analysed for their growth kinetics. These cultures were also subjected to a novel two dimensional proteomics system using free flow electrophoresis and subsequent SDS PAGE analysis to compare protein expression during the different berry growth stages. The results provided proof-of-concept that these berry organ cultures could indeed be used to study berry ripening related processes.
In conclusion, the project has been completed successfully and has reached all aims.

## 2. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

The fundamental understanding of *how grapevine works* is rapidly improving. Molecular biology tools are providing insights into the core processes involved in grapevine growth and metabolism. Grape berry ripening and the elucidation of berry physiology and metabolism have been a focus of several groups and the first comprehensive berry profiling data sets are appearing. Despite these important advances in our knowledge of the genetic potential of grapevine, very basic research questions remain unanswered. Moreover, hypothesis-driven research remains difficult in vineyard settings due to the inherently high heterogeneity in the test systems.

Grapevine plants in a vineyard setting are naturally subjected to a multitude of environmental factors, many of them negative, leading to stress signals and subsequent stress responses in the plant. The outcomes of these responses are typically altered metabolism of plant tissues, organs or the whole plant body. Understanding of the pathways involved and their regulation are imperative to anticipate and ultimately manipulate environmental stress in grapevine metabolism. Two of the most prominent negative impacts of the changing climate in our grape producing areas will be an increase in water scarcity and rising temperatures. These negative environmental influences will directly impact plant growth and physiology, as well as product quality.

**The specific aims** of this project theme related to the understanding and manipulation of core metabolic pathways in grapevine to not only provide insight into how grapevine deals with abiotic stresses, but also to provide alternative strategies and/or novel plant material more resistant to these stresses. Moreover, the project also aimed to contribute towards a fully characterised vineyard where the cause(s) and effects of environmental stresses can be separated and studied. The metabolic pathway that was studied in this project (carotenoid pathway) is also intricately involved in quality aspects and some of the aims and outcomes

relates to functional analysis of impact genes and their encoded products to improve flavour and aroma production in grapevine tissues and berries.

## 3. Workplan (materials & methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

TASK 1: THE DEVELOPMENT OF BASELINE MOLECULAR BIOLOGY INFORMATION IN GRAPEVINE TOWARDS A DEFINED MATRIX IN A VINEYARD SETTING

- Milestone 1: The application of molecular biology profiling methods to supplement viticultural analyses towards a characterised model vineyard (2008-2011)
  A highly characterised vineyard with clearly defined vigour groupings is being established as part of the Wine Science Research Niche Area (Vitimetrics project in collaboration with Albert Strever and Alain Deloire, Department of Viticulture and Oenology). This vineyard will provide a framework to define the causative factors and their effects in the vine, berry and wine. The supplementation of the extensive viticultural analyses with molecular data will provide insight regarding the intrinsic processes of the vines in reaction to the modulating factors that give rise to the characterised phenotypes. Detailed fruit analysis (biochemical, chemical as well as sensorial) and subsequent wine analysis of these grapes (biochemical, chemical and sensorial) will be conducted by other RNA partners.

TASK 2: ANALYSIS OF TRANSGENIC GRAPEVINE POPULATIONS WITH TARGETED CHANGES TO THE CAROTENOID PATHWAY

- Milestone 1: Evaluation of a berry suspension culture system to study core processes in berry metabolism *in vitro* (2008-2010)
  The molecular triggers for ripening of a grape berry are poorly known because of the challenges grapevine provide for these types of studies. Cell suspension cultures, which are rapidly dividing callus cells growing in a liquid medium, are widely used as tools to study gene expression and regulation, as well as aspects involved in plant development and metabolism. In this study, grape berry cell suspension cultures will be established from berries at different development stages and monitored with various methods to evaluate the usefulness of these cultures as “models” for berry metabolism. The cultures will be initiated by callus derived from grape berry explants. Different explants, initiated on two different media (according to available literature) will be used for callus production. In preliminary evaluations actively growing cultures could be obtained from both media, although clear cultivar-specific variation was observed. The suspension cultures will be examined for characteristic (berry) gene expression and metabolite levels. If the outcomes prove that the berry cultures maintain certain key characteristics of berry metabolism during defined developmental stages, berry cell suspension cultures could make an immense contribution to the study of berry ripening related processes.

- Milestone 2: Functional analysis of the grapevine carotenoid cleavage dioxygenases (VvCCD1) to evaluate their role in flavour and aroma production
  *Carotenoid cleavage dioxygenases*: In this study Sultana lines were transformed with a constitutively expressed grapevine CCD1. Other Sultana lines were transformed with an RNAi silencing cassette to effectively silence the native CCD1 expression. An overexpression vector was constructed using *VvCCD* under control of the 35SCaMV promoter and the OCS terminator. The gene was PCR amplified from Pinotage cDNA. The silencing vector was constructed using the pHANNIBAL plasmid. The inverted repeat that would cause silencing in the plant was PCR amplified form the Pinotage genomic DNA and was situated in the 3’UTR of *VvCCD*. These vectors, as well as an empty cassette, were used to transform *Agrobacterium tumefaciens.* Sultana lines were then transformed with the various constructs through A*grobacterium* mediated transformation.

Transgenic populations with these constructs are now available and will be analysed for transgene presence and expression/silencing. The pigment profiles of the transgenic populations will be compared to wild-type Sultana. Using Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME GC/MS), an analysis of the volatiles produced by the lines will be performed. A comparison between overexpressed, silenced and wild type lines will be made to functionally analyse the role of the cleavage dioxygenases as possible targets towards improved flavour and aroma in grapevine.

- Milestone 3: Evaluation of transgenic grapevines overexpressing *VvNCED1* for improved resistance to abiotic stresses (2009-2011).
  The grapevine NCED encoding gene has been overexpressed in grapevine plants and transgenic populations are now available to be analysed. The following steps will be included in the study:
  - Genetic analysis of putative transgenic populations and functional analysis of the transgene(s) in the grapevine lines. Multiplication of final population (end 2008)
  - Plant stress analyses (water and light stress) on the transgenic populations to identify resistance phenotypes (green house studies) (end 2009)
  - In-depth analysis of the gene-specific resistance phenotypes against the stresses to understand the mode-of-action (2010-2011). The approach would be to profile the plants (representative lines) with transcriptomic, proteomic and metabolomic tools to understand the consequences of the transgene on a whole plant and *in planta* level.

## 4. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results.
This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

| Milestone | Achievement |
|---|---|
| TASK 1: THE DEVELOPMENT OF BASELINE MOLECULAR BIOLOGY INFORMATION IN GRAPEVINE TOWARDS A DEFINED MATRIX IN A VINEYARD SETTING | A model/highly characterised vineyard has been successfully established, in collaboration with Prof Alain Deloire, in a commercial vineyard in Elgin. Light and temperature were the major variables and the sample layout was optimised to characterise and evaluate the nature of the variation, as well as provide a sound layout to obtain representative samples. Samples during berry development and ripening were taken for berry pigment and hormone profiling as well as metabolite analysis. The viticultural manipulations and characterisations are reported by Prof Deloire in Winetech project: "Influence of light and temperature at the vine and bunch level on growth and ripening of Sauvignon blanc (Vitis vinifera L.) berries".<br>• The progress made lead to a new project initiated in 2012 in collaboration with two viticulturist teams (Proff. Deloire and K. Hunter in Elgin and Robertson, respectively)<br>• Two berry profiling methods were developed and published (Lashbrook et al., 2010; Eyeghe-Bickong et al, 2012). |
| TASK 2: ANALYSIS OF TRANSGENIC GRAPEVINE POPULATIONS WITH TARGETED CHANGES TO THE CAROTENOID PATHWAY<br>Milestone 1: Evaluation of a berry suspension culture system to study core processes in berry metabolism in vitro (2008 - 2010) | The results show that berry-specific cultures can be used to study processes linked to berry development and ripening. The cultures and their analysis yielded novel results that are very promising to overcome some of the problems linked to berry heretogeneity in a |

| | |
|---|---|
| | ripening cluster. The results were published in PLoS ONE (Ramaschandra et al., 2011). |
| Milestone 2: Functional analysis of the grapevine carotenoid cleavage dioxygenase (VvCCD1) to evaluate the role in flavour and aroma production. | Functional charcterisation of this important grapevine gene was completed and the transgenic grapevine populations overexpressing and silencing the gene provided valuable insights into the functions of the encoded product.<br>• A paper is in preparation for Phytochemistry (Lashbrook et al., 2012).<br>• This work also supported a comprehensive analysis of the carotenoid metabolic pathway in grapevine during berry development and provides the most up-to-date pathway analysis available (Young et al. 2012). |
| Milestone 3: Evaluation of transgenic grapevines overexpressing *VvNCED1* for improved resistance to abiotic stresses | Cultivar Sultana was transformed with the pART27-VvNCED1 construct. A population of plants was regenerated, but due to fungal contamination, many lines were lost. The final confirmed population after genetic characterisation consists of only four independent transgenic lines. The population is hardened-off and maintained in the greenhouse, but is too small for statistical significance and additional transformations are required. This gene was previously overexpressed in *Arabidopis* and contributed to both a drought-resistant and high light stress protective phenotype. |

TASK 1: THE DEVELOPMENT OF BASELINE MOLECULAR BIOLOGY INFORMATION IN GRAPEVINE TOWARDS A DEFINED MATRIX IN A VINEYARD SETTING

- Milestone 1: The application of molecular biology profiling methods to supplement viticultural analyses towards a characterised model vineyard (2008-2009)

In collaboration with Prof Alain Deloire, a characterised vineyard/model vineyard was developed in Elgin in a Sauvignon blanc vineyard (details of vineyard and viticultural manipulations and measurements are reported in Winetech report: Influence of light and temperature at the vine and bunch level on growth and ripening of Sauvignon blanc (*Vitis vinifera* L.) berries of Prof Deloire). Characterisation of the model vineyard necessitated the evaluation and optimisation of a number of methods to extract, separate and quantify a number of key metabolites from grapevine berries throughout berry development (green through veraison to harvest) and has resulted in two method papers that were published in peer-reviewed journals. The first publication describes the systematic optimisation of the extraction and separation of all major photosynthetic pigments (carotenoids and chlorophylls) found in grapevine leaves and berries (at various developmental stages):

- Lashbrooke, J.G., P.R. Young, A.E. Strever, C. Stander & M.A. Vivier. 2010. The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling. *Australian Journal of Grape and Wine Research* 16: 349-360*).*

*Abstract:*

**Background and Aims**: Carotenoids and chlorophylls perform a number of essential roles in plants making their accurate quantification important to a variety of studies.We aimed to develop an extraction protocol to accurately determine the photosynthetic pigments in grapevine leaf and berry tissue, specifically focusing on limiting the degradation of these pigments. **Methods and Results**: An extraction protocol for grapevine leaf and berry tissue was systematically optimised by identifying a number of critical parameters. Extracted pigments were analysed using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). Specific parameters that were optimised included avoiding freeze-drying the material; the volume of acetone and the time required to extract all the pigments from the tissue; the addition of 0.1% (v/v) N-ethyldiisopropylamine to berry extracts to minimise pigment degradation during the extraction procedure; and avoiding concentration of the extracts that otherwise resulted in differential degradation of pigments. Additionally, the method of extraction and normalisation with an internal standard was adapted and improved for accuracy. The optimised protocol was validated using authentic standards and its utility shown by analysing the pigment content of berries and leaves at different growth stages. **Conclusions:** A method has been developed that is able to extract and accurately quantify, by means of HPLC profiling, the levels of

photosynthetic pigments from grape berries and leaves. The method avoided any degradation of the pigments during the extraction and was applicable to both berries and leaves in different stages of growth and development, indicating its general usefulness to vegetative and reproductive organs, even if their metabolic states are very different. **Significance of Study:** The divergence of methods used for photosynthetic pigment analysis plants, each with specific advantages and disadvantages were considered and used to optimise a number of parameters in a single method that proved to be applicable to plant organs in different developmental stages. The method is fast, applicable to vegetative and reproductive grapevine tissues, avoids degradation of pigments and ensures maximum accuracy when quantifying these important pigments.

The second publication described the optimisation of the extraction and separation of the major sugars and organic acids in grapevine berries (at various developmental stages):

- Eyeghe-Bickong H.A., Alexandersson E.O., Gouws L.M., Young P.R., & Vivier M.A. 2012 Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. *Journal of Chromatography B* 2012, 885-886: 43-49.

*Abstract:*

A high performance liquid chromatographic method was developed to profile major sugars and organic acids in grapevine berries. Sugars and organic acids in grapevine berries were extracted by chloroform/polyvinylpolypyrrolidone purification. The extracts were chromatographed on an Aminex HPX-87H on-exchange HPLC column with 5 mM sulphuric acid as mobile phase. Chromatography was visualised via a diode array detector combined with a refractive index detector. The analysis was calibrated using externa standard calibration and a novel equation was used to calculate the concentrations of malic acid and fructose from unresolved separation. For the method to be utilised for analysing a large numbers of berry samples, each sample was directly injected after sample extraction and the extraction step was downscaled to allow the use of small amounts of sample material. The concentrations of sugars and organic acids in grapevine berry samples were normalised to the internal standard concentrations obtained after extraction of an internal standard mixture. The analysis method exhibits a good precision and a high analyte recovery from samples spiked with the standard mixture and is suitable for the profiling of major sugars and organic acids in grapevine berry samples at different stages of berry development. This is the first report on the combined profiling of the major sugars and organic acids in grapevine berries using milligram amounts of plant material with direct injection after sample extraction.

The application of these analytical methods together with the transcriptomic analysis (see Figure 1) from the 2009/2010 season has resulted in a paper that has been submitted for publication and is now *in press.*

- Young, P.R., Lashbrooke, J.G., Alexandersson, A., Jacobson, D., Moser, C., Velasco R. & Vivier M.A. (2012) The genes and enzymes of the carotenoid metabolic pathway in *Vitis vinifera* L. BMC Genomics (in press).

*ABSTRACT*

**Background:** Carotenoids are a heterogeneous group of plant isoprenoids primarily involved in photosynthesis. In plants the cleavage of carotenoids leads to the formation of the phytohormones abscisic acid and strigolactone, and C13-norisoprenoids involved in the characteristic flavour and aroma compounds in flowers and fruits and are of specific importance in the varietal character of grapes and wine. This work extends the previous reports of carotenoid gene expression and photosynthetic pigment analysis by providing an up-to-date pathway analysis and an important framework for the analysis of carotenoid metabolic pathways in grapevine. **Results:** Comparative genomics was used to identify 42 genes putatively involved in carotenoid biosynthesis/catabolism in grapevine. The genes are distributed on 16 of the 19 chromosomes and have been localised to the physical map of the heterozygous ENTAV115 grapevine sequence. Nine of the genes occur as single copies whereas the rest of the carotenoid biosynthetic genes have more than one paralogue. The cDNA copies of eleven corresponding genes from Vitis vinifera L. cv. Pinotage were characterised, and four where shown to be functional. Microarrays provided expression profiles of 39 accessions in the metabolic pathway during three berry developmental stages in Sauvignon blanc, whereas an optimised HPLC analysis provided the concentrations of individual carotenoids. This provides evidence of the functioning of the lutein epoxide cycle and their respective genes in grapevine. Similarly, orthologues of genes leading to the formation of strigolactone involved in shoot branching inhibition were identified: CCD7, CCD8 and MAX1. Moreover, the isoforms typically have different expression patterns, confirming the complex regulation of the pathway. Of particular interest is the expression pattern of the three VvNCEDs: Our results support previous findings that VvNCED3 is likely the isoform linked to ABA content in berries. **Conclusions:**The carotenoid biosynthetic pathway is well characterised, and the genes and enzymes have been studied in a number of plants. The study of the 42 carotenoid pathway genes of grapevine showed that they share a high degree of similarity with other eudicots. Expression and pigment profiling of developing berries provided insights into the most complete grapevine carotenoid pathway representation. This study represents an important reference study for further characterisation of carotenoid biosynthesis and catabolism in grapevine.

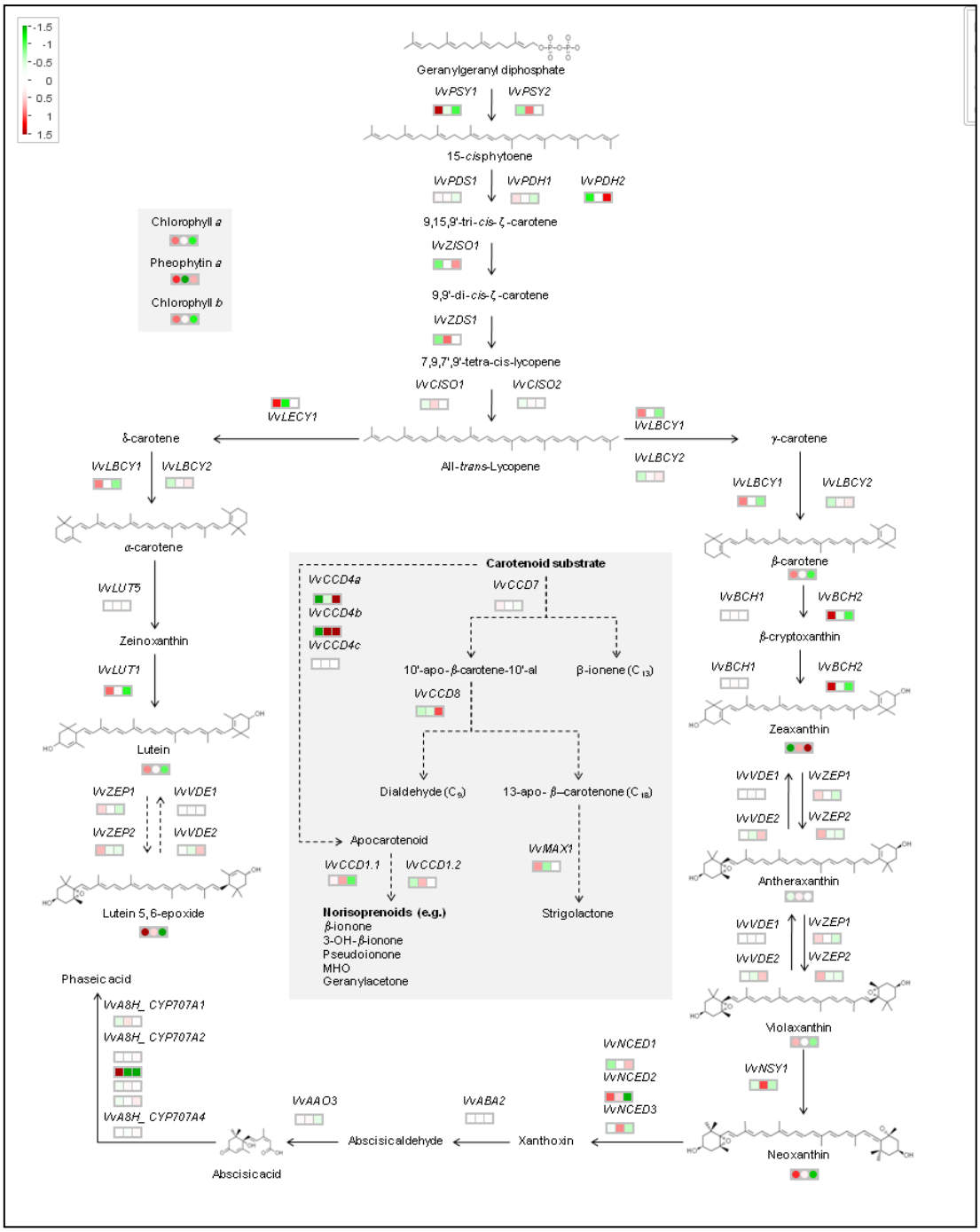


**Figure 1**. Pathway analysis of the carotenoid biosynthetic and catabolic pathways. A heatmap representation of the relative changes in gene and metabolites levels at the three stages of berry development (green, veraison and harvest). The values for the transcripts (squares) and carotenoids (circles) have been log2-scaled and mean centred. The amplitude of the carotenoid values is scaled up 100x for visualisation.

TASK 2: ANALYSIS OF TRANSGENIC GRAPEVINE POPULATIONS WITH TARGETED CHANGES TO THE CAROTENOID PATHWAY

- Milestone 1: Evaluation of a berry suspension culture system to study core processes in berry metabolism *in vitro* (2008 -2010)

Plant cell cultures are an attractive alternative source to a whole plant for various physiological and biochemical studies. Plant cells in a culture are independent of geographic,

seasonal variations and varying environmental factors. They offer a defined production system, which ensures uniform quality and rapid yield.

Berry cultures have not yet been specifically tested for their suitability to study the dynamics of berry development and ripening. To this end we developed organ-specific, synchronised cell suspension cultures from biochemically and developmentally characterised berry explants harvested from the green, véraison and ripe stages (see Figure 2).

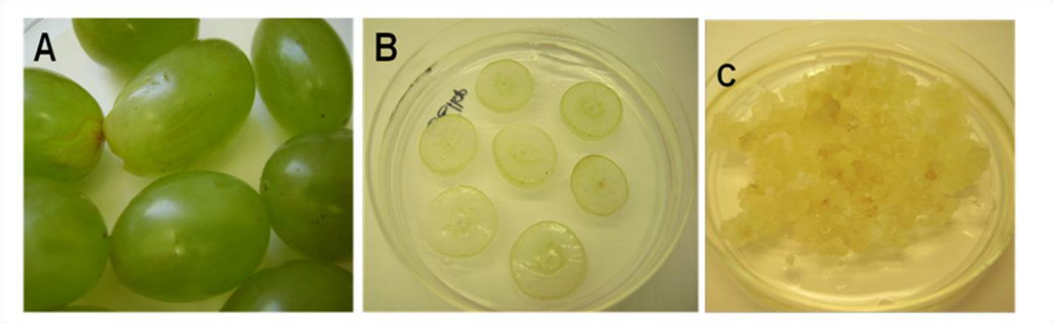


**Figure 2.** Development of berry-derived callus cultures. A. Dauphine berries as explants at the ripe stage; B. Berry explants on callus-initiation medium; C. Somatic callus cultures originating from the plated berry explants. These cultures were used to established suspension cultures.

A proteomic workflow was developed to evaluate the use of berry cell suspension cultures as a tool to study grape berry ripening. Principal component analysis (PCA) showed that the total proteome signal from the different stages, when considered as individual matrices, cluster far apart from each other, confirming that the berry cultures originating from berry explants in different developmental stages, maintained significant differences in the cultured state.

The results using berry suspension cells, combined with the liquid-phase IEF based proteomic experiments and multivariate data analysis show that berry cell cultures are promising to study grape berry ripening. The fact that berry cultures derived from berry explants in different stages of development yielded protein expression patterns that could be matched to expression profiles found in berries could provide an excellent platform for new studies. Our results indicate that this type of suspension cultures could be used to model a range of protein activities found in developing berries. The advantages of having suspension cultures that accurately mimic specific developmental stages are profound and could significantly contribute to the study of the intricate regulatory and signaling networks responsible for berry development and ripening.

The work was published by Ramaschandra, S.G., C. Stander, D. Jacobson, B.K. Ndimba & M.A. Vivier. 2010. Proteomic analysis of grape berry cell cultures reveals that developmentally regulated ripening related processes can be studied using cultured cells. *PLoS ONE*

*Abstract:*

**Background:** This work describes a proteomics profiling method, optimized and applied to berry cell suspensions to evaluate organ-specific cultures as a platform to study grape berry ripening. Variations in berry ripening within a cluster(s) on a vine and in a vineyard are a major impediment towards complete understanding of the functional processes that control ripening, specifically when a characterized and homogenous sample is required. Berry cell suspensions could overcome some of these problems, but their suitability as a model system for berry development and ripening needs to be established first. **Methodology/Principal findings:** In this study we report on the proteomic evaluation of the cytosolic proteins obtained from synchronized cell suspension cultures that were established from callus lines originating from green, véraison and ripe *Vitis vinifera* berry explants. The proteins were separated using liquid phase IEF in a Microrotofor cell and SDS PAGE. This method proved superior to gel-based 2DE. Principal component analysis confirmed that biological and technical repeats grouped tightly and importantly, showed that the proteomes of berry cultures originating from the different growth/ripening stages were distinct. A total of twenty six common bands were selected after band matching between different growth stages and twenty two of these bands were positively identified. Thirty two % of the identified proteins are currently annotated as hypothetical. The differential expression profile of the identified proteins, when compared with published literature on grape berry ripening, suggested common trends in terms of relative abundance in the different developmental stages between real berries and cell suspensions. **Conclusions:** The advantages of having suspension cultures that accurately mimic specific developmental stages are profound

and could significantly contribute to the study of the intricate regulatory and signaling networks responsible for berry development and ripening.

- Milestone 2: Functional analysis of the grapevine carotenoid cleavage dioxygenase (VvCCD1) to evaluate their role in flavour and aroma production

VvCCD1 and VvCCD1-RNAi:
*Vitis vinifera* L. cv Sultana was transformed with the pART27-VvCCD1 and pART27-VvCCD1-RNAi construct. The population was hardened-off and maintained in the glasshouse. The population (representing 8 VvCCD1 and 11 VvCCD1-RNAi individual plant lines) was genetically characterised to verify integration (via Southern hybridisations and/or PCR) and expression/silencing (via Realtime PCR) of the transgene. Results showed that the overexpression of the endogenous *VvCCD1* resulted in a general repression (silencing) of the total *VvCCD1* transcripts in photosynthetic leaf tissue. This is most probably due to homology-dependent gene silencing (HDGS). Realtime PCR was used to show that specifically the endogenous/native transcript levels of the *VvCCD1* gene were affected by the overexpression of the gene in its native background (*V. vinifera*). Although not the aim of the study; this is an interesting phenomenon that will be analysed further to gain insight into the mechanism of transgene silencing in grapevine. The population was characterised by analysing both the pigment content (chlorophyll and carotenoids) and volatiles (specifically β-ionone, the product of VvCCD1 cleavage) of the transformants relative to untransformed control plants.

Results of the 11 VvCCD1-RNAi lines showed that varying degrees of silencing (up to 80%) of endogenous *VvCCD1* expression was achieved (See Figure 3). The population was characterised by analysing both the pigment content (chlorophylls and carotenoids) and volatiles (specifically β-ionone) of the transformants relative to untransformed control plants.
Interestingly, alteration in the expression of *VvCCD1* (by overexpression or silencing) did not alter the total carotenoids (the substrates for VvCCD1) of the transgenic plants relative to the untransformed control plants in photosynthetic leaf tissue. Preliminary data showed a correlation in some plants between the *VvCCD1* expression levels (combined data from both overexpression and silencing lines), and the formation of key flavour and aroma volatiles (specifically $\beta$-ionone, 6-methyl-5-hepten-2-one (MHO) and $\alpha$-ionone).

For the population as a whole (both overexpressed and silenced), no significant correlations could be found between *VvCCD1* expression levels (overexpression or silencing) and carotenoid levels (the substrate(s) for VvCCD1 cleavage) or apocarotenoid volatiles (the product(s) of VvCCD1 cleavage) in the transgenic grapevine leaves relative to an untransformed control plant. It is important to note that since these analyses were all performed on grapevine leaf material; it is possible that the strict control that is required for photosynthesis prevents VvCCD1 cleavage of the integral photosynthetic pigments (by an unknown mechanism). It is also possible that the compartmentalisation that is found in plant tissue physically prevents the interaction of the chloroplast-localised carotenoids from the predicted cytosolically-localised VvCCD1. Currently berry formation is being attempted in the VvCCD1 transgenic grapevine population under glasshouse conditions and to subsequently analyse the transgenic berries to elucidate the *in planta* function of VvCCD1.

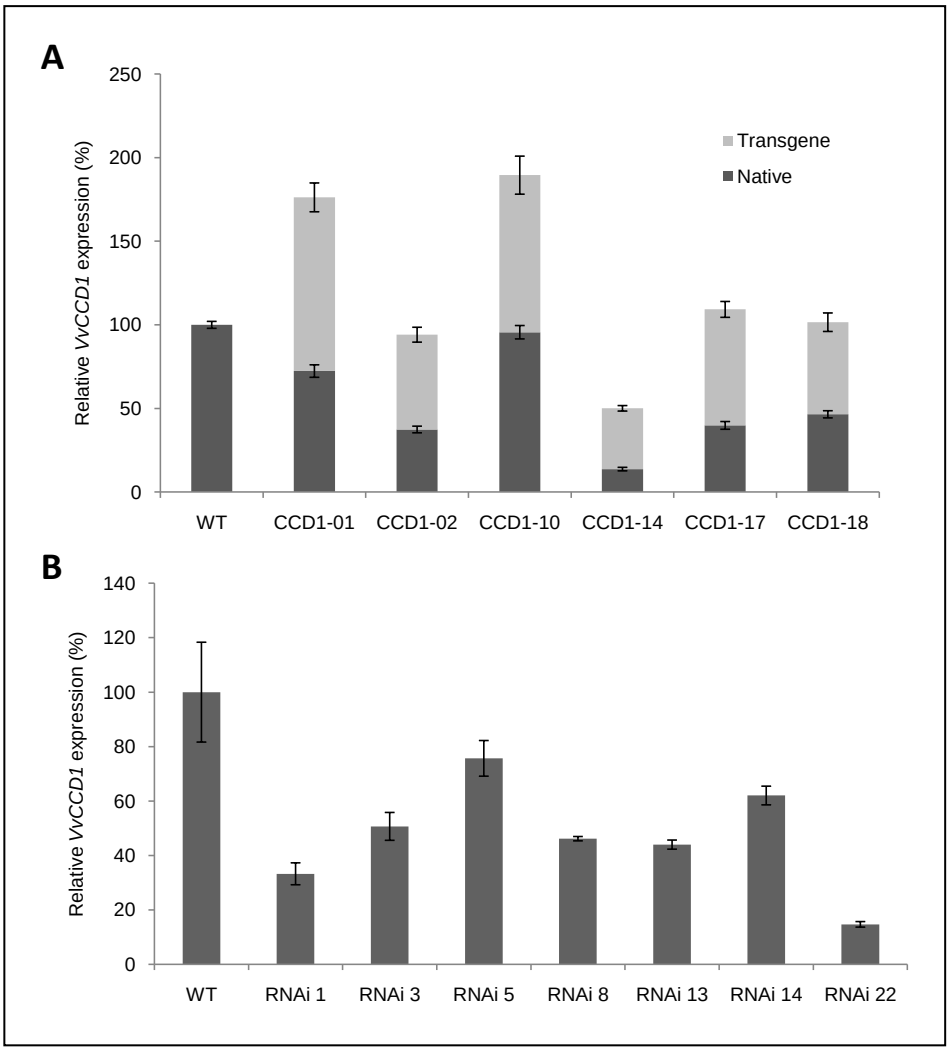


**Figure 3.** Real-time PCR analysis of VvCCD1 expression in the transgenic grapevine population. (A) Expression of endogenous (■) and transgenic (■) VvCCD1 in lines transformed with the overexpression cassette (CCD1) (n = 3). (B) Expression of VvCCD1 in lines transformed with the silencing cassette (RNAi) (n = 3). Data are expressed relative to wild-tpye (WT) expression and normalised to VvGAPDH expression.

This work is currently being compiled for a publication in Phytochemistry.

- Milestone 2: Evaluation of transgenic grapevines overexpressing *VvNCED1* for improved resistance to abiotic stresses (2009-2011).

VvNCED1*:*
*Vitis vinifera* cv. Sultana was transformed with the pART27-VvNCED1 construct. Several putative lines were lost due to fungal contamination and only a small population of putative transgenics survived. The population was hardened-off and maintained in the glasshouse. The population has been genetically characterised to verify integration (via Southern hybridisations) and expression (via northern hybridisation) of the *VvNCED1* transgene. Due to severe fungal infections and a number of clonal copies (i.e. not independent transformants), only four independent overexpressing lines were confirmed; the population is too small to analyse and additional transformations are planned to generate a larger population.
Berry formation is currently ongoing in the VvNCED1 transgenic population.

## 5. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

- Berry suspension cultures have successfully been initiated, synchronised and characterised (2008/2009)
- An HPLC method for carotenoid and chlorophyll profiling from grapevine leaves and berries has been established, validated and published (2009/2010).
- A rapid UPLC method for carotenoid and chlorophyll profiling from grapevine berries was established and was published (2010).
- An HPLC method for simultaneous profiling of the major sugar and organic acids from grapevine berries has been established, validated and published (2011/2012).
- A GC-MS method to analyse volatile aroma compounds from grapevine leaves and berries has been established and is still being optimised (2009-ongoing)
- A proteomic workflow was optimised to separate proteins from berry suspension cultures (2010
- Molecular and metabolite profiling of grapevines in vineyard settings was optimised and lead to a new project in support of viticultural teams (initiated in 2012).

Human resources development/training

Indicate the number and level (e.g. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

| | Student level (BSc, MSc, PhD, Post doc) | Cost to project (R) |
|---|---|---|
| 1. | Justin Lashbrooke (MSc: 2008-2010) | 9,500 |
| 2. | Erik Alexandersson (Post-Doc: 2010-2011) | 0 |
| 3. | Hans Eyeghe-Bickong (Post-Doc: 2011-ongoing) | 87,500 |
| 4. | Liezel Gouws (Post-Doc: 2011) | 20,000 |
| 5. | Sharath Ramaschandra (Post-Doc 2008-2010) | 26,000 |
| 6. | Samantha Dockrall (Hons:2010; MSc: 2011-2012) | 8,500 |
| 7. | | |

Publications (popular, press releases, semi-scientific, scientific)

(1) Young, P.R. & M.A. Vivier. 2010. Genetic and genomic approaches to improve grape quality for winemaking. In: Dr. A.G. Reynolds (ed.), Managing wine quality: Viticulture and wine quality, Vol. 1. Woodhead Publishing Limited, UK.

(2) Lashbrooke, J.G., P.R. Young, A.E. Strever, C. Stander & M.A. Vivier. 2010. The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling. *Australian Journal of Grape and Wine Research* 16: 349-360.

(3) Ramaschandra, S.G., C. Stander, D. Jacobson, B.K. Ndimba & M.A. Vivier. 2011. Proteomic analysis of grape berry cell cultures reveals that developmentally regulated ripening related processes can be studied using cultured cells. *PLoS ONE* 6(2), e14708.

(4) Eyeghe-Bickong H.A., Alexandersson E.O., Gouws L.M., Young P.R., & Vivier M.A. 2012 Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. *Journal of Chromatography B* 2012, 885-886: 43-49.

(5) Young P.R., Lashbrooke J.G., Alexandersson E., Jacobson D., Claudio Moser C., Velasco R. & Vivier M.A. (2012) The genes and enzymes of the carotenoid metabolic pathway in Vitis vinifera L. *BMC Genomics* (in press).

Presentations/papers delivered

(1) Lashbrooke, J.G., P.R. Young, A.G.J. Tredoux, K. Vasanth & M.A. Vivier. 2008. Functional analysis of grapevine carotenoid cleavage dioxygenase (*VvCCD1*) using over-expression and silencing strategies. Eighth International Symposium on Grapevine Physiology and Biotechnology, Adelaide, Australia. (23-28 Nov)

(2) Young, P.R., J.G. Lashbrooke & M.A. Vivier. 2008. Biotechnological potential of carotenoid biosynthetic genes isolated from *Vitis vinifera* L. Cape Biotechnology Forum, Lord Charles Hotel, Somerset West. (30 Nov – 2 Dec)

(3) Young, P.R., J.P. Moore & M.A. Vivier. 2010. The role of carotenoid genes in light and drought stress – towards a systems approach to abiotic stress tolerance in grapevine. Third meeting of FA0605 "Plant Abiotic Stress: from signalling to crop improvement", Valencia, Spain. (26-27 May)

(4) Vivier, M.A. 2010. Genetics and genomic approaches to improve grape quality for winemaking. International *Intervitis Interfructa* Congress 2010 [Sixtieth German Grape and Wine Congress], New Stuttgart Trade Fair Centre, Germany. (24 – 28 March)

(5) Vivier, M.A. & P.R. Young. 2011. GMO grapevines: excellent study models and promising (future) products. Second International Society for Horticulture Science Genetically Modified Organisms in Horticulture (GMO 2011), Nelspruit. (11-15 September) (Keynote speaker - By invitation)

(6) Young, P.R.**,** E. Alexandersson, D. Jacobson, J. Lashbrooke, Z.A. Coetzee, A.J. Deloire, M. Vivier. 2011. The molecular response of grapevine berries to an altered microclimate: the effect of leaf removal/sunlight exposure in the bunch zone on the carotenoid biosynthetic pathway. Plant Abiotic Stress - from Systems Biology to Sustainable Agriculture Conference, Limassol, Cyprus (17-19 November) (POSTER)

(7) Young, P.R., E. Alexandersson, D. Jacobson, J. Lashbrooke, Z.A. Coetzee, A.J. Deloire & M.A Vivier. 2012. The molecular and metabolite profiling of grapevine berries in a model vineyard where the microclimate of the developing bunches has been altered. South African Association of Botany (SAAB 2012), Pretoria University. (15-18 January)

(8) Lashbrooke, J.G., S.J. Dockrall, P.R. Young & M.A. Vivier. 2012. The Carotenoid Cleavage Dioxygenase (CCD) gene family gene family in *Vitis vinifera* L. South African Association of Botany (SAAB 2012), Pretoria University. (15-18 January)

(9) Vivier, M.A., P.R. Young, E. Alexandersson, D. Jacobson, J. Lashbrooke, Z.A. Coetzee & A.J. Deloire. 2012. The molecular response of grapevine berries to an altered microclimate: the effect of leaf removal/sunlight exposure in the bunch zone on the carotenoid biosynthetic pathway. SASBMB-FASBMB 2012 congress, Champagne Sports Resort, Drakensberg, KwaZulu-Natal. (29 January – 1 February) (POSTER)

## 4. Total cost summary of project

| | Year | CFPA | Deciduous | DFTS | Winetech | THRIP | Other | TOTAL |
|---|---|---|---|---|---|---|---|---|
| **Total cost in real terms for year 1** | 2008 | | R 175,000 | | R 275,000 | R 213,750 | | **R 663,750** |
| **Total cost in real terms for year 2** | 2009 | | R 297,000 | | R 297,000 | R 297,000 | | **R 891,000** |
| **Total cost in real terms for year 3** | 2010 | | R 321,000 | | R 326,700 | R 323,850 | | **R 971,550** |
| **Total cost in real terms for year 4** | 2011 | | R 343,470 | | R 347,000 | R 345,235 | | **R 1,035,705** |
| **Total cost in real terms for year 5** | 2012 | | R 0 | | R 0 | R 0 | | **R 0** |
| **TOTAL** | | | **R 1,136,470** | | **R 1,245,700** | **R 1,179,835** | | **R 3,562,005** |