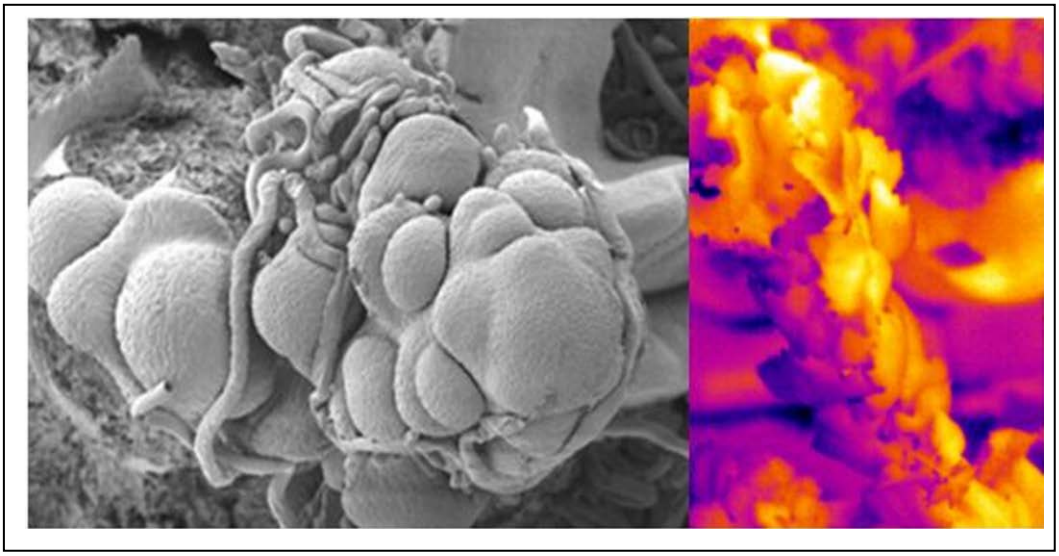
Environment - genotype interactions and the physiological processes determining fruitfulness and yield in grapevines



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **MU 08/02**

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Research Organisation: **University of Melbourne and CSIRO (PI)**

Date: **30/11/12**

Project title: Environment - genotype interactions and the physiological processes determining fruitfulness and yield in grapevines

Project Number: MU08/02

Period Report Covers: Feb 2008 – Nov 2012

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Date report completed: November 2012

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Abstract

Environment - genotype interactions during the early stages of grapevine reproduction and the physiological processes determining fruitfulness and yield in grapevines was investigated. The use of high light intensity growth cabinets proved to be invaluable in studying the effect of temperature while controlling other environmental variables. Differences in the effect of cool temperatures on flower number between propagated Shiraz and Pinot Meunier genotypes were found. Temperature effects may be due to changes in carbohydrate partitioning and/or gene expression pathways. The spatial expression of known key flowering genes *VvTFL1*, *VvLFY* and *VvFT* were explored. Increases in *VvTFL1* gene expression in latent buds was correlated with fruitfulness as determined by bud dissection and Scanning Electron Microscopy (SEM) in a selection of varieties and environments. Correlation of the genes *VvLFY* and *VvFT* was undetermined and likely reflects the complexity of the fruitfulness/environmental interaction. Over twenty new genes involved in grapevine flowering were identified by gene expression studies with four of considerable interest for further study. Temperature has a significant and complicated association with optimal bud fruitfulness and the findings reported here suggest that complexity in gene expression of known and new flowering genes reflect these associations. In order for the viticultural industry to predict and manipulate optimal fruitfulness and yield, having a solid knowledge of the role of flowering gene expression is essential. Research here has identified new genes, advanced our knowledge of temperature-physiology-gene interactions and this knowledge will assist the industry in understanding the drivers of yield and fruitfulness.

Executive Summary

Introduction: Yield instability is estimated to cost the Australian Wine Industry more than $200 million annually. Thus the prediction of yield potential and the management of yield are of utmost importance. This project aimed to explore how the environment and genotypes interact to determine fruitfulness and yield by investigating the stages of grapevine reproduction. Yield potential depends on the transition of apices to a floral state and their subsequent differentiation to an immature stage in developing grapevine buds. The early stages of reproduction are described as initiation and differentiation; they set the maximum fruitfulness and optimal yield potential and are the least understood in grapevine reproduction. For grapevine, inflorescence primordia initiation and partial development occurs in the latent bud in season 1 and largely determines yield potential as defined by inflorescence number per cane and inflorescence branch number. In season 2 the bud bursts and the inflorescence completes development and during this stage inflorescence branching and growth is completed and flower number determined. Final flower number per inflorescence contributes to yield potential but has a smaller effect compared to inflorescence primordia number. The research described in this report focused on genotypic-environmental interactions during the early stages of grapevine reproduction.

Methods: The genotype-environment interaction was investigated by using the Pinot Meunier genotypes; L2 (wild type), L1 (the microvine system) and picovine. Shiraz propagated canes were also utilized to compare genotypic-environment effects. To enable understanding of genotype-environment interactions gene expression studies were conducted on the different genotypes. Initially spatial analysis of key flowering genes *VvTFL1*, *VvLFY* and *VvFT* was conducted. Flowering gene expression in inflorescence structures, leaves, apices, tendrils and buds, at different positions along the shoot were assessed and compared. To identify novel genes Pinot Meunier genotypes L2 and picovine were selected as; (1) they showed significant differences in key flowering gene expression levels and (2) they demonstrate genetically similar backgrounds which increased the likelihood that any gene expression changes result from the difference in inflorescence development pathways. Spatial assessment of key and novel flowering genes was conducted on field grown Sauvignon Blanc buds (N+2). Bud fruitfulness was determined by light and scanning electron microscopy techniques. Climatic interactions on Sauvignon Blanc buds gene expression in vineyard environments were assessed.

Results: Cool temperatures (day/night 17/15 °C) under a high light environment allowed the highest yield potential promoting large flower numbers in propagated Shiraz cuttings. The morphology of the inflorescence structure in the cool treatment was significantly different

from the other treatments, with a shorter total inflorescence length and outer branch lengths, indicating an altering in carbon partitioning or an effect on processes determining cell number and/or size. Higher temperatures promote absolute growth rate which result in longer inflorescences while suppressing flower production. These effects are promoted by the day and not mitigated by cooler night time temperatures. When investigating genotype - environmental interaction on fruitfulness and yield, we found that the Pinot Meunier genotype L1 in the cooler temperature had reduced growth and the potential for carbohydrate production was altered by reducing shoot growth rate, relative shoot growth rate in the first 42 days, and a reduction in the number of nodes separating along the shoot. Flower number was not significantly impacted by the cool treatment but less inflorescences were produced resulting in a significantly reduced number of flowers per shoot. The cool treatment resulted in a reduced optimal yield potential. These differences may be explained by varietal differences in flowering gene expression or that the L1 genotype had relatively less resources than the cooler treatment plants in the propagated experiment. In season two, hotter temperatures advanced both shoot development and shoot growth rates. The number of inflorescence structures in the second season was promoted while lower flower number was suppressed in the cool treatment. The results from the second season showed that L1 interactions were similar to the propagated Shiraz results.

Under controlled temperature environments, the genes *VvFT*, *VvTFL1* and *VvLFY* were found to be expressed during flowering in the Pinot Meunier L1 genotype. It was found that *VvTFL1* was highly expressed in the leaves opposite inflorescences, apex, inflorescences and the tendrils. The relatively high gene expression of *VvFT* occurred in the tendrils, inflorescences and apex. The expression of *VvLFY* was relatively low in all organs tested. The Pinot Meunier picovine genotype showed significantly higher levels of expression in the Leafy gene *VvLFY* and interestingly in the flowering locus gene *VvFT*. The Pinot Meunier picovine genotype and the Pinot Meunier wild type (L2) were selected for novel gene analysis. A new gene, called *VvID3* was identifying as occurring in the picovine at a very high relative expression level. New genes called *A3*, *D*, *J* and *I* show homology to genes involved in floral development. The results suggest these newly identified genes may have a role in floral development and/or the very early stages of flower induction in grapevine. Another significant finding was that the gene *A3* is found in the grapevine inflorescence and this gene showed homology to the basic blue protein in Arabidopsis suggested to be involved in pollen tube development (Dong et al 2005). Gene *1* was identified in grapevine flowering structures for the first time. This gene is associated with copper transport and therefore could be involved in a similar floral gene expression pathway to *A3* or controlled by the same transcription regulator. Findings here suggest that further investigation into the genes *A3*, *1* and *E*, are required to determine if they represent significant components of a floral induction pathway. The identification of these new genes and further studies would allow greater insights into grapevine flowering.

To assess the impacts of temperature on bud fruitfulness, two controlled environments of day/night 27°C/30°C (hot) and day/night 17°C/15°C (cool) were compared. Expression of the flowering genes *VvLFY* and *VvTFL1* in the buds (N+2) of Pinot Meunier L1 genotype under controlled environmental conditions were similar but the relative expression of *VvFT* was very low. The relative expression of the flowering gene *VvTFL1* at node position 2 was significantly higher in the hot environment. In the field environment in Australia, this gene expression activity coincides with the period of veraison. Heightened differentiation of inflorescence primordia occurs in the latent bud during this time. *VvTFL1* is thought to be implicated in the suppression of apical dominance. Cooler temperatures resulted in reduced bud fruitfulness. Cooler temperature also reduced the expression of the genes *VvLFY* and *VvTFL1*. These finding link with the field studies findings, as conditions that reduce fruitfulness (e.g. vine development stage) are also associated with relatively lower expression of the genes *VvLFY* and *VvTFL1*. In summary the expression levels of three genes *VvTFL1*, *VvLFY* and *VvFT* known to be associated with flowering in grapevine, varied with the stage of ontogeny of reproductive development, node position of the latent bud in field grown vines from hot and cool locations.

We found a correlation (in both field and controlled cabinet experimentation) that high fruitfulness was associated with relatively high expression of gene *VvTFL1*. Whereas the genes *VvLFY* and *VvFT* were found to be expressed at both high and low levels in the field compared to controlled environmental conditions. This could be a variety specific trait as in the field we collected buds from Sauvignon Blanc whereas from the chambers we collected buds from Pinot Meunier L1 genotype. We identified high expression of the gene *VvID3* in Sauvignon Blanc buds from the field environment as well as from the picovine genotype, where flowering is prolific. This supports the role of this gene being involved in a common pathway for floral induction. Differences in anthesis and veraison bud gene expression of gene *VvID3* and *VvLFY* were found between Dookie and King Valley vineyard.

Sultana, a very low fruitful variety, was used as a comparison with Sauvignon Blanc buds to aid gene function investigations. The relative expression of the gene *VvID3* and gene *E* was higher in Sauvignon Blanc than in Sultana. Gene *D* relative expression was higher in Sultana and to date the function of this gene in other species has been implicated in seed dormancy and pollen tube development but the results reported here suggest that its function has not been fully described. We recommend that using these genes as a base point, targeted research can assist in developing an understanding in both their function in plant flowering and specifically their function in grapevine flowering.

Conclusions: We have found that temperature has a significant and complicated association with optimal fruitfulness in grapevine in both field and controlled environmental conditions. The advantage of using the high light growth cabinets described in this study is that environmental conditions can be precisely controlled with only the experimental variable

being investigated (in this case temperature) being varied to investigate environment- gene interactions. This is not the case with field studies and this was proven in this study when a hail event resulted in a forced change in site selection and the temperature differences observed between the cool (King Valley) and warm (Dookie) sites was not as great as expected when the environmental variables were measured. The advantage of using Pinot Meunier L1 genotype or dwarf continuous flowering microvine and picovine genotypes for flowering studies was proven in this study for experiments involving both short-term and long-term growth cabinet studies over several “seasons” with more experiments and “seasons” being possible in a single year than possible if the plants were grown in a field. The use of a continuous flowering Pinot Meunier L1 and picovine genotypes where tendrils are replaced with inflorescences proved valuable for gene expression studies to identify new genes associated with grapevine flowering. The identification of a large number of new genes involved in grapevine flowering reported here demonstrates the advantages of combining new high throughput molecular techniques like mRNA-seq with novel genotypes such as the picovine to discover new genes and improve our understanding of floral induction pathways. Evidence from other plant species is now emerging that components of the flowering process and flowering control are very complex as some genes have been shown to promote one activity in the flowering pathway while suppressing others and further research into grapevine fruitfulness is required if we are to better understand this complex process to improve and predict yield especially as the flowering process in grapevine occurs over two seasons.

Acknowledgments

The authors of this report would like to thank all of the hard work and excellent efforts of the staff employed in this research. Dr Tom Hill, Dr Andrea Watt, Mr Mark Foletta, Ms Sally Foletta, Mrs Carmel Lancaster, Ms Angelica Jermakow, Dr Ying Zhu and Mr Don Mckenzie. We would like to especially recognize the excellent contributions of Mrs Pat Corena who's extensive molecular biological expertise was greatly appreciated.

The project team are very grateful to the wine companies and growers who collaborated to the project by generously giving us time, plant material and their extensive knowledge of their vines. Thanks to Dookie College and vineyard manager Mr Mark Elliott. Thanks to vineyard manager Mr George Fitzgerald from Brown Brothers in the King Valley. Thanks to Helen and Johnny Cavedon vineyard managers and owners from the King Valley.

We would like to thank the Rowden White Foundation for the receipt of a large infrastructure grant proposal award in 2008. This grant allowed us to purchase the three growth cabinets that were essential for the project aims and objectives.

Thank you to the GWRDC for funding and supporting the research presented here.

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1 Background

It has been estimated that yield instability costs the Australian Wine Industry more than $200 million annually (Clingeleffer et al. 2001). Thus the prediction of yield potential and the management of yield are of utmost importance. Yield potential depends on the transition of apices to a floral state and their subsequent differentiation in developing grapevine buds. Grapevine reproduction occurs over two seasons which is a long period where environmental/management factors can influence yield potential. The early stages of reproduction are termed initiation and differentiation and these processes occur within the first year of bud development. It is at this stage where the scientific understanding of these processes is limited. The limitations to our understanding have been brought about by both the complexity of the system/environment interaction and by limited scientific technical capacity. Technical issues also include difficulties with quantitative measurement of developing buds and natural variation across bud populations within grapevines. This project combines traditional physiological and anatomical approaches with current molecular tools to improve our understanding of the variables that drive fruitfulness and the development of yield in grapevines.

The ability to combine the disciplines of microscopy and molecular biology to determine gene expression under different environmental conditions and developmental stages has been the missing link in the progression of knowledge in the area of grapevine reproductive biology. Past studies have focused either on comparative studies of vines grown in the field or in a controlled environment. Yet other studies have focused on gene expression in the grapevine over time. This study aims to span gene expression in grapevine plant parts under different growing conditions and time points and linked these results with comparative studies of inflorescence and latent bud morphology.

1.1 Industry Context

Adverse consequences of fluctuating wine grape yields are a common problem throughout the Australian wine industry for both growers and wineries. Clingeleffer et al. (2001) conservatively estimates that the level of variability (usually 15% but up to 30% or more) is costing the Australian Wine industry more than $200 million annually. A recent Mildura ASVO meeting on “Transforming Flowers to Fruit” in July 2005, a GWRDC sponsored Tatura workshop on Flowering and fruit set in grapevines and a Merbein Symposium on “Reproductive Biology in Grapevines” in June 1999 highlighted the importance of this topic to industry even in a period when the industry was experiencing a cyclical imbalance as yield directly affects most growers profit margin.

1.2 Preliminary and Previous Research

Statistical analyses of sources of variation (Clingeleffer et al. 2001) showed that fluctuating yields are primarily due to season-to-season variation in inflorescence number per bud (bud fruitfulness) and to a lesser extent inflorescence size. Hence, gaining an improved understanding of the physiology of the formation of inflorescence primordia within the latent bud and their subsequent development is crucial. Peter May in his recent 2004 book "Flowering and Fruitset in Grapevines" reviewed all the existing literature and in his concluding remarks stated that "basic studies of the physiology of the flowering process are needed" and "the use and further development of techniques of molecular biology will have to underpin such studies".

This research proposal translates Peter May's conclusions into action by combining the molecular skills of the CSIRO flowering group with the whole plant physiological skills and expertise in reproductive anatomy of The Melbourne University Viticulture research team. It is also clear from the scientific literature that the large majority of significant advances in life sciences, including plant science, now occur by combining molecular studies with more traditional physiological approaches to understand specific biological processes. This collaborative project adopts a molecular physiology approach to advance our understanding of the control of flowering in grapevines.

It is generally acknowledged that in spite of many field trial studies over many years there has been little new knowledge produced since the early pioneering studies conducted in Australia by Barnard in the 1930s and Antcliff, Buttrose, May and others in the 1960s and 1970s. The major conclusion from past studies and recent GWRDC projects (CSP00/2 Crop control for consistent supply of quality winegrapes, CSH96/1 Crop development, Crop Estimation and Crop Control to Secure Quality and Production of Major Wine Grape Varieties: A National Approach) is that seasonal (environmental) effects are greater than management effects and that different cultivars (genotypes) respond differently.

Field studies have identified that temperature and light influence aspects of fruitfulness and yield. However, a perennial problem with field experiments that attempt to elucidate plant responses to environmental factors is the confounding effect of other environmental variables. For instance, high temperature coincides with a high number of sunshine hours or cloudiness tends to increase relative humidity. In an attempt to separate the effects of temperature and light on inflorescence primordia formation Buttrose (1974) conducted a series of studies in controlled-environment growth cabinets. Buttrose identified 'windows of sensitivity' to both light and temperature and described important differences between cultivars. However, there is an apparent contradiction between the sensitivity of the stages described by Buttrose in the growth cabinet and the field studies of May and others and the statistical studies of Alleweldt and Baldwin. Furthermore, predicting wine grape yields based on the field-based approaches used by Baldwin has not been very successful.

The initial studies of Buttrose were limited and done at a time when growth cabinet facilities were expensive and light intensities much lower than in the field. However, we now possess new knowledge and tools to advance our understanding.

2 Project Aims, Overviews and Objectives

2.1 Aims

This project aims to investigate the environment - genotype interactions on fruitfulness and yield in grapevines. In particular the environmental factors investigated were temperature and light. Varieties Sauvignon Blanc, Pinot Meunier and Shiraz were used in these studies. Genotype interactions were investigated using Pinot Meunier genotypes; wild type (called L2 in this research), Pinot Meunier L1 genotype (microvine genotype developed by CSIRO) and picovine genotype. The physiological processes explored were development of inflorescence primordia and associated known flowering genes *VvTFL1*, *VvLFY* and *VvFT*, as well as exploring for novel genes. These investigations aimed to add understanding into the stages of initiation and differentiation, which are the critical processes required in early yield establishment.

2.2 Overview of the Project Research

In order to carry out the aims of this project we have utilized a suit of experimental approaches along with across discipline investigations. The environmental conditions that we utilized in this project included; environmental control facilities where we obtained state of the art technology specifically for this project to allow accurately controlling temperature while maintaining high light intensities. Investigations were conducted into the light conditions to ensure plant growth was healthy and representative in these environments we examined processes in glasshouse grown winegrapes and we conducted two seasons of experiments in the vineyard environments. In order to conduct statistically robust experimentation in the controlled environmental cabinets, we were able to utilized Pinot Meunier genotypes L1 (microvine). The Pinot Meunier L1 genotype has phenological traits that include reduced internode length which result in smaller plants that are both fruitful and can thrive in the controlled environmental conditions. The Pinot Meunier L1 genotype is unique in that floral initiation occurs both inside a latent bud similar to a normal vine and also outside the bud where inflorescence structures form instead of tendrils (Boss & Thomas 2002, Thomas unpublished). The modified flowering phenotype is due to a mutation in the *VvGAI1* gene. Berry development is similar to a normal vine (Thomas unpublished). Therefore, this unique phenotype is ideal to study the effect of different environmental conditions on inflorescence initiation and size. Propagated canes were utilised in the project. Researchers at the University of Melbourne recently refined techniques to successfully grow Shiraz ‘propagated cane’ (Mullins and Rajasekaran 1981) (Dr Watt and Dr Collins, personal communication). Pinot Meunier L1 genotype

and Shiraz canes were grown under different environmental conditions in growth cabinets and developmental and physiological techniques developed to objectively measure the vines flowering response. Instrumentation to measure environmental variables provided datasets for correlating environmental changes to flowering responses and gene expression changes in the field environment. Molecular biology technologies were used to understand how genes are linked to developmentally controlled regulation and respond to different environmental stimuli. The technologies that enable understanding of gene expression begin with methods to isolate RNA and then different analysis such as sequencing and quantitative PCR. High through put gene expression analysis by next-generation sequencing enabled a large number of genes to be examined simultaneously. This technique proved suitable for the identification of unknown genes and provided a snap shot of how genes were actively expressed in the tissue at a specific time and stage of development. Real time quantitative PCR enabled a statistically robust comparison of gene expression.

In summary, this project generated new knowledge obtained using controlled environments and associated molecular biology techniques and investigated correlations with field studies testing responses across climates, cultivars and genotypes.

2.3 Objectives

To assist in the delivery of research aims, two principle research objectives have been formulated.

1. Develop techniques to rapidly test and measure the effect of key environmental variables on grapevine flowering and yield.
2. Understand the impact of key environmental factors on genes and physiological processes that regulate inflorescence induction, size and flower number leading to the identification of specific developmental stages sensitive to environmental variables. The understanding of these environmental/gene interactions was investigated and developed using both a suite of experimental conditions such as controlled environmental cabinets, glasshouse and field conditions along with a suit of vine systems including genetic ‘model’ plants e.g. the Pinot Meunier L1 genotype called the ‘microvine model system’, plant material derived from cane propagation, a non-fruitful model system (Sultana) and winegrape material (Shiraz and Sauvignon Blanc varieties) grown in field environments.

3 Methodologies

3.1 Controlled Environment Conditions: Cabinet Testing, Methodology and Set Up

3.1.1 Cabinet Set Up

The understanding of fruitfulness, its drivers and the environmental interactions on grapevine reproductive phenology have progressed greatly over the last decade (May & Antcliff 1963; Mullins 1966; 1969; Srinivasan and Mullins 1981; Clingeleffer 2001; Lebon 2004; May 2004: Vasconcelos et al 2009; Chaib et al 2010). With the coupled advances in engineering of controlled environmental cabinets and breeding to alter vine size, it is now possible to conduct statistical robust experiments on wine grapes under high light conditions while controlling and standardizing other environmental parameters such as temperature and humidity. Due to the important role that high light radiances exhibit on some stages of fruitfulness (Srinivasan & Mullins 1980; Carmona et al 2008), we decided that it was critical to investigate previous assumptions. The difference between a low light environment and a high light environment may in part go some way to explaining the difference in phonological responses observed between controlled environmental cabinet experiments and experiments conducted in field environments. We commissioned and installed three Conviron Controlled Environmental Cabinets, CMP5000, BioLab, Industrial Technologies, Australia, (Plate 1) on the 28th Aug 2008. The cabinets were funded by a Rowden White Foundation infrastructure grant award in 2008. The functionality of the cabinets allows a high quality light spectrum while controlling temperature and humidity.

We were able to achieve a high quality light spectrum which aims to be as close as possible to the sun visible/UV spectrum by installing a selection of lightening sources, (1) High pressure sodium lamps: philips 400 W Alto, Hg, USA (K7.1, C400S51) and metal halide bulbs (Sylvania 400 W metalarc, M59/E, MS400/H0). The order of light sources can be programmed to allow the lights containing a higher proportion of blue light to come on first and the lights containing more red spectrum to go off last. The importance of this feature is that it allows us to 'mimic' sunrise and sunset more realistically. Day length was set at 16 hours for all experiments. Please refer to individual experiments for day and night temperatures, humidity and light intensity settings. During the set up of these cabinets we measured light quality and quantity with air temperatures. During plant experimentation we set cabinet temperature settings based on infra red measured bud temperatures. We found about a 5°C difference between the set cabinet temperature and the bud temperature.



Plate 1 Conviron Controlled Environmental Cabinets CMP5000.

3.1.2 Measurement of Cabinet Light Quality

Wine grapes evolved to in northern Eurasia about 65 million years ago where they adapted an evolution strategy of climbing tree canopies. Growing up rather than outwards was an evolutionary trait that gave a competitive advantage of being able to grow in what would have otherwise been a dark undergrowth, with insufficient light to survive. The importance of this evolutionary trait has ensured that grapevines, like many plants, can detect the light environment and even its wavelengths. The mechanisms which allow the detection of light is enabled by a group of compounds called the photoreceptors. In winegrapes, 6 photoreceptors from 2 families have been described. The first family is the phytochromes, where PHYA detects far red radiation and PHY B, PHY D and PHY E detect near red radiation. The second family cytochrome enables detection of blue light by CRY 1 and CRY 2. It is believed that photoreceptors play an important role in flowering as well as other processes such as circadian rhythm (a plants internal clock). In order for a response in a growth cabinet to reflect what would occur under field conditions, it is important that both light quantity (usually measure in µmol) is sufficient and very importantly light quality to allow correct functionality of the photoreceptors and associated physiological responses they control.

It should be noted that where controlled temperature experiments have been conducted to try and understand flowering, light has been reported as low, anything from 200 to 500 µmol. While grape vines can exist under this low light intensity (1/6 of the light quantity in an average summer day in Australia) they have evolved to only flower when light intensities are in full sunlight (800 µmol upwards. In research here, we are using our knowledge of grapevine evolution to test assumptions that pertain to flowering effects.

Light quality measurements were collected to determine the quality of the combined light sources and to determine if there were any low quality light areas within plant height area. Light quality and quantity spectrum measurements were only taken after the lights had been illuminated for 30 minutes and thus assumes full saturation of the light sources. The chamber floor was adjusted in each chamber so that the middle (coordinate H100, V20, D5 see Figure 1) was at a PAR of 1100 µmol enabling standisation and direct comparison of the cabinets. Within chamber B, using a miniature fiber optic spectrometer, (model EPP2000 Stellar Net Inc, USA) light measurements in the UV and visible ranges from 190-1000 nm were measured at various ‘plant’ positions within the chambers (Figure 1). Measurements were made in the scope mode, which can measure specific wavelength applications for light emission from fluorescent lamps and all types of light bulbs. Settings in this mode were: wavelength range 194 – 1110 nm measuring every 0.5 nm, 50 scans to average (Y axis smoothing), pixel resolution (X axis smoothing) was set at 560 pixels, no mathematical pixel smoothing adjustment was selected and a detector integration rate (spectra signal level) of 7 milliseconds (ms). Measurement of spectra was taken at 25°C. Measurements are recorded as counts, which represent uncompensated spectral data. Measurements at one set location was taken as a comparison in the other chambers, again where floor height had been set to a PAR of 1100 µmol. All spectra are illustrated in Figure 2 and very comparable spectra were obtained not

only across chambers but also within a chamber. There were only two locations in the chamber where the spectra were considered low in quality and these locations were not used.

A comparison of the spectrum of the sun (blue line July 2009 11:00am), probe pointed directly at sun and chamber (red line) is illustrated in Figure 3. Please note absolute quantity cannot be directly compared but relative peak intensity can be. The cabinets spectra are taken from mid right hand side of chamber 5 cm from base where the cabinet lights were set on an output of 1100 µmol m^{-2} s^{1}.

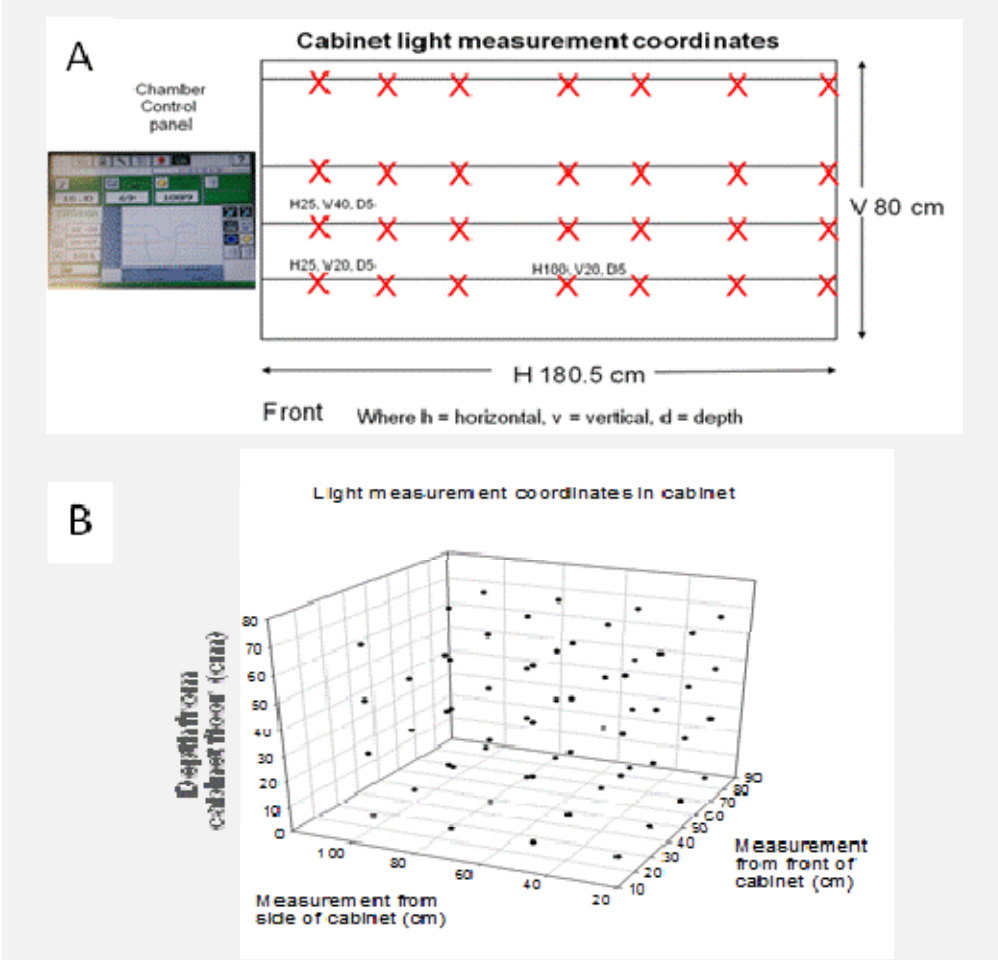


Figure 1 Illustrates map of data collection locations in the cabinet (A) and 3 dimensional visual representation of coordinates (B) for light spectrum measurement experiment

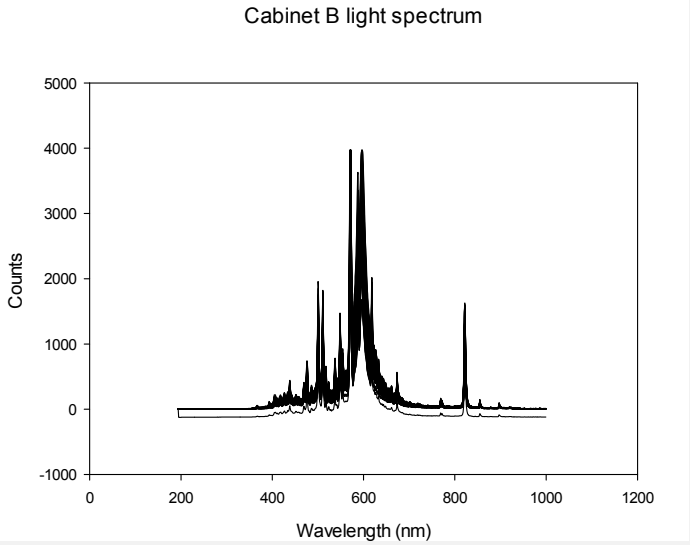


Figure 2 Light spectra all coordinates in the cabinets

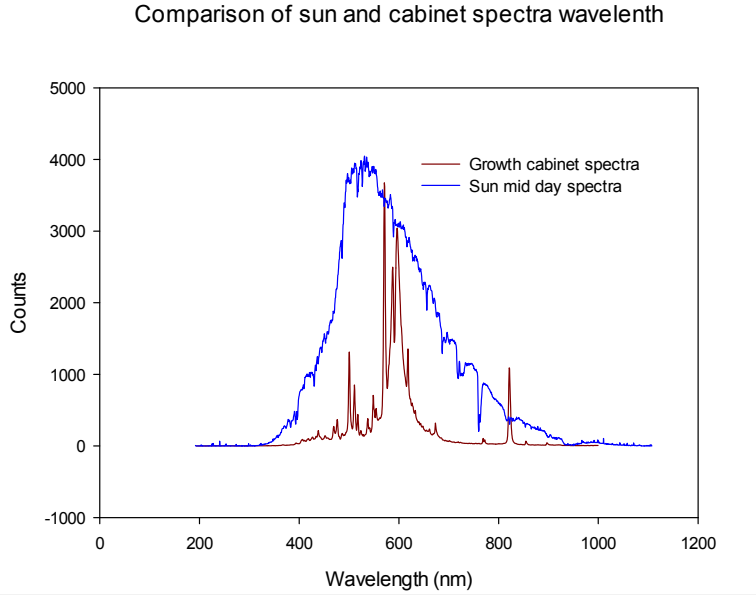


Figure 3 Illustration of sun and cabinet light spectra. Miniature Fiber Optic Spectrometer, model EPP2000 Stellar Net Inc, 14390 Carlson Circle, Tampa, FL 34677.

3.1.3 Cabinet Temperature Measurements

Cabinet temperature measurements were collected both manually and automated and these measurements were compared to ensure that the setting of the chamber temperature was reliable. In order to ensure that cabinet temperature across all units were comparable, cabinets were set up at the same temperature. A glass container with water and a temperature thermometer was placed in the middle of each chamber. A day and night temperature of 25°C was set on each chamber and temperature was monitored for a week. Thermometer and chamber recorded temperatures were comparable. A second thermometer placed in the chamber at plant height was used as a backup and to confirm cabinet temperature upon remote sensing (Plate 2).

Once we had established cabinet temperature was both reliable, accurate and comparable between all cabinets we then correlated the cabinet temperature with the winegrape bud temperature. We were able to do this experiment with the use of an Infra red camera (Plate 3). This ensured that the grapevine bud and flower temperatures were at the desired temperature both day and night. Three temperature treatments were implemented (Table 1) based on measured bud temperatures assessed using a thermal camera. Light quality variations in the cabinet were measured (using a spectroradiometer) and the high light conditions implemented were constant. These measurements have enabled us to refine experimental design and measurement technologies.



Plate 2 Cabinet control panel. Monitoring of Temperature, humidity and light conditions

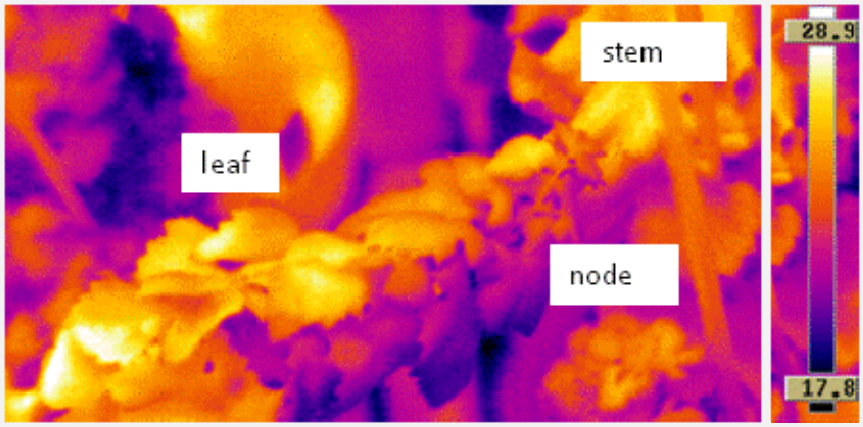


Plate 3 Illustration of the infra red temperatures measurements of Pinot Meunier L1 genotype in Cabinets

Table 1 illustrating bud tissue temperature and chamber inlet air temperatures

Bud temperature		Chamber inlet air temperature	
Day temp. (°C)	Night temp. (°C)	Day temp. (°C)	Night temp. (°C)
27	30	24	30
27	15	24	15
17	15	13	15

3.1.4 Uniform Plant Growth Experiment

Growth Cabinet commissioning was only completed once uniform plant growth experiments were conducted. The aim of these experiments was too ensure that we were able to produce healthy plants in our cabinets, that no cabinet exerted different influences on plant growth and that the light conditions were able to promote flowering. Three plant varieties were selected for these experiments, savoy cabbage (*Brassica capitata var. sabauda L*), dwarf purple bean (*Phaseolus vulgaris*) and the microvines (*Vitis* our experimental vine plant). Fifteen dwarf bean plants were potted up (pot dimensions of height 16.4 cm by top diameter 17 cm) at 2 leaf stage on the 25th March 2009. Initial plant height was recorded and all cabinet parameters were set equally. We concluded from the above testing that it was possible to grow plants in the chambers. The chamber environment was suitable for flowering conditions and that healthy grape vines could grow in this environment (Plate 6).

3.1.5 Plant Material

3.1.5.1 Propagation techniques

Canes from Shiraz vines planted in 1994 with 3.4 x 1.8 m spacing on 140 ruggeri rootstock were collected from the Dookie vineyard in 08th July 2009. Vines were subjected to a frosty night before collection. One the day of cane collection the weather was clear, sunny and cold <10°C where the collection times was conducted between 3-5 pm. About 180 canes were collected for propagation (Mullins and Rajasekaran 1981) over an area of 2.2ha and cut from node 3. Cane preparation involved trimming from the top down to 6 nodes for each (bottom cut perpendicular, top cut angled and base marked with a black line). These canes were transported in plastic bags in groups of 20 and immediately stored in the dark at 4°C. Canes were placed in a Mulliniser growing box (Plate 4) and roots were subjected to known temperatures (Figure 4) in the range of 25 - 28 °C. To ensure the buds remained dormant while root growth was taking place the container was placed at 4°C. Canes were routinely checked for root growth (Plate 5).



Plate 4 Illustration of the Shiraz vine propagation (29th July 2009).

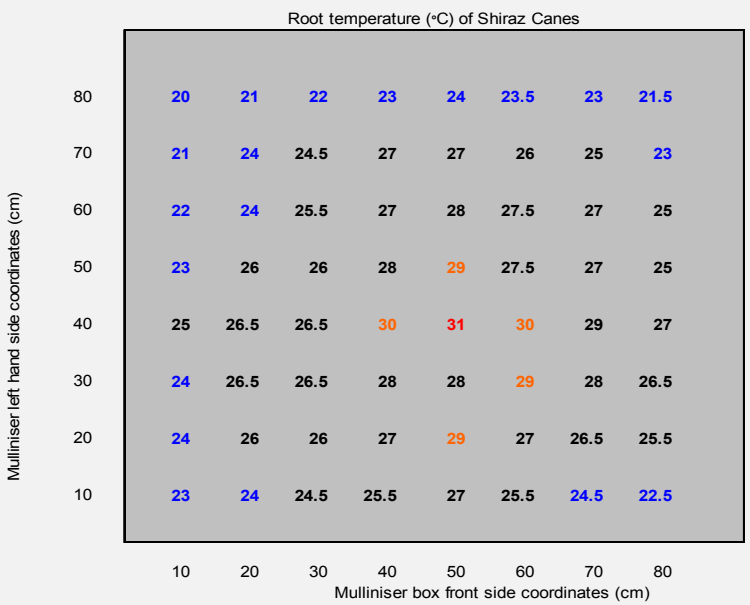


Figure 4 Root temperature of propagated Shiraz canes in "Mulliniser" box (29th July 2009). Areas below 25°C were not used.



Plate 5 Shiraz canes showing root growth (14th Aug 2009)

The number of replicate canes per treatment was determined based on analysis of data kindly supplied by Dr Watt via "Sisa' software where a continuity correction was applied, a power of 0.8 (which equals to an 80% chance of discovering a real difference) and using a double sided t-test (as increase in temperature may increase or reduce flower number (Dr Watt). When healthy root growth was established canes were transported at 4°C to the environmental cabinets **(**Plate 15**).** Refer to results Section **4.3** for details about experimental conditions.

3.1.5.2 The Pinot Meunier Genotypes

The Pinot Meunier genotypes are a selection of genotypes developed at CSIRO. The Pinot Meunier L1 genotype, also called the "microvine system" or "dwarf mutant", is a compact vine that has several attributes that make it ideal for the investigation into winegrape fruitfulness. It flowers continuously (Boss & Thomas 2002) and is able to produce 3-4 crops a year. This model vine has reproductive and berry development patterns similar to normal field grown vines (Chaib *et al.* 2010).



Plate 6 Established Pinot Meunier L1 genotype in the growth cabinets

3.1.6 Irrigation

Please refer to individual experiment for details of irrigation practice. All stock plants and vine replicates between experiments were watered twice a week (approximately 300 ml per plant but this did vary depending on plant size). Once a month all plants were soaked in water for 1 hour to allow for removal of built up salts. All plants were watered with a weak solution of seaweed solution to assist in general plant health, see fertigation section for further details.

3.1.7 Fertigation

All cabinet grown plants during experiments were grown in the following potting mix (Table 2) with a slow release fertigation application of Osmocote Plus (Table 3). The potting mix was pH adjusted 6.2 – 6.5 before planting. Sand was sieved to ¼ inch. Throughout the plant life fortnightly applications of seaweed solution were applied with irrigation except one month before experimentation where applications were increased to weekly. Seaweed Solution, Seasol supplied by Seasol International Pty Ltd, 1027 Mountain Hwy, Bayswater, Vic 3153, Australia. Week conditioning application rate used in these experiments were 150 ml per 9 L water.

Table 2 Potting mix used in all cabinet experiments.

Potting Mix	**Amounts**
Pine bark – medium, Orchid growing media, 50% composted from Debco Pty Ltd, 12 McKirdys Road, Tyabb Victoria, 3913, Australia.	20 L
Sand – grade course	10 L
Osmocote - Plus (see Table x for chemical contents and concentrations)	120 g
Ferrous sulphate hepta-hydrate	30 g
pH mix	60 g
pH mix	
lime - Calcium hydroxide Ca $(OH)_2$, Mol wt 74.09g, supplied by May and Baker	30
Gypsum is Calcium sulphate is supplied by AJAX Chemicals Ltd	30
Dolomite is fine race horse quality dolomite sourced from Bennella	110

Table 3 Osmocote Plus Scotts Australia Pty Ltd chemical content and concentrations.

Compound	%	Compound	%
Nitrogen	15	Copper	0.05
Phorophous	4.4	Total iron	0.4
Potassium	10	Manganese	0.06
Sulphate	2.5	Molybdenum	0.02
Boron	0.02	Zinc	0.015

Pot size varied for experiments and depended on vine age and root growth. Pot size for many of the experiments commenced at 17 cm height, top diameter 17 cm with an estimated volume 3.5 to 3.8 L.

3.1.8 Disease Control and Plant Health

Seaweed applications were increased to weekly for 1 month to ensure excellent plant health (see Fertigation). These strategies allowed us to control the pests and significantly limit the infestation. Monitoring of thrips occurred throughout the life time of the project.

Routine plant health maintenance included removing plants from the cabinets once a week (at the time of an irrigation event) plants were removed from the chamber and placed in a different location to ensure an even distribution of light and to limit any chamber location based impacts to growth and development.

Mancozeb fungicide was sprayed onto stem cuttings to reduce the incidence of fungal attack especially powdery mildew, as stems are exposed to a high humidity during rooting which is ideal for fungal growth. Sprays were delivered at a concentration of 15g / 10 L of water. We ensured that the spray made leaf contact. Pinot Meunier genotype L1 plants were sprayed with Cropro Stealth which is a miticide and insecticide and contains the active abamectin at a concentration of 18 g/L, the solvent N-Methyl-2-Pyrrolidone at a concentration of 265 g/L and a liquid hydrocarbon at the concentration of 58 g/L. This was supplied by PCT International Pty Ltd, 252 Bradman Street, Acacia Ridge, QLD, 4110, Australia.

3.2 Field Experimentation

3.2.1 Vineyard Details

Vines from two varieties, from Dookie College vineyard were sampled; Shiraz and Sauvignon Blanc. The Sauvignon Blanc covering an area of 1.7 ha block (Plate 7) were planted mid 1980s on mixed root stocks, with a vine spacing at 1.8 m and a row spacing of 3.4 m. While Shiraz vines on 140 ruggeri rootstock planted in 1994 cover an area of 2.2 ha at vine spacing at 1.8 m and a row spacing of 3.4 m. Vines were both mechanically and hand pruned to 40/60 nodes.



Plate 7 Dookie College vineyard Sauvignon Blanc winegrape (16th Dec 2010)

During this project we were fortunate to have access to two commercial King Valley vineyards that allowed us to collect grapevine bud material. The first was a Brown Brothers owned vineyard located at 5044 Whitfield/Mansfield Road, King Valley. We were able to sample from a 5 ha block of Sauvignon Blanc (clone 101-14) with a vine spacing of 1.2 m and row spacing of 2.2m, hand pruned where the bud number per vine ranged from 24 to 30 buds. Budburst is usually during late September/early October, anthesis is typically in December/January and harvest is typically April. This block is recognised in the vineyard for its even ripening and yielding characteristics (11-12 t/ha). Vines are planted in deep, up to 3m, volcanic soil. It was noted that budburst was at least two weeks delayed during 2010. Vines were sampled across four rows, totalling 200 vines. This vineyard is located within a Phylloxera zone and footbath/vehicle protocol was followed. Vines are illustrated in Plate 8.



Plate 8 King Valley vineyard wine grapes (16th Dec 2010)

The second vineyard was the Cavedon vineyard, 208 Peipers Lane, Whitfield, King Valley (36° 23'S, 145° 25'E), which is owned and managed by Johnny and Helen Cavedon (Plate 9). The weather recording device, Tiny Tag, was placed in the Cavedon vineyard on the 28th September 2011.

The average yield was 6 - 8 t/acre where vine spacing 2.8 m and row spacing at 2.8 m over a 4 ha block on fruiting wire at 1.5 m. The Dookie vineyard (36° 37¢S, 145° 70¢E, elevation 185 m) has a mean January temperature (MJT) of 22.8ºC heat sum and 1868 growing degree days whereas the Cavedon vineyard (36° 74'S, 146° 38'E, elevation 320m) has a MJT of about 19ºC.



Plate 9 Cavedon vineyard, King Valley

Waite vineyard Adelaide, Sultana M12 Ramsey Minimal Pruning. Anthesis commenced in the week of the 10/11/2011 and bud sampling was conducted date 11/03/2011 where positions 2 and 6 were collected. Shoot data was collected and shoots were removed from the vine and transported on ice the lab. Buds were removed and snap frozen in liquid nitrogen. Samples were stored at -80°C freezer until processing.

3.2.2 Bud Sampling and Bud Collection Methods

The latent buds (N+2) were collected *in situ* from the field environment in three locations over two seasons, commencing in season 2010/2011 and completed in season 2011/2012. Dookie College vineyard (see section 3.2.1 for details) is described as the 'warm' location and a cooler vineyard was situated in King Valley (see section 3.2.1 for details). As little knowledge is known about winegrape bud gene expression in the field environment, it was first important for us to determine the most pertinent phonological or sampling time protocol. Therefore a more complex sampling matrix was

conducted in the first season (2010/2011). For further details of collection dates and sample matrix refer to method section in individual chapters.

Bud samples from the field environment were collected from Sauvignon Blanc and Sultana whilst bud samples from the controlled environments were collected from Pinot Meunier L1 genotype.

Buds that were collected for gene expression analysis were collected using a sterile knife and immediately snap frozen on dry ice. Buds were stored at -80°C until extraction occurred. Bud transport between storage facility at Dookie College and CSIRO / University of Melbourne laboratories were transported on dry ice. Buds that were collected for microscopy analysis were place in water at 4°C until bud preparation could be conducted (usually 1 – 4 days). Refer to section Light Microscopy and Scanning Electron Microscope (SEM) for microscopy methods.

3.2.3 Climate Data

Climatic data at Dookie was collected via a Visala weather station which was situated next to the vine block. The weather station was a Vaisala (WXT520) connected to a iNTELLiTROL Network control system (MAIT Australia, Shepparton). The weather station measures wind speed and direction, relative humidity, air temperature, barometric pressure and rainfall, and is equipped with a NetRad sensor to detect radiation. Readings were taken from all sensors at ten minute intervals and data is relayed back to a computer via a wireless radio system where it can be displayed on a real-time weather consol. Climatic data at the King Valley sites was collected by both a Tiny Tag, with a collection frequency set to 1 hour during the experimental period. This information was compared with the local BOM weather station. Climatic data at the Waite Campus was recorded by a Tiny Tag with a collection frequency set to 1 hour during the experimental period.

3.3 Molecular Analysis

In order to determine quantitative gene expression, wine grape plant material must undergo a number of sequential preparation and extraction stages. Figure 5 illustrates a simplified version of these stages.

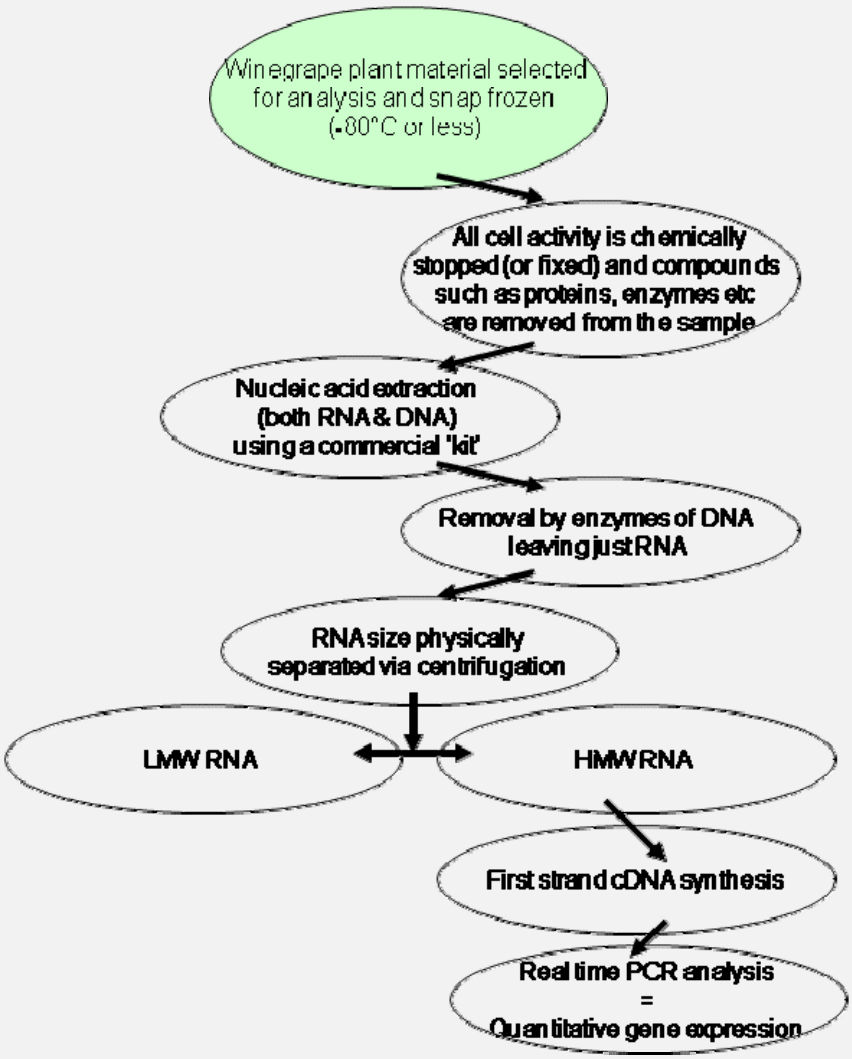


Figure 5 Sequence of winegrape preparation to obtain quantitative gene expression.

Key LMW = low molecular weight, HMW = high molecular weight, PCR = polymerase chain reaction, RNA = Ribonucleic acid and DNA = Deoxyribonucleic acid.

3.3.1 RNA Extraction

Bud samples were collected as described in section 3.2.3. Prior to RNA extraction, collected bud, inflorescence and other tissue samples (Plate 12) were transported on dry ice or stored at -80°C at all times during processing. Bud samples were ground in liquid nitrogen to a fine power and solution added to stop any nucleic acid expression and all molecules were extracted.

For gene expression analysis by real-time qPCR (RT-qPCR) total RNA was extracted using a kit and following manufactures protocols and instructions (QIAGEN® RNeasy® Kit, Australia or Spectrum Plant Total RNA Kit STRN250-1KT). In some cases total RNA was further processed to obtain high molecular weight RNA prior to RT-qPCR as described below.

For gene expression analysis by next-generation sequencing analysis (mRNA-Seq) the total RNA was separated into low (LMW) and high molecular weight (HMW) RNA for each sample. Low molecular weight RNA was extracted from 100 mg of frozen tissue using the mirPremier microRNA Isolation Kit #SNC50-1KT (Sigma). High molecular weight RNA was then extracted from the first pellet obtained during the micro isolation procedure using the method in the Spectrum Plant Total RNA Kit #STRN250-1KT. The extraction solutions and wash buffers for high molecular weight RNA extraction are also provided in #SNC50-1KT (Sigma). Contaminating DNA was removed from high molecular weight RNA by on column DNAse digestion using RNase-Free DNase Set (50) Cat N^{o} 79254.

RNA size and degradation state was accessed visually by electrophoresis running the RNA samples onto 5% (v/w) agarose gel, Agarose I^{TM}, Amresco, Australia. The gel tank preparation included soaking in 0.1 M NaOH and 0.1% sodium dodecyl sulphate detergent (SDS). Extracted RNA samples were placed in a water temperature bath set at 55°C for 5 minutes prior to being run on the gel (80 volts). Gel results were determined with the aid of UB radiation using Quantum ST4 software by Vilber Lourmat. Both low molecular weight and high molecular weight RNA quantification and integrity were assessed by NanoSpec ND-1000 (Thermo Scientific) and by Bioanalyser (Agilent Technologies). The primer pairs designed to amplify a 107 bp genomic fragment of the control gene, Ubiquitin (TC102473intronF, 5'-GTCCTCTAAGGTTTAATGAACCGG-3'; TC102473intronR, 5'-GAAAGACACAGCCAAGTTGCAC-3') was used to ensure that no contaminating genomic DNA was found after DNase I treatment. In most of the samples we were able to extract a minimum of 2 µg LMW RNA and 10 µg HMW RNA. In a small number of samples the quantity of RNA was found to be low. These samples were subjected to an ethanol precipitation. The RNA pellet was suspended in 70% w.v. Ethanol, spun and then suspended in 10 µl of sodium acetate. This was followed by 100% w.v. ethanol suspension at -20 °C for 1 hour. The sample was spun and the pellet was twice washed in 70% w.v. Ethanol. A Roto Speedy Vac (5 -7 minutes) was used to remove ethanol and dry the pellet. The pellet was resuspended in 20 µl RNase free water on ice for 1 hour. The pellet was briefly vortex and RNA concentration and quality was determined using the Nanospec ND-1000.

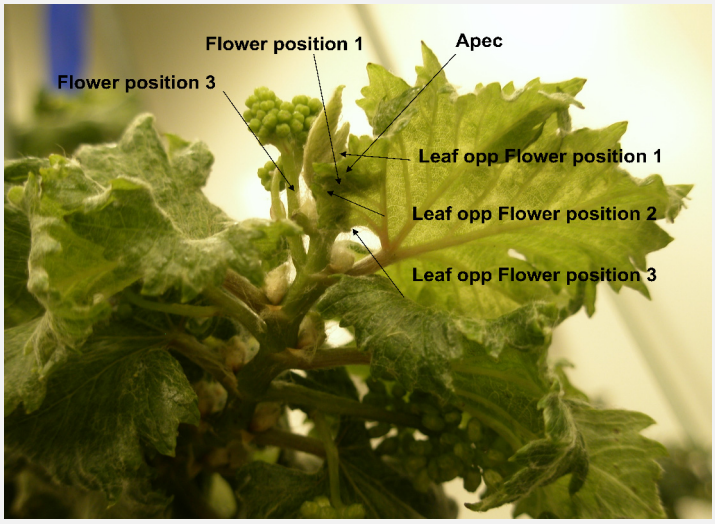


Plate 12 A. Visual representation of organ position and description of microvine sampling for RNA extraction

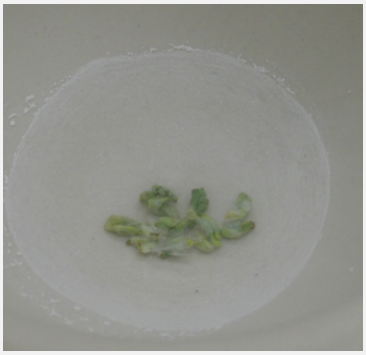


Plate 12 B Exampling of pooled inflorescences from L1 plants at sampling position 1

3.3.2 Real Time Quantitative PCR

For Quantitative real-time quantitative PCR (RT-qPCR) first strand cDNA was synthesized from 1 µg of total RNA or HMW RNA using 200U of Superscript III cat # #18080-085 (Invitrogen), 2.5 µM Oligo dT and 500 µM dNTPs and incubated at 50°C for 1 hour in a total of 20 µl. The resulting first strand cDNA was diluted 1/10 with water for RT-qPCR.

LightCycler 480 SYBR Green 1 Master 2x concentration cat # 04 887 352 001 (Roche) was used in the RT-qPCR assay at a 1x concentration along with 0.5 µM gene specific primers and 2 µl of first strand cDNA in a final reaction volume of 15 µl. Water and no reverse transcription negative controls were included in the RT-qPCR reactions (Plate 13).

PCR amplification was done in a Rotor-Gene RG-3000 (Corbett Research), 95°C for10 minutes then 50 cycles of 95°C 20 seconds, 58°C 20 seconds, 72°C 20 seconds and then a final step of 72°C for 5 minutes (Table 4).

Standard curves, melt curves and Ct values were generated for each gene and for each cDNA sample using Rotor-Gene 6000 software (Corbett Research). Relative gene expression levels between different tissue for each gene were determined using the Ct values in Biogazelle qbasePLUS2 (Integrated Sciences). Data was normalized using two reference genes, Ubiquitin and Actin and is a method that allows two processes to occur simultaneously, the amplification and the targeted quantification of nucleric acids.

Table 4 Annealing temperature for first strand cDNA synthesis

Step #	Temperature (°C)	Duration (minutes)	Function
1	65	5	Denaturing RNA
2	50	60	Superscript enzyme producing cDNA
3	70	15	Denaturing of Superscript & therefore stops reaction
4	4	1	Reduce temperature. Post this sample is placed on ice



Plate 13 Corbett Robotics used to prepare PCR reactions

3.3.3 RNA-Seq

Next-generation mRNA paired end libraries was obtained from 5 µg of HMW RNA. The Illumina protocol for sample preparation and single-end sequencing (65 b) on an Illumina Genome Analyzer II(x) was carried out by GeneWorks Pty Ltd. The result files, in FASTQ format (Cock *et al.* 2010) were assessed for quality using FastQC (Krueger *et al.* 2011). Reads were then converted from Illumina 1.5+ FASTQ into standard Sanger FASTQ using fq_all2std.pl (http://maq.sourceforge.net/fq_all2std.pl)

For genome mapping of mRNA-Seq data the 8x assembled genome of the *V. vinifera* genotype PN40024 at NCBI was used. Bowtie/0.12.7 (Langmead *et al.* 2009) and TopHat/1.2.0 (Trapnell *et al.* 2009) were used for building an index to the genome and mapping reads. Results from TopHat were converted from Sequence Alignment/Map (SAM) into BAM (SAM binary) format using samtools/1.11 (Li *et al.* 2009) and read into R using the Bioconductor Rsamtools package. A NCBI 8x transcription SQLite database was made in-house from GenBank files (chromosomes and unknown contigs) and gene2refseq files at NCBI using tools in biopython/1.57 (http://biopython.org/) to extract the sequence features exons, miscellaneous RNAs and messenger RNAs to give a final number of 25771 and provide information on splicing. The Bioconductor package edgeR (Robinson *et al.* 2010) was used for MultiDimensional Scaling to investigate variation between biological replicates and for identifying differentially expressed (DE) genes.

3.4 Microscopy

3.4.1 Aim

Yield potential depends on the transition of apices to a floral state and their subsequent differentiation in developing latent grapevine buds. Traditional approaches to studying the effects of environment and management on initiation and differentiation are limited by technical difficulties associated with quantitative measurement of developing buds and natural variation across bud populations within grapevines. Experimentation reported here combines traditional physiological and anatomical approaches with current molecular tools to improve our understanding of the variables that drive fruitfulness and yield development in grapevines.

Buds were collected for microscopic analysis in the field and glasshouse at anthesis and veraison from Sauvignon Blanc at node 2, 4 and 10 in Season 1 and from Sauvignon Blanc and Sultana at node 2 and 6 in Season 2. Once excised from the N shoot, the N+2 buds were fixed in a 5 ml vial containing 10% neutral buffered formalin. Vials were stored at 2-4°C.

3.4.2 Light Microscopy

A sample of 60-100 compound buds (N+2) from individual Sauvignon Blanc vines were collected from the field in both seasons. In season 2, 40-60 compound buds of Sultana were collected from the field at the Waite campus vineyard, Adelaide. Approximately five buds were retained at each collection time and for microscopic analysis and the remaining buds were snap frozen and stored at -80°C for RNA extraction.

Five representative buds per treatment were fixed in 10% Neutral Buffered Formalin (LabChem) in 5ml plastic vials and stored until microscopic examination. Apical meristems and inflorescence primordia were then examined under scanning electron microscope. Images were analysed to determine the average stage of bud development.

3.4.3 Scanning Electron Microscope (SEM)

Compound buds were fixed in 10% neutral buffered formalin (NBF) (Sigma chemicals) and stored in a cool-room at 2-4ºC prior to dissection. In order to quantitatively assess the stage of differentiation, the entire apex of at least five replicate primary buds with their quota of inflorescence primordia and any uncommitted anlagen were in the first instance dissected from the latent bud (N+2) under high powered light microscope (Leica IM50). The apex and intact inflorescence primordia were fixed in 2.5% gluteraldehyde (pH 6.5). Samples were then dehydrated using an ethanol dilution series (10, 30, 50, 70, 90 and twice at 100%), critically point dried (Bal-Tec CPD 030), mounted on aluminium stubs with carbon tabs and sputter coated (Edwards 5150B) before being examined under a scanning electron microscope (Philips XL30 FEG). The development of structures was assessed and measured and compared to the stages of ontogeny (Stages 0-11) (Srinivasan and Mullins 1981).

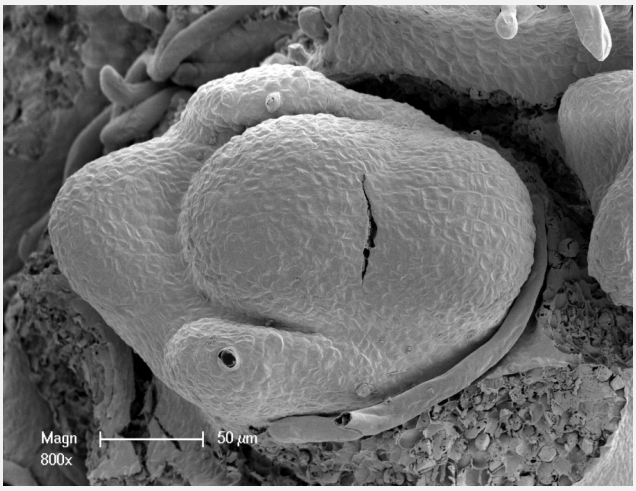


Plate 14 Example of SEM image of a Sauvignon Blanc (N+2) bud demonstrating both leaf primordia and developing inflorescence primordia.

4 Assumptions about Fruitfulness and Yield

4.1 Experimental Background

We decided to start our research investigating some assumptions about the environmental impacts on yield in wine grapes. Specifically investigating the early stages of grapevine reproduction (modified E-L stages 1 – 18, Dry & Coombe 2004), where there is less understanding of environmental impacts. We acknowledge the importance of fruit set and fruit growth (modified E-L stages 18-38 Dry & Coombe 2004) in fruitfulness and yield determination but will not be investigating these here. Therefore all findings are associated with the maximum potential of fruitfulness and yield rather than the actual.

Initially we set about to investigate how environmental conditions influenced flower number. The number of individual flower primordia from the lateral shoot (N+1) differentiate from each branch primordia as the shoot develops, inflorescence structures are formed containing flowers (modified E-L stages 4-18) and at these stages we measured the impact of climatic conditions using the controlled cabinet environments. As the reproductive pathway of a grapevine is developed over two seasons, this initial investigation represents the effect of temperature on bud development from budburst onwards (modified E-L stages 4). Mullins contributed significantly to the fundamental understanding of fruitfulness (Mullins 1960's), where the impacts of temperature and hormones on fruitfulness were investigated (Mullins 1967). During these experiments Mullins developed an effective method of propagating canes from filed grown vines, which enabled growth and flowering of field collected cane material in controlled environmental conditions. The method initially promotes root growth by exposing the roots to a warm environment while concurrently exposing the canes to cool temperatures (~5 °C) inhibiting bud break. Removing the meristem tip influences hormonal messages which promote inflorescence production once bud break occurs. In the 1960's the controlled cabinet experiments conducted by Mullins were about ¼ ambient sunlight, with a PAR less than 200 µmol. The growth cabinets that we commissioned for this research allowed us to deliver high light levels and we have assumed no impact of limiting light environment on the differentiation of inflorescence primorida. Utilizing the growth cabinets we were able to independently control both temperature and light environments. Three temperature treatments were implemented (Table 5), based on actual bud temperatures measured using a thermal infra red camera. Light quality variations in the cabinet were measured using a spectroradiometer in order to ensure similarity in high light conditions. These measurements allowed us to refine experimental design and measurement.

In our second experiment we investigated the effects of fruitfulness and yield using the grapevine Pinot Meunier L1 genotype (Section 3.1.5.2), in controlled environmental conditions. The grapevine L1 genotype was derived from the Pinot Meunier L1 cell layer. This mutation has a reduced physical size as the internode length is reduced; resulting in shorter shoots which make it ideal for controlled cabinet experimentation. The mutation is also associated with a higher production of inflorescence over tendrils. We used this L1 genotype to begin to understand the influence of temperature and light on uncommitted primordia and thus add to our potential understanding on fruitfulness. In experiments conducted here the Pinot Meunier L1 genotype was allowed to develop shoots upon which we would impose an artificial dormancy for several weeks (removal of foliage) and then new

shoot growth resulted. This enabled us to conduct experiments on the early stages of reproduction as the compound bud developed from latent buds exposed to the climatic conditions within the cabinet. The early stages of reproduction are described as initiation and differentiation, they set the maximum fruitfulness (branch number and flower number) and optimal yield potential (flower number characteristics) and are the least understood in grapevine reproduction.

4.2 Experimental Aims

Assumptions on Maximum Yield Potential:

The aim of the first experiment was to investigate the impacts of temperature at high light, on grapevine fertility specifically flower number. We selected the winegrape variety Shiraz and propagated (Mullins and Rajasekaran 1981) canes.

Assumptions on Maximum Fruitfulness Potential:

The aim of the second experiment was to investigate the impacts of temperature at high light, on branch number and flower number. We selected Pinot Meunier genotype L1 and allowed several successive seasons to allow investigation of compound and latent bud (N=1) development. Using several seasons means that we can both determine the effects of different temperatures on yield potential and fruitfulness and then investigate the impact of temperature on bud burst.

4.3 Experimental Details of Shiraz Cane Propagation to Investigate Yield

Well-ripened dormant canes from Shiraz winegrapes were collected from the Dookie vineyard on the 08th July 2009. For details of cane collection and cane propagation please refer Section 3.1.5.1. After cane roots were established the canes were transported (14th Oct 2009) from the cool room and placed in the controlled cabinets, where environmental conditions were implemented (Table 6) which were based on bud temperature IR evaluation (Table 5) and periodically tested (results not shown). During these experimentations it was assumed that adequate and non-limiting nutrition was supplied to the plants with regular fertigation. To promote inflorescence growth and avoid abortion of inflorescences, at bud burst, leaves below and adjacent to inflorescences are removed as soon as accessible and the shoot tip is excised. This shoot management allows for one of the axillary buds of one of the defoliated nodes off the main axis to produce a lateral shoot. After budburst, vines were transferred to environment conditions for the start of the experiment 21st Oct 2009, hot day cool night (Table 6). Development of the flowers and the ability for the flowers to bunch to maturity, is supported by the foliage of the lateral shoot. The cabinet environmental conditions for all treatments are summarized in Table 6. Plants grow healthy in chamber environment (Plate 15 and Plate 16) and the experimentation was completed by the 27th Nov 09. Flower structures were collected according to their phenological stage, modified E-L stage 17-18 or prior to pending cap fall.

Table 5 Measurement of bud tissue temperature and chamber inlet air temperature

Bud temp. ºC		Chamber inlet air temp. ºC	
Day temp. ºC	Night temp. ºC	Day temp. ºC	Night temp. ºC
27	30	24	30
27	15	24	15
17	15	13	15

Table 6 Controlled Environmental Treatment Parameters

Temperature Treatments	Hot	Hot day cool night	Cool
Environmental Parameter			
Day temperature (°C)	27	27	17
Night temperature (°C)	30	15	15
Photo period (hr)	16	16	16
Humidity (%)	45-60	45-60	45-60
PAR (µmol)	1200	1200	1200

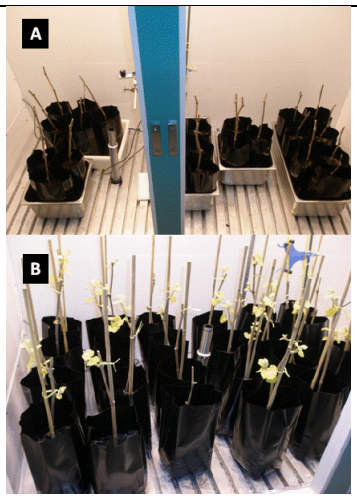


Plate 15 Propagation of Shiraz canes (A) placed in growth cambers after cool room 14th Oct 2009 and (B) is the 27/30 treatment cabinet 21st Oct 2009.



Plate 16 Propagation of Shiraz canes illustrating the 30 / 15 treatment (Nov 09)

This is defined by Coombe (1995) where 12-14 leaves are separated, single flowers separated but caps still in place. Collected Inflorescences were stored in 10% neutral buffer formalin. Once counting could be conducted, inflorescences were transferred between three water washes, where wash 1 was 200 ml distilled water for 1 hour, wash 2 and 3 were both 30 ml of distilled water for 30 minutes. Flower per inflorescences were counted and morphological aspects of branch length in the

inflorescence was measured and included the length of the hypoclade, the inner (also cited in the literature as primary branch) and the outer arms (or wing) were measured. Leaves per lateral shoot were counted at harvest date.

4.4 Results of Shiraz Cane Propagation – Environmental interactions on Yield Potential

The impacts of temperature on grapevine fertility was investigated (25th Aug 09 – 27th Nov 09) on field grown Shiraz (Sept – Dec 09) where canes were propagated (section 3.1.5.1 and 4.3). Environmental conditions were controlled using the cabinets (Table 6) and the light environment constituted of a long day, 16 hour photo period and high light quantity where PAR was set to 1200 µmol (Table 6). The most fertile bud temperature regime in this experiment was the cool treatment, where daytime temperature was 17 ºC and night time temperature was 15 ºC which resulted in a total flower number averaging at 541 ± 152 per node position per cane (Table 7). The majority of flowers were located on the primary branches (84%) and not on the wings. The morphology of the inflorescence structure in the cool treatment was significantly different from the other treatments, with a shorter total inflorescence length and outer branch lengths, indicating an altering in carbon partitioning (Plate 17). Buds exposed to higher temperatures such as 27 ºC during the day and 30 ºC at night resulted in significantly (P=0.002) lower fertility with a total flower number 338 ± 85 and longer inflorescence structures, than the cooler treatment. The data suggests that it is the high daytime temperatures that promoted the reduction in fertility and not the cooler night time temperatures (15 ºC where total flower number was 380 ± 92,Table 7). The daytime temperature impacts significantly on the growth of the fruiting bud. A representative image depicts the large difference in growth rates of the lateral shoot and inflorescence structure (Plate 18), where these images were taken 14 days after the start of the experiment. Where directly comparable, these findings support the findings from Mullin's data (1960's experimentation) suggesting that light levels do not impact at this stage but temperature has a significant effect. No significant difference in leaf number or leaf lamina length per lateral was found, suggesting equal availability of source carbohydrates.

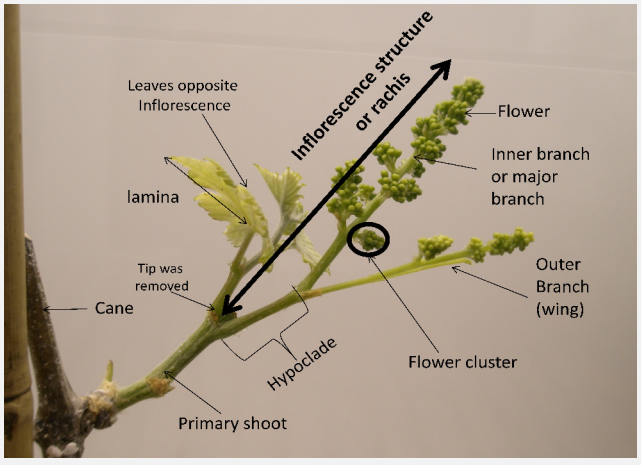


Plate 17 Shiraz inflorescence structure description and definitions

Table 7 Effect of environment on propagated Shiraz cane flower number and morphology at harvest

	Day / Night Bud Temperature Treatments		
	Hot	**Hot day cool night**	**Cool**
	27 / 30 (°C)	**27 / 15 (°C)**	**17 / 15 (°C)**
Flower Number			
Total flower #	338 ± 85 a	380 ± 92 a	541 ± 152 b
Flower # on inner branch	282 ± 52 a	303 ± 51 a	455 ± 97 b
Flower # on outer branch (wing)	56 ± 60 a	77 ± 63 a	86 ± 73 a
Inflorescence Structural Morphology			
Total inflorescence length (mm)	81 ± 21 ab	95 ± 23 a	77 ± 11 b
Inner branch length (mm)	66 ± 17	76 ± 23	60 ± 10
Hypoclade length (mm)	16 ± 5	20 ± 6	18 ± 3
Outer branch (wing) length (mm)	38 ± 12 b	43 ± 15 a	30 ± 10 c
Leaves on lateral shoot			
Leaf #	2.7 ± 1.2		3.2 ± 7.2
Largest leaf lamina length (mm)	16 ± 6		15 ± 5



Plate 18 Representative image depicting Shiraz shoot and inflorescence development after 14 days of temperature treatment where 17/15 (17 °C day & 15 °C night), 27/15 (27 °C day & 15 °C night) and 27/30 (27 °C day & 30 °C night).

4.5 Experimental Details of the Investigation of Environmental and Genotypic interactions on Fruitfulness and Yield

The impacts of temperature on grapevine fruitfulness and early yield indicators was investigated (Sept 2009–10) on Pinot Meunier genotype L1 (Section 3.1.5.2). Single green leaf cane cuttings were collected (25th Sept 2009) from stock plants. Base node of the cane were removed along with all leaves expect for the leaves at the apex and 2/3 nodes down the cane. The base of canes were dipped in Clonex purple (Growth Technology, Australia) at a concentration of 3 g/L and planted in 50:50 perlite : vermiculite mix. To prevent fungal infection cuttings were sprayed with mancozeb (1.5g/L) and placed into 70% humidity 30 °C glasshouse environment. Once root growth had commenced, cuttings were transferred to soil (Section 3.1.7, 3L mix placed in each of 72 avocado bags, 35 replicates) and transferred to a controlled cabinet where initially a hot day/cool night of 27/15 °C was delivered (08th Oct 2009) for a duration of 21 days. The light environment constituted of a long day, 16 hour photo period and high light quantity where PAR was set to 1200 µmol (Table 6). During these experimentations it was assumed that adequate and non-limiting nutrition was supplied to the plants with regular fertigation. Plants were allowed to establish and moved to treatment conditions (Table 5 and Table 6) on the 29th Oct 2009. Upon experiment commencement, the lowest nodes were tagged from apical meristem (usually the second node) and all inflorescences removed. Details of dates and assessment are located in the Appendix Table 20. The day and night temperature treatments are summarised in Table 8. Season 1 commenced on the 29th Oct 2009. Assessments occurred 21 days after the start on the 19th Nov 09 and finally 42 days after the start on the 10th Dec 09. E-L modified as described by Coombe 2004 for each treatment at each time point. Pinot Meunier L1 genotype was pruned on the 02nd April 2010 (Plate 19) and plants were placed into season 2 temperatures (Table 8). The 'hot-warm' and the 'warm-hot' treatments mean that buds have developed in one temperature treatment but burst in a different temperature treatment. In the hot, warm and cool temperature treatments buds have developed and burst under the same temperature conditions (Table 8). In the second season harvest date was determined by 10% cap fall stage.

Table 8 Pinot Meunier L1 Genotype Treatment Conditions

Code	Stage 1 (Season 1) day / night temperature (°C)	Stage 2 (Season 2) day / night temperature (°C)
Hot	27 / 30	27 / 30
Hot - warm	27 / 30	27 / 15
Warm	27 / 15	27 / 15
Warm - Hot	27 / 15	27 / 30
Cool	17 / 15	27 / 15



Plate 19 Pinot Meunier L1 Genotype season 1 pruning.

4.6 Results of Investigation into Pinot Meunier L1 Genotype Fruitfulness and Yield

Pinot Meunier genotype L1 in controlled environmental conditions was exposed to 3 temperature treatments hot, warm and cool in the first season (Section 4.5; Table 8) and 5 in the second season, to determine the effect on fruitfulness and yield.

4.6.1 Season 1, effects on yield potential

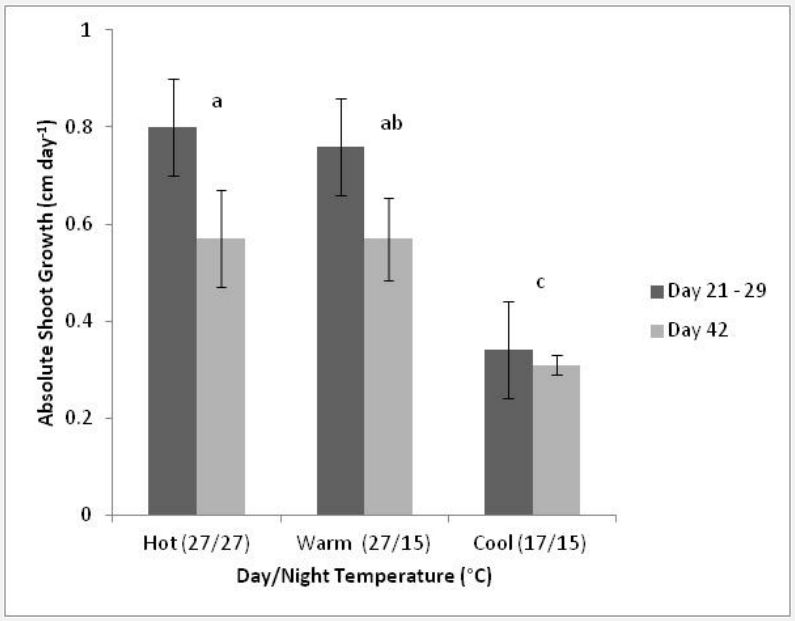


Figure 6 Pinot Meunier L1 genotype season 1 shoot growth rate at hot, warm and cool temperature treatments.

Mean (n=15-4) and error bars indicate standard deviation. Letters a, b and c indicate significant differences (P > 0.001)

No significant difference in shoot growth rate was found between the hot and the warm temperature treatments at either collection periods (Figure 6). The cool treatment had significantly lower shoot lengths at both time points compared to the warm and the hot treatments (Figure 6). Optimal relative shoot growth rate was found after 29 days in the hot treatment (Figure 7). Relative shoot growth rate is significantly reduced as the temperature reduces (Figure 7). After 42 days of the treatments, relative shoot growth rate had significantly decreased in the warm treatment (Figure 7). This trend was observed in all of the treatments (Figure 7). The number of nodes that separated along the shoot was reduced by the cool temperature treatment (Figure 8). Node separation was most promoted with the warm treatment in the first 29 days of the treatment (Figure 8).

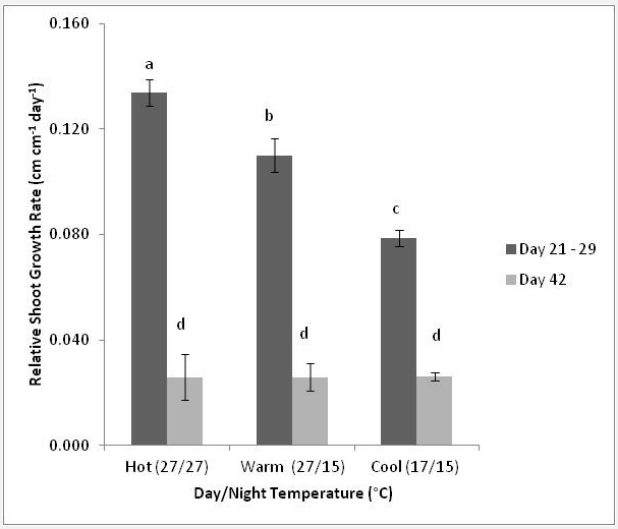


Figure 7 Pinot Meunier L1 genotype season 1 relative shoot growth rate at hot, warm and cool temperature treatments.

Mean (n=15-4) and error bars indicate standard deviation. Letters a, b and c indicate significant differences (P > 0.001).

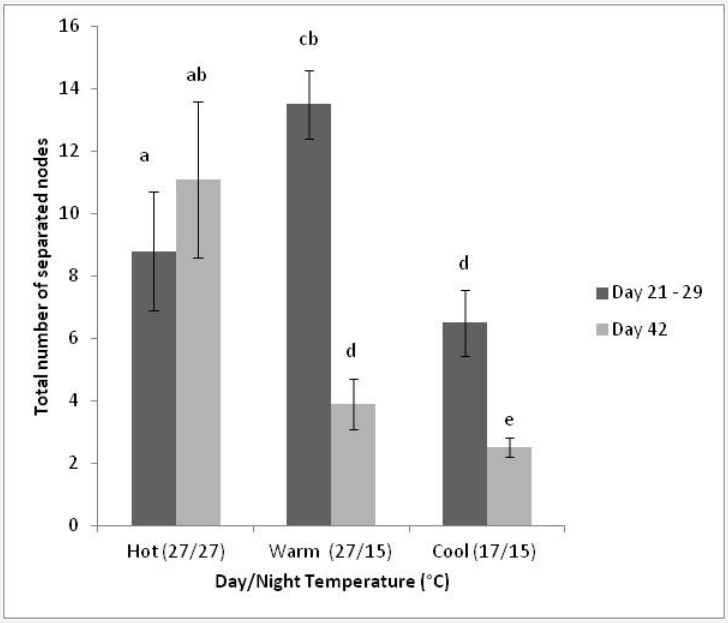


Figure 8 Pinot Meunier L1 genotype season 1 number of nodes separated along the shoot at hot, warm and cool temperature treatments.

Mean (n=15-4) and error bars indicate standard deviation. Letters a - e indicate significant differences (P > 0.001).

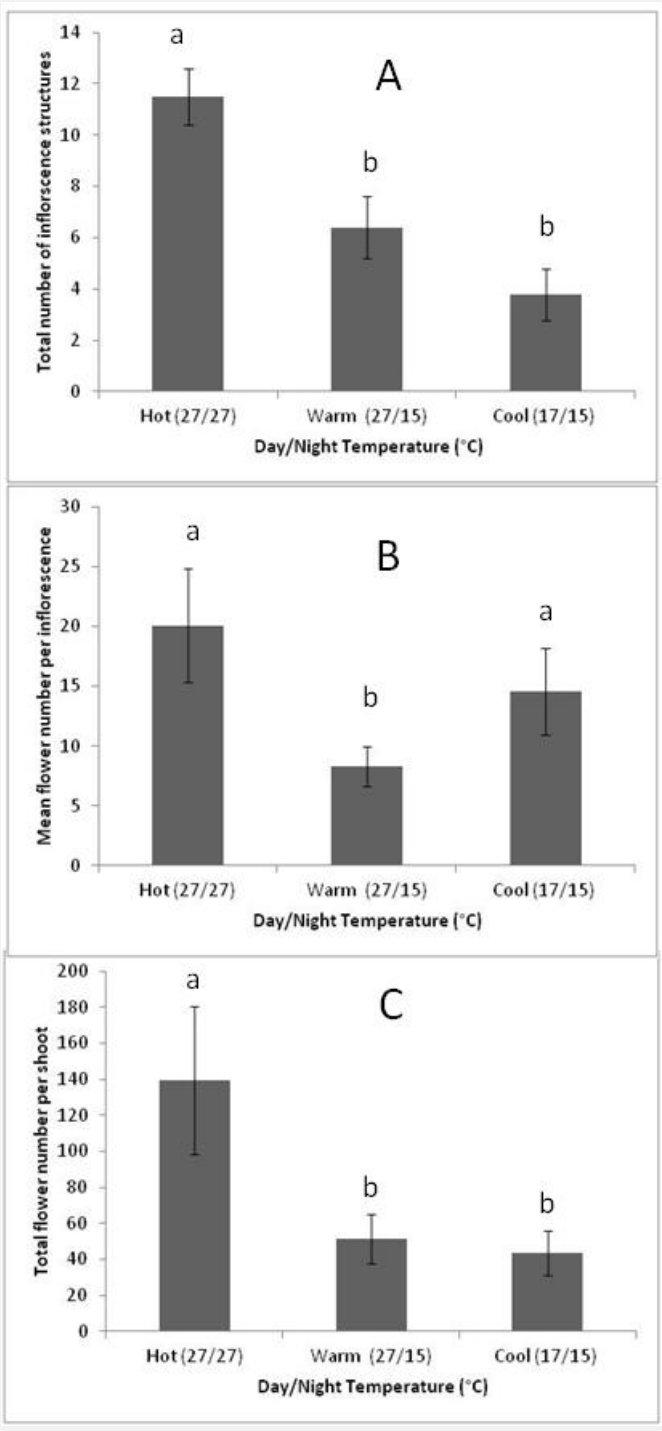


Figure 9 Pinot Meunier L1 genotype season 1 assessment of fruitfulness at hot, warm and cool temperature treatments. (A) total number of inflorescence structures on the shoot, (B) mean flower number per inflorescence and (C) total flower number per shoot.

Mean (n=15-4) and error bars indicate standard deviation. Letters a, b and c indicate significant differences (P > 0.01).

Inflorescence number per shoot was significantly different with each temperature treatment, showing a trend of the higher the temperature treatment the more inflorescence number per shoot were observed in season 1 (Figure 9 A). The average flower number per inflorescence was not impacted by temperature treatment (Figure 9) whereas total flower number per shoot was reduced. The higher the temperature the more aborted structures were observed on the shoot (Figure 10 A). Leaf and tendril numbers were also impacted by temperature, with hot temperature having a greater number of leaves and tendrils than cooler temperatures (Figure 10 B).

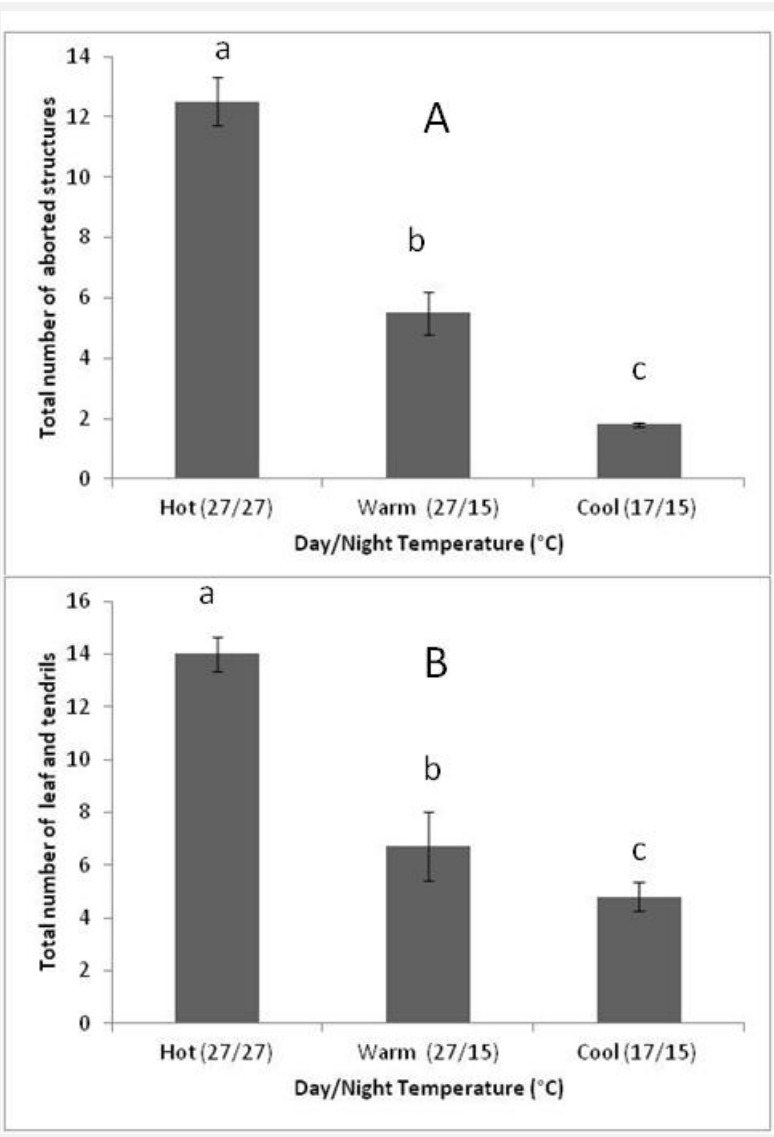


Figure 10 Pinot Meunier L1 genotype season 1 assessment of fruitfulness at hot, warm and cool temperature treatments. (A) Total number of aborted structures (inflorescences/tendrils) and (B) total number of leaves and tendrils.

Mean (n=15-4) and error bars indicate standard deviation. Letters a, b and c indicate significant differences (P > 0.001).

4.6.2 Season 2, effects on fruitfulness

The treatments can be ranked according to phenological development, where the hot treatment (day/night 27/30 °C) was most advanced (Figure 11A) followed by hot-warm (where season 1 was day/night 27/30 °C and season 2 day/night 27/15 °C), warm, warm hot and the less developed was the cool treatment. The reduced development rate in the cool treatment is observed at all time points (Figure 11). The hot-warm and warm treatments demonstrated the most advanced phenological development before flowering was complete (Figure 11).

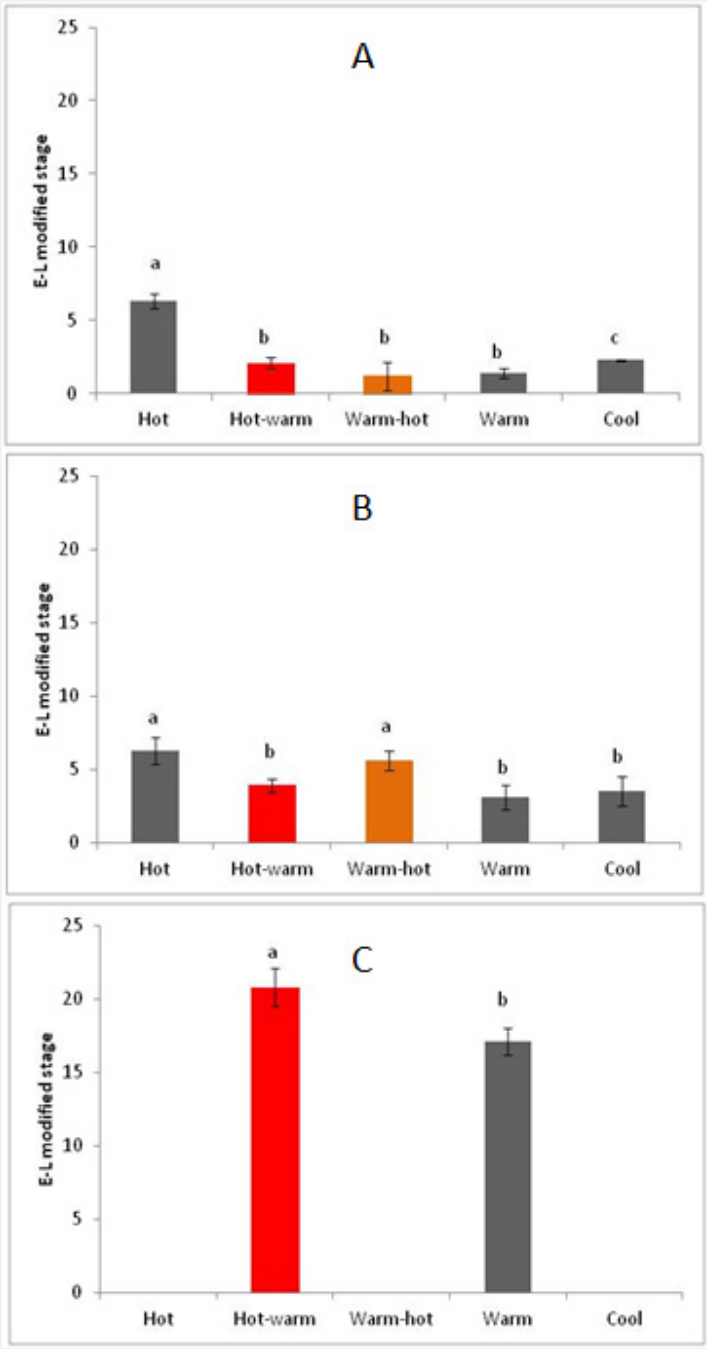


Figure 11 Pinot Meunier L1 genotype Season 2 modified E-L assessment at five temperature treatments, where (A) 12 days, (B) 27 days and (C) 44 days after bud break.

Mean (n=7-4) and error bars indicate standard deviation. Letters a, b and c indicate significant differences (P > 0.001).

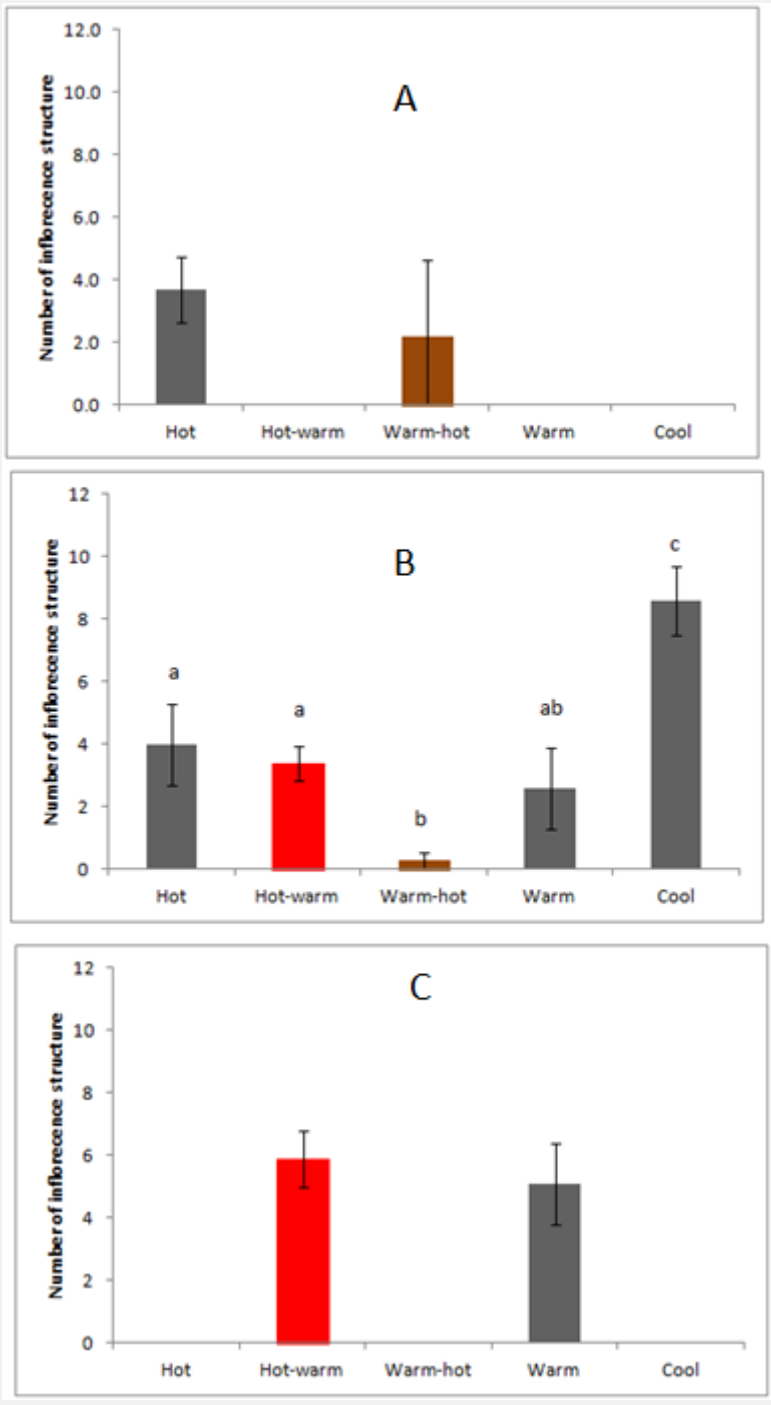


Figure 12 Pinot Meunier L1 genotype Season 2 inflorescence number at five temperature treatments, where (A) 12 days, (B) 27 days and (C) 44 days after bud break.

Mean (n=7-4) and error bars indicate standard deviation. Presence of letters a, b and c indicate significant differences (P > 0.001).

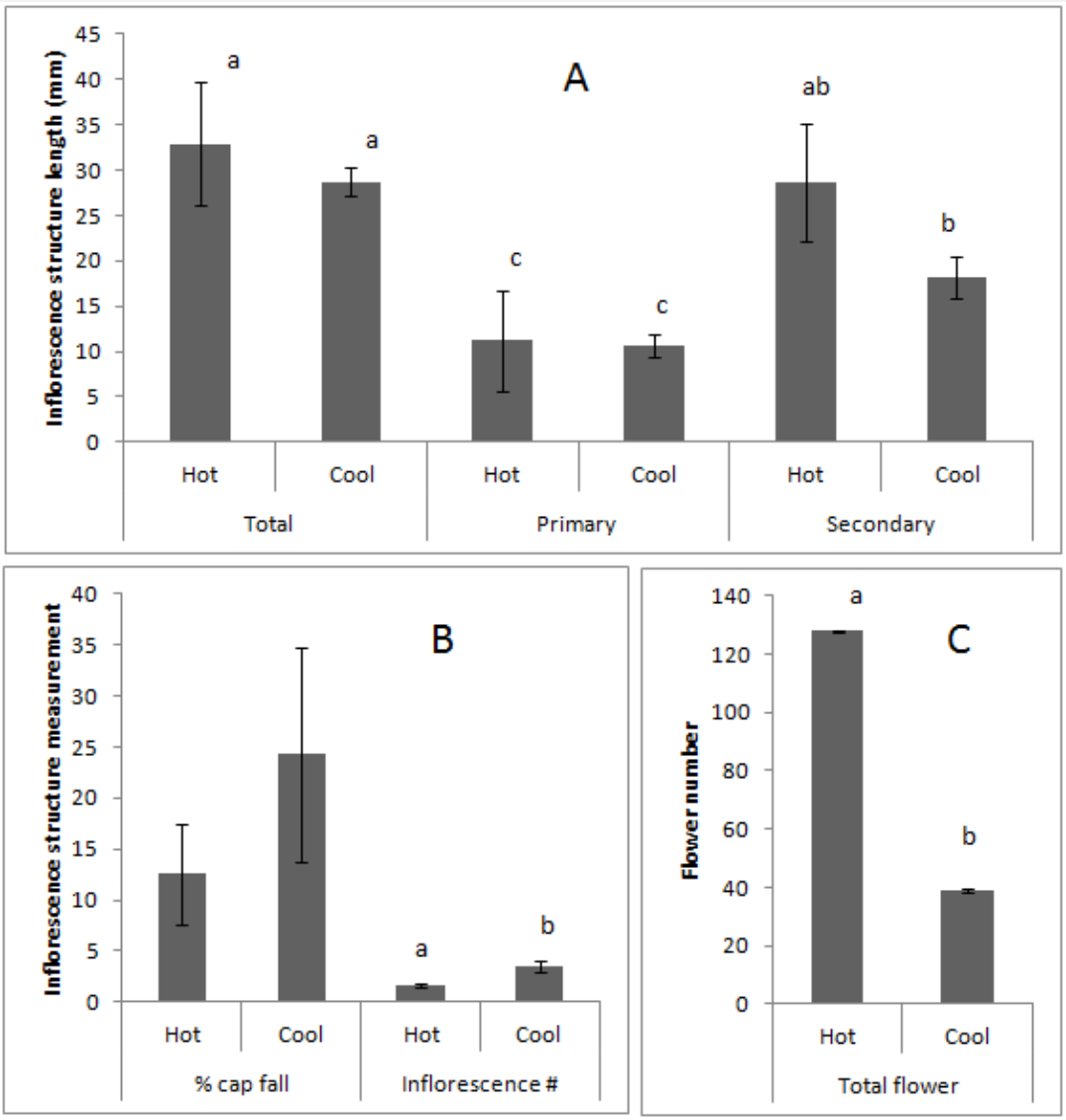


Figure 13 Pinot Meunier L1 genotype Season 2 inflorescence structure measurements comparing the hot and cool temperature treatments at harvest, where (A) inflorescence length comparing total, primary and secondary (B) inflorescence % cap fall and inflorescence number and (C) flower number per inflorescence.

Mean (n=7-4) and error bars indicate standard deviation. Presence of letters a, b and c indicate significant differences (P > 0.05).

Harvest date for each treatment was based on the flowering stage cap fall. The first treatment to be harvested was the hot treatment and the last was the cool treatment. Significant differences between inflorescence total length, primary branch length and secondary branch length were observed (Figure 13A) but no significant difference between the hot and cool temperature treatments within these measurements. Significantly more inflorescences structures were found in the cool treatment (Figure 13B) compared to the hot treatment at harvest. Flower number per inflorescence was significantly higher in the hot treatment compared to the cool treatment (Figure 13B).

4.7 Discussion on Assumptions Fruitfulness and Yield

No consistent statement of the relationship of temperature on fruitfulness and yield has been made to date. This is because the relationship is complicated and can depend on the cultivar, vineyard management practice, the interaction of temperature relative to the reproductive development stage, the period of temperature at each stage and the influence of other environmental parameters. It can be difficult to evaluate evidence collected from field experimentation on grapevine reproductive development, as exposure to complex environmental interactions over two seasons is often compounding. It is also important to understand the underlying assumptions presumed during experimentation. Controlled environmental conditions coupled with specifically designed experimentation, may result in a better understanding of the underlying mechanism which in turn will allow for a better understanding and perhaps prediction in the field and commercial environment.

4.7.1 Initiation and differentiation of latent bud inflorescence primordia

Observations from morphological studies suggest a requirement for high temperatures for the early stages (in the summer of the first year) of inflorescence formation in grapes (Huglin, 1958; Alleweldt, 1963; Baldwin, 1964). These findings were further supported by a close positive correlation (r = 0.82) between mean air temperature and the percentage of fruiting shoots (Dimitrieva, 1970). The formation of inflorescence primordia of latent buds, which is an important driver of fruitfulness, is promoted by higher temperature. This has been demonstrated both in the European summer months by Alleweldt (1963) who observed a positive relationship between temperature and the number of clusters appearing on the shoot in the following season in the cvs Riesling and Sylvanner, and in Australia by Baldwin (1964) on cv. Sultana fruitfulness. The critical period of high temperature driven effects seems to be three weeks before the formation of anlagen by the apices of the latent buds (Buttrose 1969, Buttrose 1970). Shiraz has been shown to initiate inflorescence with temperatures as low as 20 °C (Buttrose 1970; Okamoto et al 1977). Both light and temperature are important in early inflorescence development. The shiraz cane propagation conducted here, used dormant compound latent shoots (N+1 – N+6) collected from the vineyard where the mean ambient January (summer months 2008) temperature at Dookie was 32.5°C and the lowest daily temperature being 20.0 °C, and therefore it can be concluded that temperature was not a limiting impact. It was concluded in the review on flowering by Srinivasan and Mullins (1981) that a high temperature and a high light quality were requirements for maximum fruitfulness of latent buds (Alleweldt, 1963; May and Antcliff, 1963; Baldwin, 1964; Wagner, 1966; Buttrose 1970, Ruffber & Kliewer 1975, Zelleke & Kliewer 1979, Moncur et al. 1989, Dunn & Martin 2000, Petrie & Clingeleffer 2005). In our experimentation the light environment was unlikely to be limiting too as the mean solar radiation was 35.2 MJ/m^2 (January 2008). Smart et al. (1982a) suggested that the leaves subtending the bud act as a light receptor to the bud. In order to account for canopy light microclimate Shiraz canes from the external canopy were collected for experimentation. Persistent water stress can reduce the fruitfulness of latent buds (Winkler 1965, Huglin 1960). Rainfall is limiting at the Dookie vineyard as the total rainfall for January (2008) was 56.4mm, however these vines are irrigated and received 3

hour irrigation events every 4-5 days. Fertigation requirements of Shiraz Dookie vineyard vines are monitored and delivered after analysis of petiole, it is assumed that an optimal supply of Nitrogen was delivered to the vines to allow inflorescence primordium formation and for the differentiation of flowers (Alleweldt 1964), phosphorous to promote bud fruitfulness (Isoda 1964) and potassium to promote inflorescence formation (Srinivasan 1968; Srinivasan et al. 1974).

4.7.2 Differentiation of inflorescence primordium

The onset and speed of bud break is influenced by temperature and it may be the temperature pre bud break that is the determinant factor (May 1964). Controlled temperature conditions of day/night 17/15 °C followed by a warm bud break condition (28/15°C) resulted in low flower number but increased inflorescence structures. It has been cited that a temperature of 27 °C was more favourable to bud break than 15 °C (May 1964) where an 8 day delay was recorded on Sultana. Shiraz bud break was delayed (5-8 days) in the cooler 15 °C compared to the warmer 27 °C treatments. While higher temperatures promote bud break they elicit negative impacts on the number of inflorescence per shoot and the number of flowers per inflorescence (Pouget 1981) in Merlot and Cabernet Sauvignon comparing temperatures of 12 and 25 °C. The significantly reduced flowers per inflorescence reported here on Shiraz when comparing 15 and 25 °C under high light reflect closely to Pouget (1981) findings with a difference in light quantity of Pouget experiments being conducted under relatively low light quantities. Benismail & Ejnaoui (2004) manipulated bud temperature by covering with plastic-covers and observed a reduction in fertility of Cardinal vines which they attributed to physiological and anatomical changes induced during flower formation. Their experimental treatments resulted in an increase of flower number with cooler temperatures (Benismail & Ejnaoui 2004).

The differentiation of inflorescence primordia can influence the number of flowers per inflorescence and is not associated with the number of inflorescences (which become bunches later on in the developmental pathway). Root and cane stored assimilates (sugars from photosynthesis) are used by the vine during bud burst for the development of new shoots and any associated structure (tendril and inflorescences). It has been reported that the carbohydrate availability is important in the timing and the duration of flowering (Bennett et al 2005). Limiting carbohydrates seem to inhibit slower shoot development, fewer inflorescences per shoot, fewer flowers per inflorescence and reduced vine yield (Bennett et al 2005). Using propagated Shiraz canes we artificially created a situation where the carbohydrate sources were assumed consistent between all treatments. Leaf number per lateral shoot was measured and no significant difference between treatments were found. The lamina length (at longest point) of the largest leaves present on each lateral shoot was measured and again no significant difference was detected. It should be noted that photosynthetic measurements on these leaves were not possible due to the small and delicate nature of these leaves. If carbohydrates were limiting due to a reduced photosynthetic rate, which is most likely in the 17/15 °C day/night temperature treatment, a lower inflorescence per shoot should have been observed if comparison of Bennet et al (2005) findings can be made. This was not the case and it is unlikely that carbohydrates were limiting. That said, we cannot rule out a situation of limiting

carbohydrates, therefore two explanations for how temperature may be influencing flower number per inflorescence will be discussed. If the assumption is made that carbohydrates are not limiting, then signal compounds (such as the plant hormones e.g. gibberellins and cytokinins) are controlling and initiating floral induction pathways in the meristemic primordia which are leading to gene expression changes, resulting in change in the number of inflorescences formed on each shoot and therefore determining inflorescence number. If however the assumption is that carbohydrates are limiting, then a simple reduction of available carbohydrates are limiting flower number per inflorescence, which may explain the cool treatment effects in the genotype/environmental interaction experiment. The third possibility is that the operating mechanism is explained by an interaction of these affects. The remaining discussion in this chapter will explore these explanations in more detail.

4.7.3 Carbohydrate limiting causing reduction of differentiation of inflorescence primordium

Higher temperature promote shoot and inflorescence growth rate (increasing cell division and cell elongation) in a relatively short time. The mechanism that mobilises the carbohydrate source supply (that is sugars from the roots and canes) is not fast enough to keep up with the growing demand of the branching inflorescence. The reduced energy becomes a controlling step and inhibits the number of flowers per inflorescence which in turn contributes to a reduced maximum yield potential. Interestingly treatments in these experiments suggested that the daytime temperature and not the night time temperature, elicited the reduced flower per inflorescence in Shiraz. While the cooler temperature inhibits shoot and inflorescence development allowing enough carbohydrates to be mobilised for optimal flower per inflorescence development. The carbohydrates which are believed to mobilise at bud break and translocate to the developing inflorescence each hour are sucrose [50 µM], glucose [250 µM] and fructose [250 µM] via both xylem and phloem sap (Glad et al. 1992a&b; Campbell & Strother 1996). Gene expression in flowers have been shown for the genes encoding membrane sucrose- or hexose transporters, these include *VvSuc11*, *VvSuc12* and *VvSuc27* genes (Davies et al. 1999). The role of these genes appears to be involved in grapevine sucrose/H+-transport and are differentially expressed in plant tissues. Sugar fluctuations during flower development in grapevine appear to be variety specific (Lebon et al., 2004). Gewurztraminer exhibited higher concentrations of starch and sucrose in the inflorescences compared to those of Pinot noir, where higher levels of glucose and fructose were recorded (Lebon et al. 2004). When the reserve replenishment is not optimal, reproductive parameters of the following year are impacted. Due to the chronology of reproductive organ formation, both the number of inflorescences per plant and the number of flowers per inflorescence are affected (Duche^ne et al. 2003a & b; Bennett et al. 2005).

4.7.4 Non -carbohydrate limiting causing reduction of differentiation of inflorescence primordium

The gene Terminal flower 1 in grapevine or *VvTFL1* (homologous gene in Arabidopsis is Terminal flower 1 (*TFL1*)) has not had its function confirmed in grape vine but it is believed to be a repressor of flowering. In spatial studies the gene *VvTFL1* in grapevine (Carmon et al. 2002) was expressed at the end of inflorescence branches were it is believed to suppress flower production. If the evidence of reduced flower number is considered alongside the observed architectural modifications to the inflorescence in high temperature environments, the gene *VvTFL1* is a likely candidate which may be either directly or indirectly influencing the uncommitted primordia. Carmon et al. (2002) demonstrated that *VvTFL1* was expressed in vegetative meristematic tissues as well as floral meristems suggesting it plays a general role in flowering too. Refer to sections 5, 6 and 7 for findings on genotype/environmental impacts on grapevine flowering genes.

5 Identification of Genes Involved in Inflorescence and Floral Development

5.1 Background

The aim of this study was to discover new genes with a putative role in floral development. A series of experiments were conducted into genotypes of the Pinot Meunier variety. The wild type Pinot Meunier is referred to here as the L2 and regenerated from the L2 cell layer (Franks et. al. 2002). The L1 genotype is a mutant derived from a somatic mutation of *VvGAI1* in the L1 layer of the apical meristem which is divided into two distinct layers, L1 and L2 of Pinot Meunier. The Pinot Meunier grape L1 genotype (3.1.5.2) has been demonstrated to be suitable for floral genetic studies (Chaib et al 2010). The L1 genotype results in phenotypic differences, such as reduced internodes and increased flowering. Uncommitted primordia differentiate into inflorescences on actively growing shoots in the resulting L1 plant whereas the wildtype phenotype is retained in the L2 plant (Boss & Thomas 2002). The phenotype of L1 is of short stature and the development of inflorescences instead of tendrils thus making it a useful mutant for the discovery of genes involved in floral induction. Genetic studies showed that the L1 mutant could be self crossed to produce progeny with two copies of the *Vvgai1* allele which also produce rapid flowering and are even shorter in stature (Boss & Thomas 2002). The resulting plant is called the picovine genotype.

5.2 Spatial Gene Expression Analysis of Pinot Meunier

In order to discover new genes with a putative role in floral development some spatial gene expression studies on the Pinot Meunier genotypes were required. This would allow initial identification of which genotype would be the most suited 'model' plant along with identifying the appropriate tissues to conduct gene expression studies on. Phenotypic and gene expression differences can be compared and understanding developed. In the initial 'model' plant investigation, a series of experiments were conducted to compare multi-factorial responses of phenotypic, gene location and gene expression. Plants were established and grown in the glasshouse at Adelaide, Waite Campus. The apex, inflorescence, tendril and leaves opposite these organs were selected from these genotypes (Table 9).

Table 9 Novel and known flowering gene assessment matrix for Pinot Meunier genotypes.

Spl #	Genotype	Tissue type from both micro & HWM RNA	Tissue position
1	L2	Tendril	1
2	L2	Leaf (opposite tendril)	1
11	L2	Tendril	2
12	L2	Leaf (opposite tendril)	2
11	L2	Tendril	2
12	L2	Leaf (opposite tendril)	2
3	L1	Tendril	1
4	L1	Leaf (opposite tendril)	1
13	L1	Tendril	2
14	L1	Leaf (opposite tendril)	2
5	L1 Chambers 30 / 15	Tendril	1
6	L1 Chamber 30 / 15	Leaf (opposite tendril)	1
7	L1 Chambers 30 / 15	Inflorescence	1
8	L1 Chamber 30 / 15	Leaf (opposite inflorescence)	1
15	L1 Chambers 30 / 15	Tendril	2
16	L1 Chamber 30 / 15	Leaf (opposite tendril)	2
17	L1 Chambers 30 / 15	Inflorescence	2
18	L1 Chamber 30 / 15	Leaf (opposite inflorescence)	2
9	Picovine	Inflorescence	1
10	Picovine	Leaf (opposite inflorescence)	1
19	Picovine	Inflorescence	2
20	Picovine	Leaf (opposite inflorescence)	2

Table 10 *Comparison of Pinot Meunier L1 and Picovine Genotype (Glasshouse) flowering gene in inflorescence structures, were node number is the position along the shoot from the tip (apex).*

	***LEAFY* (*VvLFY*) grapevine**	
Node	**L1**	**Pico**
1	0.0003	0.06
2	0.0005	0.04
3	-	0.007

	***TERMINAL FLOWER* 1 (*VvTFL*)**	
Node	**L1**	**Pico**
1	0.02	0.005
2	0.02	0.004
3	0.03	0.0005

	***FLOWERING LOCUS T* (*VvFT*)**	
Node	**L1**	**Pico**
1	0.008	0.10
2	0.05	0.2
3	0.07	0.10

Table 11 Pinot Meunier L1 genotype Microvine chamber 27/15 °C

L1 27 / 15 °C	χ Ratio *VvFT*	χ Ratio *VvTFL1*	χ Ratio *VvLFY*
Total	0.02 a	0.03 a	0.0006 b ***
Apex	0.01 b *	0.1 a *	0.002 a *
Flowers	0.04 ab *	0.03 c *	0.0004 b **
Tendrils	0.06 a *	0.04 b *	0.001 a *
Leaves opposite tendrils	0.002 c ***	0.002 e **	0.0005 b **
Leaves opposite flowers	0.002 c ***	0.02 d **	0.0001 b **

The gene *VvFT*, *VvTFL1* and *VvLFY* were found to be expressed in flowering in the Pinot Meunier L1 genotype. The average gene expression of *VvFT* or *VvTFL1* is about 40 times more than *VvLFY* (Table 10 and Table 11 $P > 0.001$). There is no significant difference of expression comparing the genes *VvFT* to *VvTFL1*. The highest expression levels of *VvFT* occur in the tendrils, inflorescences and apex. *VvFT* is expressed about 10 times less in the leaves and there is no difference if the leaves are either opposite a flower or a tendril. *VvTFL1* relative expression is very high in the apex and expression is significantly different in each organ sampled suggesting a functional role for *VvTFL1* in different organs. The high relative expression in the leaves opposite inflorescences, apex, inflorescences and the tendrils suggests an important role of this gene. *VvLFY* relative gene expression is low in the Pinot Meunier L1 genotype. The highest expression is in the apex and tendrils and may indicate a functional role in these organs.

An investigation into the gene expression of the flowering genes *VvFT*, *VvTFL1* and *VvLFY*, illustrated that the Pinot Meunier picovine genotype showed significantly higher levels of expression in the Leafy gene *VvLFY* and interestingly in the flowering locus gene *VvFT*. These preliminary and novel findings were followed up genetic studies to investigate the roles of novel flowering genes (see below Sections 5.3 and 5.4).

5.3 Novel Gene Identification Techniques

The objective of this study was to discover new genes with a putative role in floral development by comparing differences in gene expression between the picovine inflorescence structures with the tendril structures of the L2 wildtype (Section 5.1) at identical node position using a technology called Whole Transcriptome Shotgun Sequencing (WTSS). In the studies described here we conducted the sequencing on messenger RNA only. This accepted terminology for applying the technique of WTSS on messenger RNA is called “mRNA-Seq”. This is a high-throughput sequencing technology to sequence cDNA in order to get information about a sample's messenger RNA content. Vannozzi et al

(2012) used this technique to conduct a genome-wide analysis of stilbene synthesis in Vitis mutants. Validation of selected genes was then performed using RT-qPCR. The Pinot Meunier genotypes L2 (wild type) and the pico vine, were chosen for this study due to their genetically similar backgrounds which increases the likelihood that any gene expression changes result from the difference in inflorescence development pathways (Section 5.2).

5.4 Results and Discussions

Total RNA was extracted from tissue collected from different stages of inflorescences, tendrils and leaves (Figure 14) from the Pinot Meunier wild type (L2) and the picovine genotype (Section 5.1). Leaves directly opposite each tendril or inflorescence were also collected and included in the analysis as a negative control. Total RNA was processed and mRNA-Seq analysis was undertaken as described in the methods section (Sections 5.3 and 3.3).

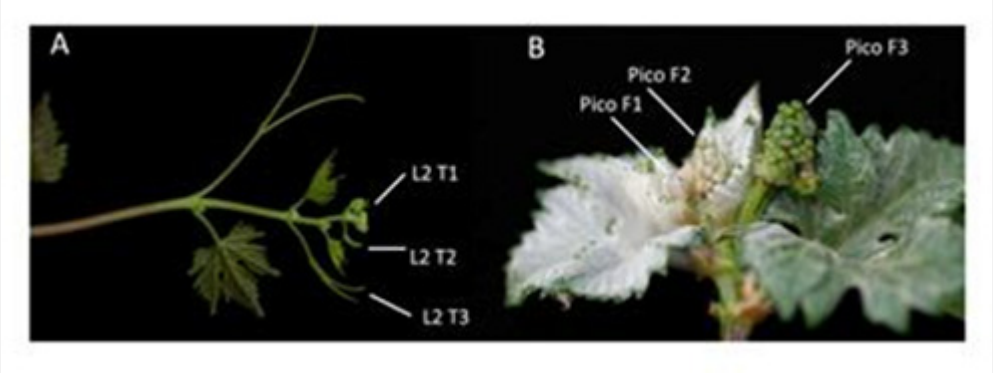


Figure 14 Tissue collected for total RNA extraction. A Pinot Meunier L2 wildtype T1, T2 and T3 are tendril positions from the shoot apex; B picovine (V46) F1, F2 and F3 are floral positions from the shoot apex.

Bioinformatic methods were used to screen out anomalies, count each sequence and then group unique sequences based on the NCBI 12X *Vitis vinifera* assembly to determine expression levels of expressed genes. Expression levels of these genes were calculated as RPKM (reads per kilobase of transcript per million mapped reads). The RPKM value for a given sequence is a gene expression value which can be compared between genes within and across samples. Sequences from mRNA-Seq data were screened for specificity to picovine inflorescence structures and sequences detected in tendrils or leaves were excluded. The resulting list of inflorescence specific genes was sorted by expression level calculated as mean RPKM (Table 12). Gene specific primers designed for selected inflorescence-specific genes were used to determined gene expression levels by RT-qPCR in order to validate the results (Figure 15). Pearson coefficient R was calculated to compare mRNA-Seq and RT-qPCR data for each validated gene.

A total of 20 genes showing significant expression across flowering stages (F1, F2 and F3; Figure 14) were selected for validation with RT-qPCR (Table 12). Figure 15 shows a RT-qPCR plot for one of the validated genes showing specific gene expression detection in the floral tissues. Pearson coefficient R calculations showed strong correlations for 19 of the 20 genes as shown in Table 13. Gene expression was detected in leaf and tendril by RT-qPCR in Gene *H* and thus was not considered a gene specifically expressed in floral tissue. Figure 16 illustrates mRNA-Seq and RT-qPCR data for genes with different expression patterns for the flowers stages and supported via a significant correlation between the two techniques. The correlation between mRNA-Seq and RT-qPCR data results demonstrate that mRNA-seq is a reliable technique for gene discovery.

The putative gene function of the selected genes using the UniProtKB database is listed in Table 14. Results demonstrate the success of mRNA-Seq technology in identifying genes involves in flower development. Genes *A3*, *D*, *J* and *I* show homology to genes involved in floral development. In particular, Gene *I* shows homology to a gene found to be expressed in floral meristems in the carpel primordia (Yamaguchi et al 2004) and may indicate a functional role in the very early stages of flower induction. The discovery of this gene is novel.

Moreover other genes identified can be associated to floral induction by their interactions according to the findings in the Protein Knowledgebase (UniProtKB). For example *A3* shows homology to basic blue protein in *Arabidopsis* involved in pollen tube development (Dong et al 2005). Inflorescence specificity occurs in the pistils, papilla cells, embryo sacs, sepals and stamen filament and expression is undetected in petals, anthers and pollen (Dong et al 2005). Interestingly, this gene has been found to be down regulated by microRNA 408 (miR408) via AGO1 and AGO2 in response to low copper availability (Abdel-Ghany et al 2008; Maunoury et al 2011). Gene *1* in Table 14 is involved in copper transport and therefore could be involved in a similar floral gene expression pathway to A3. Moreover, Gene *1* function may also be associated with sugar transport (Antony et al 2010) and Gene E shows homology to a sugar transporter. Further investigation is required to confirm if Gene *A3*, Gene *1* and Gene *E* are involved in a common pathway for floral induction.

In the grapevine, unlike Arabidopsis, floral induction occurs early on in the uncommitted primordia of latent buds during spring and summer and experience winter dormancy before bud burst and flowering in the following spring (Boss & Thomas 2002). The ability to use the Pinot Meunier genotypes L1 and picovine to investigate gene expression of inflorescence development outside of a latent bud has allowed us to discover inflorescence-specific genes that can be evaluated within latent buds to shed more light on the roles of these genes in early floral induction.

Table 12 Expression levels (RPKM) of twenty inflorescence-specific genes across 3 developmental stages (F1-F3) identified by mRNA-Seq. Mean reads per kilobase of transcript per million mapped reads (RPKM) were calculated.

Gene ID	F1	F2	F3	mean RPKM
A3	14.3	193.5	900.1	262.3
B	0.8	57.9	783.1	211.5
C	1.8	16.5	239.1	70.0
D	0.3	28.2	178.8	61.1
E	1.3	40.7	179.3	60.9
F	0.1	0.2	286.4	59.7
G	2.4	49.5	156.5	53.2
H	40.5	62.7	40.0	48.9
I	20.0	36.6	34.2	35.6
J	4.9	35.7	34.1	24.8
1	0.2	1.0	86.4	19.5
2	2.2	17.3	24.4	15.5
3	11.3	18.9	6.8	15.3
4	0.2	9.6	52.0	14.3
5	0.0	0.8	37.9	13.7
8	0.4	13.1	15.7	11.6
11	3.1	16.5	1.0	10.9
12	3.3	16.8	4.0	10.8
13	8.4	15.8	8.2	10.1
21	16.7	2.4	2.5	5.3

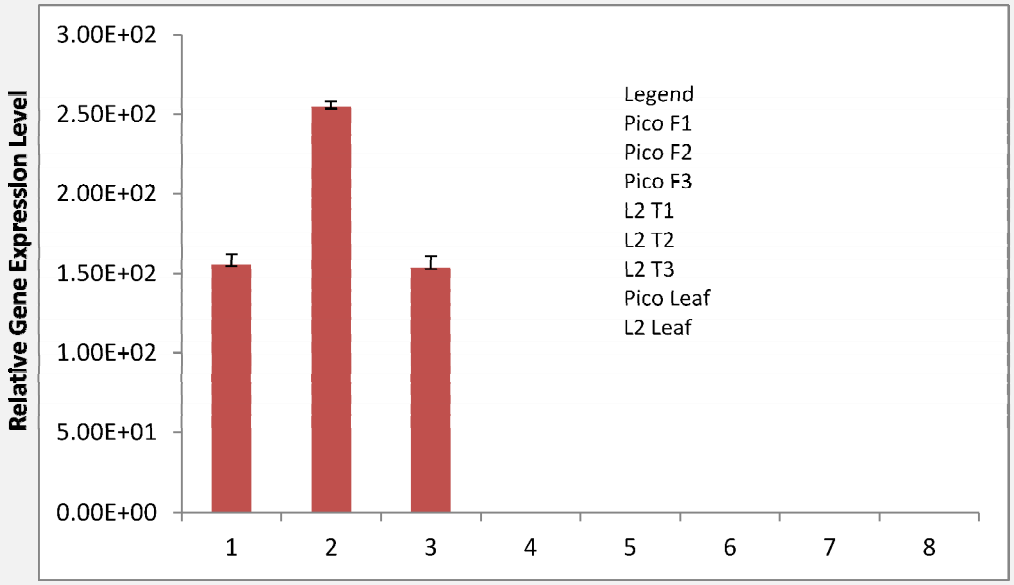


Figure 15 RT-qPCR results showing gene expression of *VvID3* specific to floral tissue of Pinot Meunier genotypes.

Key: F = inflorescence structure with position #, T = tendril + # identifying location on shoot. L2 = wild type genotypes.

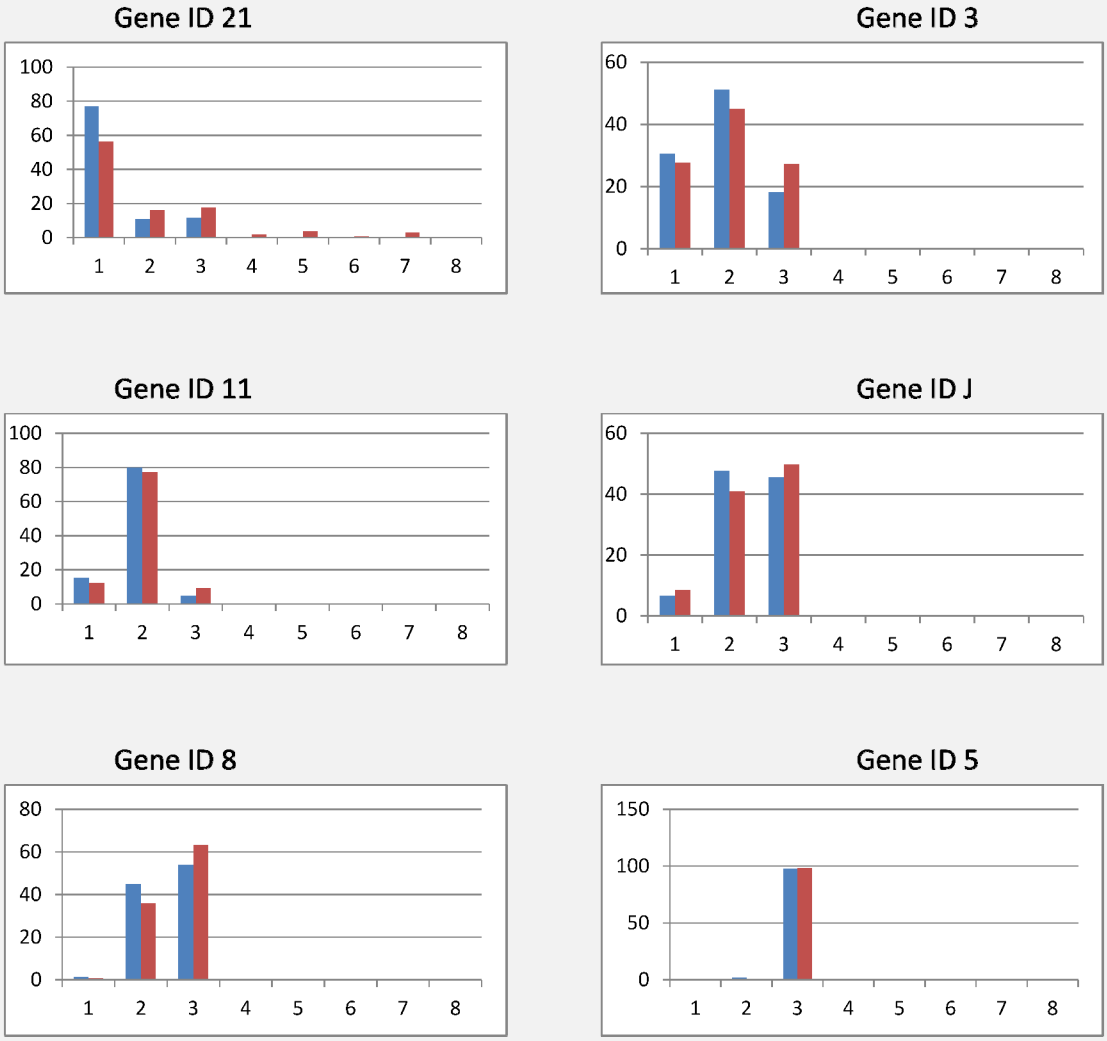


Figure 16 Comparison of mRNA-Seq (blue) and RT-qPCR (red) gene expression data of putative floral-specific genes. 1. Pico genotype F1, 2. Pico genotype F2, 3. L1 genotype (V46) F3, 4. L2 genotype T1, 5. L2 genotype T2, 6. L2 genotype T3, 7. L1 genotype (V46) leaf and 8. L2 genotype leaf.

Key F1 = inflorescence position 1, F2 = inflorescence position 1, L1 = leaf at position 1 and T1 = tendril at position 1.

Table 13 Statistical correlation results between mRNA-Seq and RT-qPCR data

Gene ID	Pearson Correlation Coefficient R
A3	0.998
B	1.000
C	0.999
D	0.999
E	1.000
F	0.993
G	0.998
H	0.032
I	0.914
J	0.989
1	1.000
2	0.998
3	0.977
4	0.998
5	1.000
8	0.979
11	0.997
12	0.985
13	0.959
21	0.983

Table 14 Putative gene function based on protein homology

Gene ID	Gene description	Gene Name	Organisms	Putative function & tissue specificity
A3	Basic blue like protein	ARPN	*Arabidopsis thaliana*	Fertilization, pollen tube growth Expressed in inflorescences
B	Uncharacterized locus			
C	Inactive hydroxysteroid dehydrogenase-like protein 1	HSDL1	*Homo sapiens*	Highly expressed in human sex organs
D	Probable glucan endo-1,3-beta-glucosidase A6-like	Glucan endo-1,3-beta-glucosidase A6	*Zea Mays*	Embryo & anther development & pollen exine formation
E	Bidirectional sugar transporter SWEET5-like	SWEET5	*Oryza sativa*	Sugar transporter
F	Probable xyloglucan endotransglucosylase/ hydrolase protein 23-like		*Brassica oleracea*	Cell wall construction in growing tissue
G	Uncharacterised locus			
I	Protein DROOPING LEAF-like	DL	*Oryza sativa*	Regulates carpel specification in floral development. Expression early in the infloresence when carpel primordia develop.
J	Transcription factor ABORTED MICROSPORES-like	AMS	*Arabidopsis thaliana*	Plats a crucial role in tapetum development, male fertility and pollen differentiation First observed in the bud stage.
1	Copper transporter	COPT1	*Oryza sativa*	Involved in copper transport in co-operation with sugar transporters.
2	Probable sulfate transporter 3.4-like	SULTR3;4	*Arabidopsis thaliana*	Involved in sulphate assimilation
3	ABC transporter A family member 8-like	ABC transporter A family member 8	*Arabidopsis thaliana*	Cell membrane transport
4	17.6 kDa class I heat shock protein 3-like	HSP17.6A	*Arabidopsis thaliana*	Chaperone activity seed development and germination
5	auxin-induced protein 5NG4-like		*Pinus taeda*	Auxin mediated signalling in hypocotyls roots & stems
8	Uncharacterised locus			
11	Uncharacterized locus			
12	Uncharacterized locus			
13	Uncharacterized locus			
21	Transcription factor HBP-1b(c1)-like	RCOM_0353720	*Ricinus communis*	DNA binding transcription factor

6 Impact of Climatic Variables on Fruitfulness and Gene Expression under Controlled Climatic Conditions

6.1 Experimental Background and Aims

Controlled environments that allow manipulation of temperatures were used to assess the gene expression in buds and thus allow a comparison of bud gene expression in field environments where climatic conditions cannot be controlled. Bud microscopy was conducted to assess bud fertility and development stage and these were compared with relative gene expression of *VvFT*, *VvTFL1* and *VvLFY* grapevine flowering genes were measured.

6.2 Experimental Details

Pinot Meunier genotype L1 plants were allowed to grow in controlled climatic conditions where the vine tissue day temperature was 27°C and the night temperature was 15°C (27/15 °C), PAR 1200 µmol and humidity 45 - 60% were maintained for two simulated seasons (dormancy was achieved by pruning back shoots and removing all leaves). Buds were removed and a sub-sample (n=5) was processed for microscopy to identify fruitfulness and ontogeny (Section 3.4; Section 6.3). Remaining buds were snap frozen and stored at -80 °C for gene expression analysis. Results from preliminary experimentation (Section 5.1) on Pinot Meunier genotypes allowed identification of genes of interest; *VvFT*, *VvTFL1* and *VvLFY*. Bud RNA was extracted (section 3.3.1) and real time quantitative PCR conducted (Section 3.3.2; Section 6.4).

6.3 Results of Microscopy

Images of Pinot Meunier L1 genotype illustrate that growing this genotype at different temperatures produces very different development of the latent bud (N+2) (Plate 19). The buds that developed at a temperature of 27 °C during the day and 30 °C at night (27/30 °C) were more fruitful (80% Table 15) where inflorescence primordia, bract primordia and primary branch primordia had developed (Plate 19 A). These buds were at stage 7 ontogeny (Plate 19). Where the L1 genotype was grown in the cooler day and night temperatures of 17/15 °C buds displayed no visible signs of inflorescence development, had a fruitful percent of 0 and only leaf primordia were visible (Plate 19). Ontogeny of these buds were at stage 0 and very different from the ontogenetic development found in the 27/30 °C treatment.

Measurements from Pinot Meunier L1 genotype that developed at a day and night temperatures 27/30 °C illustrate that buds at position node 2 were less fertile than buds at position node 4 and 10 (Table 15). Apex, inflorescence primordia, bract primordia and primary branch primordia are observed across all buds, with a slight separation of these structures (Plate 21).

Table 15 Temperature Comparison of Inflorescence Primordia (% score) of Pinot Meunier L1 Genotype

	Inflorescence Primordia (% score)	
	Temperature Day / Night (°C)	
Node	27 / 30	17 / 15
2	80	0
4	100	0
10	100	n/a

Note mean from 5 replicates per node per temperature treatment

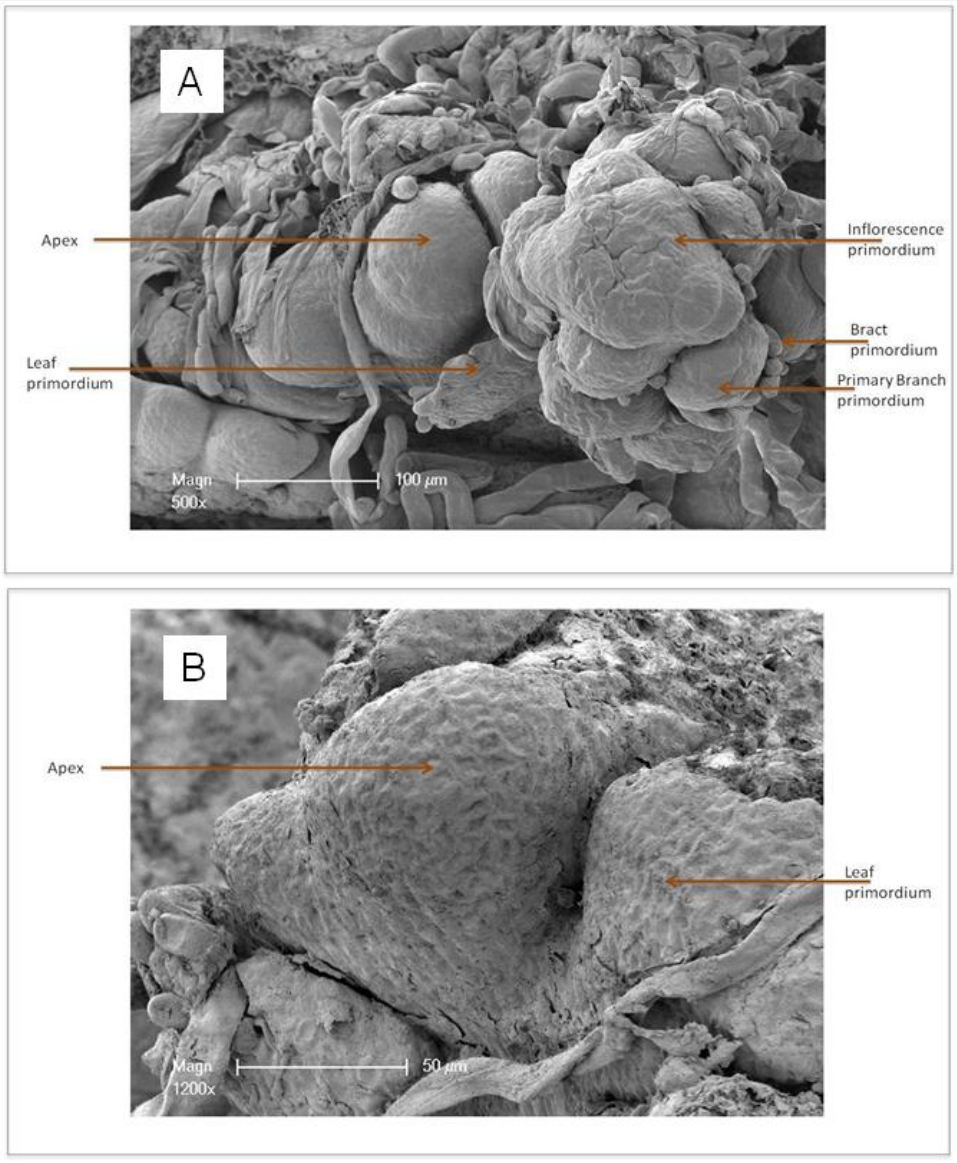


Plate 20 The effect of temperatures on node 2 Pinot Meunier L1 genotype (A) day/night 27/30 °C stage 7 ontogeny and (B) day/night 17/15 °C stage 0 ontogeny.

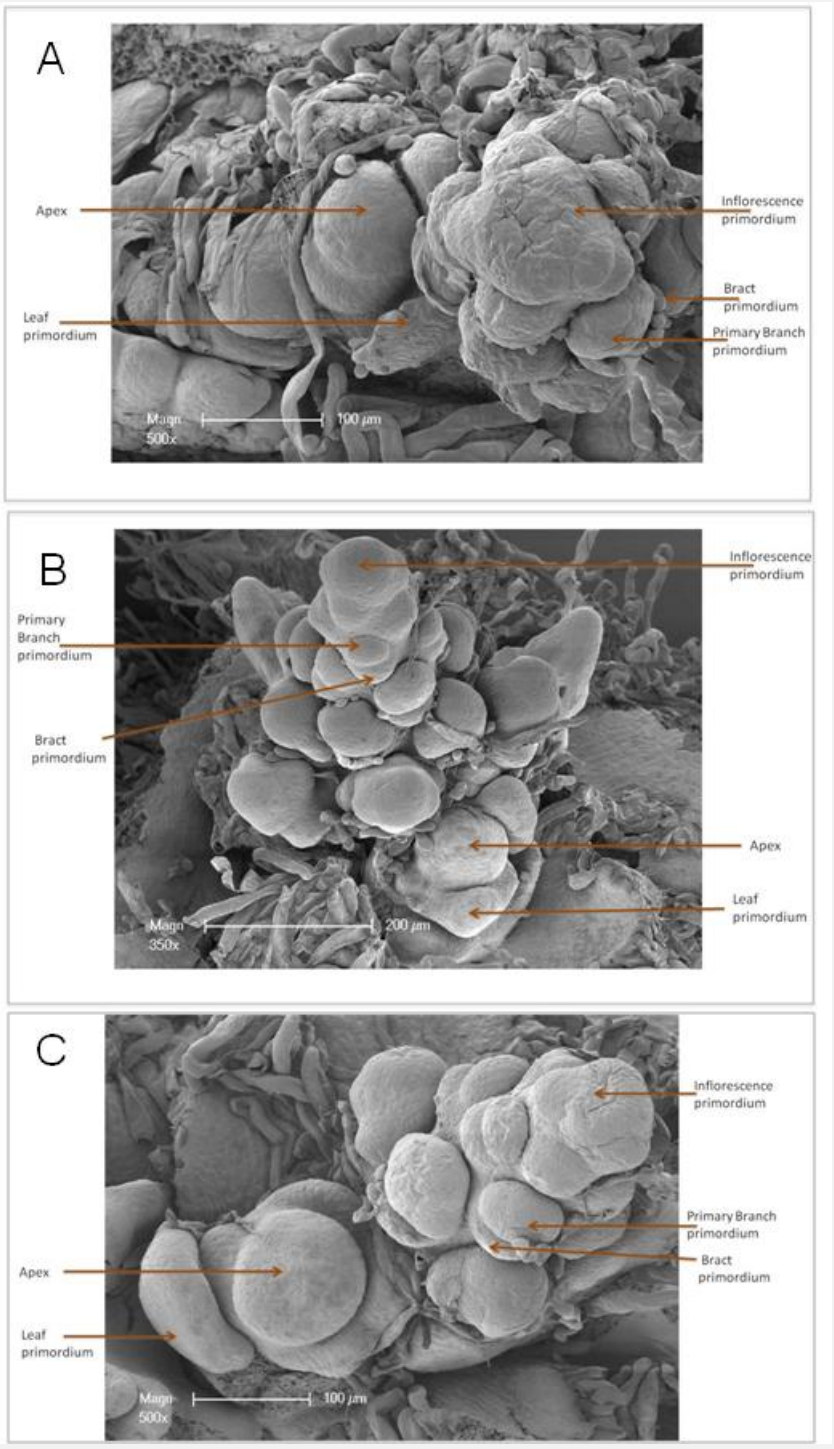


Plate 21 Pinot Meunier L1 genotype in controlled environments with day/night temperatures at 27/30 °C (A) node 2 (B) node 4 and (C) is node 10. All nodes at stage 7 Ontogeny.

6.4 Results and Discussion of Quantitative Gene Expression

The relative expression levels of the flowering genes *VvLFY* and *VvTFL1* were found to be similar to each other in the developing latent buds (N+2) at node positions 2, 4 and 10 of Pinot Meunier L1 genotype. The relative expression of the flowering gene *VvLFY* was very low in all buds regardless of position along the shoot (Figure 17).

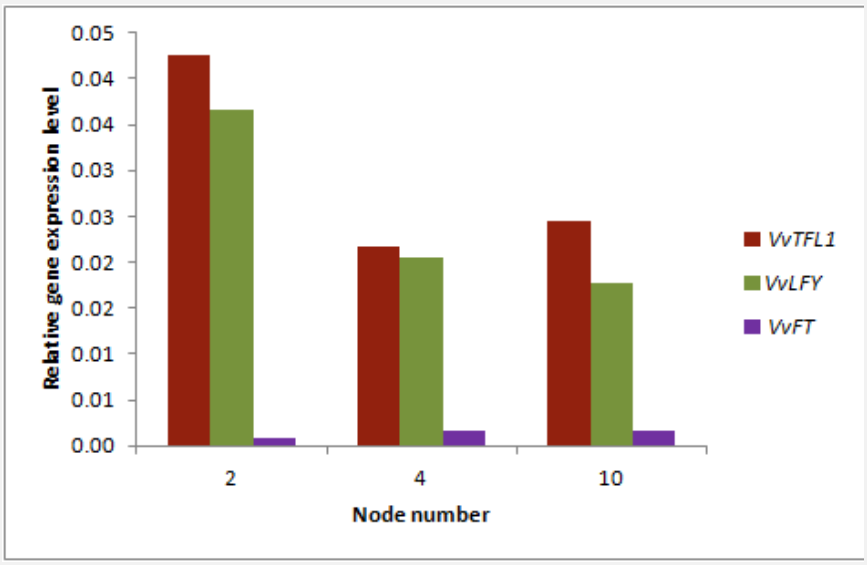


Figure 17 Pinot Meunier genotype L1, flowering gene relative expression at different nodes along a shoot at day/night 27/30 (°C).

Gene identification *VvTFL1* (red), *VvLFY* (green) and *VvFT* (purple) where 100% inflorescence primordia.

When the temperature of the bud was set to a cooler regime of day/night 17/15 °C, gene expression in all of the flowering genes tested reduced significantly (Figure 18). This reflected a reduction in fruitfulness and bud development found in this treatment (Table 15 and Plate 21). These findings suggest that lower temperatures can reduce expression of *VvLFY* and *VvTFL1* and that these genes play a role in fruitfulness in the grapevine bud.

In summary the Impact of climatic variables, specifically the role of temperature was investigated on bud fruitfulness and gene expression. Utilising controlled climatic conditions, findings demonstrate the possible functional role and importance of the genes *VvLFY* and *VvTFL1*. These findings have developed our understanding of gene expression and inflorescence development in the early stages of bud development. This research is important in providing a basis to understand gene expression in vineyards that demonstrate low fruitfulness under low temperature conditions. Further research targeting these genes would be recommended.

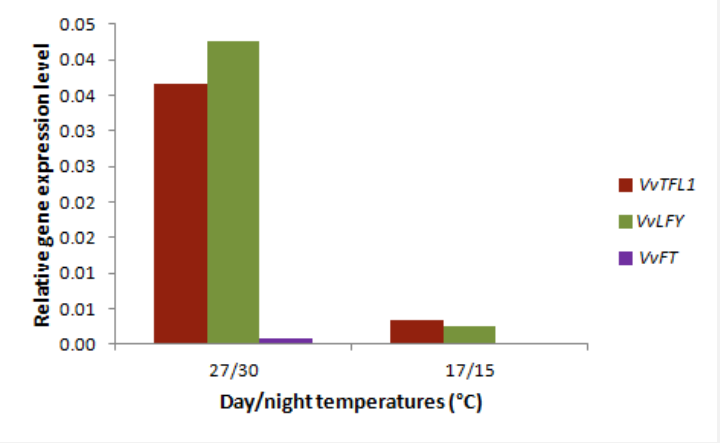


Figure 18 Pinot Meunier genotype L1 flowering gene relative expression at different temperatures.

Gene identification *VvTFL1* (red), *VvLFY* (green) and *VvFT* (purple) where 100% inflorescence primordia.

7 Impact of Climatic Variables on Bud Gene Expression of Field Grown Vines

7.1 Background

Yield potential depends on the transition of apices to a floral state and their subsequent differentiation in developing latent grapevine buds (Boss & Thomas 2002). Traditional approaches to studying the effects of environment and management on initiation and differentiation are limited by technical difficulties associated with quantitative measurement of developing buds and natural variation across bud populations within grapevines. Experimentation reported here combines traditional physiological and anatomical approaches with current molecular tools to improve our understanding of the variables that drive fruitfulness and yield development in grapevines.

7.2 Experimental Aims

To study the expression of genes that may be involved in latent bud floral induction and development by assessing the gene expression level in buds sampled from different climates and genotypes and make an assessment for the presence or absence of inflorescence primordia. Combining climate data, gene expression data, microscopy information and genotype information may further advance our understanding of the impact of genotypic differences and environment on the expression of the floral induction genes.

The outcomes of this experimentation allowed an industry protocol to be developed for bud collection in the field environment.

7.3 Experimental Details

A comparison of bud development and gene expression at different phenological stages of *Vitis vinifera* L. cv. Sauvignon Blanc took place over two seasons, Season 1 (2010/2011) and Season 2 (2011/2012) in south-eastern Australia.

The vine sampling population consisted of 200 vines (4 x rows of 50 vines) at each site, where the first row of each block and the first 2 panels of each row were excluded. Random vine selection across the four rows and then random shoot selection across a 100 cm transect for those vines (50cm either side of crown). Where possible shoots were left *insitu* and latent buds excised from the

shoots. On-site weather data was collected from each site, and details can be found in section 3.2.3 Climate Data and Section 7.4.

Table 16 Climatic variables on bud expression: Bud collection details

Season	Variety	Vineyard	Location	Collection dates (EL stage)	Node collections
2010/2011	Sauvignon Blanc	Dookie	Warm	T1 – 29/10/10 (16) T2 – 15/11/10 (18) T3 – 23/11/10 (21) T4 – 14/12/10 (33) T5 – 10/01/11 (35)	Node 2 Node 4 Node 10
	Sauvignon Blanc	King Valley	Cool	T1 – 22/11/10 (17) T2 – 15/12/10 (32) T3 – 6/01/11 (33)	Node 2 Node 4 Node 10
2011/2012	Sauvignon Blanc	Dookie	Warm	T1 – 17/11/11 (23) T2 – 9/01/12 (21)	Node 2
	Sauvignon Blanc	King Valley	Cool	T1 – 19/12/11 (25) T2 – 8/03/12 (35)	Node 2
	Sultana	Adelaide - Field	Warm	T1 – 3/11/11 (23)	Node 2

At the start of each growing season, 4-6 shoots were tagged to monitor shoot growth within 4 rows of fifty vines (200 vines total). Each shoot was measured for shoot length and node number at each collection point. Field collections of buds (N+2) were taken at various time points during the growing season (Table 16). Buds were collected from shoots matching the tagged vines and were randomly selected from vines across two or more rows in each vineyard. Results from Sauvignon Blanc season 1 (2010/2011) bud temporal and spatial experimentation, informed phenological collections times for season 2 (2011/2012).

Protocol for bud collection for genetic analysis

Once excised from the N shoot, the N+2 buds were frozen on dry-ice before being stored in vials at -80°C. Frozen buds were then transported by air on dry ice to Adelaide CSIRO, Plant Industry, Urrbrae and stored at -80°C. The E-L stage was recorded at each collection time (Coombe 1995). Putative floral genes detected by mRNA-Seq (Section 5) and the genes *VvTFL1*, *VvLFY* and *VvFT* were assessed in buds by RT-qPCR to determine an association between the presence of inflorescence primordia and significant gene expression changes within one cultivar (Sauvignon Blanc) grown in two different vineyards Dookie and King Valley. The same genes were assessed to detect gene expression differences between Sauvignon Blanc buds collected in the Dookie vineyard with those of Sultana that had developed in the Waite vineyard, Adelaide, to identify genes associated with the variation in inflorescence primordia development due to genotype.

Protocol for bud collection for microscopy

Buds were collected for microscopic analysis in the field and glasshouse at anthesis and veraison from Sauvignon Blanc at node 2, 4 and 10 in Season 1 and from Sauvignon Blanc and Sultana at node 2 in Season 2. Once excised from the N shoot, the N+2 buds were fixed in a 5 ml vial containing 10% neutral buffered formalin. Vials were stored at 2-4°C.

Bud preparation

A sample of 60-100 compound buds (N+2) from individual Sauvignon Blanc vines were collected from the field in both seasons. In season 2, 40-60 compound buds of Sultana were collected from the field at Waite vineyard, Adelaide. Approximately five buds were retained at each collection time for microscopic analysis and the remaining buds were snap frozen and stored at -80°C for RNA extraction.

Extraction of RNA from bud material

RNA was extracted from compound bud. Buds from individual treatments were pooled and ground in liquid nitrogen until the powder was consistent with fine talc. For details of RNA preparation, cDNA synthesis and RT-PCR analysis please refer to section 3.3.

In the first season (2010/2011) bud dissection was conducted on 3 nodes located at positions; 2, 4 and 10 where collection was possible. In the first season buds from Dookie College vineyard and the King Valley vineyard were collected resulting in a total of 540 buds analysed. In the second season (2011/2012) bud dissection were conducted on one node position at anthesis and veraison. Buds were collected from three vineyards, Dookie, King Valley and the Waite vineyard.

7.4 Climatic Data

Within vineyard climate data (temperature and humidity) was collected for each vineyard in each season (Season 1 = 2010/2011 and Season 2 = 2011/2012).

At the Dookie vineyard a Vaisala model WXT510 weather station was installed according to (Allen et al 1998 FAO Irrigation and drainage paper 56). Data was collected every 15 minutes every 24 hours. Daily and monthly averages were calculated from the data. At the King Valley vineyard a Tiny tag sensor was placed in a centre row. Data was collected every 30 minutes every 24 hours. Daily and monthly averages were calculated from the data. Long term averages (LTA) were obtained from the Australia Bureau of Metrology (BOM) website (www.bom.gov.au).

The minimum and maximum temperature at Dookie vineyard was similar to the long term average (LTA) across both seasons of the bud sampling (Figure 19).

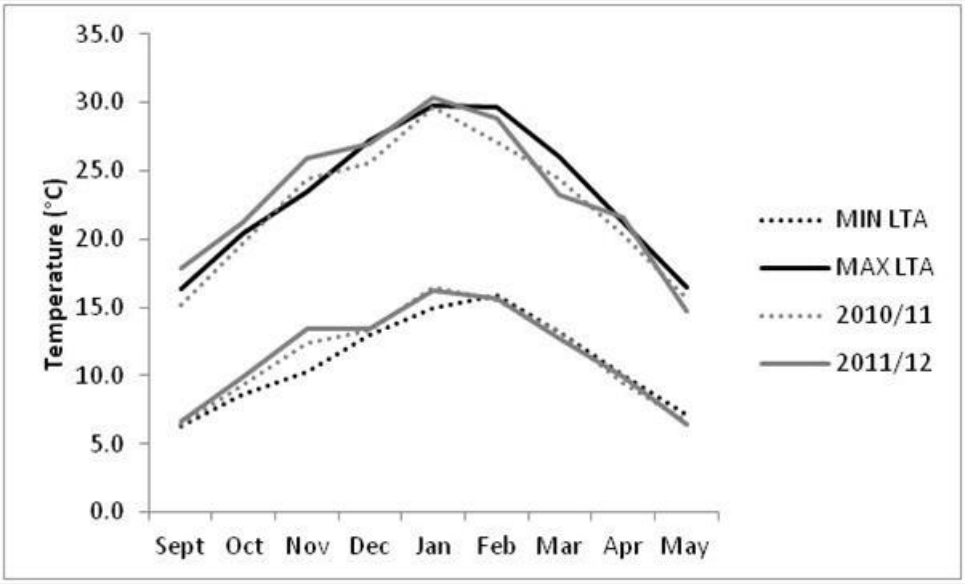


Figure 19 Dookie Vineyard Minimum and Maximum Temperature Long Term Averages (LTA), Season 1 (2010/2011) and Season 2 (2011/2012) Comparison

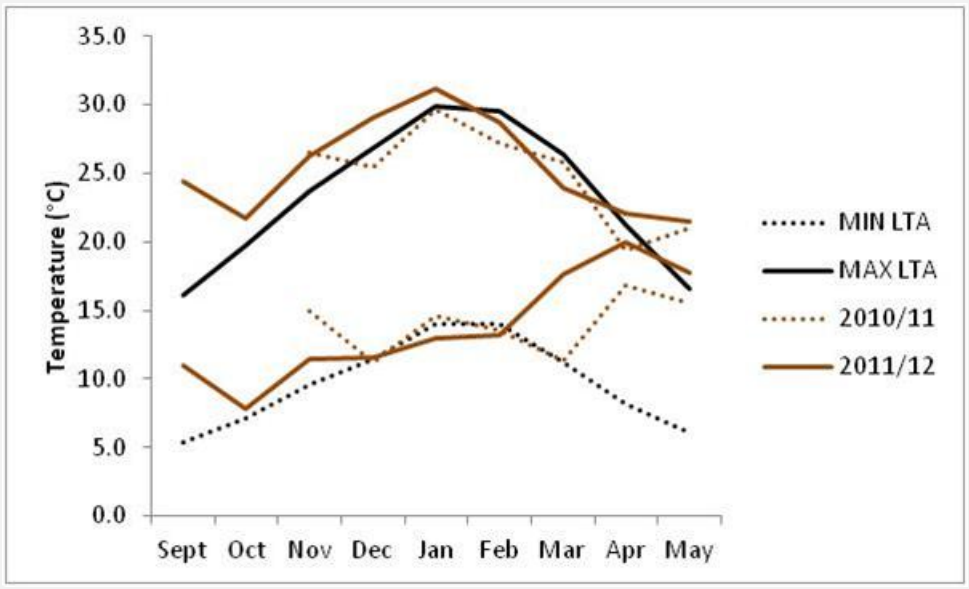


Figure 20 King Valley Minimum and Maximum Temperature Long Term Averages (LTA), Season 1 (2010/2011) and Season 2 (2011/2012) Comparison

The King Valley vineyards illustrate similar responses to the long term average (LTA) in the average maximum temperature across both seasons (Figure 20). The minimal temperature in both seasons (2010/2011 and 2011/2012) was considerably warmer than the LTA in March to May (Figure 20).

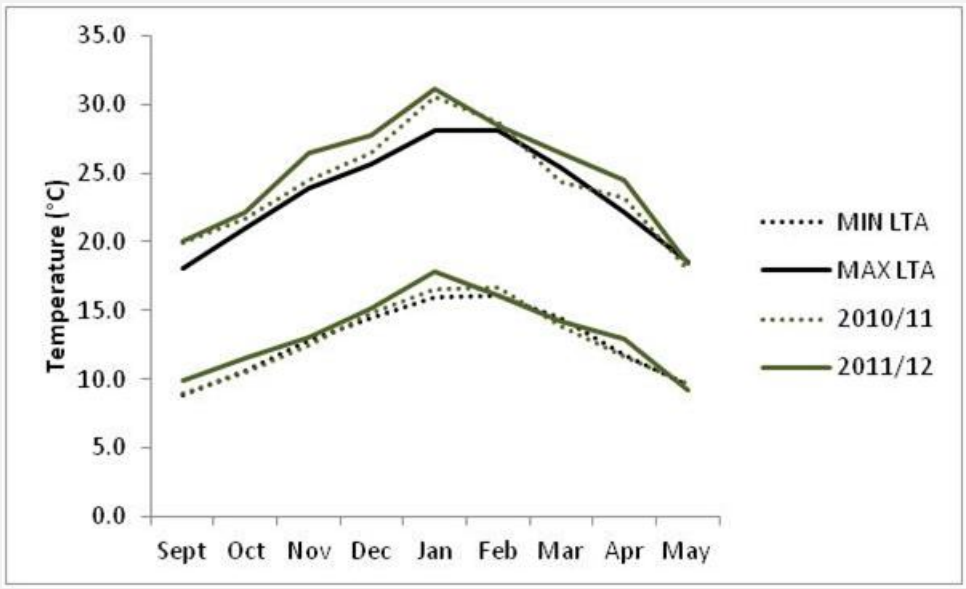


Figure 21 Waite Vineyard Minimum and Maximum Temperature Long Term Averages (LTA), Season 1 (2010/2011) and Season 2 (2011/2012) Comparison

The Waite vineyard minimum and maximum temperature was similar to the long term average (LTA) across both seasons of the bud sampling (Figure 21).

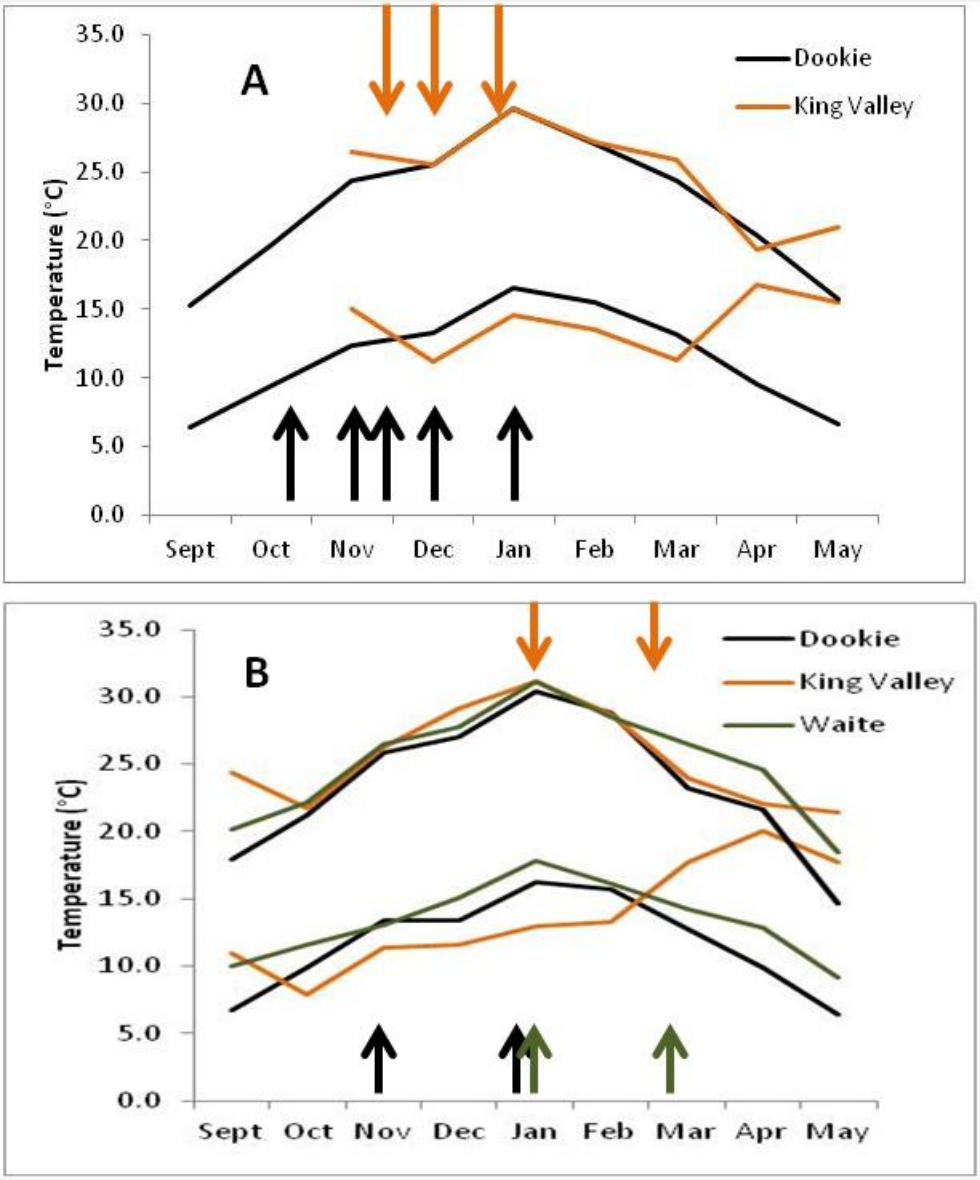


Figure 22 King Valley and Dookie Vineyard Minimum and Maximum Temperature (A) Season 1 (2010/2011) and (B) Season 2 (2011/2012). Arrows indicate sampling dates; black for Dookie, orange for King Valley and green for the Waite vineyard.

Comparison of minimum and maximum temperature at Dookie and King Valley vineyard (Figure 22) illustrate that the maximum temperature was similar in both seasons. The minimum temperature at King Valley was slightly cooler than Dookie in the sampling months and then becomes considerably warmer from March to May across both seasons. The Waite vineyard minimum and maximum temperature was similar to that of Dookie Vineyard (Figure 22B).

King Valley vineyard had a more humid environment in season 1 (2010/2011) than Dookie Vineyard (Figure 23). This trend was similar for maximum humidity in the following season (2011/2012) but the opposite effect for minimum humidity compared to Dookie (Figure 23). The Waite vineyard minimum and maximum humidity was similar to that of Dookie Vineyard (Figure 23).

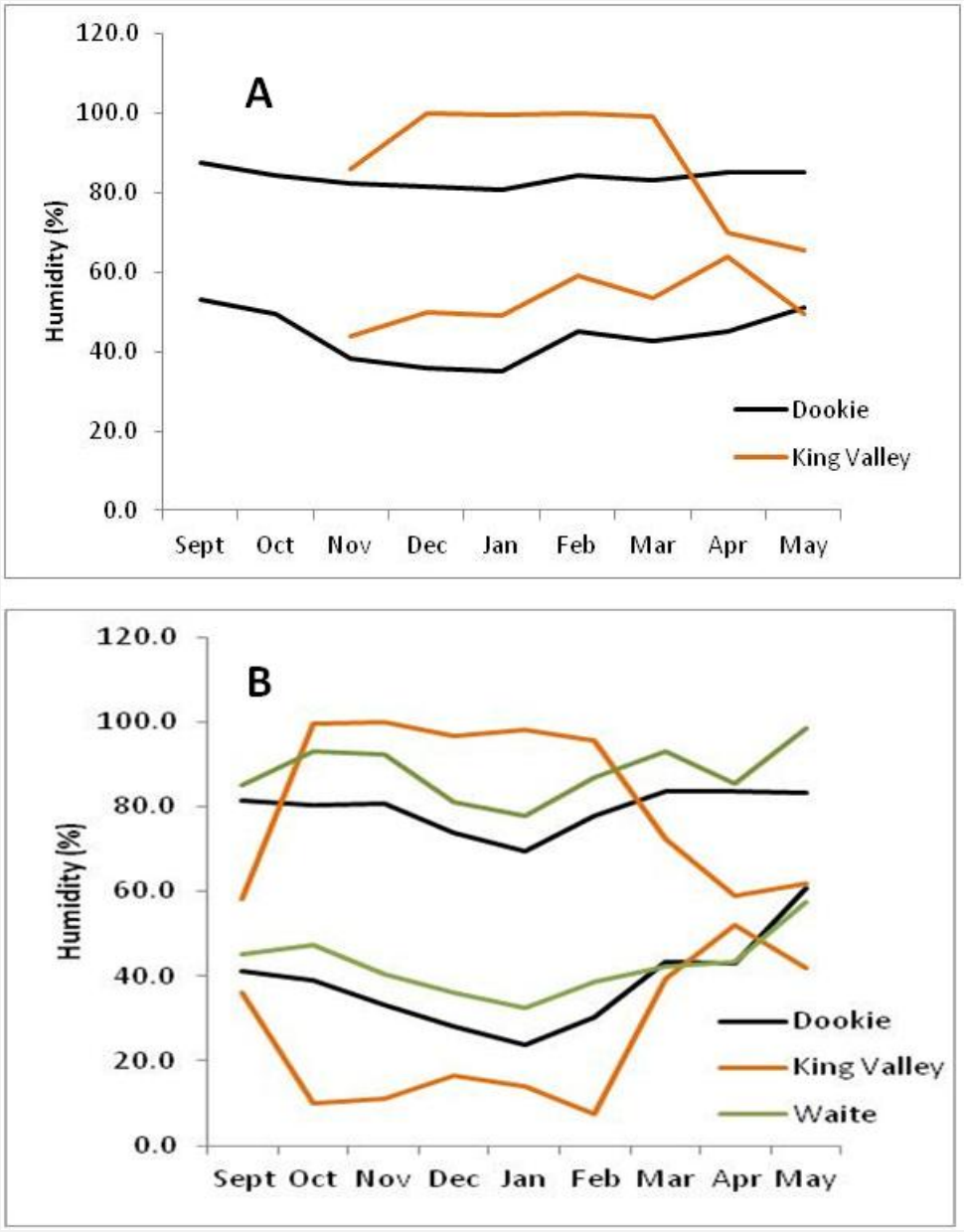


Figure 23 King Valley and Dookie Vineyard Minimum and Maximum Humidity (A) Season 1 (2010/2011) and (B) Season 2 (2011/2012)

7.5 Phenology and Bud Fruitfulness Assessment

Sauvignon Blanc phenology and bud development was assessed at two vineyards Dookie and King Valley (Section 3.2) over a two year consecutive seasons commencing in September 2010. All results described in this chapter on bud development refer to the latent bud (N+2). Selected shoot lengths were measured and the number of buds separated along each shoot was recorded at bud collection times. Vine developmental stages were assessed according to the system described by Coombe (1995) and defined as EL stages (Table 1 and Table 17). Growth of shoot length and separated nodes was linear throughout the bud collection period (Figure 24).

At anthesis in season 1 there were more nodes separated (11.35 ± 1.2) and longer shoot lengths (30 ± 5.4 cm) at King Valley vineyard compared to those at Dookie vineyard (Table 17). Buds were more developed at King Valley vineyard than at Dookie. Bud fruitfulness was assessed by light and scanning election microscopy (SEM) with developing inflorescence primordia identified and then counted (Table 18). In the developing buds leaf primordia were visible at anthesis (Plate 22). Spatial assessment of bud fertility at anthesis showed that bud fruitfulness was influenced by node position, with node 4 having the lowest fertility at both vineyards (0% and 40% Table 18). Ontogeny stage at both vineyards was assessed to be 0 in node 4 at anthesis. Anthesis occurred on different calendar dates at both sites during season 1 and this resulted in developing buds being exposed to cooler minimum and maximum temperatures at the Dookie vineyard compared to the King Valley vineyard (Figure 22). Season 1 temperatures at both sites for this early period in canopy development in season 1 were similar to the long term averages (Figure 19 and Figure 20). Humidity at King Valley vineyard was higher than at Dookie vineyard throughout the sampling period (Figure 23) during season 1. Temporal assessment of bud development showed that fruitfulness increased throughout the season obtaining 100% at EL stage 33 at Dookie vineyard and one EL stage earlier at the King Valley vineyard (EL stage 32 Table 18). During this period nodes continued to separate along the shoot and shoot lengths increased (Table 17). The King Valley vineyard had more node separation (30 ± 2.9) and longer shoots (158 ± 34 cm) than the Dookie vineyard. At véraison leaf primordia are visible and ontogency stage 7 was reached at both vineyards detailing bract, primary branch and inflorescence primordia (Plate 23). The minimum temperature at King Valley was slightly cooler than Dookie for the sampling time/phonological stages and then becomes considerably warmer from March to May across both seasons (Figure 22). These climatic differences have not impacted on visible bud development (Plate 23).

During season 2 (2011/2012) Sauvignon Blanc phenology and bud development were assessed at the Dookie and King Valley vineyards. An additional variety Sultana, planted at the Waite vineyard, was added to allow gene expression comparison to a genotype with low fruitfulness and inflorescence primordia. Sultana is notorious for its low fertility.

Table 17 Bud experiment shoot phenology measurements and assessment

Variety	Climate / Vineyard	Collection dates (EL stage)	Node Position	Number of nodes separated	Shoot length (cm)	EL Stage
Season 2010 / 2011						
Sauvignon Blanc	Warm / Dookie	T1 – 29/10/10 (16)	2 , 4, 10	11.35 ± 1.2	30 ± 5.4	16 ± 0.7
		T2 – 15/11/10 (18)	2 , 4, 10	13.5 ± 1.0	62 ± 5.0	18 ± 0.6
		T3 – 23/11/10 (21)	2 , 4, 10	15.9 ± 1.6	70 ± 11.9	21 ± 2.6
		T4 – 14/12/10 (33)	2 , 4, 10	24.9 ± 1.2	96 ± 15	33 ± 0.5
		T5 – 10/01/11 (35)	2 , 4, 10	27.4 ± 2.2	119 ± 19	35 ± 0
Sauvignon Blanc	Cool / King Valley	T1 – 22/11/10 (17)	2 , 4, 10	13 ± 1.7	52 ± 8.6	17 ± 0.9
		T2 – 15/12/10 (32)	2 , 4, 10	22 ± 1.8	127 ± 17	32 ± 0
		T3 – 6/01/11 (33)	2 , 4, 10	30 ± 2.9	158 ± 34	33 ± 0.5
Season 2011 / 2012						
Sauvignon Blanc	Warm / Dookie	T1 – 17/11/11 (21)	2	17 ± 0.8	71 ± 5	23 ± 0
		T2 – 9/01/12 (35)	2	19 ± 4.3	84 ± 7	35 ± 0
Sauvignon Blanc	Cool	T1 – 19/12/11 (25)	2	18 ± 0.5	102 ± 15	23 ± 0
	King Valley	T2 – 8/03/12 (35)	2	32*	150*	35 ± 0
Sultana	Warm /	T1 – 3/11/11 (21)	2	14 ± 0	83 ± 19	21 ± 0
	Waite	T2 – 11/01/12 (23)	2	33 ± 7	191 ± 79	23 ± 0

*Measurements assessed

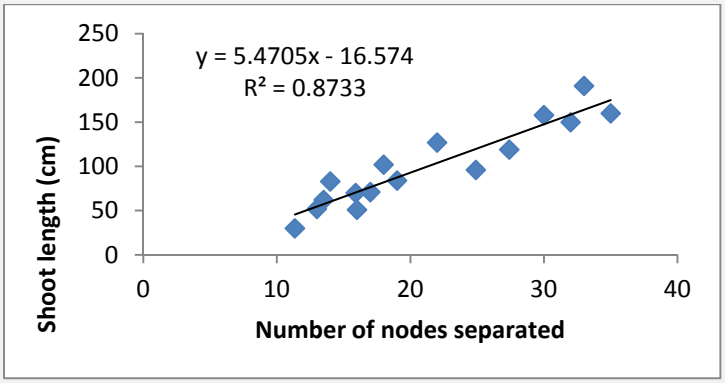


Figure 24 Relationship of shoot length and number of nodes separated

Table 18 Inflorescence primordia bud assessment

Season 2010 / 2011 Inflorescence primordia (% score)						
Vineyard / Variety	**Node**	**EL stage**				
		16 - 17	18	21	32-33	33-35
Dookie / Sauvignon Blanc	2	20	20	20	40	100
	4	0*	20			100*
	10		20			
King Valley / Sauvignon Blanc	2	100			100	100*
	4	40*			100	100*
	10	100			100	100

Season 2011 / 2012 Inflorescence primordia (% score)					
Vineyard / Variety	**Node**	**EL stage**			
		21	23	33	35
Dookie / Sauvignon Blanc	2	0*			100*
King Valley / Sauvignon Blanc	2	0*			100
Waite vineyard / Sultana	2	0*			20*

* SEM micrographs illustrated

*

The number of nodes that separated on shoots at anthesis during season 2 was similar at both Dookie and King Valley vineyards whereas shoot length was longer at King Valley vineyard (Table 7). Bud fertility at Dookie and King Valley vineyards were at 0% and ontogeny was at stage 0. In the developing bud leaf primordia and apexes are visible during season 2 anthesis (Plate 24 and Plate 25) but no visible inflorescence structures. At veraison visual assessment of bud development showed advanced leaf primordia along with 100% fruitfulness, ontogeny stage 6, with bract, primary branch and inflorescence primordia. Branching was more advanced in buds at the King Valley vineyard than at Dookie vineyard (Plate 26 and Plate 27). Sultana nodes that developed at the Waite vineyard are significantly less developed with no inflorescences at anthesis and only 20% inflorescence primordia at EL stage 35 (Table 18 & Plate 28).

In summary Season 1 (2010/2011) bud development tended to become fruitful earlier than during season 2 (2011/2012). Therefore Sauvignon Blanc fruitfulness was plastic across the seasons and King Valley vineyard was more fruitful at an early phonological EL stage. It is worth noting that Dookie anthesis is about one month earlier than vines at the King Valley vineyard. The time difference of one month resulted in 'like' phenology stages being exposed to different climatic conditions at the two vineyards. Therefore the vines from the Dookie vineyard were subjected to lower temperatures at early EL stages (post anthesis and anthesis) compared to the King Valley vineyard (Figure 22). In conclusion, the results demonstrate over both seasons and at all vineyards we were able to select bud development that allowing comparison of gene expression where there were no visible inflorescence primordia and buds that had visible inflorescence primordia after bud dissection.

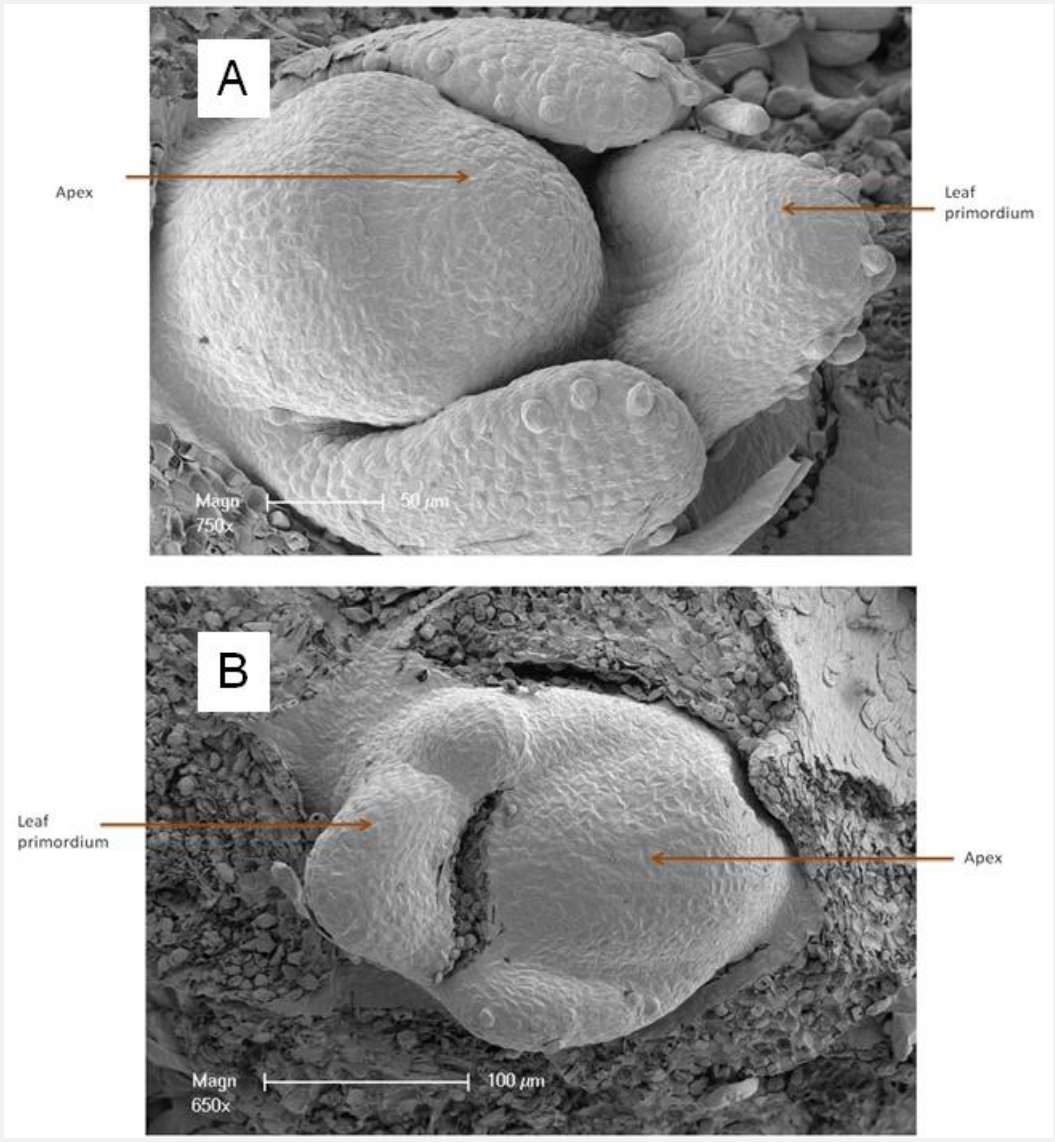


Plate 22 Sauvignon Blanc bud development at anthesis season 1 (A) Dookie vineyard* and (B) King Valley vineyard*. No visible inflorescence primordia.

*Note: Season 2010/2011 Node 4 Stage, EL 16-17, 0 Ontogeny

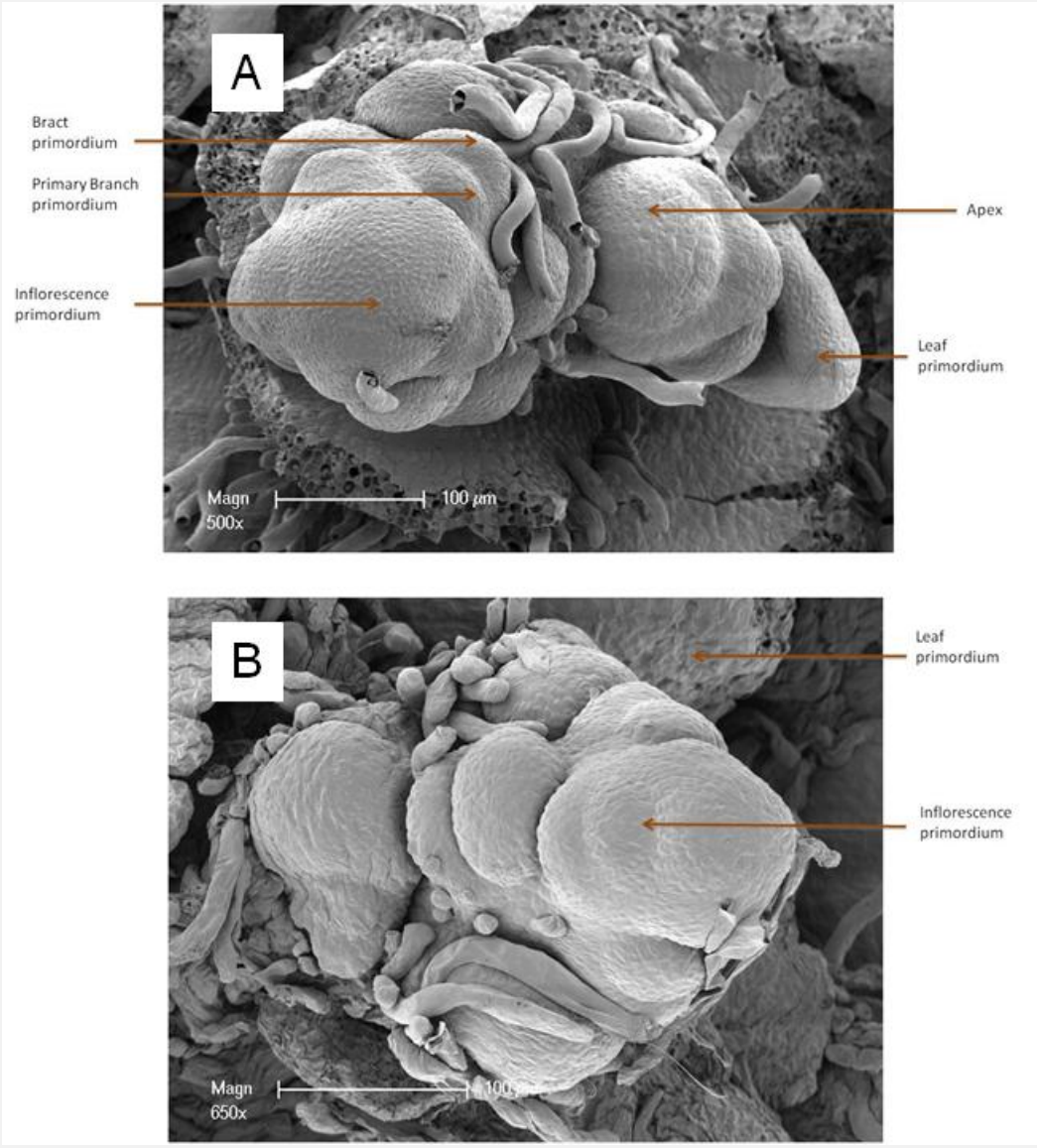


Plate 23 Sauvignon Blanc bud development at veraison season 1 (A) Dookie vineyard* and (B) King Valley vineyard*. Iinflorescence primordia visible.

*Note: Season 2010/2011. Node 4, EL 33/35, Ontogeny Stage 7, 100% fruitfulness

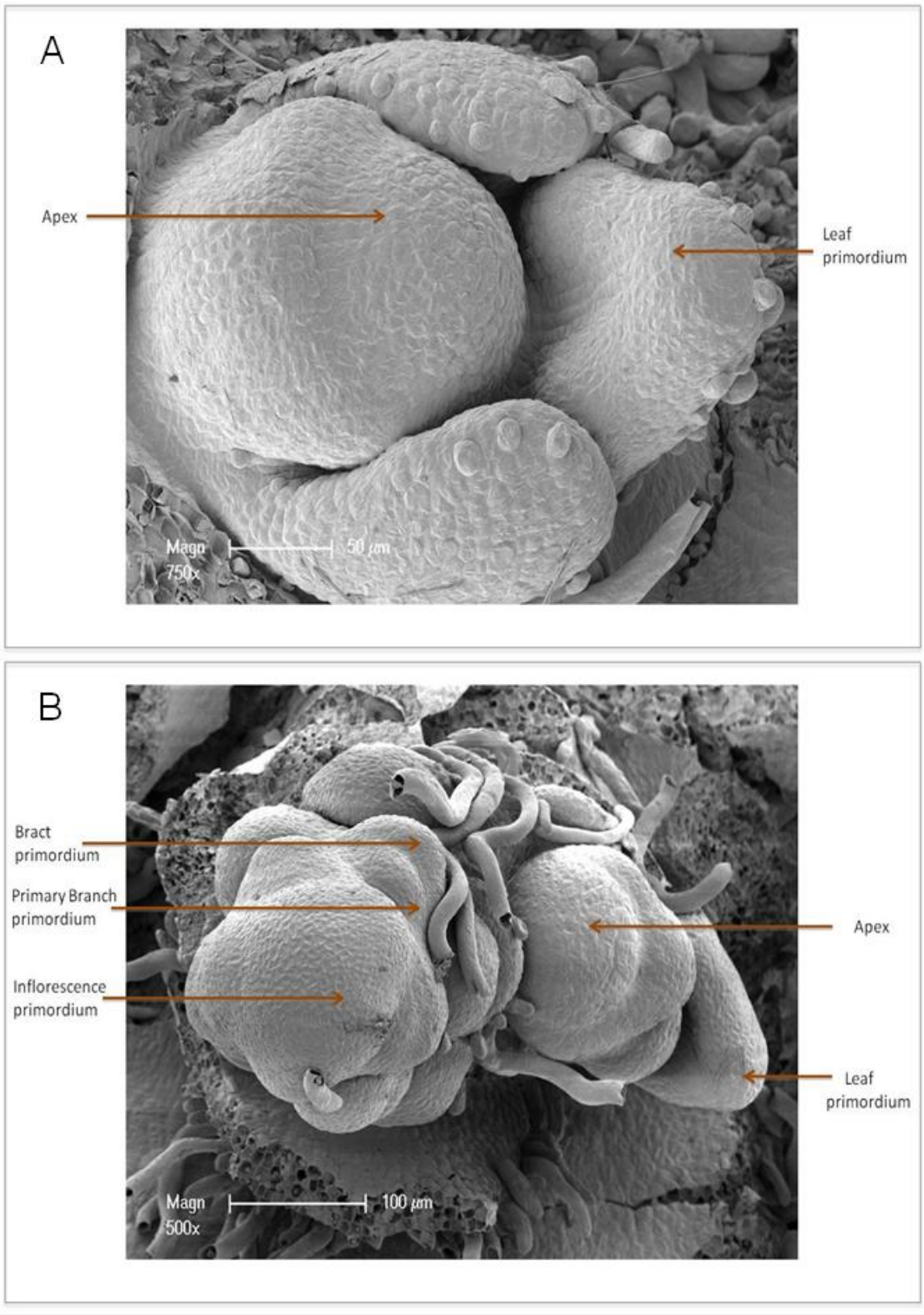


Plate 24 Sauvignon Blanc season 2 bud development at Dookie Vineyard season 2 (A) anthesis no inflorescence primordia* and (B) veraison illustrating inflorescence primordia.

*(A) node 4 EL 16, ontogeny 0, Fruitful 0%

** (B) node 4 EL 35, ontogeny 7, Fruitful 100%. Season 2011/2012.

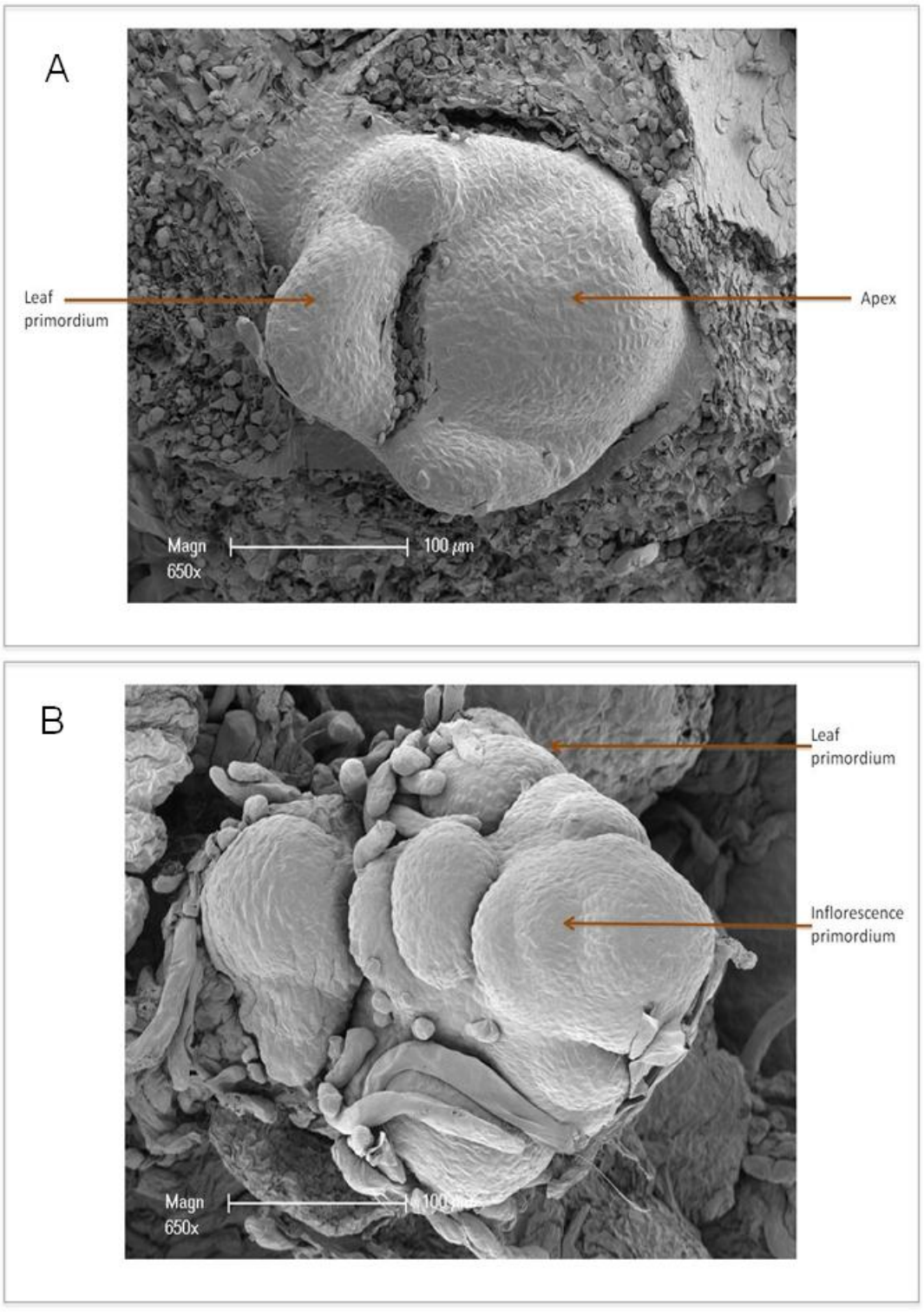


Plate 25 Sauvignon Blanc season 2 bud development at King Valley vineyard season 2 (A) anthesis no inflorescence primordia* and (B) veraison illustrating inflorescence primordia.

*(A) node 4 EL 17, ontogeny 0, Fruitful 0%

** (B) node 2 EL 35, ontogeny 7, Fruitful 100%. Season 2011/2012.

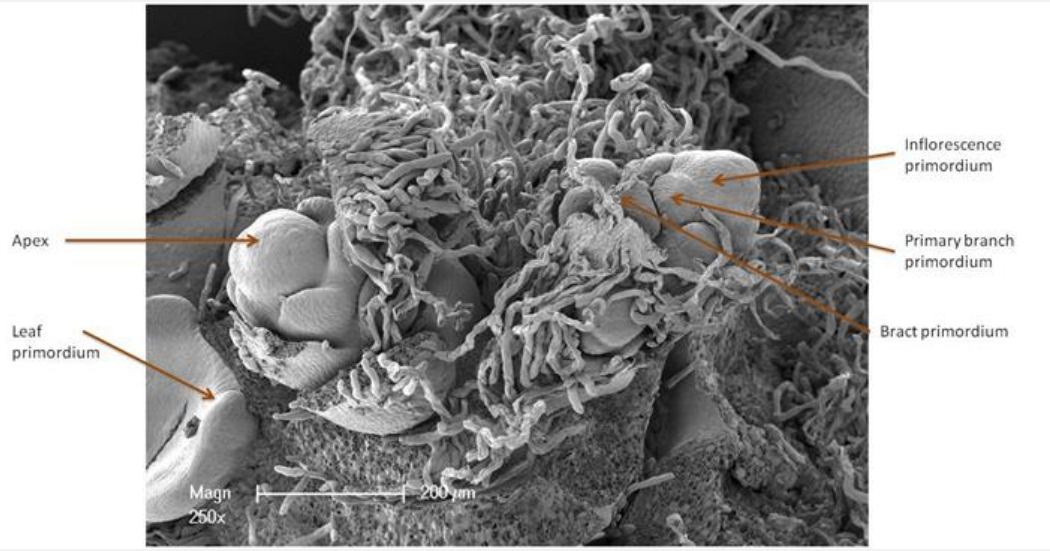


Plate 26 Sauvignon Blanc developing nodes illustrating inflorescence primordia Season 2 veraison at Dookie vineyard*

* Season 2011/2012 EL Sate 35 stage 7 ontogeny, node 2, 100% fruitfulness

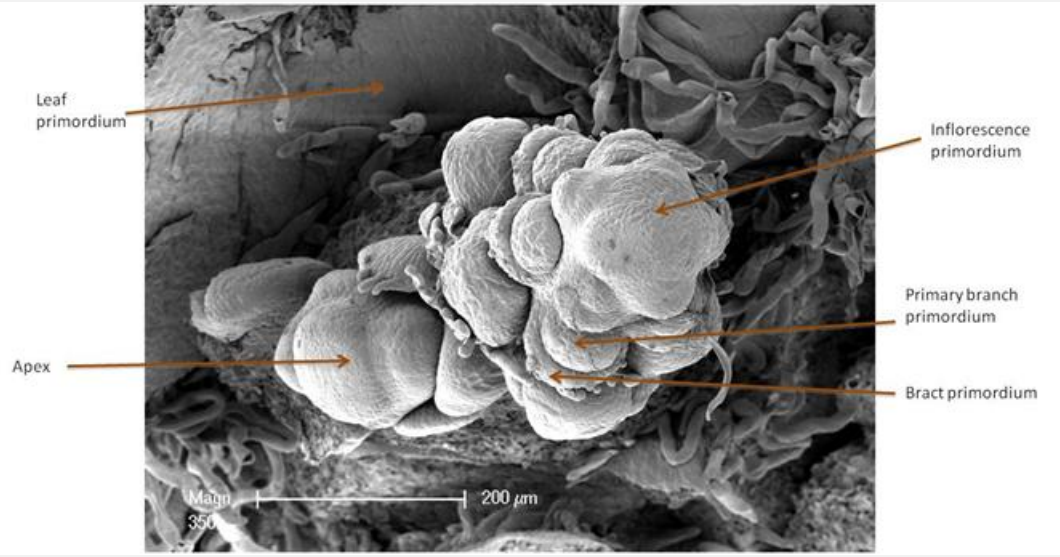


Plate 27 Sauvignon Blanc developing nodes illustrating inflorescence primordia Season 2 veraison at King Valley vineyard*

* Season 2011/2012 EL Sate 35 stage 7 ontogeny, node 2, 100% fruitfulness

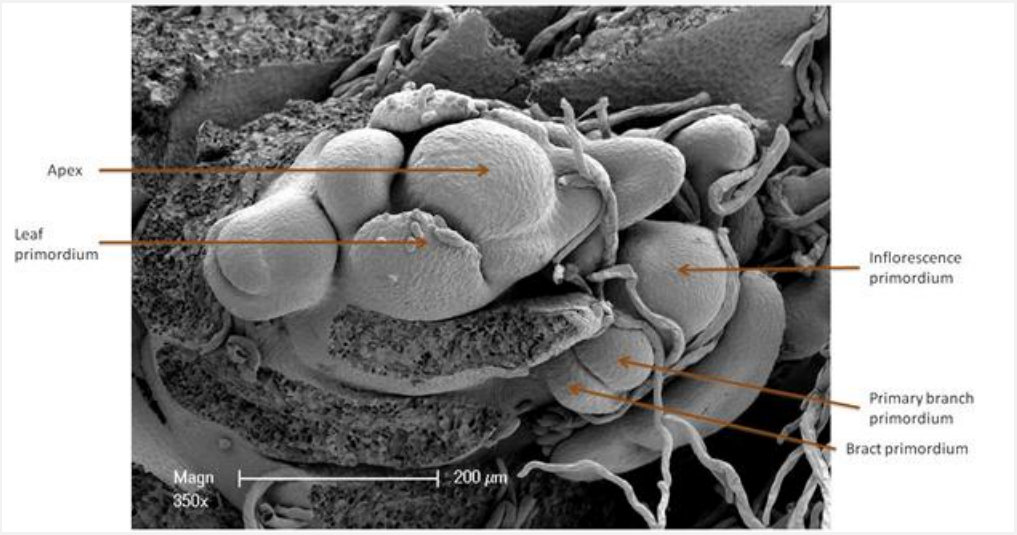


Plate 28 Sultana developing nodes illustrating limited inflorescence primordia Season 2 veraison at Waite vineyard*

* **Season 2011/2012 EL Sate 35 stage 7 ontogeny, node 2 20% fruitfulness**

7.6 Gene Expression

Statistical analysis of RT-PCR gene expression

Genes were assessed by RT-qPCR and significant gene expression differences between buds with and without inflorescence primordia were evaluated for the different sites. Table 19 shows p-values for each gene compared in this way. RNA extracted from buds showed low gene expression levels for many genes. To accurately assess gene expression at low levels three reference genes were used in the assay including a new reference gene developed specifically for this study. Ubiquitin, Actin & E7 were reference genes used to normalise gene expression data and showed minor gene expression variability across bud samples. E7 is a newly identified reference gene and shows homology to the eukaryotic initiation factor 4 A-11. More than one reference gene for normalising RT-qPCR data is desirable as it increases the robustness and precision of the assay. Table 19 lists the genes investigated for gene expression differences between buds with and without inflorescence primordia. The Ct value is the PCR cycle number at which the gene expression level is determined hence the lower the Ct value, the higher the gene expression level. The no transcriptase control (NTC) had no reverse transcriptase (SuperscriptIII) added to the first strand synthesis reaction. It is an indicator of residual DNA contamination and also gives a cut off value for the maximum Ct for accurate gene expression measurement. The NTC shows a median Ct of 34.2. The high Ct value indicates no residual genomic DNA contamination. Overall, bud samples showed low gene expression levels for the putative floral genes when compared to the reference genes. Genes with a median Ct of >34.2 were not analysed for gene significant differences. The p-values for the individual sites were calculated using a two-way unpaired t-test whereas the p-values looking for significant differences at more than one vineyard site were calculated from a two way Mann-Whitney test. Gene expression changes of $p<0.05$ were considered significant (Figure 25). When observed gene expression changes showed a similar response between two or more sites, the p-values were calculated as one-way tests.

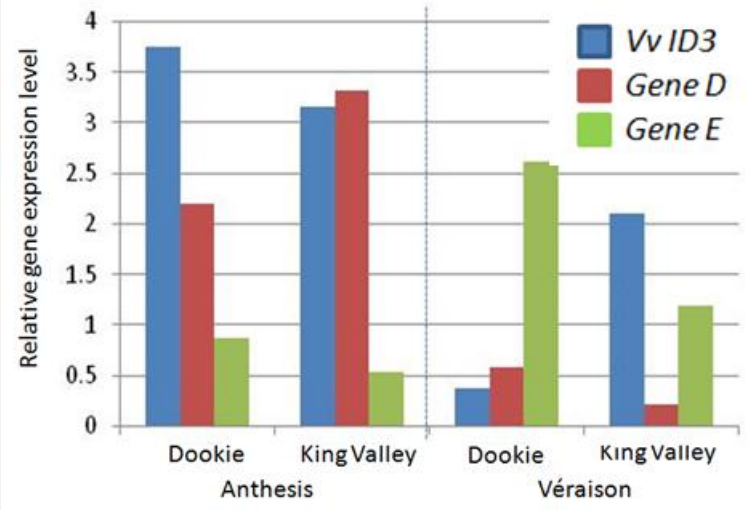


Figure 25 Season 2 relative gene expression level changes associated with Sauvignon Blanc inflorescence primordia development at Dookie and King Valley.

Gene VvID 3 (blue), *VvTFL1* (red), *VvLFY* (green), Anthesis 0% inflorescence primordia, veraison 100% inflorescence primordia.

Gene expression and inflorescence primordia development

Season 1 Sauvignon Blanc bud relative gene expression levels were compared between Dookie and King Valley vineyard. At anthesis about 0-40% inflorescence primordia are visible and relative gene expression in *VvTFL1* is similar at both vineyards. The gene expression trend for genes *VvLFY* and *VvFT* are similar at both vineyards with King Valley having a higher quantity. Mean maximum temperatures at both vineyards were similar and above 20 °C, while King Valley vinearyd mean minimal temperatures were slightly cooler than at the Dookie vineyard. higher humidity was observed at the King Valley vineyard. The differences in climatic conditions did not impact on gene expression at anthesis. At veraison, where 100% inflorescence primordia are visible, gene expression patterns are similar in *VvTFL1* and *VvFT* but considerably different in *VvLFY* relative expression (Figure 26). The mean minimal temperature was considerably higher at King Valley than at Dookie and may be promoting the VvLFY gene. At anthesis in season 1, relative gene expression of different node positions along the shoot was described (Figure 27). Genes are equally expressed in *VvTFL1*, *VvLFY* and *VvFT*. Node 2 are typically not as fruitful and relatively low expressions of *VvTFL1* reflect this. Interestingly high relative expression levels of *VvFT* were found with node 10 (Figure 27).

In season 2 Sauvignon Blanc Gene identification 3 (termed here as *VvID3*) showed a gene expression pattern where the level of expression was reduced in bud tissue with 100% inflorescence primordia whereas the *VvLFY* gene showed an increase in gene expression in buds with 100% inflorescence primordia (Figure 25). Some interesting trends were observed in these genes over time and vineyard. Differences in anthesis and veraison bud gene expression of gene VvID3 and *VvLFY* were found between Dookie and King Valley vineyard. At anthesis gene *VvID3* gene expression was higher than at veraison in buds at both vineyards. *VvLFY* gene expression was lower at anthesis and increased at veraison in buds at both vineyards. It is interesting to note that the expression levels in *VvID3* are significantly different at Dookie than King Valley vineyard at veraison (Figure 25).

VvID3 showed homology to an ABC transporter (Table 14 Chapter 5) which belongs to a superfamily of both membrane bound and soluble proteins with a broad range of putative functions primarily involved in transport. This gene superfamily is a new area of investigation (Federica 2000; Verrier 2008) and their function has yet to be elucidated. There is evidence that *VvTFL1* is associated with the maintenance of meristem indetermination (Carmona et al. 2007) which could explain why its expression decreased in buds with 100% inflorescence primordia. The detected increase in *VvLFY* at 100% inflorescence primordia is consistent with findings that *VvLFY* was shown to be expressed in lateral meristems that give rise to inflorescences. However *VvLFY* expression is also detected in the lateral meristems of tendrils and also in leaf primordia that suggests a possible general role in cell proliferation (Carmona et al. 2002).

Gene expression differences attributed to genotypic differences in response inflorescence primordia development

Figure 28 shows genes with significant difference in gene expression between Sauvignon Blanc buds with and without inflorescence primordia. The gene expression changes for Sultana were investigated at the same bud positions as a comparison with a less fruitful genotype.

VvID3 expression was seen to be lower in the fruitful bud consistent with findings for Sauvignon Blanc inflorescence primordia grown in two different environments. Similarly Gene *E*, was lower in buds with inflorescence primordia and is a putative sugar transporter that could possibly be involved in a floral development pathway (see discussion in Section 6.4). Gene *D* expression, however, is lower in Sauvignon Blanc fruitful buds but increases in Sultana non-fruitful buds. This gene is implicated in seed dormancy and pollen tube development but its function has not been fully investigated.

Isolating these genes of interest is an initial step in gaining a better understanding of the processes involved in inflorescence primordia formation within the latent bud which in turn can provide information to ultimately improve bud fruitfulness in different climatic conditions and reduce crop yield variability. This study has identified genes with a putative role in floral induction and/or the early stages of inflorescence development. Further studies to confirm gene function is possible using functional genomics approaches. In summary the presence and absence of visible inflorescence primoridia and gene expression were demonstrated.

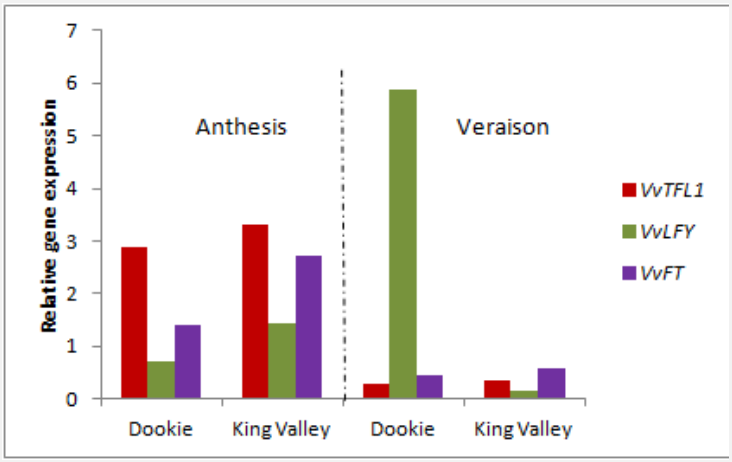


Figure 26 Season 1 Sauvignon Blanc bud relative gene expression level comparison of King Valley and Dookie vineyards at 0-40% (anthesis) and 100% inflorescence primordia (veraison).

Gene identification *VvTFL1* (red), *VvLFY* (green) and *VvFT* (purple).

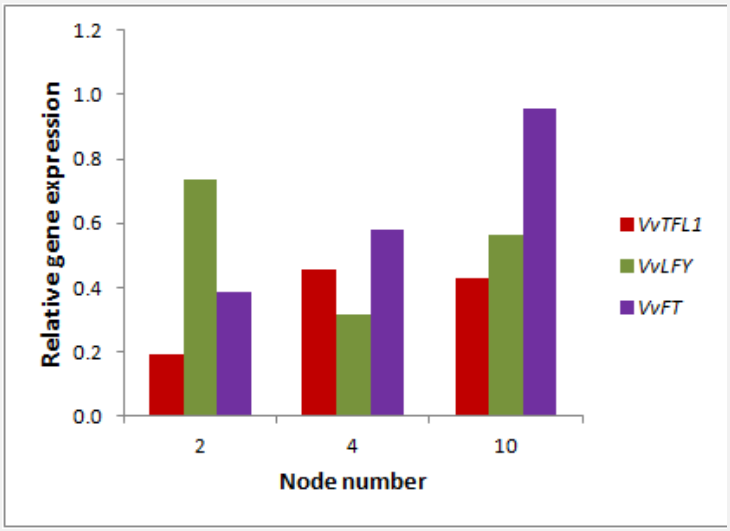


Figure 27 Season 1 Relative gene expression level bud position comparisons at King Valley vineyard at anthesis 40% (bud 4) and 100% (2 and 10) inflorescence primordia Sauvignon Blanc.

Gene identification *VvTFL1* (red), *VvLFY* (green) and *VvFT* (purple).

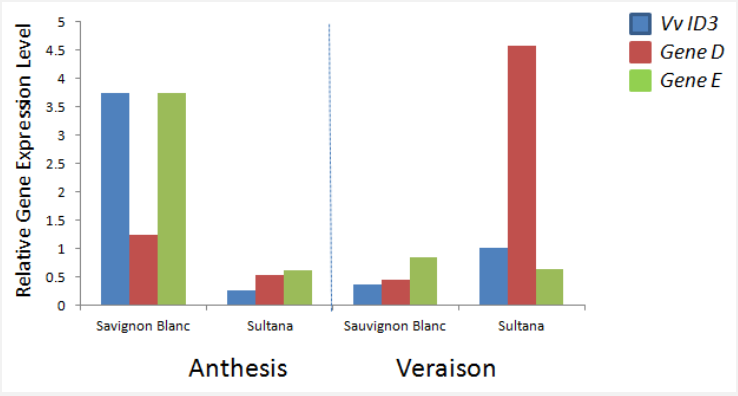


Figure 28 Season 2 Relative gene expression level comparisons between Sauvignon Blanc (SB) and Sultana (SU) with fruitfulness associations.

For genes which are associated with differences in bud fruitfulness (presence of inflorescence primordia). *VvID3* (blue), *VvTFL1* (red), *VvLFY* (green), Anthesis 0% inflorescence primordia, veraison 100% inflorescence primordia for SB and 20% for SU.

Table 19 Statistical evaluation of gene expression in latent buds at node 2 between time point 1 (0% inflorescence primordia) and time point 2 (20% or 100% inflorescence primordia)

Gene ID	Median Ct	p-value Dookie	p-value King Valley	p-value Avelstoke	p-value Dookie & King Valley	p-value Dookie, King Valley & Avelstoke	p-value Dookie & Avelstoke
UBIQUITIN	18.4	N/A	N/A	N/A	N/A	N/A	N/A
E7	19.6	N/A	N/A	N/A	N/A	N/A	N/A
ACTIN	19.6	N/A	N/A	N/A	N/A	N/A	N/A
3	23.2	0.008	0.04	0.2	0.014	0.2	0.057
13	25.2	0.9	0.6	0.2	0.7	0.937	0.7
VVTFL	25.8	0.01	0.009	0.1	0.01	0.001	0.3
A3	25.9	0.5	0.2	0.3	1	0.2	0.9
F	26.1	0.3	0.8	0.8	0.7	0.6	0.7
1	26.2	0.5	0.9	0.4	0.7	0.2	0.7
5	26.7	0.4	0.2	0.8	0.1	0.4	0.1
8	27.3	0.7	0.3	0.6	0.3	0.6	0.9
VVFL	27.7	0.02	0.02	0.02	0.014	0.001	0.2
4	27.7	0.7	0.6	0.2	0.9	0.31	0.3
21	28.1	0.8	0.9	0.6	0.9	1	0.9
VVFT	28.4	0.5	0.0004	0.03	0.06	0.004	0.6
C	28.7	0.7	0.8	0.4	0.9	0.7	0.7
J	30.7	0.8	0.4	0.6	1	0.8	0.5
11	31.6	0.7	0.5	0.4	0.3	0.2	0.7
D	31.6	0.002	0.079	0.087	0.01	0.35	0.2
E	32.4	0.091	0.003	0.5	0.01	0.02	0.2
G	32.8	0.4	0.7	0.4	0.3	0.9	0.7
*NTC	34.2	N/A	N/A	N/A	N/A	N/A	N/A
B	34.9	N/A	N/A	N/A	N/A	N/A	N/A
I	35.1	N/A	N/A	N/A	N/A	N/A	N/A
12	35.2	N/A	N/A	N/A	N/A	N/A	N/A

8 Summary and Conclusions

8.1 Scientific Outcomes

Some key findings in the understanding of physiology of grapevine fruitfulness were identified in these studies. Cool temperatures (day/night 17/15 °C) under a high light environment allowed the highest yield potential promoting large flower numbers in propagated Shiraz. A literature review conducted by Buttrose (1974) concluded that temperature impacts more on inflorescence primorida formation than the impact of a low light environment. Findings presented in this report conclude that conducting experimentation in controlled cabinets under high light environments supports this conclusion. For optimal fruitfulness temperatures need to exceed 20 °C (Buttrose 1970; Kasimatis et al 1975; Zelleke & Kliewer 1979; Moncur et al. 1989, Dunn & Martin 2000; Clingeleffer 2005) and the cool treatment used in these experiments was below this threshold. The optimal temperature conditions for flowering of different varieties however present a significant range according to different studies, from cooler temperatures 16/17 °C to warm/hot temperatures of 20-30 °C (Winlker et al 1974; Kozma 2003; May 2004). The morphology of propagated Shiraz inflorescence structure in the cool treatment was significantly different from the other treatments, with a shorter total inflorescence length and outer branch lengths, indicating an altering in carbon partitioning or an effect on processes determining cell number and/or size. Higher temperatures promote absolute growth rate which result in longer inflorescences while suppressing flower production. These effects are promoted by the day and not mitigated by cooler night time temperatures. When investigating genotype - environmental interaction on fruitfulness and yield, we found that the Pinot Meunier genotype L1 in the cooler temperature had reduced growth and the potential for carbohydrate production was altered by reducing shoot growth rate, relative shoot growth rate in the first 42 days, and a reduction in the number of nodes separating along the shoot. Flower number was not significantly impacted by the cool treatment but less inflorescences were produced resulting in a significantly reduced number of flowers per shoot. Pouget (1981) in controlled environmental conditions had demonstrated a reduction in inflorescences per node with cooler treatments (12 °C) and a reduction in flower number, where the cool temperature was 5 °C cooler than temperatures used here. The cool treatment resulted in a reduced optimal yield potential. A reduction in flower number per inflorescence in cooler field environments has been reported on several occasions (Dunn & Martin 2000; Clingeleffer 2005). Differences in flower number response between the propagated plants and the genotypic interaction, in the effect of temperature on flower number were interesting. This may be explained by varietal differences in flowering gene expression or that the L1 genotype had relatively less resources than the cooler treatment plants in the propagated experiment. The correlation of low yields caused by slower shoot development, reduction in flowers per inflorescences and reduced inflorescences per shoot have been observed in vineyard environments (Trought 2005). Pinot Meunier genotype L1 exposed to hotter temperatures during the second season, advanced both shoot development and shoot growth rates. The number of inflorescence structures in the second season was promoted while lower flower number was suppressed in the cool treatment. The cooler treatment in the second season had a daytime

temperature over 20 °C but a night time temperature of 15 °C. The results from the second season genotypic L1 interactions were similar to the propagated Shiraz results and may be explained by alterations of carbohydrate partitioning or by changes to gene expression patterns in the developing inflorescence primordia. These findings support the observation that warmer bud break temperatures can compensate for exposure to cooler temperatures during initiation and differentiation.

Genes play a critical role in the control of flowering and understanding the spatial and temporal expression patterns may elucidate many of the varied field-cultivar-management interactions. Advances in the understanding of grapevine genetic influence in flowering have been based on comparison with genes found in Arabidopsis (a model plant). While some similarities in the flowering process have been found there are many differences between the two species (Boss et al 2003; Carmona et al 2003). There is still great requirement for studies on the biological function of genes in the grapevine. One mechanism available for understanding winegrape gene function is to use the genetic diversity in different genotypes and this was the approach that we selected in studies reported here. The important component of yield determination is bunch number per vine (Keller et al 2004; Clingeleffer 2006) and the potential for this stage is determined genetically during initiation in the bud. To date little has been understood about the grapevine flower regulatory pathways and how environment and management effects may influence these pathways. Three genes of particular interest in the grapevine have been identified. *LEAFY* (in the grapevine called *VvLFY or VFL*) is a flower meristem identity gene (Mendal et al 1992; Carmona et al 2002; Joly et al 2004; Boss et al 2006). The gene *FT or Flower time* has been identified in characterised in grapevines *VvFT* (Joly et al 2004; Boss et al 2006; Sreekantan and Thomas 2006) and is associated with floral induction in latent buds and with the development of inflorescences, flowers and fruits (Sreekantan and Thomas 2006; Carmona et al 2007). The gene *TFL1* or terminal flowering locus 1 is believed to be a member of the same sub family as *FT* gene and has been shown to be expressed in flowering in grapevines (Boss et al 2006; Carmona et al 2007). The gene *TFL1* is described in grapevine as *VvTFL1*. Recently Pin & Nilsson (2012) suggested that during the evolution of this gene it undertook multifaceted roles across several plant systems. Under controlled temperature environments, the genes *VvFT*, *VvTFL1* and *VvLFY* were found to be expressed in floral development in the Pinot Meunier L1 genotype. It was found that *VvTFL1* was highly expressed in the leaves opposite inflorescences, apex, inflorescences and the tendrils. The expression of *VvTFL1* in the leaves opposite inflorescences or tendrils has not been previously reported to the best of our knowledge where expression of *VvTFL1* in the inflorescences has been previously found (Carmona et al 2007; Yao et al 2012). The relatively high gene expression of *VvFT* occurred in the tendrils, inflorescences and apex. *VvFT* has been shown previously to be highly expressed in inflorescences, flowers, tendrils and weakly in the apex (Sreekantan and Thomas 2006; Carmon et al 2006). The expression of *VvLFY* was found to be relatively low in all organs tested in spatial gene studies reported here. *VvLFY* has been found to be highly expressed in early stages of tendril development or as bud development progresses and organs form, expression drops (Maizel et al 2005; Yao et al 2012), as we observed in results reported here. In other woody plants, the high expression of the *LFY* gene is believed to be associated with time of flower meristem formation (Walton et al 2001). Pinot Meunier picovine genotype showed significantly higher levels of expression in the LEAFY gene *VvLFY* and interestingly in the flowering locus gene *VvFT*. The Pinot Meunier picovine genotype and the Pinot Meunier wild

type (L2) were selected to identify new genes involved in floral development. A new gene, called *VvID3* was identifying as occurring in the picovine at very high relative expression levels. New genes called *A3*, *D*, *J* and *I* showed homology to genes involved in floral development. The results suggest these newly identified genes may have a role in floral development and/or the very early stages of flower induction in grapevine. Another significant finding was that the gene *A3* was found to be expressed in the grapevine inflorescence and this gene showed homology to basic blue protein in Arabidopsis involved in pollen tube development (Dong et al 2005). Gene *1* was identified in grapevine flowering structures for the first time. This gene is associated with copper transport and could be involved in a similar floral gene expression pathway to *A3* or controlled by the same transcription regulator. Our findings suggest that further investigation into the genes *A3*, *1* and *E*, are required to determine if they represent significant components of a floral induction pathway. The identification of these new genes and further studies would allow great insights into grapevine flowering.

To assess the impacts of temperature on bud fruitfulness, two controlled environments of day/night 27°C/30°C (hot) and day/night 17°C/15°C (cool) were compared. Expression of the flowering genes *VvLFY* and *VvTFL1* in the buds (N+2) of Pinot Meunier L1 genotype under controlled environmental conditions were similar but the relative expression of *VvFT* was very low. The relative expression of the flowering gene *VvTFL1* at node position 2 was significantly higher in the hot environment. In the field environment in Australia, this gene expression activity coincides with the period of veraison. Heightened differentiation of inflorescence primordia occurs in the latent bud during this time. *VvTFL1* is thought to be implicated in the suppression of apical dominance. Recently it has been suggested that Cytokinin may induce the expression of *VvTFL1A* and thus control fruitfulness (Crane et al 2012). Cooler temperatures resulted in reduced bud fruitfulness. Cooler temperature also reduced the expression of the genes *VvLFY* and *VvTFL1*. These finding link with the field studies findings, as conditions that reduce fruitfulness (e.g. vine development stage) are also associated with relatively lower expression of the genes *VvLFY* and *VvTFL1*. In summary the expression levels of three genes *VvTFL1*, *VvLFY* and *VvFT* known to be associated with flowering in grapevine, varied with the stage of ontogeny of reproductive development, node position of the latent bud in field grown vines from hot and cool locations.

There was some evidence that *VvLFY* may be up regulated with warm temperatures at veraison at the King Valley vineyard. We found a correlation (in both field and controlled cabinet experimentation) that high fruitfulness was associated with relatively high expression of gene *VvTFL1*. Whereas the genes *VvLFY* and *VvFT* were found to be expressed at both high and low levels in the field compared to controlled environmental conditions. This observation may be a variety specific trait as in the field we collected buds from Sauvignon Blanc whereas from the chambers we collected buds from Pinot Meunier L1 genotype. We identified high expression of the gene *VvID3* in Sauvignon Blanc buds from the field environment as well as from the picovine genotype, where flowering is prolific. This supports the role of this gene being involved in a common pathway for floral induction. Differences in anthesis and veraison bud gene expression of gene *VvID3* and VvLFY were found between Dookie and King Valley vineyard.

Sultana, a very low fruitful variety (Buttrose 1970), was used as a comparison with Sauvignon Blanc buds to aid gene function investigations. The relative expression of the gene *VvID3* and gene *E* was higher in Sauvignon Blanc than in Sultana. Gene *D* relative expression was higher in Sultana and to date the function of this gene in other species has been implicated in seed dormancy and pollen tube development but the results reported here suggest that its function has not been fully described. We recommend that using these genes as a base point, targeted research can assist in developing an understanding in both their function in plant flowering and specifically their function in grapevine flowering.

In conclusion we have found that temperature has a significant and complicated association with optimal fruitfulness in grapevine in both field and controlled environmental conditions. The advantage of using the high light growth cabinets described in this study is that environmental conditions can be precisely controlled with only the experimental variable being investigated (in this case temperature) being varied to investigate environment- gene interactions. This is not the case with field studies and this was proven in this study when a hail event resulted in a forced change in site selection and the temperature differences observed between the cool (King Valley vineyard) and warm (Dookie vineyard) sites was not as great as expected when the environmental variables were measured. The Pinot Meunier genotypes; where L1 is a dwarf continuous flowering microvine system and the picovine has more prolific flowering was used in this study. The advantages of using these genotypes for flowering studies was proven in this study for experiments involving both short-term and long-term growth cabinet studies over several “seasons” with more experiments and “seasons” being possible in a single year than possible if the plants were grown in a field. The use of a continuous flowering Pinot Meunier L1 and picovine genotypes where tendrils are replaced with inflorescences proved valuable for gene expression studies to identify new genes associated with grapevine flowering. The identification of a large number of new genes involved in grapevine flowering reported here demonstrates the advantages of combining new high throughput molecular techniques like mRNA-seq with novel genotypes such as the picovine to discover new genes and improve our understanding of floral induction pathways. Evidence is now emerging that components of the flowering process and flowering control are very complex (Pose et al 2012) as some genes have been shown to promote one activity in the flowering pathway while suppressing others and further research into grapevine fruitfulness is required (Section 8.1) if we are to better understand this complex process to improve and predict yield especially as the flowering process in grapevine occurs over two seasons.

8.1 Further Research

Further fundamental research is required into understanding both the impact of carbohydrate balance and gene expression pathways when trying to understand early impacts of the environment on yield and yield components. To understand these processes, we recommend a genotype – controlled environment interaction. This system elucidates effects and allows greater fundamental understanding than field studies. Once fundamental understanding has been achieved, we recommend testing in field conditions, to confirm these findings.

Evidence is now emerging that components of the flowering process and flowering control are very complex (Pose et al 2012) as some genes have been shown to promote one activity in the flowering pathway while suppressing others. Further research into grapevine fruitfulness is required if we are to better understand this complex process to improve and predict yield especially as the flowering process in grapevine occurs over two seasons. These investigations are critical for develop strategies when dealing with variable climatic situations.

8.2 Potential for Adoption by Viticulture Industry

8.2.1 Industry Insights

In findings reported here gene expression studies illustrated in one case a simple specific correlation, with high fruitfulness associated with high expression of the gene *VvTFL1*. Other genes showed more complicated responses to fruitfulness and environmental interactions. There is growing evidence in the literature that gene expression and flowering control is a very complicated process. The complication in this process and the climatic and management interactions influencing this gene expression may help us to understand yield variations and ultimately one day this knowledge may help us to predict yield and obtain consistent yields. Temperature has been shown as a significant driver of yield potential. The research here has demonstrated how key flowering genes may respond to these temperature changes and it is suggested that temperature during the initiation of inflorescence primordia in the bud has a greater effect than management during this season 1 stage. However, management has a large effect on yield per hectare in season 2 by determining how many buds to leave for the following season by pruning to a specific bud number. Not knowing the yield potential of buds (inflorescence primordia presence due to gene – temperature interactions) prior to pruning is likely a major cause of seasonal yield variation. There has been much debate about how carbohydrate partitioning and vine balance play a role in yield variation. Outputs from this research have demonstrated that gene expression also plays a critical role in this process.

It was proven in this study that high light growth cabinets represent a suitable system for investigating environment effects on grapevine physiological processes and gene expression. These cabinets can replace field investigations as they provide a more precise and controlled way of investigating the effect of a single environmental variable such as temperate by keeping other variables such as humidity, light quality and day length constant.

8.2.2 Adoption Scenarios

This project has produced two industry adoption scenarios:

1. Knowledge that results in better ways of measuring potential yield and the effects of environmental. The project has demonstrated that gene expression studies can be conducted in field situations, which allow a better understanding of gene interactions in vineyards, perhaps understanding these traits will assist in managing future extreme climates.

2. Knowledge of gene–environment interactions controlling fruitfulness is expected to lead to improved planting material with more consistent yields due to the production of new genotypes with lower sensitivity to environmental conditions. Adoption is likely to occur over the long-term and as part of a larger CSIRO grape improvement program.

9 References Cited

Abdel-Ghany SE & Pilon M (2008) MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in Arabidopsis. Journal of Biological Chemistry 283:15932-15945.

Antony G, Zhou J, Huang S, Li T, Liu B, White F, Yang B, Yuan M, Chu Z, Li X, Xu C & Wang S (2010) Plant Cell 22:3164-3176.

Alleweldt G (1963) Einfluss von Klimafactoren auf die Zahl der Infloreszenzen bei Reben. Wein-Wiss., 18:61-70.

Allen R.G, Pereira LS, Raes D & Smith M (1998) Crop evapotranspiration – Guidelines for computing crop water requirements. FAO Irrigation and Drainage Paper 56. FAO, Rome.

Baldwin JG (1964) The relation between weather and fruitfulness of the Sultana vine. Aust. J. agric. Res., 15:920-8.

Benismail MC & Ejnaoui A (2004) Effects of supplement heating on grapevine (Vitis vinifera L.) 'Cardinal' cultivated under mild climate in Morocco. Proceedings Of The 1st International Symposium On Grapevine Growing, Commerce And Research Ed DeSequeira, OA & Sequeira, JC. Acta Horticulturae, 652:273-279.

Bennett J, Jarvis P, Creasy GL & Trought MCT (2005) Influence of defoliation on overwintering carbohydrate reserves, return bloom, and yield of mature Chardonnay grapevines. American Journal of Enology and Viticulture 56:386–393.

Boss PK & Thomas MR (2002) Association of dwarfism and floral induction with a grape 'green revolution' mutation. Nature 416:847- 850

Boss PK, Sreekantan L & Thomas MR (2006) A grapevine TFL1 homologue can delay flowering and alter floral development when overexpressed in heterologous species. Functional Plant Biology 33(1):31-41.

Buttrose MS (1969a) Fruitfulness in grapevines: effects of light intensity and temperature. Bot. Gaz., 130: 166-73.

Buttrose MS (1969b) Fruitfulness in grapevines: effects of changes in temperature and light regimes. Bot. Gaz., 130:173-9.

Buttrose MS (1969c) Vegetative growth of grapevine varieties under controlled temperature and light intensity. Vitis, 8: 280-5.

Buttrose MS (1970a) Fruitfulness in grapevines: development of leaf in buds in relation to bud fruitfulness. Bot. Gaz., 131:183.

Buttrose MS (1970b) Fruitfulness in grapevines: the response of different cultivars to light, temperature and day length. Vitis, 9: 121-5.

Buttrose MS (1974) Climatic factors and fruitfulness in grapevines. Horticultural Abstracts 44:319-326.

Chaib J, Torregrosa L, Mackenzie D, Corena P, Bouquet A & Thomas MR (2010) The grape microvine - a model system for rapid forward and reverse genetics of grapevines. Plant Journal, 62(6):1083-1092.

Chatelet P, Laucou V, Fernandez L, Sreekantan L, Lacombe T, Martinez-Zapater JM, Thomas MR & Torregrosa L (2007) Characterization of Vitis vinifera L. somatic variants exhibiting abnormal flower development patterns. J. Exp. Bot. 58: 4107-4118

Clingeleffer, P. (2001) Crop development, crop estimation and crop control to secure quality and production of major wine grape varieties: A national approach. Final Report, Project CSH 96/1, Grape and Wine Research and Development Corporation, Adelaide.

Campbell JA & Strother S (1996) Seasonal variation in pH, carbohydrate and nitrogen of xylem exudate of Vitis vinifera. Australian Journal of Plant Physiology 23:115–118.

Carmona MJ, Cubas P & Martinez-Zapater JM (2002) VFL, the grapevine FLORICAULA/LEAFY ortholog, is expressed in meristematic regions independently of their fate. Plant Physiology 130:68-77.

Carmona MJ, Calonje M & Martinez-Zapater JM (2007a) The FT/TFL1 gene family in grapevine. Plant Molecular Biology 63:637–650.

Carmona MJ, Cubas P, Calonje M & Martinez-Zapater JM (2007b) Flowering transition in grapevine (Vitis vinifera L.). Canadian Journal of Botany 85:701–711.

Carmona MJ, Chaı J, Martı´nez-Zapater JM and Thomas MR (2008) A molecular genetic perspective of reproductive development in grapevine. Journal of Experimental Botany 59(10):2579–2596.

Cock PJA, Fields CJ, Goto N, Heuer ML, Rice (2010) The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Research, 38:1767-1771.

Cooley NM, Clingeleffer PR and Walker R (in press). Effects of water stress on grapevine carbohydrate reserves. 'Vine Carbohydrate Dynamics and Source-Sink Relationships'. GWRDC workshop proceedings.

Coombe BG (1995) Growth Stages of the Grapevine: Adoption of a system for identifying grapevine growth stages. Australian Journal of Grape and Wine Research 1(2):104–110.

Crane O, Halaly T, Pang X, Lavee S, Perl A, Vankova R & Or E (2012) Cytokinin-induced VvTFL1A expression may be involved in the control of grapevine fruitfulness. Planta 235(1):181-192.

Davies C, Wolf T & Robinson SP (1999) Three putative sucrose transporters are differentially expressed in grapevine tissues. Plant Science 147, 93–100.

Dunn GM & Martin SR (2000) Do temperature conditions at budburst affect flower number in Vitis vinifera L. cv. Cabernet Sauvignon? Australian Journal of Grape and Wine Research 6:116-124.

Dry P & Coombe BG (2004) Grapevine growth stages – The modified E-L system. Viticulture 1 – Resources. Winetitles 2nd edition 2004:153.

Dimitrieva KV 1970. The effect of meteorol09ical conditions on fruitfulness in vines. Met. Klim Gidrol., mezved. nauc. Sborn. No.5, pp.150-3 (Hort. Abstr., 40, No.8112).

Duche^ne E, Jaegli N, Sabler R & Gaudille`re . (2003a). Effects of ripening conditions on the following season's growth and yield components for Pinot noir and Gewurztraminer grapevines (*Vitis vinifera* L.) in a controlled environment. Journal International des Sciences de la Vigne et du Vin 37:39–49.

Duche^ne E, Monamy C, Langellier F, Jaegli N, Sabler R, Meluc D & Panigai L. (2003b) Effects of the ripening conditions in the vineyard on growth and yield components in the following season. Journal International des Sciences de la Vigne et du Vin 37:103–116.

Dong J, Kim ST & Lord EM (2005) Plantacyanin plays a role in reproduction in Arabidopsis. Plant Physiol. 138:778-789.

Frederica L. Theodoulou (2000) Plant ABC proteins. Biochimica et Biophysica Acta (BBA) - Biomembranes 1465(1–2):9–103.

Franks T, Botta R & Thomas MR. (2002) Chimerism in grapevines:Implications for cultivar identity, ancestry and genetic improvement. Theoretical and Applied Genetics 104:192–199.

Hellemans J, Mortier G, De Paepe A, Speleman F & Vandesompele J (2007) qBse relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8:R19.

Huglin P (1958) Recherches sur les bourg eons de la vigne: Initiation florale et developpement vegetatif. Annls Amel. Pl. 8:113-272.

Huglin P (1960) Causes determinant les alterations de la floraison de la vigne. Ann. Amelior. Plant. 10:351-8.

Glad C, Regnard JL, Querou Y, Brun &, Morot-Gaudry JF (1992a) Flux and chemical composition of xylem exsudates from Chardonnay grapevines: temporal evolution and effect of recut. American Journal of Enology and Viticulture 43:275–282.

Glad C, Regnard JL, Querou Y, Brun O & Morot-Gaudry JF (1992b) Phloem sap exudates as a criterion for sink strength appreciation in Vitis vinifera cv. Pinot noir grapevines. Vitis 31:131–138.

Isoda R (1964) The effects of N, P, K and Ca fertilizer applications on the growth and yield of grapevines planted in granitic soil and diluvial soil derived from granite. Journal of Japanese Society Horticulture Science 33:221-6.

Kasimatis AN, Lider LA & Kliewer WM (1975) Influence of trellising on growth and yield of 'Thompson Seedless' vines. Journal of Enology and Viticulture 26:125–129.

Keller M, Mills LJ, Wample RL & Spayd SE (2004) Crop load management in concord grapes using different pruning techniques. American Journal of Enology and Viticulture 55:35–50.

Kozma HA (2003) Exploration of flower types in grapes (Vitis vinifera). In: Kozma P, Nyéki J, Soltész M, Szabó Z, editors. Floral Biology, Pollination and Fertilisation in Temperate Zone Fruit Species and Grape. Budapest: Akamémiai kaidó. pp. 75-225.

Krueger F, Andrews SR & Osborne CS (2011) Large Scale Loss of Data in Low-Diversity Illumina Sequencing Libraries Can Be Recovered by Deferred Cluster Calling. Plos One, 6:e16607.

Langmead B, Trapnell C, Pop M & Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biology, 10:R25.

Lebon G, Duchêne E, Brun O, Magné C & Clément C (2004) Flower abscission and inflorescence carbohydrates in sensitive and non-sensitive cultivars of grapevine. Sexual Plant Reproduction 17:71–79.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G & Durbin R (2009) Proc GPD: The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078-2079.

Maunoury N & Vaucheret H (2011) AGO1 and AGO2 act redundantly in miR408-mediated Plantacyanin regulation. PLoS ONE 6:E28729-E28729.

May P (1964) Ober die Knospen - und Infloreszenzentwicklung der Rebe. Wein-Wiss., 19:457-85.

May P (2004) Flowering and fruitset in grapevines. Lythrum Press, South Australia.

May P & Antcliff AJ (1963). The effect of shading on fruitfulness and yield in the Sultana. Journal of horticultural Science 38:85-94.

Maizel A, Busch MA, Tanahashi T, Perkovic J, Kato M, Hasebe M, Weigel D (2005) The floral regulator LEAFY evolves by substitutions in the DNA binding domain. Science 308:260–263.

Mandel MA, Gustafsonbrown C, Savidge B & Yanofsky MF (1992) Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature 360:273–277.

Moncur MW, Rattigan K, Mackenzie DH & Mc Intyre GM (1989) Base Temperatures for Budbreak and Leaf Appearance of Grapevines. Am. J. Enol. Vitic. 40:21-26.

Mullins MG (1966) Test-plants for investigations of the physiology of fruiting in *Vitis vinifera* L. Nature, Lond. 209, 419—20.

Mullins MG (1967a) Morphogenetic effects of roots and of some synthetic cytokinins in *Vitis vinifera* L. Journal of Experimental Botany 18:206-14.

Mullins MG (1967b) Regulation of Inflorescence Growth in Cuttings of the Grape Vine (*Vitis vinifera* L.). Journal of Experimental Botany 19 (60):S32-543.

Mullins MG & Rajasekaran K (1981) Fruiting cuttings: revised method for producing test-plants of grapevine cultivars. American Journal of Enology and Viticulture 32:35–40.

Okamoto G, Konishi Y & Shimamura K (1977) The retardation of lateral shoot secondary growth and stimulation of inflorescence primordia development by spraying with CCC after harvest in Neo

Muscat grapevines in a heated plastic greenhouse. Scientific Reports of the Faculty of Agriculture, Okayama University, 21–26.

Petrie PR & Clingeleffer PR (2005) Effects of temperature and light (before and after budburst) on inflorescence morphology and flower number of Chardonnay grapevines (*Vitis vinifera* L.). Australian Journal of Grape and Wine Research 11(1)59-65.

Percival DC, Fisher KH & Sullivan JA. (1994) Use of Fruit Zone Leaf Removal With *Vitis-Vinifera* L Cv Riesling Grapevines .1. Effects On Canopy Structure, Microclimate, Bud Survival, Shoot Density, and Vine Vigor. American Journal of Enology and Viticulture, 45(2):123-132.

Petrie PR & Clingeleffer PR (2005) Effects of temperature and light (before and after budburst) on inflorescence morphology and flower number of Chardonnay grapevines (Vitis vinifera L.). Australian Journal of Grape and Wine Research 11:59–65.

Petrie PR & Clingeleffer PR (2006) Crop thinning (hand versus mechanical), grape maturity and anthocyanin concentration: outcomes from irrigated Cabernet Sauvignon (*Vitis vinifera* L.) in a warm climate. Australian Journal of Grape and Wine Research 12(1)21-29.

Pin P. A. & Nilsson O (2012) The multifaceted roles of FLOWERING LOCUS T in plant development. Plant, Cell and Environment (2012) 35:1742–1755.

Pose D, Yant L & Schmid M (2012) The end of innocence: flowering networks explode in complexity Current Opinion In Plant Biology 15 (1):45-50.

Pouget R (1981) Action de la température sur la différenciation des inflorescences et des fleurs durant les phases de prédébourrement et de post-débourrement des bourgeons latents de la vigne. Connaissance Vigne et Vin 15:65–79.

Robinson MD, McCarthy DJ & Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26:139-140.

Ruffner HP & Kliewer WM (1975) Phosphoenolpyruvate carboxykinase activity in grape berries. Plant Physiol. 56(1):67-71.

Smart RE, Shaulis NJ & Lemon ER (1982) The effect of Concord vineyard microclimate on yield II. The interrelation between microclimate and yield expression. American Journal of Enology & Vicitulure 33:109-116.

Srinivasan C (1968) Effect of certain fertilizer treatments with reference to the time of application in grapes (*Vitis vinifera* L.) var. Anab-e-Shahi. M.S. diss., Univ. Madras (1968).

Srinivasan C and Mullins MG (1981) Physiology of flowering in the grapevine A review. Journal of Experimental Botany 59:2579-2596.

Srinivasan C, Muthukrishnan CR, Shanmugam A & Chellam CV (1974) Phosphorus metabolism in grape buds during floral initiation. Experientia, 30:762-3.

Sreekantan L, Torregrosa L, Fernandez L, Thomas MR (2006) VvMADS9, a class B MADS-box gene involved in grapevine flowering, shows different expression patterns in mutants with abnormal petal and stamen structures. Functional Plant Biology 33:877-886.

Trapnell C, Pachter L, Salzberg SL: TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009, 25:1105-1111.

Trought MCT (2005) Fruitset - possible implications on wine quality. ASVO Proceedings. Transforming Flowers to Fruit Workshop; Mildura, Australia. 32-36.

Vannozzi, Alessandro; Dry, Ian B.; Fasoli, Marianna et al 2012 Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and abiotic stresses. Bmc Plant Biology, 12 (130) DOI: 10.1186/1471-2229-12-130.

Vasconcelos, M.C., Greven, M.M., Winefield, C.S., Trought, M.C.T., and Raw, V. 2009. The flowering process of Vitis vinifera: A review. Am. J. Enol. Vitic. 60 (4): 411-434.

Verrier PJ, Bird D, Burla B, DassaE, Forestier C, Geisler M, Klein M, Kolukisaoglu Ü, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EP and Yazaki K (2008) Plant ABC proteins - a unified nomenclature and updated inventory. Trends in Plant Science 13 (4):151–159.

Wagner R (1966) Effects d'un ~clairage d'appoint sur la fertilite des bourgeons de la Vigne. C.R. Acad. Agric. Fr., 52: 670-3.

Walton EF, Podivinsky E, Wu RM (2001) Bimodal patterns of floral gene expression over the two seasons that kiwifruit flowers develop. Physiologia Plantarum 111:396–404.

Winkler AJ (1965) General Viticulture. Univ. Calif. Press, Berkley and Los Angeles, pp.633.

Winkler AJ, Cook JA. Kliewer WM and L Lider LA (1974) General Viticulture. p143-144. University of California Press, Berkeley .

Yamaguchi M, Shimizu M, Ino H, Terai H, Hayashi K, Kaneda T, Mabuchi H, Sumita R, Oshima T, Hoshi N, Higashida H. (2004) Compound heterozygosity for mutations Asp611-->Tyr in KCNQ1 and Asp609-->Gly in KCNH2 associated with severe long QT syndrome. Clinical Science (London) 108 (2):143-50.

Yao A, Wang J, Zhan S, Yang Y, Zhou L& Hu J (2012) The expression patterns of the genes VvTFL1, VFL, VAP1, VvAG1 and VvSEP3 during bud and flower development in the “Xiangfei” grapevine (*Vitis vinifera* L.). African Journal of Agricultural Research 7(2):240-249

Zelleke A & Kliewer WM (1979) Influence of Root Temperature and Rootstock on Budbreak, Shoot Growth, and Fruit Composition of Cabernet Sauvignon Grapevines Grown under Controlled Conditions. American Journal of Enology & Viticulture 30:312-317.

Appendix 1 Communications

Cool Climate Conference Tasmania 2012. “Environmental – genotype interactions and the physiological processes determining fruitfulness and yield in grapevines from hot and cool climates” 2012 - AM Watt, MR Thomas, P Corena, GM Dunn and NM Cooley. Abstract http://www.winetasmania.com.au/files/Watt_poster.pdf

Appendix 2 Intellectual property

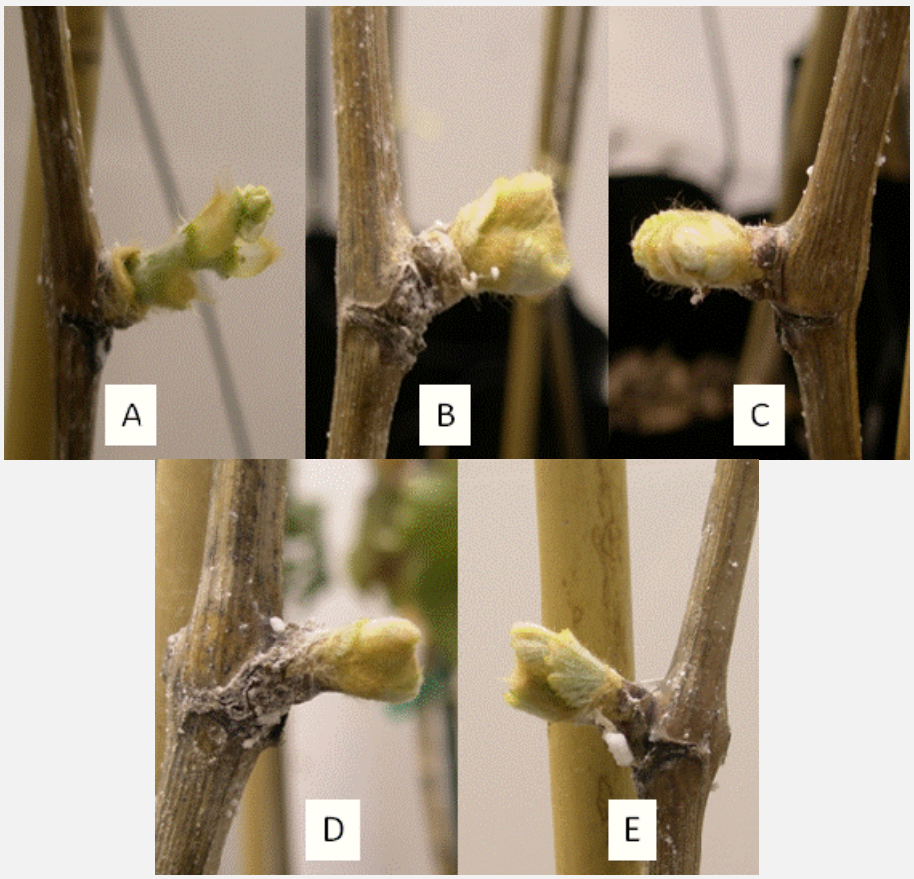
Appendix 3 Staff

University of Melbourne Associate Dr Nicola Cooley, Dr Andrea Watt, Assoc. Prof. Greg Dunn, Dr Tom Hill Professor, Ms Carmel Lancaster and Mr Mark Foletta.

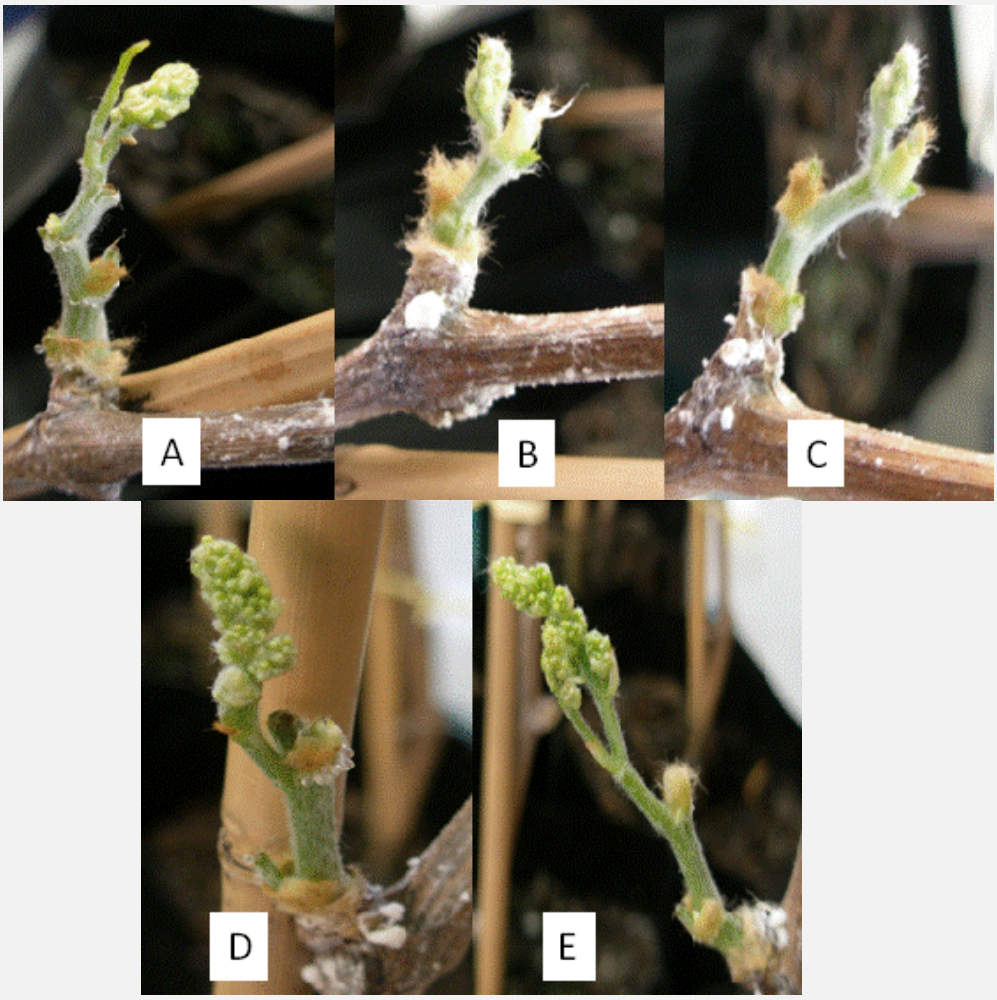
CSIRO Plant Industry, Dr Mark Thomas, Mrs Pat Corena, Mr Don Mackenzie, Dr Ying Zhu and Ms Angelica Jermakow.

Appendix 4 Data

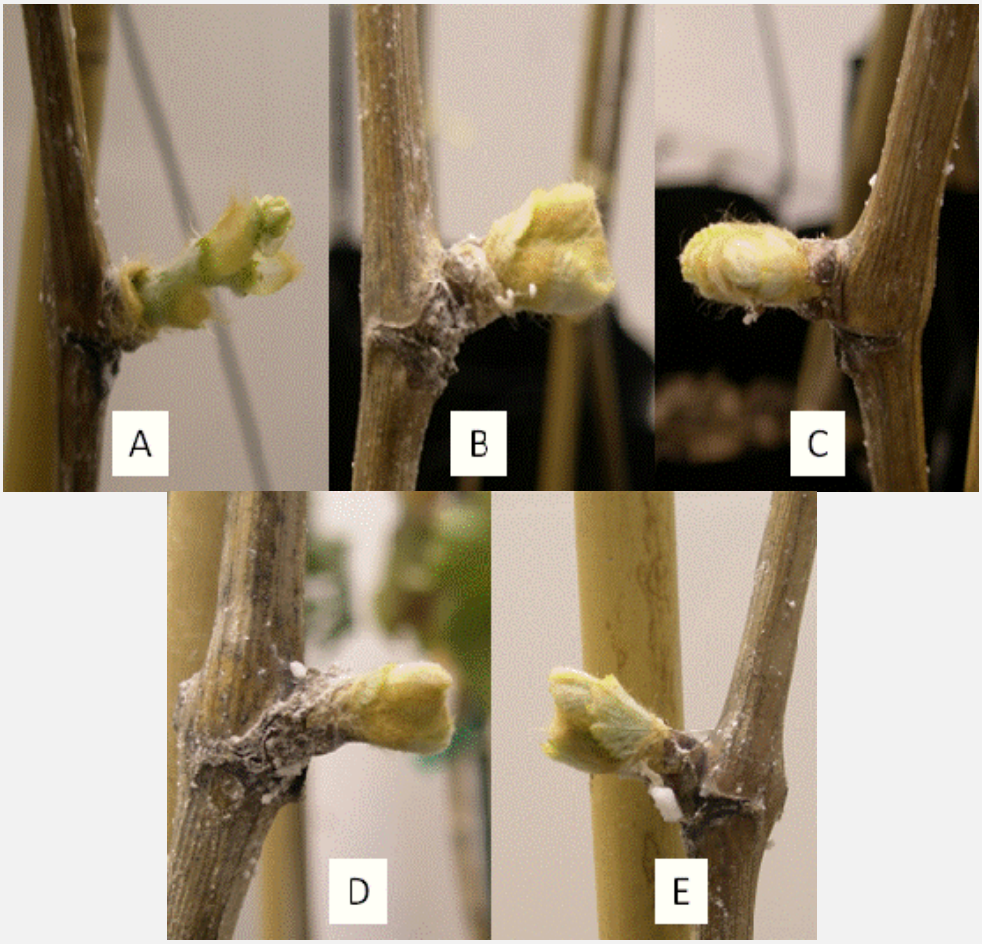
9.1 Plates from the Assumptions About Fruitfulness and Yield: Visual Assessment of the Environment Interactions on Propagated Shiraz Canes



Appendix 4 Plate 1 Representative image depicting Shiraz shoot and inflorescence development after 14 days of temperature treatment 17/15 (17 °C day & 15 °C night) where A to E are five randomly selected replicates.



Appendix 4 Plate 3 Representative image depicting Shiraz shoot and inflorescence development after 14 days of temperature treatment 27/15 (27 °C day & 15 °C night) where A to E are five randomly selected replicates.



Appendix 4 Plate 4 Representative image depicting Shiraz shoot and inflorescence development after 14 days of temperature treatment 27/30 (27 °C day & 30 °C night) where A to E are five randomly selected replicates.

Table 20 Chapter 4 Details of experiment assessment and treatments

Treatment	Date (exp duration days)	Assessment
27 / 30	19th Nov 09 (42)	Development
27 / 15	27th Nov 09 (50)	Development
17 / 15	27th Nov 09 (50)	Development
27 / 30	10th Dec 09 (63)	Development
27 / 15	10th Dec 09 (63)	Development
17 / 15	10th Dec 09 (63)	Development
27 / 30	6th Jan 10 (69)	Development
27 / 15	6th Jan 10 (69)	Development
17 / 15	6th Jan 10 (69)	Development
27 / 30	8th Jan 10 (71)	6 plants in this treatment rated
27 / 30	5th Feb 10 (99)	5 plants rates in this treatment
27 / 15	18th Feb 10 (112)	Sampled for RNA analysis
27 / 15	24th Feb 10 (118)	Sampled for RNA analysis, apec, flower & adjacent leaf
27 / 30	24th Feb 10 (118)	Pruned back and wooded material
27 / 15	24th Feb 10 (118)	Pruned back and wooded material
17 / 15	24th Feb 10 (118)	Not phonologically ready to be pruned
27 / 30	02nd April 10 (155)	All foliage removed. Pruned at node 10. Node tagged.
27 / 15	02nd April 10 (155)	All foliage removed. Pruned at node 10. Node tagged.
17 / 15	02nd April 10 (155)	All foliage removed. Pruned at node 10. Node tagged.
27 / 30	02nd April 10 (155)	7 plants moved into 27 / 15 chambers and became hot / warm
27 / 15	02nd April 10 (155)	5 plants moved into 27 / 30 chambers
17 / 15	02nd April 10 (155)	All plants remained in this treatment

Appendix 5 – Financial Statement

Project Title:	Environment - genotype interactions and the physiological processes determining fruitfulness and yield in grapevines

GWRDC Project Number	**Project Start Date**	**Project End Date**
MU 08/02	01/07/2008	30/11/2012

Financial Year (e.g. 2006/07)	**(a)** ***Funds brought forward from previous year***	**(b)** **#Approved GWRDC Budget (full year)**	**(c)** **Total budget available** **(a)+(b)**	**(d)** **Actual expenditure ($)**	**(e)** **Difference ($)** **(c)-(d)**
Year 1: 2008/09	**n/a**	145,001.00	145,001.00	37,192.67	107,808.33
Year 2: 2009/10	107,808.33	149,340.00	257,148.33	193,027.43	64,120.90
Year 3: 2010/11	64,120.90	86,759.00	150,879.90	122,543.04	28,336.86
Year 4: 2011/12	28,336.86	67,081.00	95,417.86	95,417.86	0.00
Year 5: 20__/__	0.00		0.00	0.00	0.00
TOTAL		448,181.00		448,181.00	**0.00**

* "Funds brought forward" refers to any unspent GWRDC funds from the previous year and approved by the GWRDC to be spent the following year (i.e. column *(e)* from previous year).

"Approved GWRDC Budget" is the funding amount specified in the Project Agreement, including any approved Variations after Project commencement.

Was funding expended as approved in the Project Agreement? Yes ☒ No ☐

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

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

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

I hereby certify that this statement is true and accurate.

Signature of duly authorised representative)

……

Orazio Francavilla CPA	Senior Research Accountant
……	…….. ……
Name:	*Title:*
	Date: