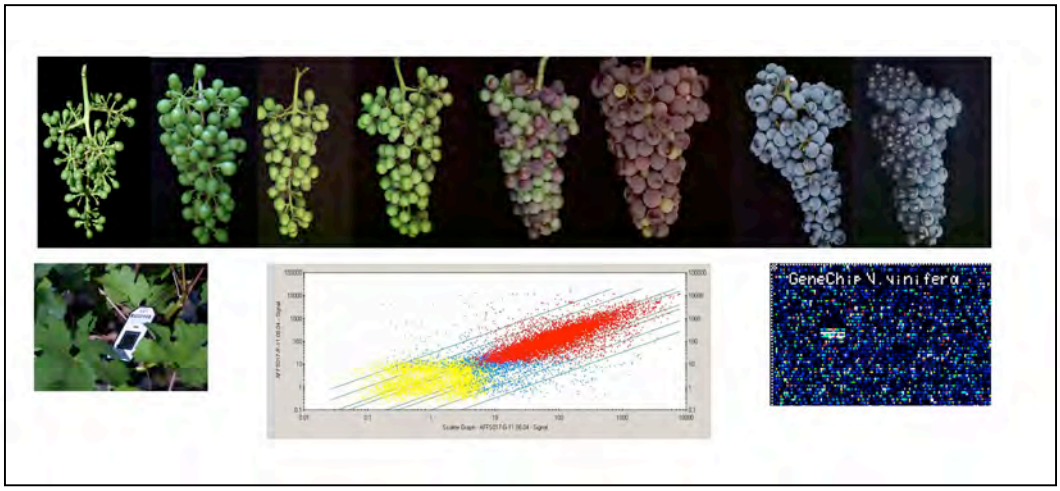
Cooperative Research Centre for Viticulture

Australian Government
Grape and Wine Research and Development Corporation

Understanding and improving quality through a genomics approach



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number**: CRCV 03/08S**

Principal Investigators: **Drs. Mark R. Thomas & Chris Davies**

Research Organisation: Cooperative Research Centre for Viticulture

Date: **March 2007**

Project Title: Genomics and generic technologies: Grapevine gene discovery

CRCV Project Number: 3.4.5

Period Report Covers: November 2003 – December 2006

Author Details: Dr. Mark R. Thomas & Chris Davies
CSIRO Plant Industry
PO Box 350, Glen Osmond, SA 5064, Australia

Phone: 61 (0)8 83038600
Fax: 61 (0)8 83038601
Mobile:
Email: mark.r.thomas@csiro.au
christopher.davies@csiro.au

Date report completed: March, 2007

Publisher: Cooperative Research Centre for Viticulture
ISBN OR ISSN:

Copyright:
© Copyright in the content of this guide is owned by the Cooperative Research Centre for Viticulture.

Disclaimer:
The information contained in this report is a guide only. It is not intended to be comprehensive, nor does it constitute advice. The Cooperative Research Centre for Viticulture accepts no responsibility for the consequences of the use of this information. You should seek expert advice in order to determine whether application of any of the information provided in this guide would be useful in your circumstances.

The Cooperative Research Centre for Viticulture is a joint venture between the following core participants, working with a wide range of supporting participants.

Table of Contents

	Page
Abstract	**2**
Executive Summary	**3**
Background	**5**
Project Aims and Performance Targets	**7**
Method	**8**
Results & Discussion	**19**
Measuring changes from flowering to harvest that influence grape berry development and composition.	**19**
Chalk Hill Wines, Slate Creek Vineyard, Willunga 2003/2004 (WIL-04)	**19**
Hardy Wine Company, Clare 2003/2004 (CLA-04), 2004/2005 (CLA-05)	**54**
Wingara Wine Group, Deakin Estate, Wingara 2004/2005 (WIN-05)	**65**
Linking Gene Expression Changes to Measurements of Berry Development & Composition/Quality Across Three Sites	**76**
Outcomes & Conclusion	**101**
Recommendations	**103**
Appendix 1: Communication	**104**
Appendix 2: Intellectual Property	**105**
Appendix 3: References	**106**
Appendix 4: Staff	**109**
Appendix 5: Additional Data	**110**
Appendix 6: Budget reconciliation	

Abstract
The interaction between the vine, viticultural practices and the environment is a complex one in which changes in gene expression play a central part. The developmental program which 'designs and runs' berry growth and ripening is largely mediated through changes in gene expression. In addition many of the interactions that occur between the berry and the environment and which impact on berry characteristics are also reliant on changes in gene expression. We have used microarray technology, which allowed us to assess the expression of thousands of genes at once to develop maps of gene transcription to describe berry development and identify potential molecular markers for each stage of development. To relate gene expression data to berry development and composition as well as wine quality a holistic approach was taken. Sensory analysis was performed on the wine, and berry development and composition parameters i.e. berry weight, brix, sugars, anthocyanins, acids, inorganic elements and amino acids were measured throughout development. The microarray grape GeneChip used in these studies contained nearly 16000 gene features that represented between one third to one half of all the protein coding genes expected to be present in the grapevine genome. Our work showed that there are surprisingly frequent changes in the expression of many genes throughout berry development. Some of these changes, such as those associated with the initiation of ripening, appeared to be part of the plants inbuilt developmental program. Few significant gene expression changes were found when viticulture management treatments (irrigation or pruning) were compared. More numerous gene expression changes were found between berries from different sites suggesting that the expression of a group of genes within the berry are markedly influenced by environmental factors. By studying the changes in gene expression that occur in berries grown under different conditions we have developed a greater understanding of the key genes that control berry growth and berry composition and how they are regulated.

Executive Summary

We have used the newly developed technique of microarray analysis to determine and objectively measure the expression of many thousands of genes at once to study grape berry development. The switching on and off of genes and their expression level determines how a berry grows and the biological and biochemical processes that occur within the berry. By relating changes in the expression of genes to the stage of berry development and the changes in physical and biochemical characters of the berry we have begun to unravel the complex causes and effects that result in the 'finished' berry at harvest.

To relate gene expression data to berry development and composition as well as wine quality a holistic approach was taken with sensory analysis performed on the wine, and berry development and composition parameters measured throughout development including; berry weight, brix, sugars, anthocyanins, acids, inorganic elements and amino acids. The microarray grape GeneChip used in these studies contained nearly 16000 gene features that represented between one third to one half of all the protein coding genes expected to be present in the grapevine genome. This is the first time such a study has been attempted.

The project was designed to investigate gene expression similarities and differences during berry development of Cabernet Sauvignon on own roots between three sites (Willunga, Wingara, Clare) and two viticultural treatments; one at Clare, spur versus machine pruned vines, and one at Wingara, control irrigation versus prolonged deficit irrigation. This allowed an evaluation of the technology under commercial conditions. Part of this evaluation involved the assessment and development of statistical tools for the investigation of the large datasets that were produced within the project.

It was found that microarray technology represented a relatively robust and objective measure of gene expression changes that occurred during berry development. Out of the nearly 16000 genes on the chip it was found that for all three sites and treatments a similar number of genes were identified as having a significant ($p<0.0000001$) expression pattern; 6263 (Wingara control irrigated vines), 8046 (Wingara prolonged deficit treatment), 7215 (Willunga), 7608 (Clare spur pruned vines) and 7425 (Clare machine pruned vines). This indicates that gene expression patterns are quite fluid during berry development as a large percentage of the genes assayed change significantly during development. Some genes were found to have similar expression patterns across all three sites indicating that these genes are developmentally programmed by the plant and external factors had little effect on their expression. External factors such as viticultural treatments and the environment might influence berry development and composition by affecting gene expression levels. The results of the viticulture treatment studies showed that within the same site there were very little gene expression differences detected with eight genes showing significant ($p<0.0000001$) differences between the pruning treatments at Clare and eight genes showing significant ($p<0.0000001$) differences between the irrigation treatments at Wingara. Sensory analysis of the wine and berry composition measurements confirmed that treatment differences were minor for the years studied. In contrast there were a large group of genes that showed different expression patterns between the three sites indicating that external environmental factors may have a larger effect on berry development than the viticulture treatments studied. It is suggested that further use of microarray studies will lead to an improved understanding of the important internal

plant factors and external factors controlling berry development and composition and the key genes involved. With this technology and resulting new knowledge grapevine researchers will be better able to predict, test, measure and evaluate the effect of new varieties, viticultural treatments and environmental factors on berry development, composition and wine style/quality. For viticulturalists the technology may eventually lead to better informed decision making when comparing the financial worth of one viticulture practice over another and the likely influence of short-term seasonal and longer-term climate change effects on berry composition and wine style/quality.

Microarray technology and gene expression is increasingly becoming the preferred method of choice in human health studies for helping to diagnose illness such as cancer and in determining the best treatment. This increasing emphasis placed on molecular diagnostic tools for making informed decisions in the health area is likely to eventually extend to animals and plants. The South Australian SME, GeneWorks Pty. Ltd., was involved in this project as a product developer of molecular diagnostic tools and kits. This study investigated the use of the grape GeneChip for identifying diagnostic molecular markers of berry development. Despite being extensively studied, progress has been slow in establishing an objective method for identifying berry development stages. Coombe (1995) proposed the modified E-L system as a standardised method for determining growth stages however, it is subjective and does not allow the fine subdivision of pre and post-veraison stages. The pre-veraison green stage is especially problematic. It was found that unique gene expression patterns could be associated with certain stages of berry development including the important fruit set stage, later stages of pre-veraison, veraison and post-veraison. Further research is required to determine if some of genes are robust diagnostic indicators and predictors of specific stages of commercial interest. What role these genes have in berry development and composition still remain to be determined but it does represent a new way of classifying berry development based on gene expression and may in the future form an important component of a new standard system for determining specific stages of berry development.

In conclusion, this study has demonstrated that microarrays are a powerful technology for objectively measuring gene expression changes of thousands of genes in grapevine. The datasets produced in this study represent a valuable resource that can be mined for information over the foreseeable future by grape researchers. Because of the large and complex datasets produced it is suggested that future studies involve multi-disciplinary teams that combine viticultural, molecular and bioinformatic expertise. The expected release in 2008-9 of a 2nd generation microarray grape GeneChip containing all of the grapevine genes present in the genome will offer the opportunity to conduct new studies that will allow, at the gene level, a complete in-depth understanding of berry development and composition.

This project was financially supported by the GWRDC, CRCV, CSIRO Plant Industry and GeneWorks Pty Ltd.

Background

This project was developed as a CRCV supplementary bid proposal to further investigate the technology and outcomes of the GWRDC/CRCV project CRCV3.4.2 "Grape genomics: grapevine gene discovery". In this project, microarray technology was investigated as an objective method for studying gene expression changes occurring during berry development and how these gene changes relate to developmental stages, berry composition and wine quality. The gene microarray method also allowed us for the first time to study how "terroir", or the environment and management practices, influences gene expression during berry development.

The progress of grape berry development, and in particular the process of berry ripening, is determined by three interacting factors; the genome of the grapevine (intrinsic to the identity of the cultivar); the environment, including weather and climate effects; and viticultural practices that are used to manage and manipulate grapevine growth. These factors do not act independently but interact in a complex and dynamic manner. The composition of fruit at harvest is the outcome of these interactions over the entirety of berry development. Previous studies have indicated an effect of the environment on grapevine growth and berry development. For example, some studies have demonstrated links between light and temperature and the development of colour in some varieties. The effects of various viticultural practices, such as pruning and irrigation regimes have also been extensively investigated in a range of cultivars. However, the genotype of the plant has a greater influence on berry composition than the other factors and has not previously been studied in depth due to a lack of suitable technology and knowledge about the genes in the grapevine genome. As there are a large number of genes present in plant genomes molecular research has until recently tended to focus on isolated and relatively small parts of metabolism. The grapevine genome is estimated to contain approximately 30,000 genes. It is the timing, location and level of the expression of the proteins derived from these different genes that in a large part determines berry growth and composition ('style or quality'). Studying the changes in levels and activities of many thousands of proteins at one time is currently not technically possible and even modest experimental efforts in this area are extremely expensive. In contrast the expression of the majority of genes is determined by the level of its corresponding mRNA (i.e. the intermediate messenger molecule that transmits genetic information from the DNA of the genome to the proteins that are encoded by the genes) and new microarray technology (Schaffer *et al*., 2000) can be used to investigate thousands of genes at one time in an objective manner. Not only do the differences between genes contained in the genomes provide intrinsic varietal variation (i.e. the production of specific compounds peculiar to a variety), they also vary the way in which each cultivar responds to its environment and viticultural practices.

The development of new technologies to study changes in gene expression has opened up new opportunities for improving our understanding of biology. The human genome project has had a significant impact on our knowledge of human development and is proving vital in the development of new strategies for human disease diagnosis and management. The techniques, tools and knowledge developed through the human genome project have also had wide-ranging applications in other fields of biology including the study of plant development. For example, microarrays have been used to better understand plant response to drought and cold stress (Seki *et al*., 2001). Using this approach it is not only possible to develop 'maps' of global changes in gene expression during a particular event but single genes involved in specific processes have

also been identified by using these technologies as a gene search tool. For example, microarrays have been used in strawberry to identify a gene involved in flavour biogenesis (Aharoni *et al*., 2000).

The technique of microarray analysis allows the changes in the expression of thousands of genes to be assayed at one time. As a part of a preceding project (CRCV/GWRDC project 3.4.2) we developed a small, in-house, cDNA microarray that demonstrated some of the potential for the use of this approach in grapevines. Many unexpected gene expression patterns were observed providing a wealth of new knowledge about berry development. Analysis of berries from different cultivars showed that there were some genes with differential expression patterns that are candidates for investigating varietal differences. In the current project we have used the Affymetrix microarray technology that consists of a GeneChip with approximately 16,000 features allowing the determination of the expression of 14,000 *Vitis vinifera* transcripts (which includes 10,500 unigenes) and 1,700 transcripts from North American *Vitis* species transcripts, at one time. The grapevine Affymetrix chip was developed by the GeneChip Consortia Array Program as a result of the expressed sequencing tag (EST) sequencing initiative promoted by the International Grapevine Genome Project (IGGP) (http://www.vitaceae.org/). The grapevine EST sequencing project, CRCV/GWRDC project 3.4.2, was a part of this international effort providing both EST sequences and high quality cDNA libraries. In the case of the Affymetrix GeneChip, the 16,000 gene features were designed from the EST information and probes consisting of short specifically designed oligonucleotides (25mers) were synthesised directly onto the chips by microlithographic techniques.

In addition to knowing when and where each gene is expressed through the use of using microarray technology we can often also determine the putative function of the protein encoded by the gene in question through computer assisted analysis. By combining the putative function and expression data we can build a picture of what processes are occurring within grapevine cells/organs at any particular time. This approach can then be used to gain new insights into the processes involved in berry development. The approach also allows for the identification of genes involved in key processes and those that may act as molecular markers for key events during berry growth. Once identified the identified molecular markers have the potential to become predictors of fruit development, composition and quality as well as fruitfulness and plant health.

Project Aims and Performance Targets

The project aims were:

- Collection and analysis of microarray gene expression data of fruit from different sites and regions.
- Development of statistical tools for identifying genes associated with fruit of differing quality.
- Identification of gene predictors for fruit development and quality.

Performance targets were:

1. Tissue collected, RNA extracted and microarray data collected from fruit of differing ripeness and from different regions.
2. Statistical methods developed and evaluated and the best method chosen for microarray data analysis.
3. Gene markers with potential for predicting fruit development and quality identified and evaluated.
4. International collaborations explored.
5. Grape microarray data and methodology made available to other grape researchers for their investigations.
6. Discussions and decision by GeneWorks Pty Ltd on whether to proceed with development of a molecular diagnostic kit for fruit ripeness/quality.
7. Publications.

The delay in the signing of the CRCV Variation Agreement resulted in this CRCV Supplementary Bid project starting in November 2003 instead of July 2003. Achievement of milestones was delayed accordingly and the project completed in December 2006.

Method:

Sampling of grapevine tissues for microarray and biochemical analysis

An extensive grapevine sampling program was undertaken over three seasons to supply tissue for the preparation of RNA for gene expression analysis using microarrays and for the measurement of various biochemical parameters. Different sampling regimes were used depending on the experiment (see below) but sampled tissues were always frozen immediately in liquid nitrogen and stored at -80^{o}C until used. All studies were conducted using Cabernet Sauvignon vines grown on own roots in the field. Cabernet Sauvignon was chosen as the cultivar for these studies in line with the international collaborative effort, promoted by the IGGP, which resulted in GeneChips designed largely around EST sequences from Cabernet Sauvignon.

Plant material preparation

A representative sub-sample of the berries was ground using the Analytical Batch Mill A11 IKA 9 (Wilmington, NC). The ground berry sub samples were used for total RNA extraction for microarray experiments as well as for anthocyanin, amino acid and elemental analyses.

Developmental series

To study changes in gene expression throughout berry ripening four sampling programs were conducted using field grown Cabernet Sauvignon vines at three locations. A series of samples were taken throughout development. Three replicates were sampled for each treatment or series and comprised of pooled berries taken from bunches on either side of rows at different positions within the vineyard. To ensure the uniformity of the collected berries, bunches were tagged at the start of the sampling season as a reference of developmental stage. Photos were taken of the tagged bunches as a record of the sampled berries for the particular time point. Sampling was completed between 0930 and 1430 hrs. The progress of fruit development was monitored by measuring berry weight and ^{o}Brix as described below. Only two of the three biological replicates were used in microarray analysis.

Chalk Hill Wines, Slate Creek Vineyard, Willunga 2003/2004 (WIL-04)

This trial consisted of a single treatment where vines were grown under conditions of usual commercial practice in this vineyard. Flower and fruit samples were collected on a weekly basis (commencing 20/11/2003) throughout the growing season with the last collection at commercial fruit harvest 25/3/2004 (Table 1). Triplicate biological replicate samples were collected from both sides of 20 vines in North/South oriented rows. Berries were sampled randomly from all parts of the canopy. Between 120 and 200 berries were sampled per time point depending on the developmental stage (higher numbers earlier in development when the fruit were smaller). Berries were deseeded for the WIL-04 time course experiment at 4 weeks post flowering (WPF). Tissue samples were immediately frozen and stored as described above. A subset of berries was collected at each sample time, weighed and ^{o}Brix determined.

Table 1. Willunga 2003-2004 sampling.

Tissue	Developmental stage (wpf)	Date of sampling
Flowers (caps on and caps off)	0	20/11/2003
Berries	1	27/11/2003
Berries	2	04/11/2003
Berries	3	11/12/2003
Berries	4*	18/12/2003
Berries	5	24/12/2003
Berries	6	31/12/2003
Berries	7	08/01/2004
Berries	8	15/01/2004
Berries	9	22/01/2004
Berries	10	29/01/2004
Berries	11	05/02/2004
Berries	12	12/02/2004
Berries	13	19/02/2004
Berries	14	26/02/2004
Berries	15	04/03/2004
Berries	16	11/03/2004
Berries	17	18/03/2004
Berries	18	25/03/2004

*From this development stage onwards, seeds were removed from berries prior to RNA extraction

Hardy Wine Company, Clare 2003/2004 (CLA-04) and Clare 2004/2005 (CLA-05)

Own-rooted Cabernet Sauvignon vines from a Leasingham Wines trial site at Clare managed by Hardy Wine Company were used. Grape samples from this specifically designed pruning trial were obtained for two consecutive seasons (Table 2). During the trial, two different pruning regimes spur were analysed. Samples were collected in triplicate from vines watered at the usual level of application. Two treatments were sampled i.e. Spur pruned and Machine pruned vines. Flower and fruit samples were collected on a weekly (spur pruned) and fortnightly (machine pruned) basis during the 2003/2004 season commencing on 24/11/2003 and finishing on the 8/3/2004. The last sample from which usable RNA was extracted was taken on the 9/2/2004, shortly after veraison (Table 2). Analysis of a full developmental series was not possible as a very hot spell of weather severely damaged the vines and fruit and RNA could not be extracted from the fruit harvested after 9/2/2004 (see Results and Discussion). During the 2004/2005 season a full developmental series from flowering to commercial harvest was sampled from 23/11/2004 to the 16/03/2005. Again both the spur and machine pruned vines were sampled. Tissue samples were immediately frozen and stored as described above. A subset of berries was collected at each sample time and their weight and ^{o}Brix determined. The pruning treatments applied by Hardy Wine Company at the start of each season was 50 nodes per vine for the Spur treatment and approximately 85 nodes per vine for the Machine pruned treatment. Harvest data supplied by the Hardy

Wine Company for the 2004/2005 season (CLA-05) showed that average bunch number per vine was 59.40 for the Machine treatment and 46.08 for the Spur pruned treatment, average bunch weight (grams) was 33.5 g for Machine and 33.5 g for Spur and average berry number per bunch was 130.40 for Machine and 98.90 for the Spur treatment. Total yield (tonnes per hectare) in the 2004/2005 season was 5.85 t/ha for the Machine pruned treatment and 3.58 t/ha for the Spur pruned treatment.

Table 2. Clare 2003-2004, Clare 2004-2005 sampling.

Tissue	**Developmental stage (wpf)**	**Date of sampling**
Flowers Spur^&Mach#	0	24/11/2003
Berries Spur&Mach	1	01/12/2003
Berries Spur	2	08/12/2003
Berries Spur&Mach	3	15/12/2003
Berries Spur	4	22/12/2003
Berries Spur&Mach	5	30/12/2003
Berries Spur	6	05/01/2004
Berries Spur&Mach	7	12/01/2004
Berries Spur	8	19/01/2004
Berries Spur&Mach	9	27/01/2004
Berries Spur	10	02/02/2004
Berries Spur	11	09/02/2004
Tissue	**Developmental stage (wpf)**	**Date of sampling**
Flowers Spur^&Mach#	0	23/11/2004
Berries Spur&Mach	1	01/12/2004
Berries Spur&Mach	2	07/12/2004
Berries Spur&Mach	3	16/12/2004
Berries Spur&Mach	4	23/12/2004
Berries Spur&Mach	5*	30/12/2004
Berries Spur&Mach	6	06/01/2005
Berries Spur&Mach	7	13/01/2005
Berries Spur&Mach	8	20/01/2005
Berries Spur&Mach	9	27/01/2005
Berries Spur&Mach	10	03/02/2005
Berries Spur&Mach	11	11/02/2005
Berries Spur&Mach	12	16/02/2005
Berries Spur&Mach	13	23/02/2005
Berries Spur&Mach	14	02/03/2005
Berries Spur&Mach	15	09/03/2005
Berries Spur&Mach	16	16/03/2005

*From this developmental stage onwards, seeds were removed from berries prior to RNA extraction

^ spur pruned vines

machine pruned vines

Wingara Wine Group, Deakin Estate, Wingara 2004/2005 (WIN-05)

Own-rooted Cabernet Sauvignon, planted in 1995 at a density of approximately 1366 vines per ha (2.44 m within row and 3 m between rows) on Nookamka sandy loam (Hubble and Crocker 1941) located in the Sunraysia region of Victoria, Australia were used. Vines were trained in east-west orientated rows, on a two wire vertical trellis and mechanically hedged. The trial commenced during season 2002/2003. During this trial two watering regime treatments were analysed. A standard irrigation treatment, which

was irrigated to full point during treatments, was compared with prolonged deficit irrigation (PD) treatment. Treatments in 12 vine plots were arranged in a random design down the rows with a total of 12 replicates. In total the experimental site extended over 36 rows (i.e. 2 guard rows and one measured row). The PD treatment commenced initially as a regulated deficit irrigation (RDI) practice. RDI duration was determined based on visual assessment of tendrils, canopy leaf area, vine canopy to crop ratio and soil water monitoring data. The RDI treatment was imposed pre-veraison for a period of about three weeks depending on seasons. After the RDI practice a period of non-irrigation was imposed and extended for up to three weeks after the RDI period and represents an extreme deficit irrigation treatment. Water was applied *via* drip irrigation and was similar for all treatments from budburst until after flowering (i.e. up to the commencement of RDI) and from the completion of PD until leaf fall. The timing, duration and volume of water applied for each treatment during managed stress periods and in total are presented in Table 3. The RDI treatment commenced on the 22nd November 2004 with the duration a result of commercial decisions taking into account seasonal factors, soil moisture and vine growth. The timing and duration of the PD period was also the result of commercial decisions and commenced on the 20th December 2004. During the period between the completion of the RDI treatment and start of the PD treatment the soil water profile was restored by 3 irrigation treatments (10th December 2004, 8 hours applying 12.48 mm; 13th December 2004, 8 hours applying 12.48 mm; 17th December 2004, 8 hours applying 12.48 mm). The water applied to the control treatment in the PD period ranged from 25 – 50 mm. Total application of water (ML/ha) applied as irrigation is shown in Table 3.

Table 3. Irrigation treatment durations, volumes applied during managed stress periods and total season irrigation applied.

Irrigation treatment	**RDI period Irrigation applied**				**PD period irrigation applied**				**Season total water applied (ML per ha)**
	ET[1] (%)	**ON**	**OFF**	**Water applied (mm)**	**ET[1] (%)**	**ON**	**OFF**	**Water applied (mm)**	
Standard	100	22-Nov-04	09-Dec-04	34	100	20-Dec-04	03-Jan-05	50	4.5
PD	50	22-Nov-04	09-Dec-04	18	0	20-Dec-04	03-Jan-05	0	3.8

Abbreviations: **RDI** regulated deficit irrigation, **PD** prolonged deficit irrigation and **ET** evaporative transpiration. [1] **ET** was based on soil water.

Samples for microarray analysis were taken over the 2004-2005 season on a weekly basis (Table 4). For each vine, ten berries per bunch were randomly sampled from five bunches. Bunch location was selected based on the method reported by Krstic *et al.* (2003). A total of 50 berries per vine were immediately frozen and stored as described above. From the twelve vines per treatment sampled, 4 replicates (from 4 row sections down the block) were bulked together and three sub-samples of 50 berries were used for microarray analysis. The first flower sample was taken on 10/11/2004 and the last berry sample was harvested 2/03/2005. Berries were deseeded from 3 weeks post flowering. Tissue samples were immediately frozen and stored as described above. Measurement of total soluble solids, berry fresh and dry weight, berry total anthocyanins and

phenolics, berry juice pH, titratable acidity and organic acids concentration was monitored on a weekly basis from veraison to harvest.

Table 4. Wingara 2004-2005 sampling.

Tissue	**Developmental stage (wpf)**	**Date of sampling**
Flowers Con^&PD#	0	10/11/2004
Berries Con&PD	1	16/11/2004
Berries Con&PD	2	23/11/2004
Berries Con&PD	3*	30/11/2004
Berries Con&PD	4	07/12/2004
Berries Con&PD	5	14/12/2004
Berries Con&PD	6	21/12/2004
Berries Con&PD	7	29/12/2004
Berries Con&PD	8	04/01/2005
Berries Con&PD	9	10/01/2005
Berries Con&PD	10	18/01/2005
Berries Con&PD	11	24/01/2005
Berries Con&PD	12	01/02/2005
Berries Con&PD	13	08/02/2005
Berries Con&PD	14	14/02/2005
Berries Con&PD	15	22/02/2005
Berries Con&PD	16	02/03/2005

*From this developmental stage onwards, seeds were removed from berries prior to RNA extraction

^Con = Standard irrigation treatment

#PD = Prolonged deficit irrigation treatment

Diurnal series (DIU-05)

A series of samples were taken over the 24 day/night cycle in order to assay changes in gene expression corresponding to diurnal effects i.e. rhythmic changes in gene expression that occur on a daily basis and reflect a cyclic interaction with the vines environment. The diurnal samplings were done on Cabernet Sauvignon vines at Chalk Hill Wines, Slate Creek Vineyard, Willunga during 2004/2005 (DIU-05). Sampling was done both 4 weeks post flowering and 16 weeks post flowering. All berries were deseeded. Tissue samples were immediately frozen and stored as described above. A subset of berries was collected at each sample time and their weight and ^{o}Brix determined.

Pre-veraison sampling

Cabernet Sauvignon vines in North/South oriented rows were sampled at 4 weeks post flowering for this experiment. The fruit was approaching veraison as a few berries on a few bunches were showing some colour. To determine diurnal variations in gene expression 10 bunches of a similar size, condition and position in the canopy were chosen for each of three biological replicates and tagged for sampling. For each of the time points 5 berries were sampled per bunch, therefore 50 berries were collected at all points except for the final sampling where 10 berries were taken per bunch. Of the 100 berries 50, were taken at random and ^{o}Brix and weight measurements were done for each berry. An additional 6 bunches were chosen for an experiment to determine circadian rhythms. 3.5 hrs before the commencement of sampling for the diurnal series (see below) these 6 bunches were wrapped in 2 layers of aluminium foil to exclude all light. These bunches were sampled at two time points (see below) when 50 berries were taken off each of three replicate bunches. The first 'diurnal' sampling time point was at 0750 hrs ACDT (11/1/2005). Further samples were taken at 2400, 0500, 1000 and 1500 hrs ACDT (12/1/2005). Samples were taken from the light excluded bunches at 0500 and 1500 hrs ACDT (12/1/2005). Sunset for 11/1/2005 was at 2034 hrs ACDT while sunrise on 12/1/2005 was at 0614 hrs ACDT (data from Geoscience Australia), http://www.ga.gov.au/bin/gazmap_sunrise?placename=willinga&placetype=0&state=SA). Sunrise and sunset are defined as the instant in the morning/evening under ideal meteorological conditions with standard refraction of the sun's rays, when the upper edge of the sun's disk is coincident with an ideal horizon.

Post-veraison sampling

Sampling for the 2004/2005 diurnal experiment was completed as described above at 16 weeks post flowering except the first sampling time was 1850 pm ACDT 2/3/2005, the second sample was taken at 2400 hrs ACDT, the third at 0500 hrs ACDT and the last sample was taken at 1000 hrs ACDT. Sunset for 2/3/2005 was at 1953 hrs ACDT and sunrise for 3/3/2005 was at 0704 hrs ACDT (data from Geoscience Australia), http://www.ga.gov.au/bin/gazmap_sunrise?placename=willinga&placetype=0&state=SA).

Extraction of grape berry total RNA

Frozen ground berry tissue (1 g) was incubated with 8 ml of extraction buffer (0.2 M Tris pH 8.3, 2% polyethylene glycol 4000 (PEG 4000), 5 M sodium perchlorate, 1% sodium dodecyl sulphate (SDS), 8.5% insoluble polyvinylpolypyrrolidone (PVPP), 1% beta-mercaptoethanol) for 40 minutes at room temperature with constant agitation. After the incubation, the solid material was separated from the extract by filtration through silanised glass wool and the filtrate was collected as a clear liquid by low speed centrifugation at 4^{o}C for 10 minutes. The crude total RNA was precipitated by the addition of 2.5 volumes of 100% ethanol for 60 minutes at
–20^{o}C. The RNA was collected as a pellet by centrifugation and then was washed in 70% ethanol and recollected by centrifugation. The RNA was further purified using a modified RNeasy Plant Mini Kit (Qiagen catalogue #74904) method which included a DNAase treatment step (Qiagen, #79254). The SUPERase RNase inhibitor cocktail (Ambion catalogue # 2694) was added to each RNA sample before long-term storage at -80^{o}C. The concentration and quality of the undiluted total RNA was measured by spectrophotometry. The quality of the total RNA was confirmed by visualisation on a 1% agarose gel containing 1 ug/ul of ethidium bromide in 0.5X TBE. Prior to shipment

of RNA to AGRF Melbourne for micoarray hybridisation assays, the RNA was precipitated in ethanol, washed twice in 70% ethanol and dried under vacuum.

Preparation of probes for microarray analysis, hybridisation of probes to Affymetrix Vitis vinifera gene chips

The preparation of biotin labelled probes from 5 ug of RNA and their hybridisation to GeneChips was conducted by AGRF (Melbourne) using protocols and instrumentation supplied by Affymetrix (http://www.affymetrix.com). The quality of RNAs to be labelled and the quality of the probe labelling process was monitored by AGRF using an Agilent Bioanalyser system. Where potential problems with either RNA quality of probe labelling were detected the labelling reaction was repeated. This frequently involved the preparation of freshly purified RNA. Affymetrix grapevine GeneChips (Part# 520054; purchased from Millenium Sciences, Surry Hills, Victoria), were hybridised with the prepared probes, washed (Affymetrix GeneChip Fluidics Station) and scanned (GeneChip Scanner 3000 using GeneChip Operating Software GCOS version 1.1).

The order in which the GeneChips were hybridised and washed was conducted through a balanced randomisation process to reduce any potential problems with bias in the efficiency of chip hybridisation and washing. The raw data from these GeneChips was then put through the quality assurance procedure described below. Where poor quality data was detected the chip analysis was repeated but such cases were rare. When all the data was deemed satisfactory it was analysed by the statistical methods described below.

Statistical methods

Quality assessment of Affymetrix GeneChip data after hybridisation

Data analysis was performed in R using Bioconductor (Gentleman *et al*., 2004) packages from the Bioconductor website (http://www.bioconductor.org/) and in house scripts. Hybridisations were quality controlled by using the affy (Gautier *et al*., 2004) and affyPLM package (Bolstad *et al*., 2004). RNA digestion plots were examined for evidence of RNA degradation and PM density plots were examined for unusual intensity distributions. NUSE boxplots, RLE Boxplots and pseudoimage plots of weights were examined for the presence of artifacts or poor quality hybridisations. The percentage of probes on an array with PLM weights less than 0.75 was used as the quality ranking criteria. GeneChip data which did not meet specific criteria were removed from the data set and the hybridization was repeated with a new GeneChip and a fresh RNA sample.

Data analysis

The Limma package of Bioconductor was used for data analysis (Smyth, 2004). Normalisation was performed separately for each experiment using the gcrma (Zhijin *et al*., 2004) package.

Moderated t-tests (Smyth, 2004) were applied to identify genes differentially expressed in the transition from flower to berry. That is, between the first and second time points of the developmental series. Genes were ranked according to the FDR (False Discovery Rate; Reiner *et al*., 2003) adjusted p-values with a threshold of $p<0.001$ for the CLA-05, CLA-04, WIN-05 experiments and $p<0.05$ for the WIL-04 experiment.

To identify genes potentially associated with the presence of seeds, pairs of time points where the seeds had been removed from the second but not the first were analysed. Removal of seeds occurred at week 6 for CLA05, week 4 for WIN05 and week 5 for WIL-04. Moderated t-tests were applied to identify differentially expression and genes were ranked according to the FDR adjusted p-values with a threshold of $p<0.001$ for CLA-05 and WIN-05 and $p<0.01$ for WIL-04.

Genes with expression determined by a daily clock or daylight were investigated over 24 hours. For the diurnal and circadian data, separate analyses were performed for 24 hour time course datasets and analysis of circadian effects. For each of the week 4 (Green Berry: GB) and week 16 (Post-veraison: PR) 24 hour time courses, a linear model was applied to estimate the successive difference between time points and empirical Bayes adjustment of standard errors performed. Genes with FDR (Reiner *et al*., 2003) adjusted F-statistic p-values of less than 0.0025 were selected for further analysis. For each of the GB and PR circadian datasets a 2x2 factorial analysis was conducted to estimate the change over time from 5am to 3pm in the control (light) vine, the initial difference between the light and dark vines at 5am, and the interaction of treatment by time. The two treatment groups were analysed using the time course package. Genes showing evidence of differential change between treatments from 5am to 3pm were selected on the basis of time course ranks of < 1000, interaction LODs > -3, and with the absolute value of the interaction parameter being greater than the minimum absolute change from 5am to 3pm in either the light or dark treatment group. Genes with FDR(6) adjusted p-values of less than 0.2 for the interaction parameter (LOD >0) in the GB dataset were also selected.

For each of the berry developmental series CLA-05, WIN-05, WIL-05, a nested F-test was used to identify differential expression over time. Genes were selected for further analysis using FDR adjusted p-values with threshold $p<0.0000001$. The expression profiles were standardised to lie between 0 and 1 and clustering was applied separately to the data from each site using CLARA (Kaufman *et al*., 2005).

Climate weather data, other field data

Air temperature and humidity data was obtained at 10 minute intervals using the Tinytag Plus TGP-1500 data logger for Clare and Wingara sites for the 2004-2005 season. Air temperature and humidity readings (taken every hour) were obtained from the Australian Bureau of Meterology (http://www.bom.gov.au) for the Willunga site for the 2004 season.

Assaying total anthocyanin levels by spectroscopy

To prepare samples for total anthocyanin determination, 0.1 g of powdered samples were added to 1 mL of methanol containing 1% (v/v) HCl and the anthocyanins extracted at room temperature in the dark on a rotating mixer for one hour. The tissue was pelleted by centrifugation at 15,000 rpm for 15 min and the supernatant retained. Total anthocyanins were measured spectrophotometrically by reading absorbance at 520 nm immediately following centrifugation.

Analysis of anthocyanins and flavonols by high-performance liquid chromatograph (HPLC)

Anthocyanins and flavonols were extracted from ground berries from the time course experiments (Table 1). Triplicate 200 mg ground grape samples were extracted by vortexing in 1 mL of 50% methanol followed by sonication for 20 mins at room temperature. Samples were spun for 10 mins at 12,000 rpm in a microfuge and loaded into autosampler vials. Samples were then run on a Hewlett Packard HP 1100 (HPLC) with Chemstations software with a C18 RS, 3 um, Wakosil (150 x 4.6 mm) column (# 2066052) at a flow rate of 1 mL per min. A 10% formic acid (Solvent A) and a methanol gradient (Solvent B; filtered HPLC grade) was run (see gradient table). Data was collected at 280, 320, 353 and 520 nm.

GRADIENT	
Time	**Solvent B**
0	17
1	17
15	35
40	37
42	100
48	100
50	17
52	17

Elemental analysis

The ion content of the developmental berries series (see above) was determined by CSIRO Land and Water Analytical Services essentially as described by Zarcinas *et al*. (1983). Approximately 1.5 g of powdered grape berry was mixed with concentrated nitric acid. The slurry was then heated to 140°C with stepwise increases over eight hours. The temperature was maintained until the sample boiled down under reflux to a volume of 1 mL. The sample was allowed to cool and the residue diluted to 20 mL with water. After mixing the sample was filtered and the solution analysed for macro and micro nutrient content by inductively coupled plasma optical emission spectroscopy (Spectro Modula). The level of potassium, boron, calcium, copper, manganese, magnesium, sodium, phosphorous, sulphur and zinc were determined for each sample. Wines produced from CLA-05 and WIN-05 were also analysed to determine any differences between treatments.

Winemaking and sensory analysis

Small scale wine lots (50 kg per replicate) were made from samples harvested from the developmental series studies from Clare 2005 vintage (CLA-05) and Wingara 2005 vintage (WIN-05). The CLA-05 wines were from berries from either Spur or Machine pruned vines and the WIN-05 wines were also made from either the standard or prolonged irrigation treatment. These wines were made in triplicate by Provisor for CLA-05 grapes and by CSIRO Merbein for WIN-05 grapes. Wines were made under standard conditions (see Appendix 5a for a flow chart that outlines the fermentation protocol used by Provisor). Fruit received and wine bottling reports are included in Appendix 5b,c.

Sensory analysis was also completed by Provisor using trained sensory panels to measure the properties of each trial wine. Generation of these results consisted of the following stages: Informal assessment; Difference testing and Descriptive analysis. Additional details of the methodology used can be found in the Provisor sensory reports Appendix 5b,c.

Organic acids and sugar analysis

Juice was extracted by berry pressing in a mortar and pestle and then processed through a sieve and clarified by centrifugation. The berry juice was then diluted 1 in 4 in water and processed through Strata C18-E (Phenomenex Part 8B-5001-EAK) and PTFE (Alltech Part # 2973) filters. The samples were then measured for tartaric acid, malic acid, succinic acid, citric acid, glucose and fructose by HPLC at CSIRO Merbein according to the method reported in GWRDC project report "Integrated strategies to manage seasonal variation in winegrape maturation" (Project Number CRV 01/01).

Amino acids analysis

Ground deseeded berries were analysed for amino acid concentration. The slurry from ground deseeded berries was centrifuged to pellet the solid matter and the supernatant was removed. This process was repeated twice. The supernatant was then diluted 1 in 5 in water. Free physiological amino acids were derivatised using the EX:faast kit (Phenomenex cat # KH0-7337) for each berry sample in the berry development time course experiments. Standard concentrations of amino acids were also derivatised to prepare a standard curve for the measurement of amino acid concentration. All samples were analysed by LC/MS by Provisor.

Results & Discussion

Measuring changes from flowering to harvest that influence grape berry development and composition.

An improved and detailed understanding of the processes that occur during berry development, and in particular during berry ripening, is key to the production of improved grapevine planting material and to the development, testing and monitoring of improved grapevine management practices. The physical changes that occur during berry development such as size increase and skin colouration are manifest to the naked eye but result from a complex series of interrelated reactions and processes that occur on the subcellular scale. One way of gaining considerable knowledge of what these processes are, how they are controlled, how they interact with each other and how they are modified by external influences is to understand the changes that occur in gene expression levels. We have used the newly developed technique of microarray analysis that allows us to determine and measure the expression of many thousands of genes at once to study grape berry development. By relating changes in the expression of genes with known or predicted function to the stage of berry development and the changes in physical and biochemical characters of the berry we can begin to unravel the complex causes and effects that result in the 'finished' berry at harvest.

One way to understand the changes that occur during berry development is to follow the changes in gene expression that occur from flower to ripened fruit. To this end four sampling programs were conducted using field grown Cabernet Sauvignon vines at three locations where a series of samples were taken throughout development. At two of the sites two different viticultural treatments (pruning, irrigation) were compared. The changes in 15,700 grape genes on the Affymetrix GeneChip (14,000 *V. vinifera* transcripts and 1,700 transcripts from other *Vitis* species) were determined in these samples by microarray technology. To relate this information to berry development and composition, measurements of berry weight, sugars, organic acids, anthocyanins, amino acids and mineral elements were also collected in addition to wine sensory analysis for some treatments.

Chalk Hill Wines, Slate Creek Vineyard, Willunga 2003/2004 (WIL-04)
This trial consisted of a single treatment where vines were grown under conditions of usual commercial practice in this vineyard. Flower and fruit samples were collected on a weekly basis (commencing 20/11/2003) throughout the growing season with the last collection at commercial fruit harvest (25/3/2004) (Table 1).

Measurements of berry development & composition
The progress of berry development was followed by measuring berry weight and the levels of total soluble solids. Berry development progressed with the expected bi-sigmoidal pattern of berry growth with two phases of rapid berry growth separated by a lag phase that extended from 7 to 9 weeks post-flowering (Fig. 1). Berry growth stopped some time before harvest with no significant increase between 13 and 18 weeks post-flowering. The curves for ^{o}Brix and colour (as measured by absorbance at 520 nm) are remarkably similar with increases occurring from 9 weeks post-flowering (wpf) (Fig. 1). Both curves were biphasic with relatively sharp increases from 9 to 13 wpf followed by a reduced rates of increase from 13 wpf to 18 wpf. Not surprisingly the curves for the hexoses glucose and fructose are similar to that for ^{o}Brix with both curves

being biphasic (Fig. 2). The level of glucose was less than that for fructose for each of the samples taken after week 12. This may indicate that glucose is preferred over fructose as a metabolic substrate.

Organic acid accumulation

To develop a more in depth picture of the progress of berry development some more detailed analysis of biochemical changes was also completed. HPLC analysis of organic acid levels showed that, similar to previous reports regarding Cabernet Sauvignon, malic and tartaric acids are the most significant contributors to total acid levels (Fig. 3). As tartrate is a stronger acid than malate (pK 2.98 versus 3.40) it is clear that tartaric acid is the most important contributor to total acidity at harvest. Tartrate and malate levels are at their highest at around the time of veraison (veraison was determined to be at 9 wpf on the basis of the curve for ^{o}Brix, Fig. 1). After version the levels of both acids decreased sharply. The ratio of tartrate to malate changed significantly throughout berry development with tartrate being more than three fold higher in concentration at harvest (Fig. 3). Citric acid is the third most abundant organic acid in grapes but occurs at much lower levels than malic and tartaric acids. Citric acid made a smaller but measurable contribution to the total organic acid pool (Fig. 4). As for malate and tartrate the maximal level of citrate was at veraison. The curve for total acids level (the summation of malate, tartrate and citrate) shows that after reaching maximal levels at veraison the acids decrease fairly sharply until 14 wpf after which there was little change for the next four weeks until harvest.

Anthocyanin accumulation

HPLC analysis was used to measure the levels of the various forms of anthocyanins during berry development. Measurable amounts of the glucosides, acetylglucosides and coumaroylglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin were detected. Malvidin glucoside was the most abundant single species (63.29% of all glucosylated anthocyanins, 39.69% of total anthocyanins at harvest) (Table 5, Fig. 5). The summed malvidin species contributed 68.2% of the total anthocyanins at harvest. The levels of the combined anthocyanin species increased steadily from 10 weeks post-flowering but the levels stabilised at around 15 weeks post-flowering and remained approximately the same until harvest at 18 weeks post-flowering (Fig. 5).

Table 5. Summary of Willunga 2003/2004 berry series, anthocyanin species (mg/g) at harvest (25/03/2004) for three replicates.

Delphinidin glucoside	Cyanindin glucoside	Petunidin glucoside	Peonidin glucoside	Malvidin glucoside	Total Glucosides
0.0844 ± 0.0169	0.0162 ± 0.0046	0.0531 ± 0.0090	0.0523 ± 0.0099	0.3552 ± 0.0546	0.5612 ± 0.0950
Delphinidin acetylglucoside	Cyanidin acetylglucoside	Petunidin acetylglucoside	Peonidin acetylglucoside	Malvidin acetylglucoside	Total Acetylglucosides
0.0207 ± 0.0031	0.0039 ± 0.0008	0.0172 ± 0.0024	0.0151 ± 0.0020	0.1915 ± 0.0265	0.2483 ± 0.0347
Delphinidin coumaroylglucoside	Cyanidin coumaroylglucoside	Petunidin coumaroylglucoside	Peonidin coumaroylglucoside	Malvidin coumaroylglucoside	Total Coumaroylglucosides
0.0051 ± 0.0005	0.0037 ± 0.0006	0.0046 ± 0.0007	0.0082 ± 0.0012	0.0637 ± 0.0103	0.0853 ± 0.0133
Total Delphinidin	Total Cyanidin	Total Petunidin	Total Peonidin	Total Malvidin	Total Anthocyanins
0.1102 ± 0.0205	0.0238 ± 0.0060	0.0749 ± 0.0120	0.0756 ± 0.0131	0.6103 ± 0.0914	0.8948 ± 0.1430
Quercetin glucoside					
0.0177 ± 0.0026					

Amino acid accumulation

Amino acid levels were also measured throughout berry development. Fifteen different amino acids were measured reliably in all samples even though the levels of some were very low, the remainder were at such low levels that they were at the limits of detection in one or more of the samples and so the data has not been included. The patterns of amino acid accumulation can be divided into four classes. The majority of amino acids (alanine, glutamate, histidine, isoleucine, phenylalanine, proline, serine, valine) had relatively stable levels prior to veraison but the levels increase fairly steadily at veraison, or shortly after, and continue to increase until harvest (Figs. 6a,d,g,h,i,j,k,o). The second group consists of arginine, glycine and threonine as although they increased in concentration after veraison their levels peaked before harvest after which they decreased somewhat (Figs. 6b,f,l). Aspartate, tryptophan and tyrosine form a group which were generally higher in level post-veraison but whose levels appeared to oscillate considerably over the season (tryptophan and tyrosine are at very low levels) (Figs. 6c,m,n). A single amino acid, glutamine comprises the fourth group whose level was higher early in berry development but by 5-6 weeks post-flowering the level decreased and stabilised until harvest (Fig. 6e).

The most abundant amino acid is proline, which was at 1.84 mg/kg at harvest and comprised 30.48% of the total amino acids. Proline concentration has been suggested to increase in response to stress and act as a compatible solute assisting in the management of osmotic pressures within the cell. Presumably this occurs in grape berries in response to the large increase in the levels of glucose and fructose that occurs after veraison. The increase in proline levels may allow the berry cells to continue to function despite the sharp increase in osmotic pressure. Alanine is the next most abundant amino acid at harvest (7.09%), but is at levels (0.19 mg/kg) approximately ten fold lower in concentration than proline. The large increase in proline (and to a lesser extent arginine) levels after veraison may be correlated to the lack of increase in glutamine levels as glutamate may be preferentially diverted to proline (and arginine) synthesis.

When plotted on a per berry basis it is notable, but not unexpected given the amino acid concentration data, that there is little amino acid accumulation in the berries until after veraison (Fig. 6). One notable exception is the amount per berry of glutamine that actually increased early in berry development then decreased towards veraison.

Elemental analysis

Ten elements were detected in developing grape berries (Fig. 7) and their patterns of their accumulation on a concentration basis can be divided into groups. The element with the highest concentration was potassium (Fig. 7g) which decreases from high levels early in berry development until 4 weeks post-flowering after which the levels rose gradually until harvest when the concentration was 2575 mg/kg. Boron followed a similar pattern but was at much lower levels at harvest (7.2 mg/kg) (Fig. 7a). Another group of elements which includes calcium, copper, magnesium, manganese, phosphorus, sulphur and zinc followed a similar pattern to that described above but after decreasing to lower levels early in berry development their levels tended to remain low rather than gradually increasing (Figs. 7b,c,d,e,f,i,j). Sodium accumulation is a little more complex as levels were higher in the early stages and then decreased during development and then increased again later in ripening after about 13 weeks post-flowering (Fig. 7h).

As will be discussed later the levels of potassium in berry musts can be important in winemaking due to the formation of insoluble potassium tartrate. The presence of elevated levels of potassium can therefore cause a reduction in must acidity that has to be corrected by the addition of tartaric acid.

Measurements of gene expression by microarray analysis

Quality of data

Various programs from the Bioconductor Project site (http://www.bioconductor.org/) were used to analyse the quality of the results obtained from Grapevine Affymetrix GeneChips that had been hybridised to probes prepared from the RNA samples described above. These programs used a range of methods to analyse the GeneChips for the presence of spatial artefacts and effects due to poor hybridisation. Due to the care taken in the preparation of RNA, probe preparation and chip hybridisation and washing there were only low numbers of failures and a high percentage of the GeneChips were found to have produced data of sufficient quality to be used in subsequent statistical analyses of gene expression. Various, usually minor spatial effects, such as edge effects (Fig. 8), were observed by examining Prove Level Model (PLM) Pseudo-Images. In general these small spatial defects did not greatly compromise the data judged by other statistical criteria. In some cases the overall quality of hybridisation was judged to be below the set criteria and new RNA preparations had be made and new GeneChips hybridised. The GeneChips with poor hybridisation show an abnormal intensity distribution in PLM Pseudo-Image plots (e.g. Fig. 8) and stood out in other quality tests such as plots of Perfect Match (PM) log intensities (Fig. 9) and plots of Normalised Unscaled Standard Errors (NUSE) (Fig. 9). After examining the data in detail it was decided that the best method for discriminating chip quality was to reject GeneChips with more than 25% of probes with PLM weights < 0.75. This was done for all experiments and chip hybridisations were redone where necessary to ensure high quality data was used for analysis.

Gene expression changes influenced by diurnal effects

A series of diurnal samples (DIU-05) were taken over the 24 day/night cycle in order to assay changes in gene expression corresponding to diurnal effects i.e. rhythmic changes in gene expression that occur on a daily basis. These changes could be a result of the berry responding to changes in sunlight quantity and quality as well as an internal clock (circadian rhythm). Experiments were designed to identify whether the expression of a gene was influenced by environmental factors (daylight/dark) or by an internal clock within the plant. This was achieved by excluding light reaching the berries during the sunlight period. Sampling was done for both pre- and post-veraison berries to see if there were differences between berry developmental stages.

It was found that for the pre-veraison berries there were a group of genes that responded to light by changing their expression pattern during the daylight period in response to light. As an example, gene 1607227_s_at showed a significant increase in expression from 5:00 AM (sunrise) and expression increases throughout the day (Fig. 10). This gene is clearly regulated by light and not by a circadian (internal) clock as the treatment where light was excluded (Circadian treatment; red lines) showed no increase in gene expression during the same period (Fig. 10). The function of this gene is unknown. In pre-veraison berries 250 genes were identified that showed a significant change in gene expression in response to light from sunrise to 3:00 PM while there were 153 genes in post-veraison berries that showed significant changes in gene expression during the same period.

Some genes were also identified in pre-veraison berries that appeared to be regulated by an internal circadian clock within the plant (Fig. 11). The gene shown in Fig. 11 has the same rising expression pattern from sunrise to 3:00 PM regardless of berry exposure to the presence or absence of sunlight. The function of this gene is unknown. For pre-veraison berries 465 genes were identified that showed a significant change in gene expression that appeared to be regulated by a circadian clock while there were 487 genes in post-veraison berries that showed similar significant changes in gene expression during the same period.

The data from the pre- and post-veraison berries were compared to determine if the stage of berry development influenced the response of a gene to diurnal effects (sunlight or a circadian clock). It was found that 438 genes had a significant different diurnal response depending on the stage of berry development (Fig. 12). For both genes shown in Fig. 12 expression rapidly increases in pre-veraison berries from sunrise (5:00 AM). In contrast the same genes in post-veraison berries had a constant low expression throughout the same time period (Fig. 12). While the function of the gene in Fig. 12a is unknown the putative function of the gene in Fig. 12b has been identified as a chlorophyll a/b binding protein CP24.

The conclusion from this diurnal gene expression study is that in the berry a group of genes display changing expression levels during a 24 hour day/night cycle. This group of genes can be classified into those that respond to light or those that are regulated by an internal circadian clock within the berry. In addition, a gene's response to diurnal rhythms is influenced by berry development stage. As a result of these findings it is clear that the time of berry sampling in the vineyard is important for biochemical, physiological and gene studies to reduce errors and variation as a result of diurnal

effects. For the experiments reported below the experimental design incorporated sampling of berries within specific time windows.

The flower to fruit transition

The GeneChip expression data was examined to identify specific genes at flowering that were involved in the transition from fertilisation of the ovule to the start of berry development. The difference between the second and first time point, corresponding to the transition from flower to berry, was examined for each of the four developmental time course data sets (Willunga 2003/2004, WIL-04; Wingara 2004/2005, WIN-05; Clare 2003/2004 and 2004/2005, CLA-04 and CLA-05). This one week period from anthesis was targeted for investigation because this is a critical period for fruit set and initiation of berry development.

Two main groups of genes were identified which significantly decreased or increased from time point 1 (anthesis) to time point 2 (one week after anthesis). The group of genes that decreased during this period would be expected to be involved in processes specific to the flower and anthesis. Two examples are shown in Figs. 13, 14 and it is seen for both genes that a similar expression profile was observed across all 4 developmental time course sets and sites. Tentative annotation of the genes suggest that one gene is a YABBY-like transcription factor (Fig. 13) that is involved in the regulation of other genes and the other gene (Fig. 14) is similar to S-linalool synthase which is involved in pathways linked to flavour and aroma. The other class of genes that display a statistically significant rapid increase in expression after anthesis are represented by the example genes 1613542_at and 1614877_at (Figs. 15, 16). One of these genes has an unknown function (Fig. 16) while the other (Fig. 15) has a tentative annotation of caffeic acid 3-O-methyltransferase which is involved in the stilbene, coumarine, flavonoid and lignin biosynthesis pathway.

There were 4,960 genes identified which, in at least in one of the developmental series, showed a significant expression difference between anthesis and one week of berry development (adjusted p-value thresholds of $p<0.05$ for WIL-04 samples or $p<0.001$ for the samples from other sites). Like the two examples shown in Figs. 14, 15 that are involved in pathways responsible for aroma and flavonoids some of these other genes are associated with pathways associated with berry composition/quality as well as growth and development.

The results presented in Figs. 13-16 also provided an indication of the variation between sample replicates. Collecting field samples from vineyards can be problematic if the experimental design does not take into account variation present in the vineyard and it is very important to gain a good representation across the whole trial (Krstic et al., 2003). Values for replicates were very close across all time points and sites except for the anthesis time point for WIL-04. The reason for the WIL-04 time point 1 replicate variation was traced to the samples used for analysis with one sample derived from flowers which had intact caps while the other replicate represented flowers which had undergone cap drop. The high degree of agreement observed between replicates indicated that our vineyard sampling protocol was of a suitable standard and that there was very low technical variation introduced into the data as a result of RNA isolation and hybridisation of this labelled RNA to the GeneChip. The high reproducibility between replicates was also observed throughout all the experiments detailed in this report. This, in addition to the quality checks detailed above, gave us a high level of

confidence that the microarray protocols and technology used in this study represented a relative robust and objective measure of gene expression changes that occur during berry development.

Effect of seeds in berry samples used for GeneChip analysis
A potential factor that might affect gene expression results when comparing berry profiles over a developmental series is the presence or absence of seeds in berry samples used for RNA extraction. While seeds were removed from most berry samples prior to RNA extraction the small size of seeds precluded this for samples collected during the early stages of berry development.

Seed specific genes were identified in WIL-04 by measuring the difference in gene expression levels between the time points which correspond to the transition of berry samples containing seeds and those without. Genes showing a significant drop in expression after seed removal (time point 5) were identified as potentially seed specific in their expression. Statistical analysis was performed on the M-values as detailed in the Method section with time points 2-4 (1-3 weeks post-anthesis) representing RNA extracted from berries with seeds and time point 5 onward representing berries where seeds had been removed prior to RNA extraction. Two representative gene expression profiles are shown in Fig. 17 where a significant drop in gene expression occurs between the two time points. This suggests that these two genes may be seed specific. To determine if this drop in gene expression was seed-specific or due to some other factor the expression levels of the identified genes in WIL-04 were compared with gene expression levels for the other two sites before and after seed removal to look for similar changes. . There were only four genes identified that showed a significant expression difference (adjusted p-values with a $p<0.001$ for CLA-05 and WIN-05 data sets and $p<0.01$ for the WIL-04 dataset.) between the two time points representing berries with and without seeds. An example of one of these genes is shown in Fig. 18.

The conclusion from this study is that genes that show seed-specific expression are a very rare class of the nearly 16,000 genes on the GeneChip and nearly all of the gene expression differences that are likely to be detected over a berry developmental series will be genes that are expressed in the berry flesh and skin.

Gene expression changes during berry development
Berry samples were collected at weekly intervals until commercial harvest and the RNA extracted from each sample used for microarray analysis. Data from the 16,000 genes on the GeneChip were analysed to identify genes that showed significant expression changes. A summary of the results shows that thousands of genes have altered expression levels of more than two fold throughout berry development (Fig. 19). Of particular interest is the similar pattern observed for genes that were either down-regulated or up-regulated (Fig. 19).

To investigate the changes in more detail a nested F-test was used to identify differential expression over time. Genes were selected for further analysis using FDR adjusted p-values with a threshold of $p<0.0000001$. Out of the nearly 16000 genes on the chip, 7215 were identified as having a significant differential expression pattern below this threshold p-value. To group these significant genes into similar patterns during berry development the clustering algorithm CLARA was used. The number of clusters was either set to 50 or 100. Briefly, the gene expression levels were rescaled

from 0.0 to 1.0 and involved a gene specific linear transformation to each expression profile so that the range of expression values was the same for all profiles while shape was preserved. Applying CLARA to the rescaled data allocated genes to clusters according to the shapes of their profiles because the effect of overall expression levels was eliminated. Rescaling will lead to genes being allocated to the same cluster if they have the same expression pattern but different magnitude of variation, however, ranking by p-value within clusters can be used to bring to the fore those genes with the largest magnitude of variation. The results of a 50 cluster CLARA analysis on the Willunga (WIL-04) berry development dataset is shown in Figs. 20-22. The flower samples were removed from the analysis. The cluster in Fig. 20a contains 158 genes and the mean profile of all the genes in the cluster is represented in Fig. 20b. It can be seen that this group of genes in cluster 4 has low expression pre-veraison but expression levels rapidly increase from veraison onwards. This suggests that these genes have a role in veraison and post-veraison processes that may influence berry growth and composition. As it is easier to interpret cluster profiles by looking at the mean profile, Fig. 22 contains all 50 clusters showing the major gene expression changes that occurred during berry development from one week post-anthesis to harvest. Ordering of the profiles in Fig. 22 was based on Principal Component Analysis (Fig. 21) and manual examination of mean expression profiles.

An examination of all 50 clusters showed that there are many unique gene profiles occurring during the pre-veraison period as well as the post-veraison period and may represent indicators of important processes occurring in the berry. Up until now it has been difficult to accurately measure key changes that occur during the pre-veraison stage. Using microarray technology we have been able to show that this berry stage is very dynamic. For the pre-veraison stage there are groups of genes (clusters 1, 12, 35) that are expressed during the very early stages but are turned off by time point 4 while other sets of genes (clusters 36, 17) are not down regulated until timepoint 8 immediately prior to veraison (Fig. 22a). Another class of genes (clusters 40, 13) have increasing expression until timepoint 6-8 then expression decreases after verasion (Fig. 22 a,b). Genes that are highly expressed until veraison are represented by clusters 3, 5 and 14 (Fig. 22b). Veraison is characterised by groups of genes (clusters 49, 38) that show decreased expression just before and after veraison and other groups of genes (clusters 2, 4) where the expression increases rapidly at verasion (Fig. 22d). The post-veraison period is mainly characterised by gene clusters whose expression profiles gradually increase over this period (Figs, 22d,e). Interestingly there are some cluster expression patterns that fluctuate markedly from anthesis to harvest (Fig. 22f,g). It appears that some of the observed gene expression patterns in Fig. 22 represent indicators of developmental events within the berry and that some of the fluctuating expression patterns may represent a response due to external factors such as management treatments or the environment. This is investigated and discussed further in the following sections as well as relating gene expression changes to quality traits like organic acids, colour and proline.

Figure 1.

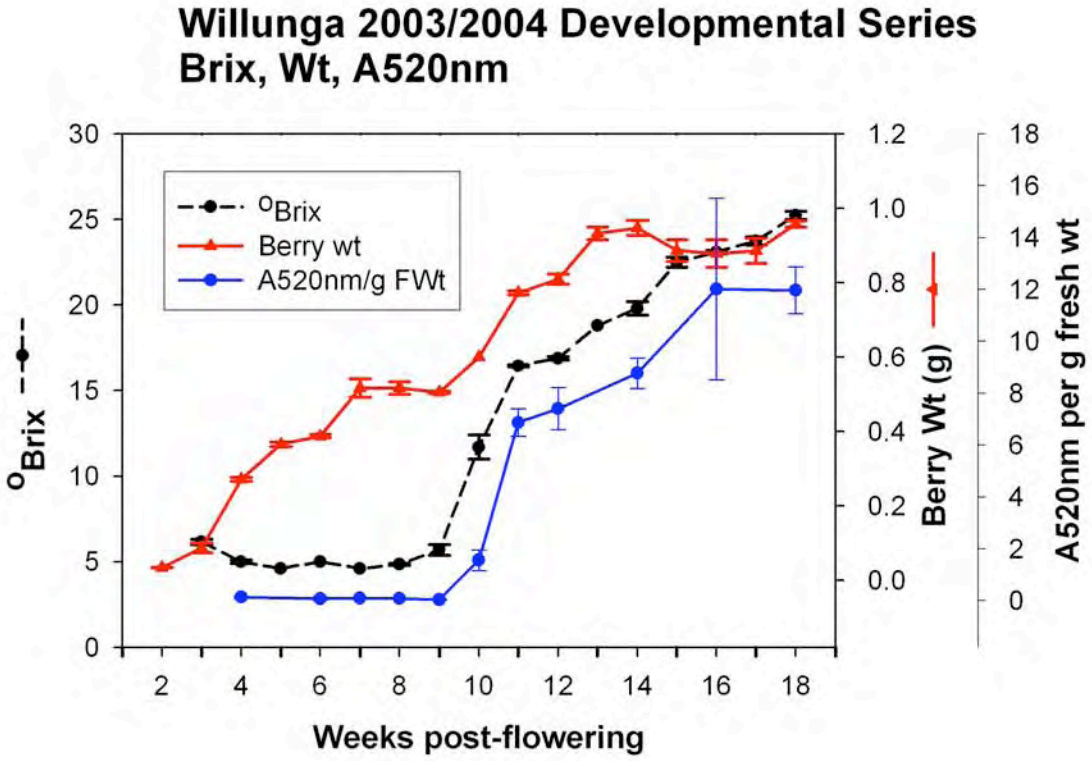


Figure 2.

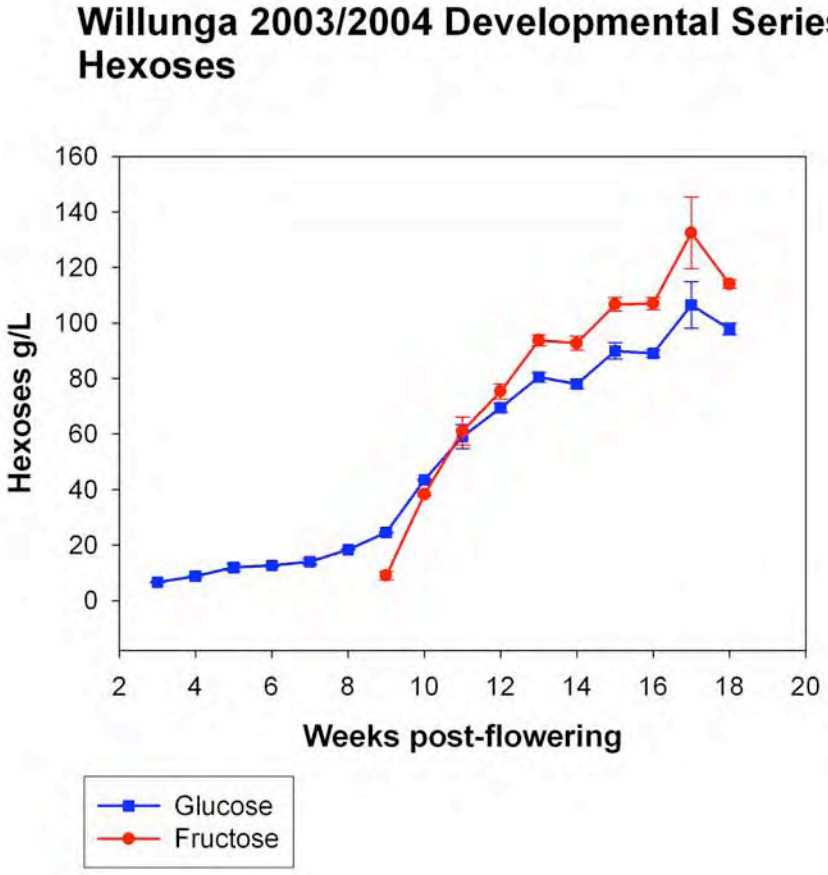


Figure 3.

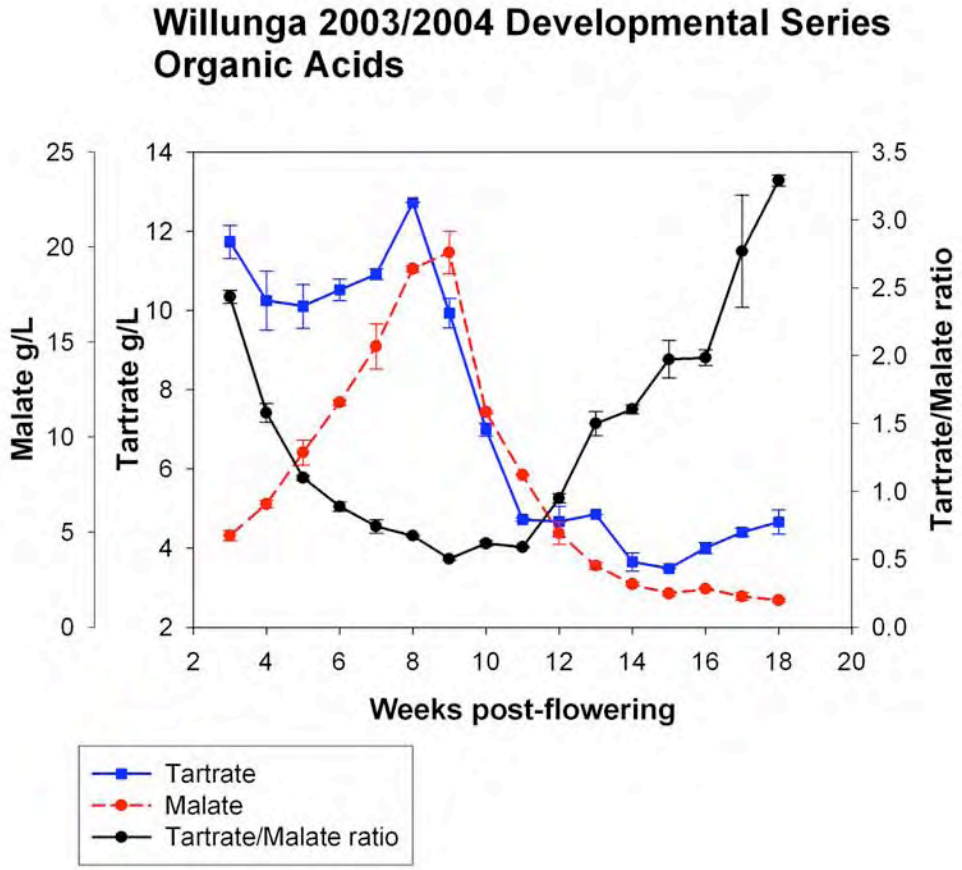


Figure 4.

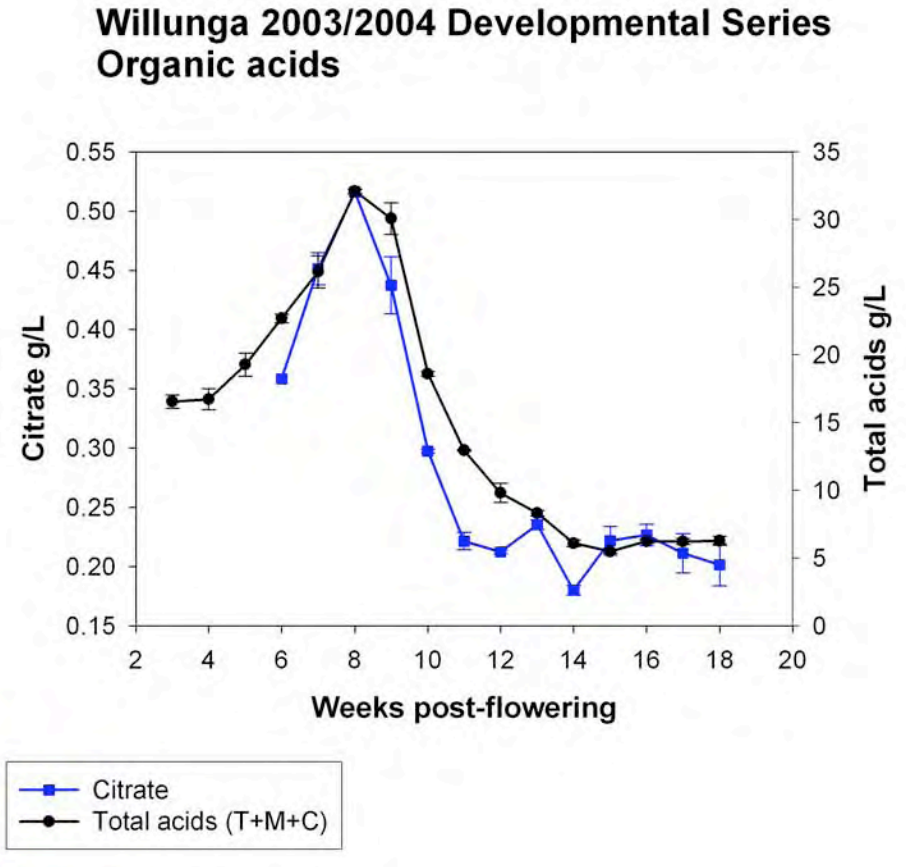
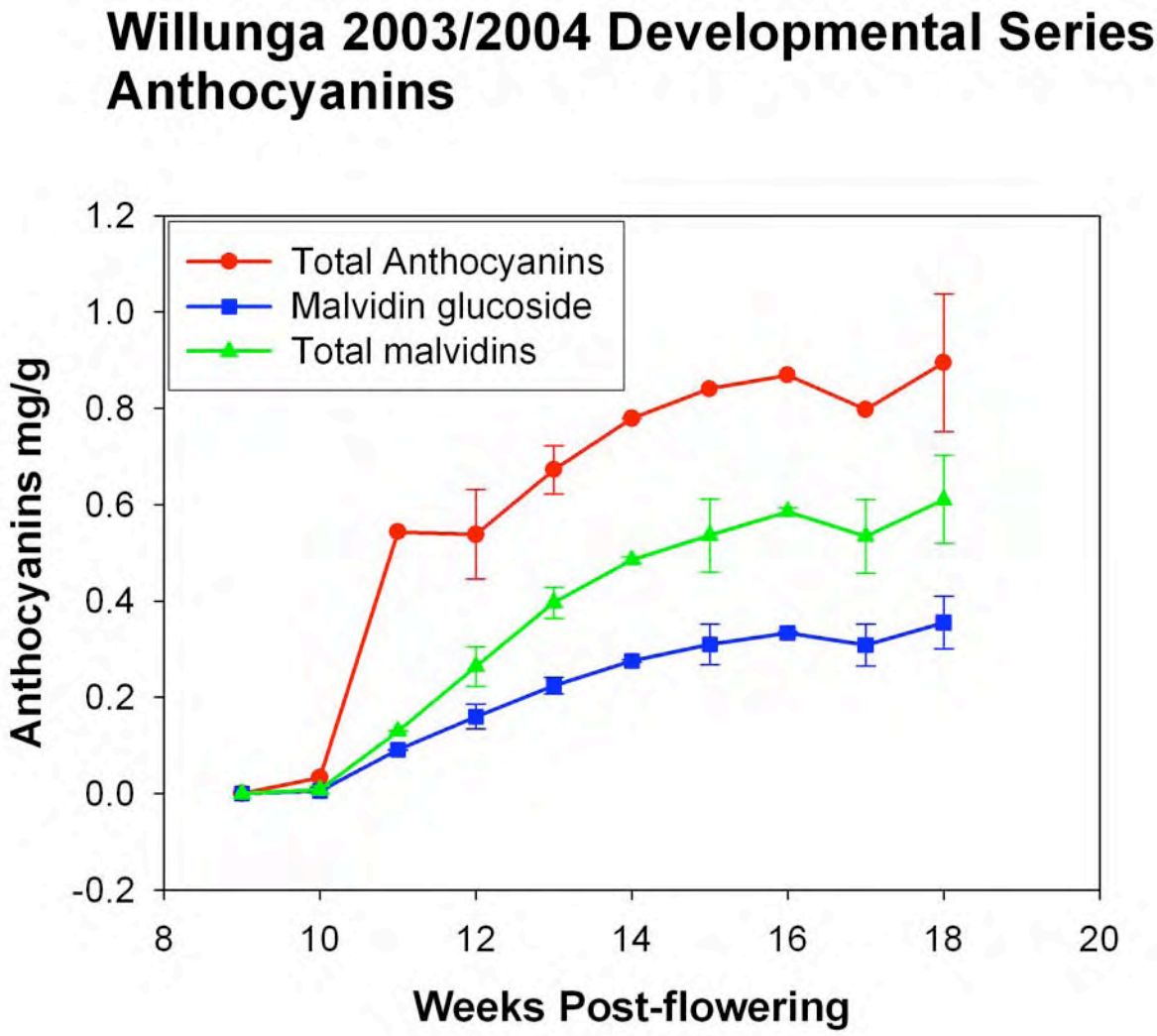
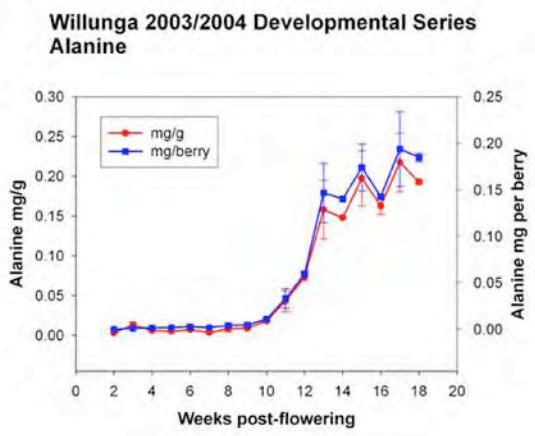


Figure 5.

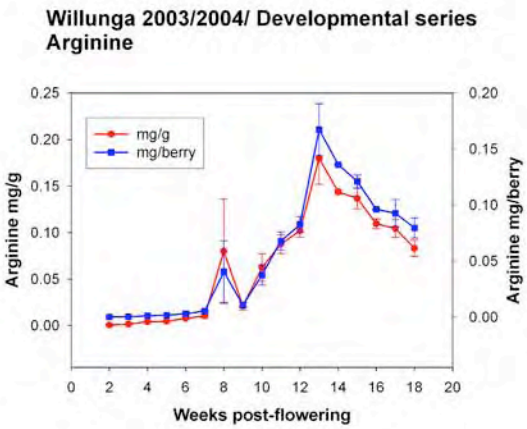

Willunga 2003/2004 Developmental Series
Anthocyanins
Total Anthocyanins
Malvidin glucoside
Total malvidins
Anthocyanins mg/g
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
8
10
12
14
16
18
20
Weeks Post-flowering

Figures 6a-f.

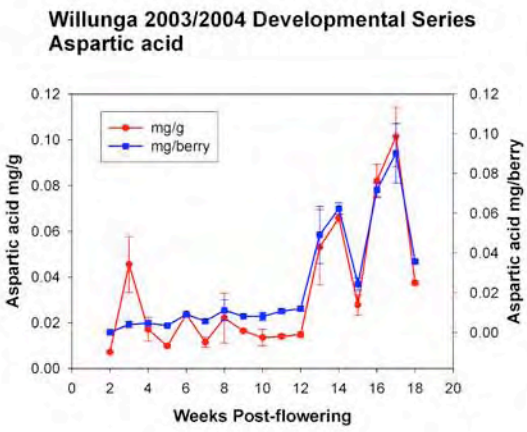
(a)



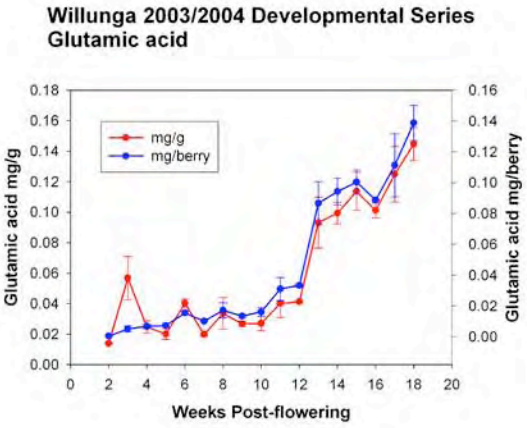
(b)



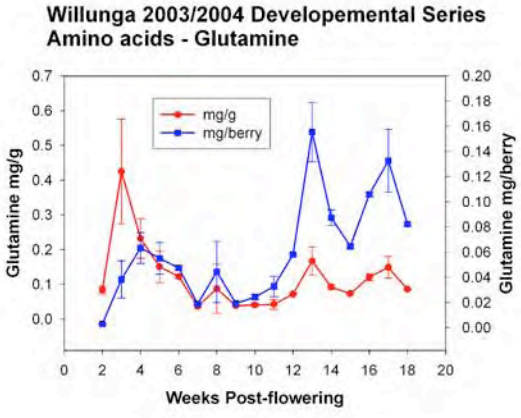
(c)



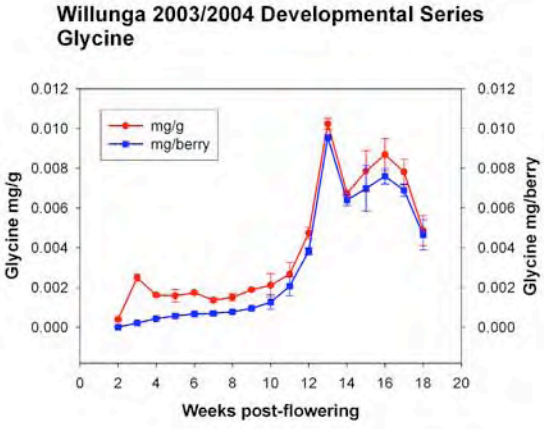
(d)



(e)

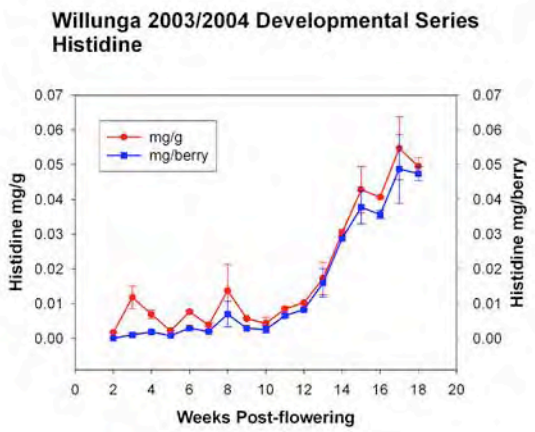


(f)

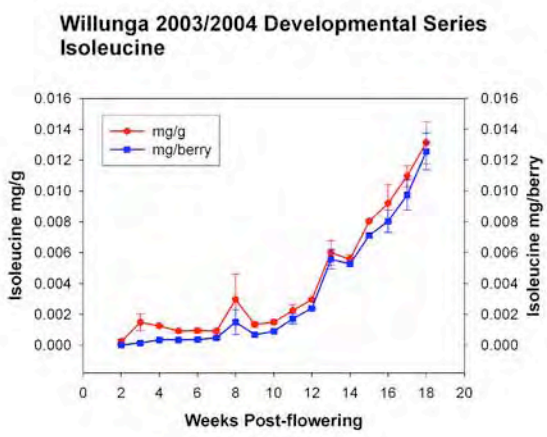


Figures 6g-l.

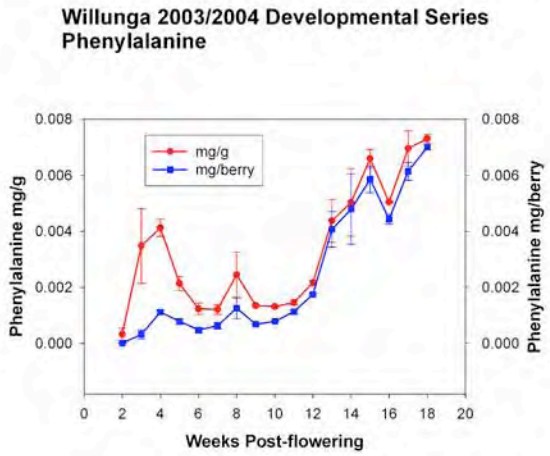
(g)



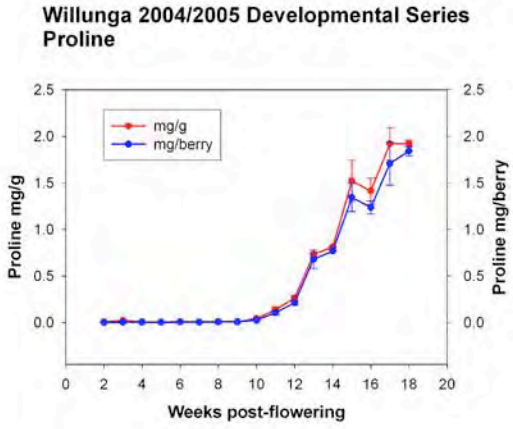
(h)



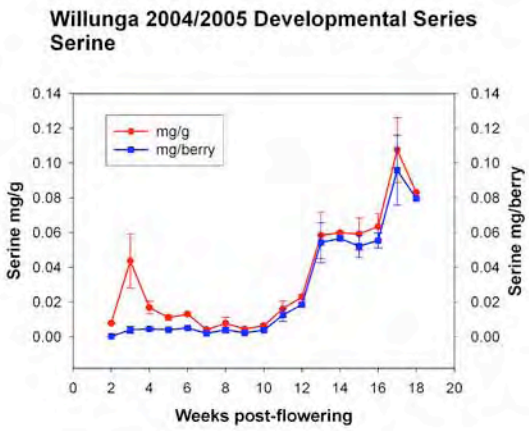
(i)



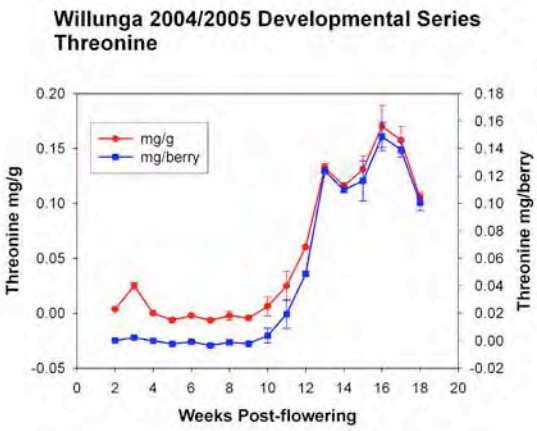
(j)



(k)

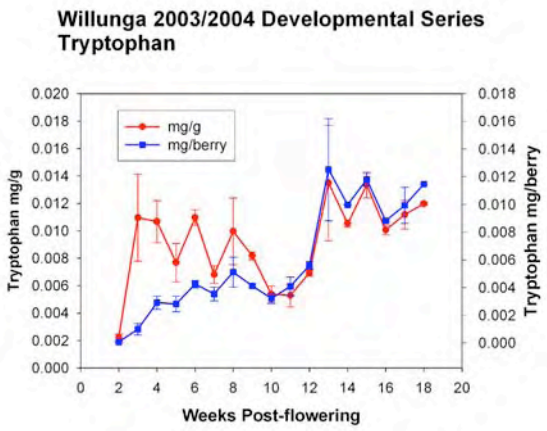


(l)

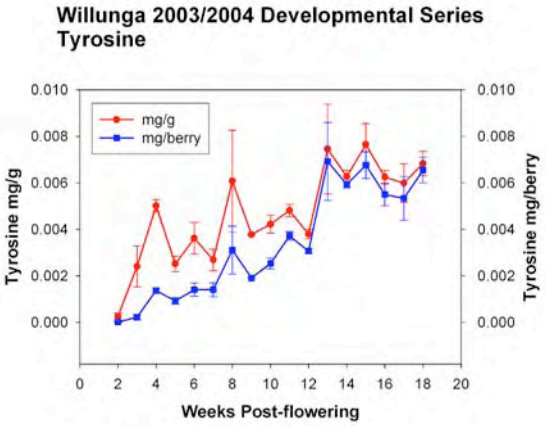


Figures 6m-o.

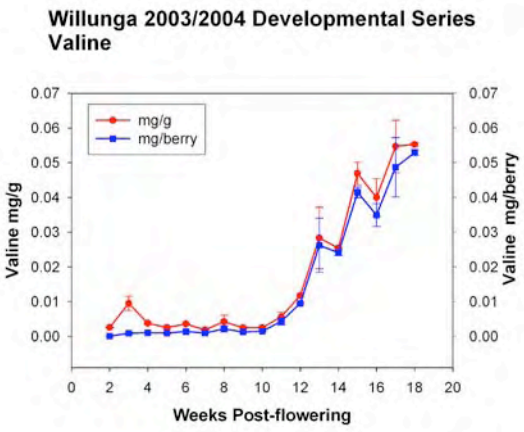
(m)



(n)

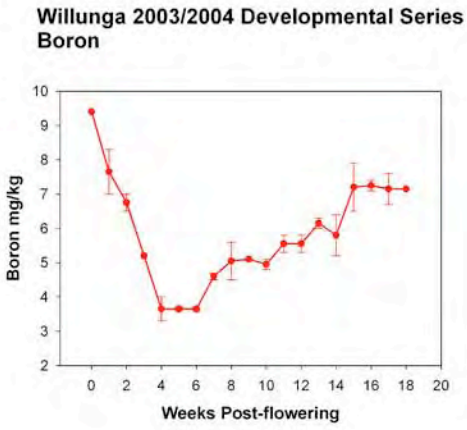


(o)

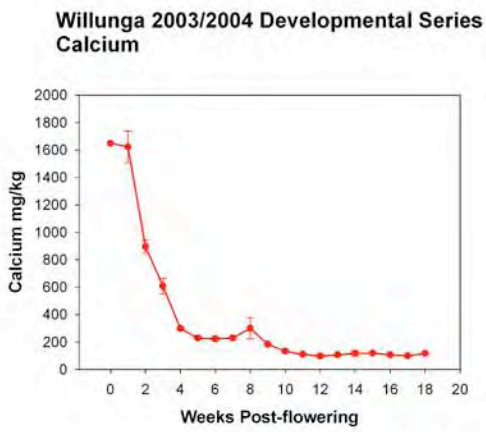


Figures 7a-f.

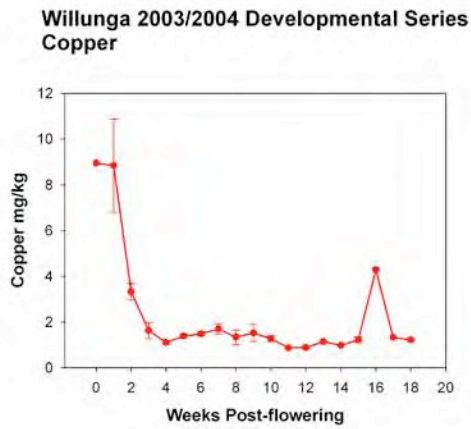
(a)



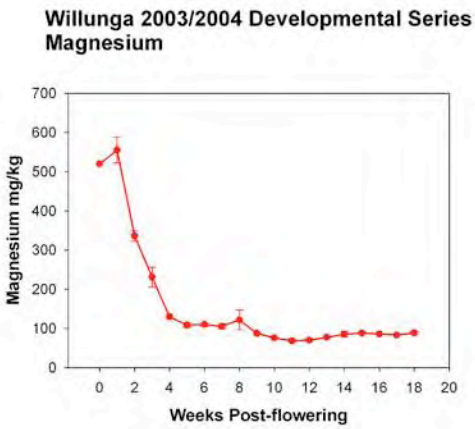
(b)



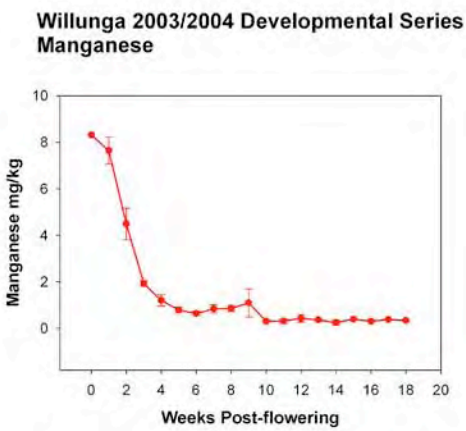
(c)



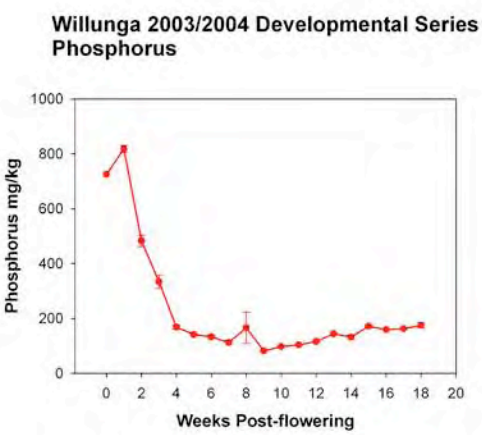
(d)



(e)

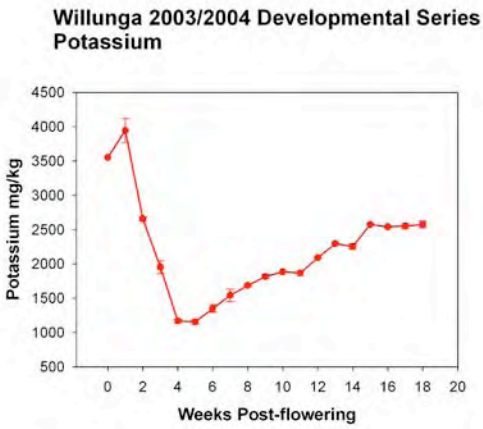


(f)

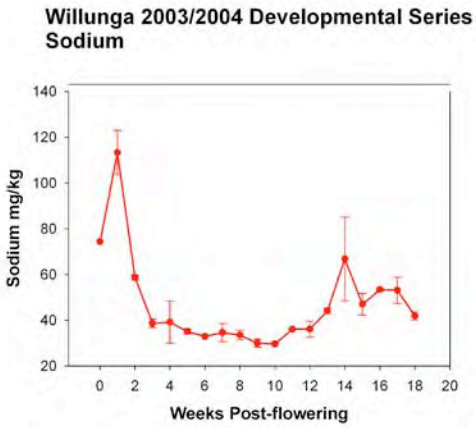


Figures 7g-j.

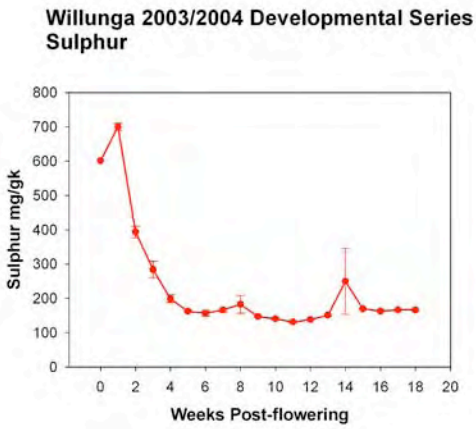
(g)



(h)



(i)



(j)

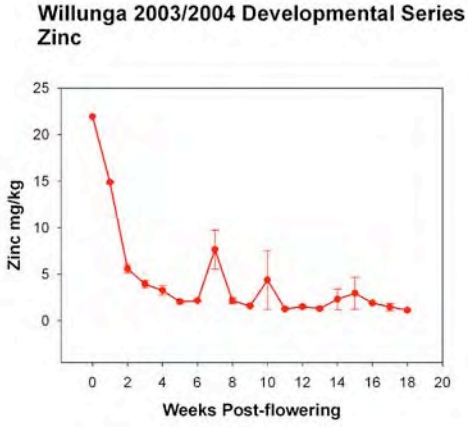


Figure 8. Quality control assessment of Affymetrix grapevine GeneChips.

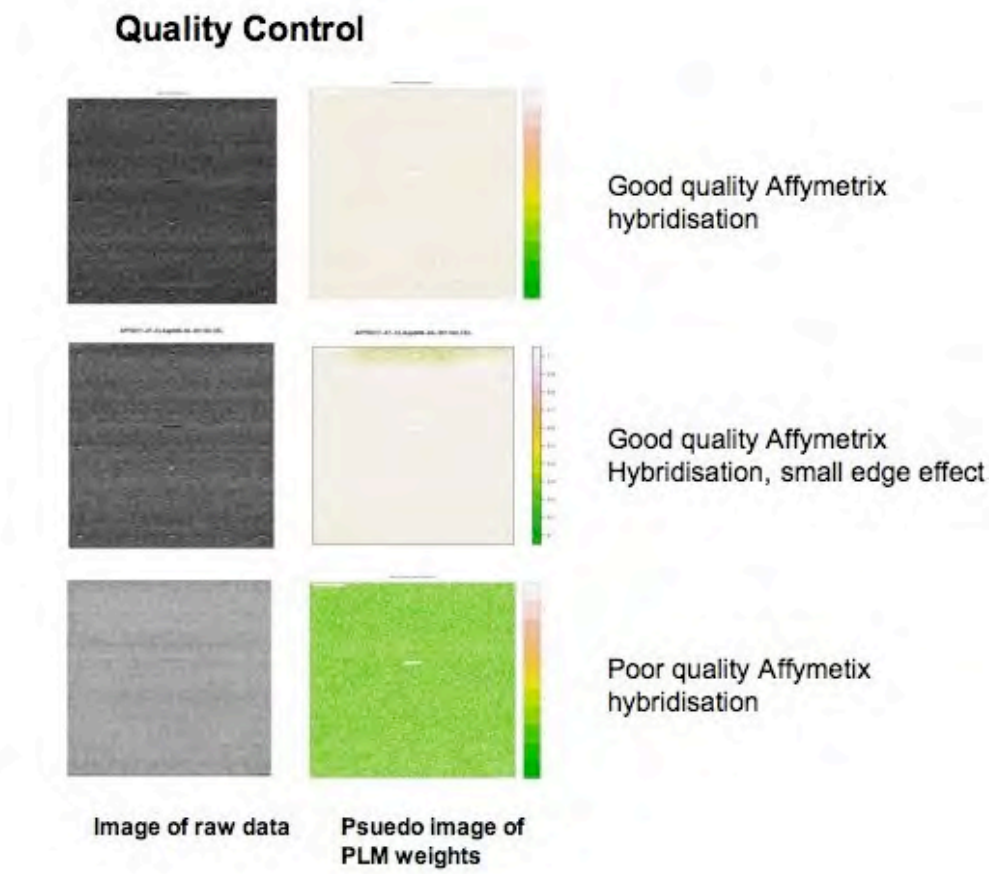


Figure 9. Quality control assessment of GeneChips hybridizations.

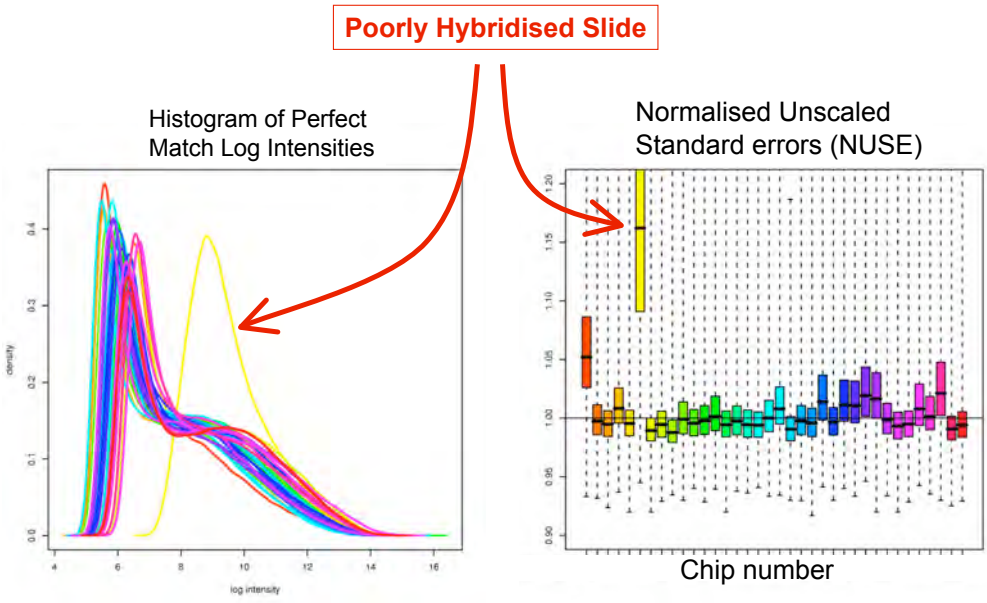


Figure 10. An example of a pre-veraison berry gene whose expression is regulated by light during a 24 hour period.

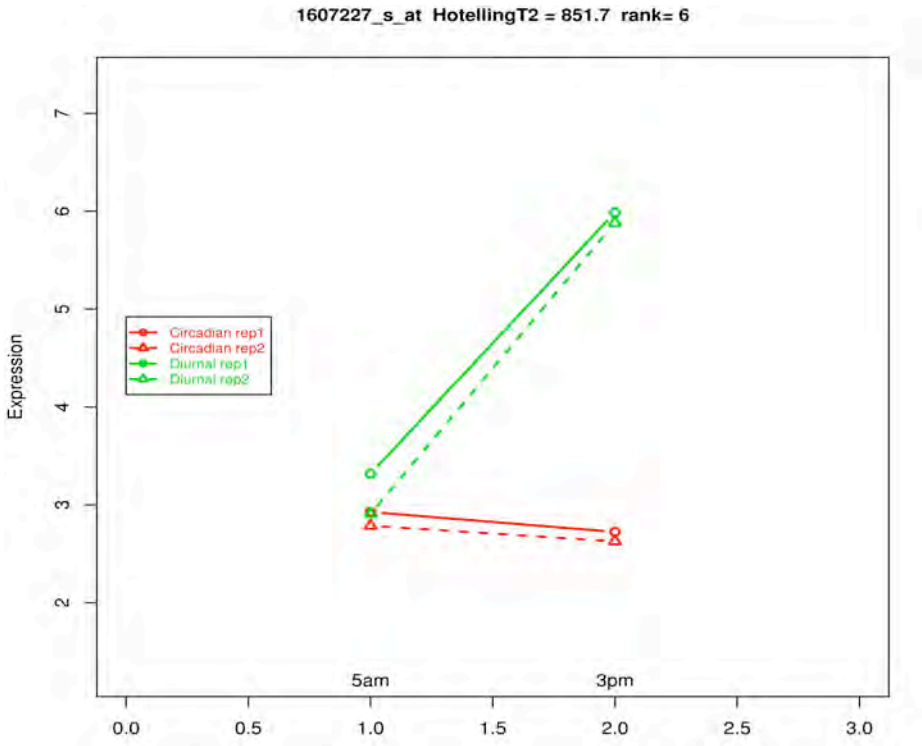


Figure 11. An example of a pre-veraison berry gene whose expression is regulated by an internal clock instead of light during a 24 hour period.

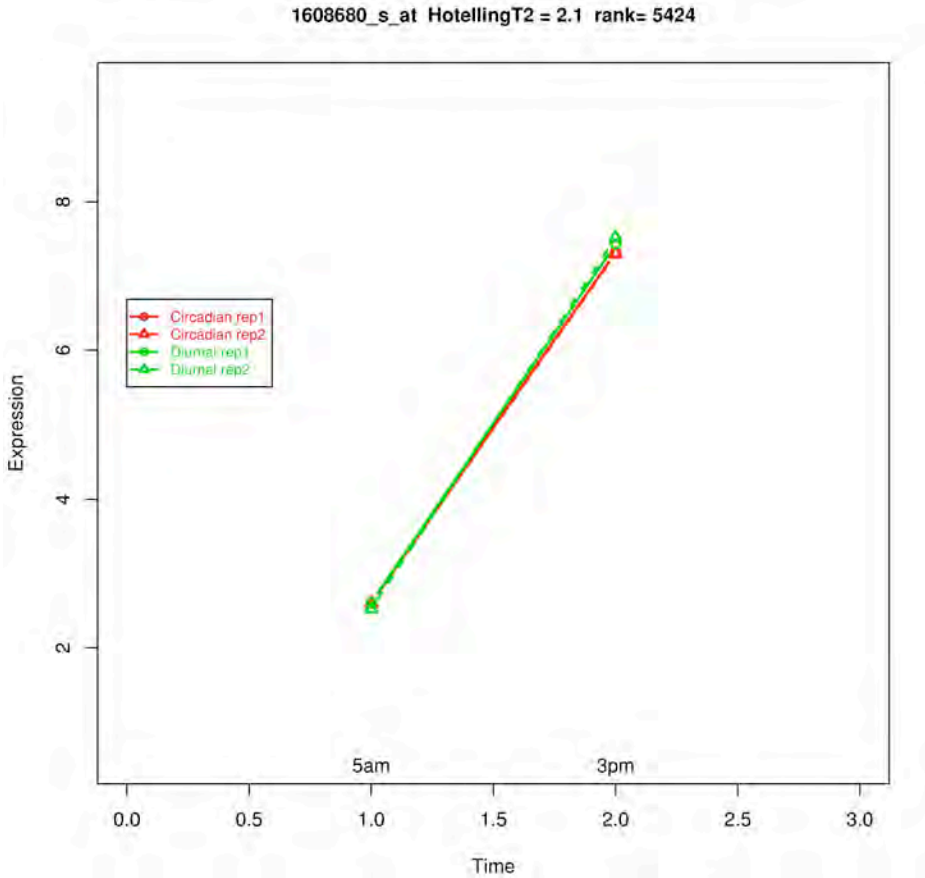
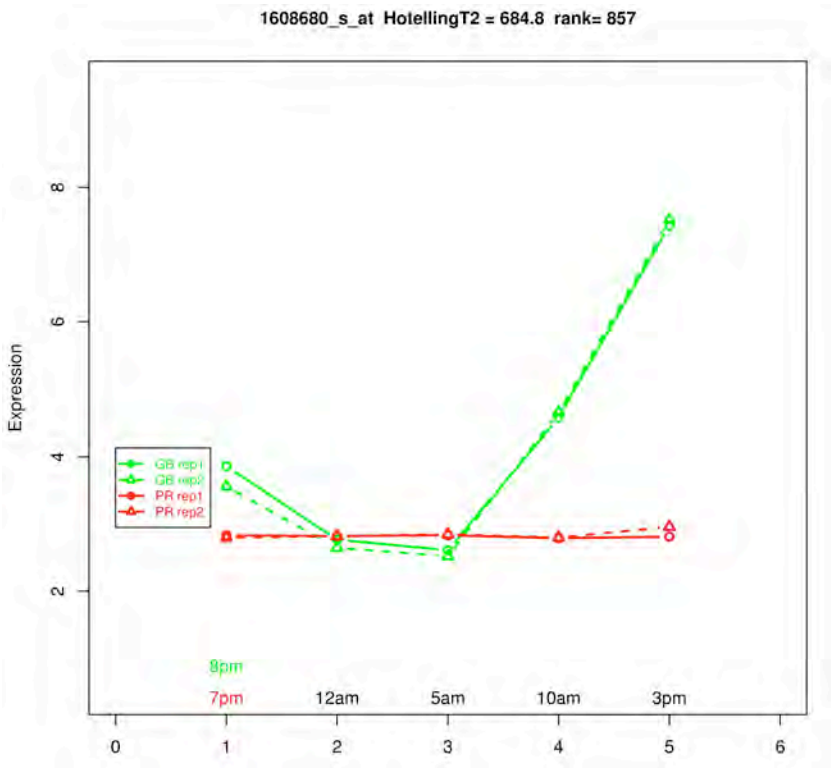


Figure 12. An example of two genes whose expression during a 24 hour period differs significantly dependin on the stage of berry development. GB, pre-veraison; PR, post-veraison.

(a)



(b)

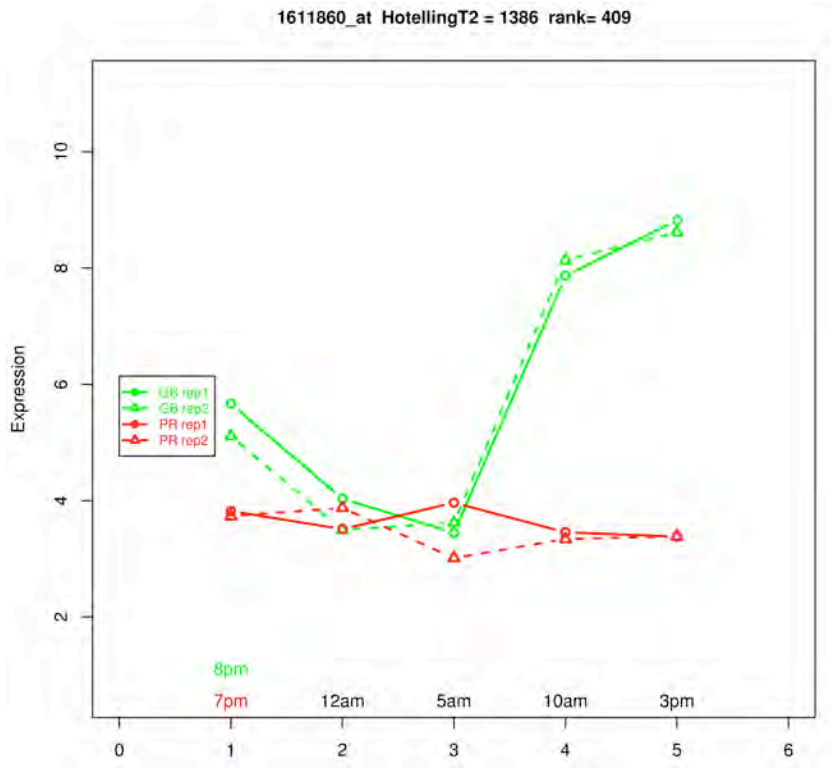


Figure 13. Flower to berry transition gene changes. An example of a gene expression profile that that shows a significant rapid decrease after anthesis (time point 1). Time points represent weekly intervals post-anthesis. Data from 4 separate developmental series.

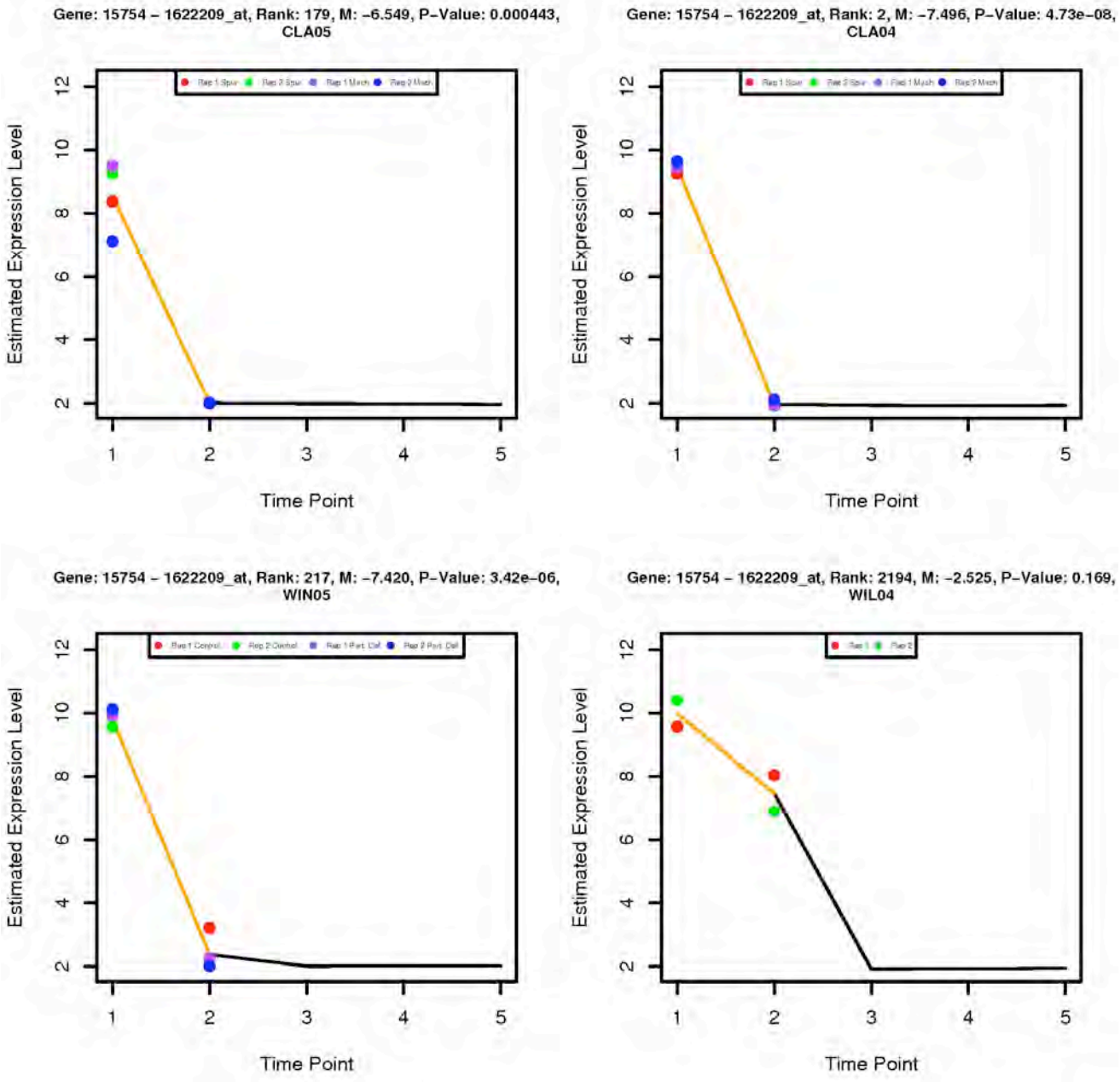


Figure 14. Flower to berry transition gene changes. An example of a gene expression profile that that shows a significant rapid decrease after anthesis (time point 1). Time points represent weekly intervals post-anthesis. Data from 4 separate developmental series.

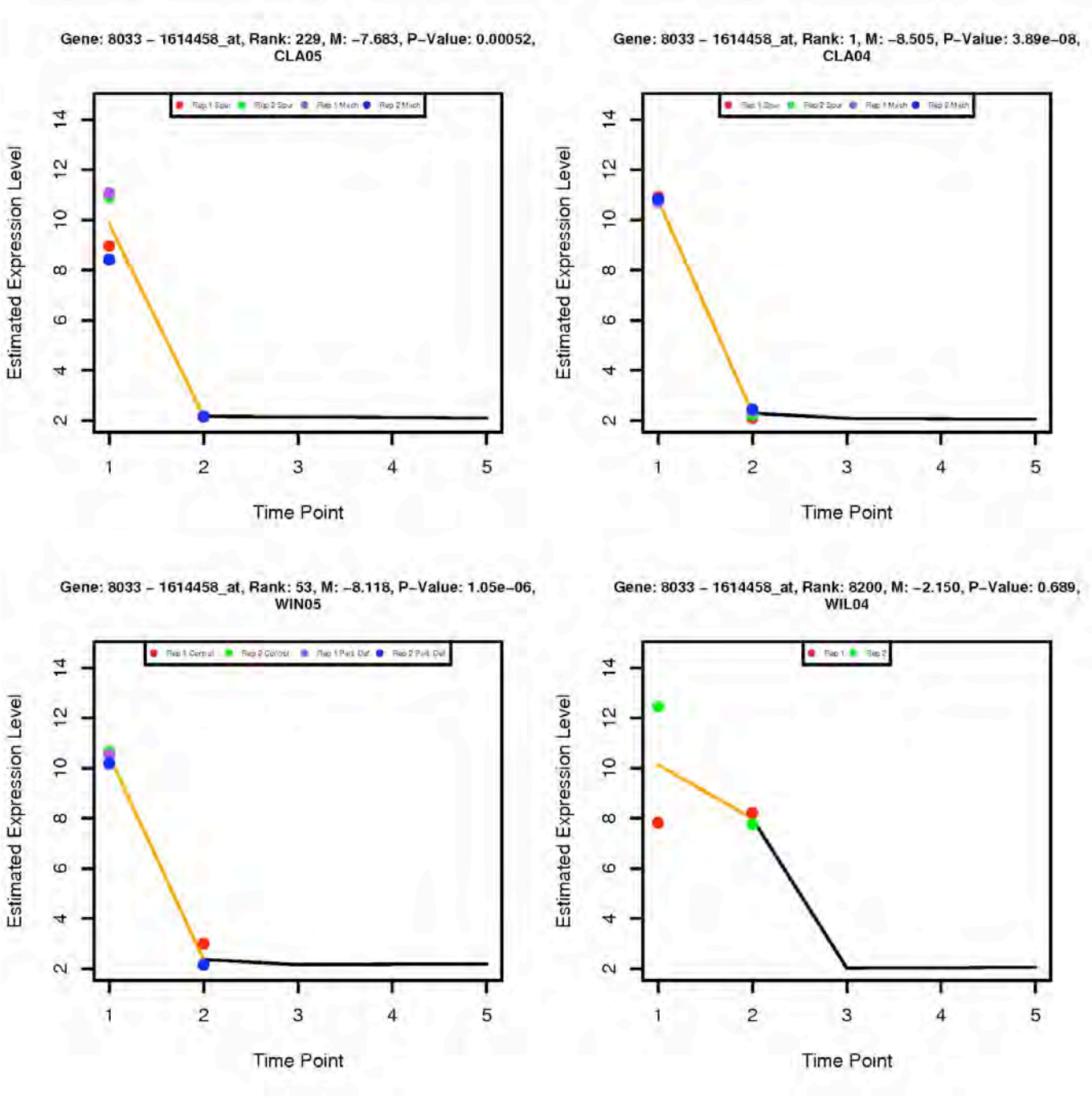


Figure 15. Flower to berry transition gene changes. An example of a gene expression profile that shows a significant rapid increase after anthesis (time point 1). Time points represent weekly intervals post-anthesis. Data from 4 separate developmental series.

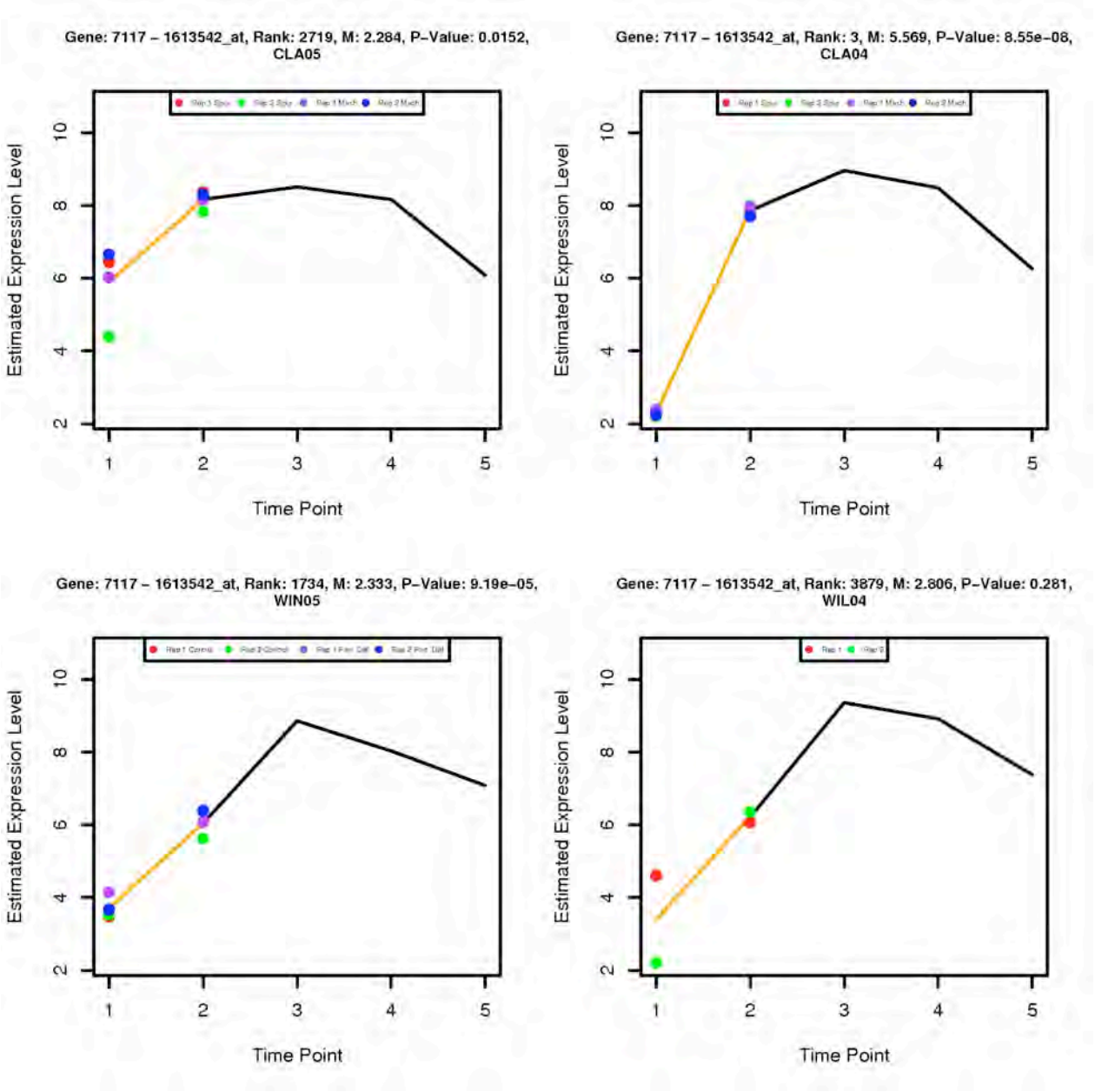


Figure 16. Flower to berry transition gene changes. An example of a gene expression profile that shows a significant rapid increase after anthesis (time point 1). Time points represent weekly intervals post-anthesis. Data from 4 separate developmental series.

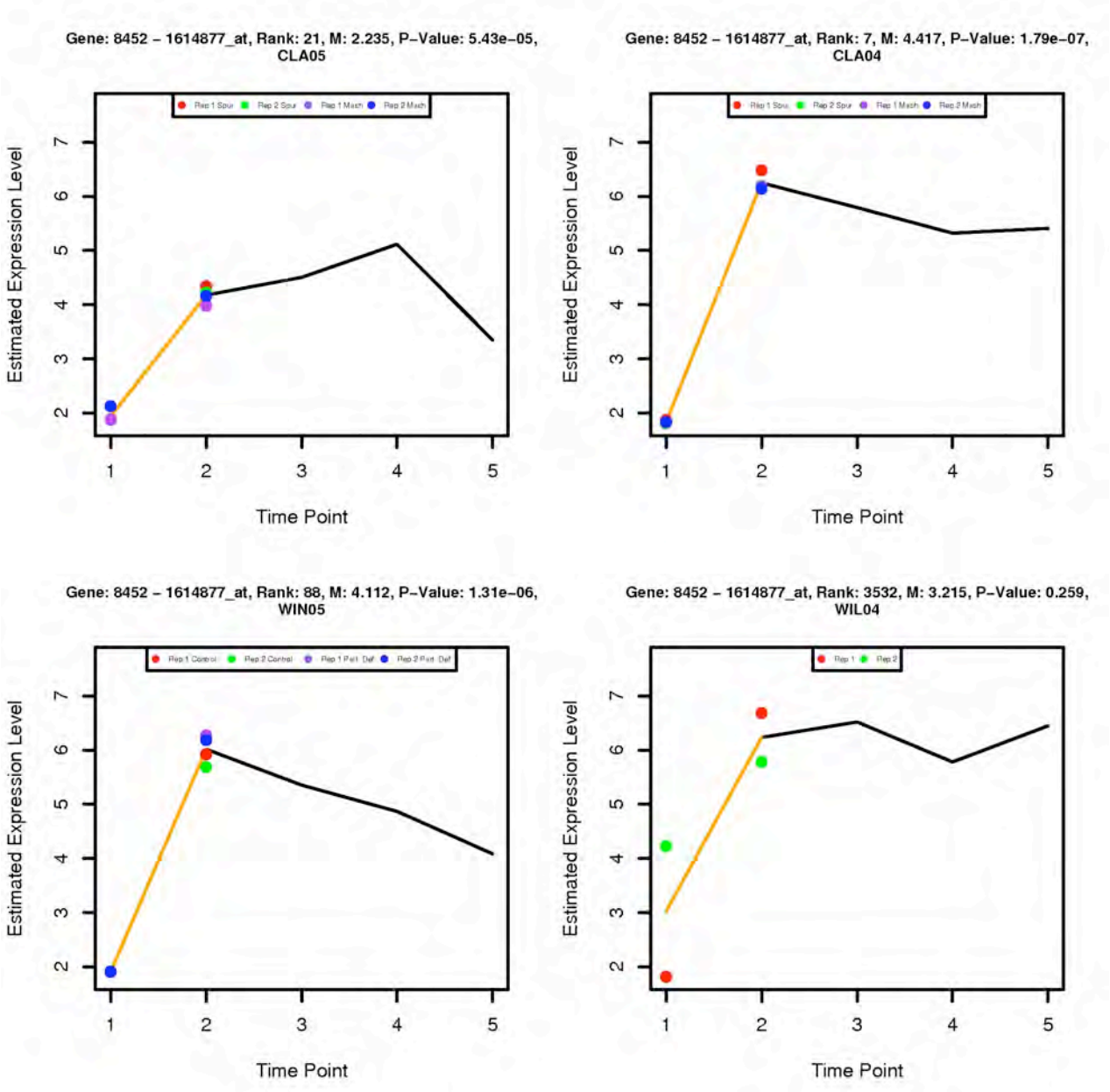
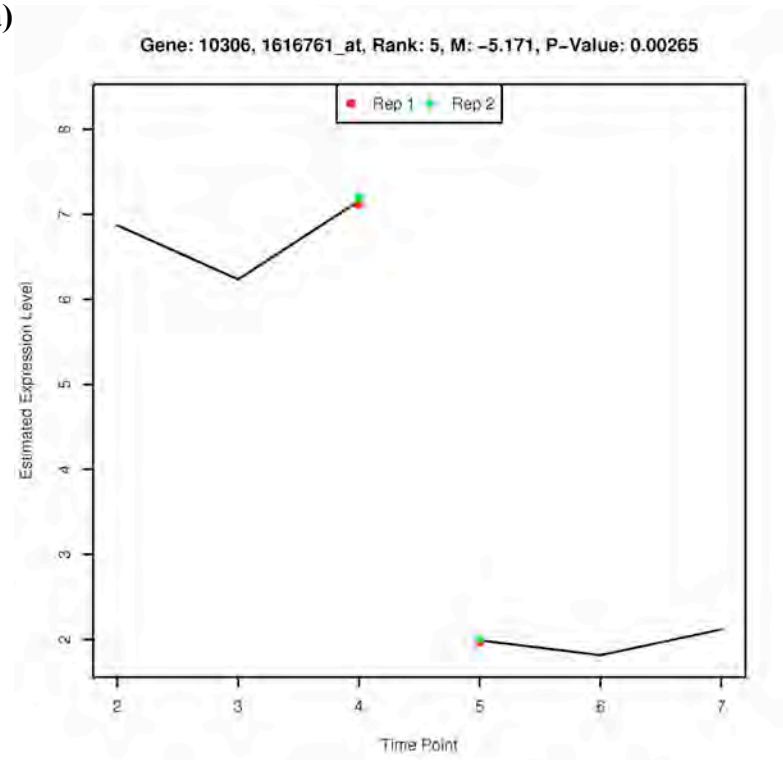


Figure 17. Examples of gene expression profiles that showed a significant decrease in expression after seed removal . The break in the plot lines represents the transition time points from seeded berries to berries without seeds. Data from Willunga (WIL-04) berry developmental series.

(a)



(b)

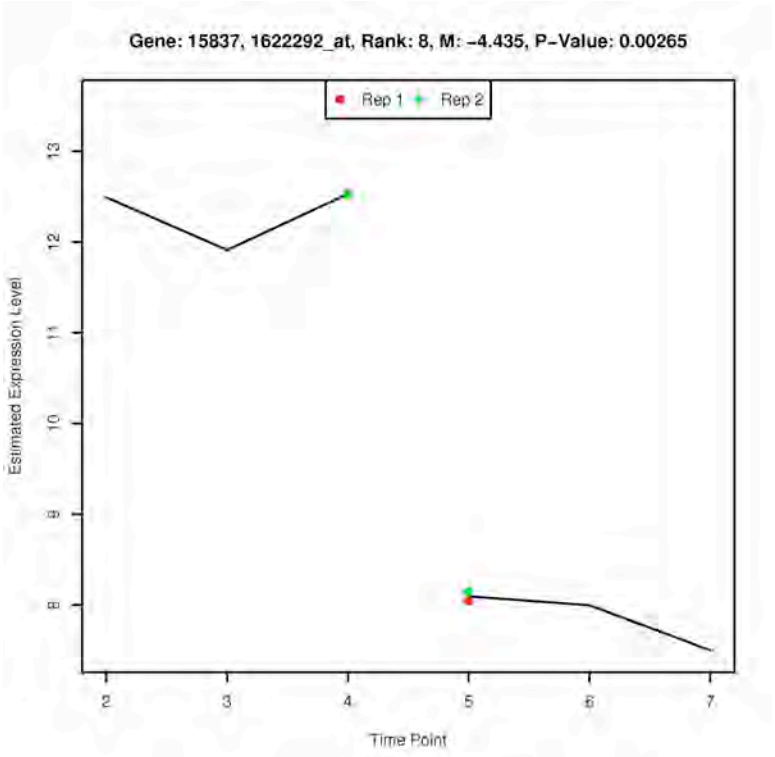


Figure 18. Examples of gene expression profiles that showed a significant decrease in expression after seed removal . The break in the plot lines represents the transition time points from seeded berries to berries without seeds. Data from Clare (CLA05), Wingara (WIN05) and Willunga (WIL04) berry developmental series.

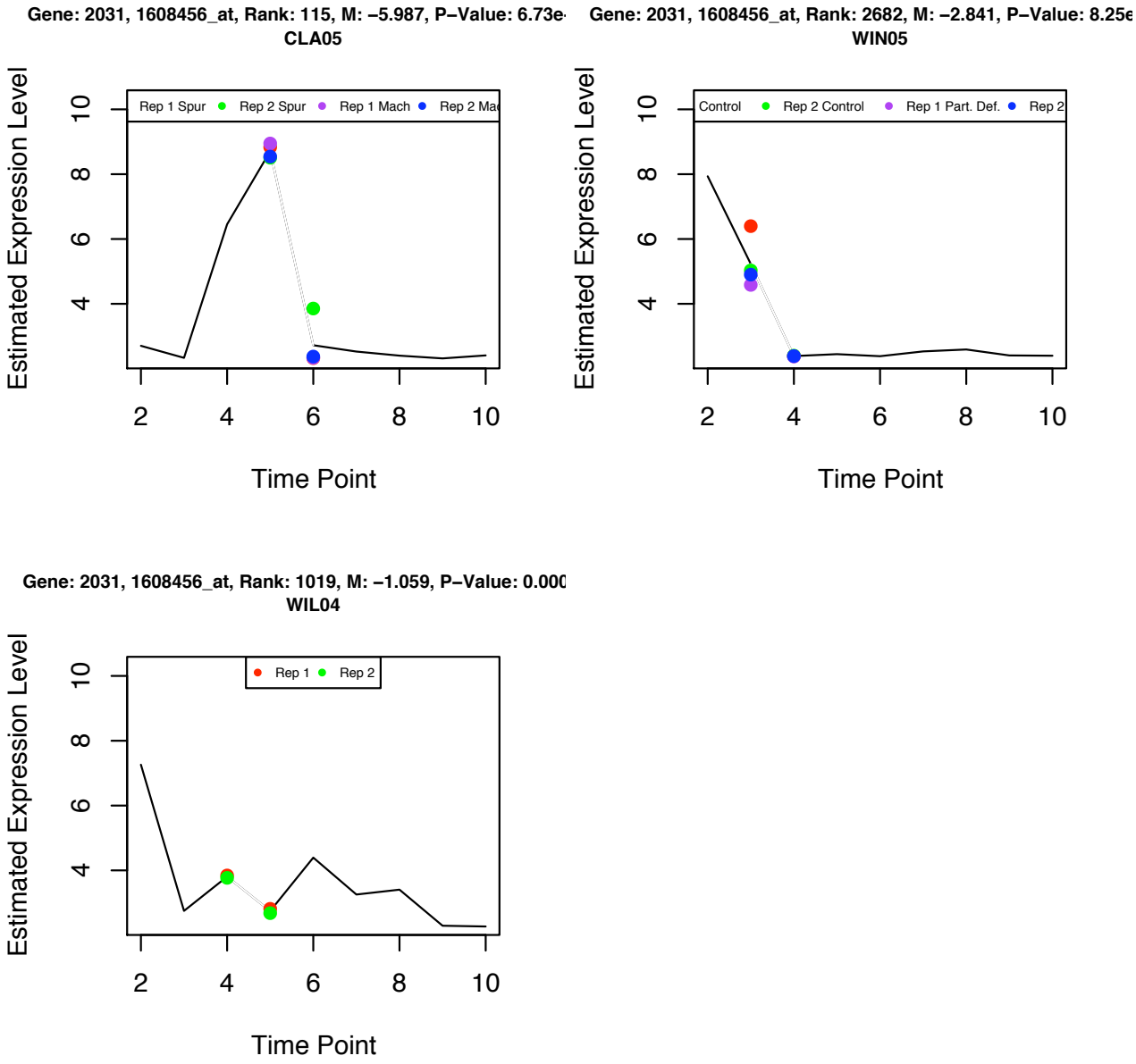


Figure 19. **Number of Genes that Significantly Change Expression Levels During Berry Development - Willunga 2003/2004**

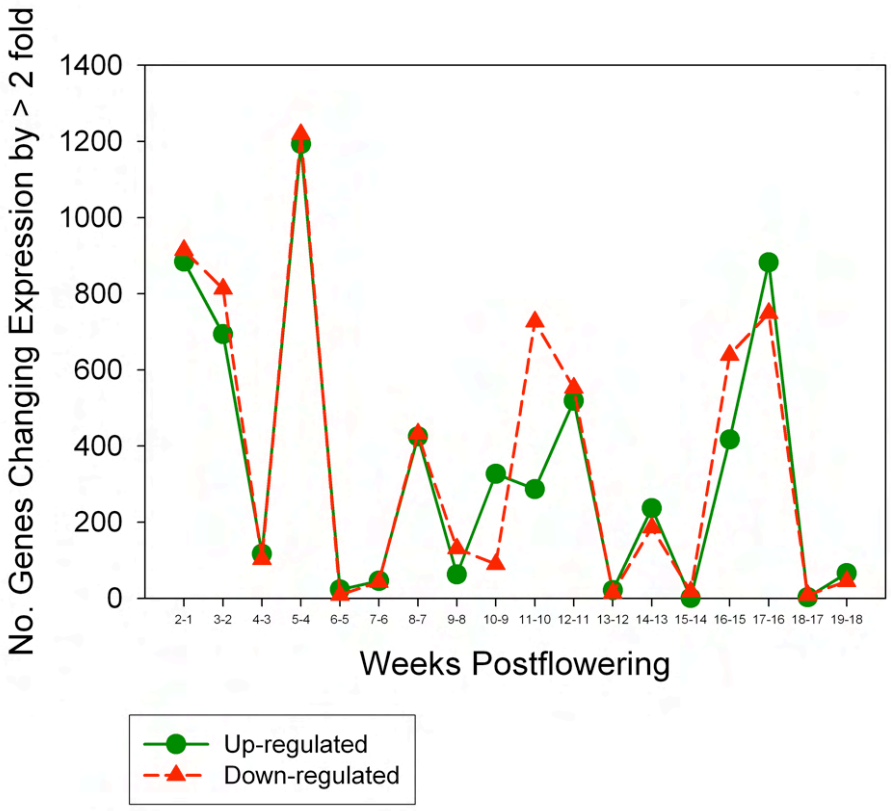
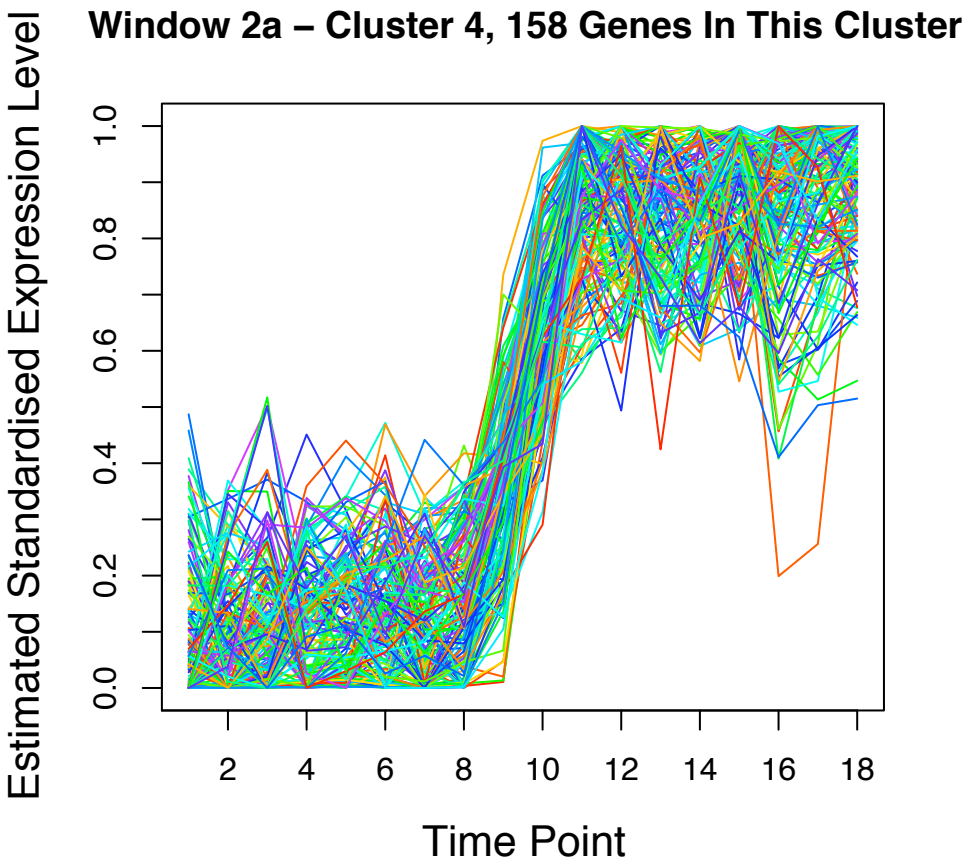


Figure 20. An example gene expression cluster after CLARA clustering for 50 clusters. (a) Cluster showing all 158 genes assigned to the cluster. (b) Mean profile of cluster 4. Data from the Willunga (WIL04) berry developmental series.

(a)



(b)

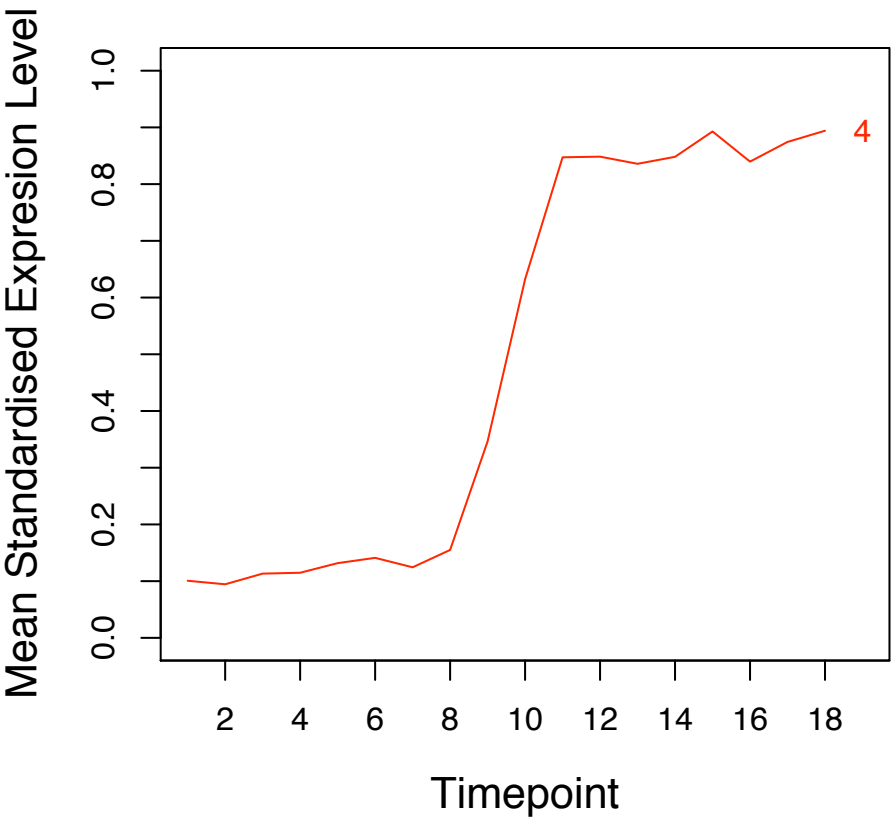


Figure 21. Principal Component Analysis of the 50 CLARA clusters to identify similar expression profiles. Data from the Willunga (WIL04) berry developmental series. Cluster numbers that are near each other represent clusters with similar profiles.

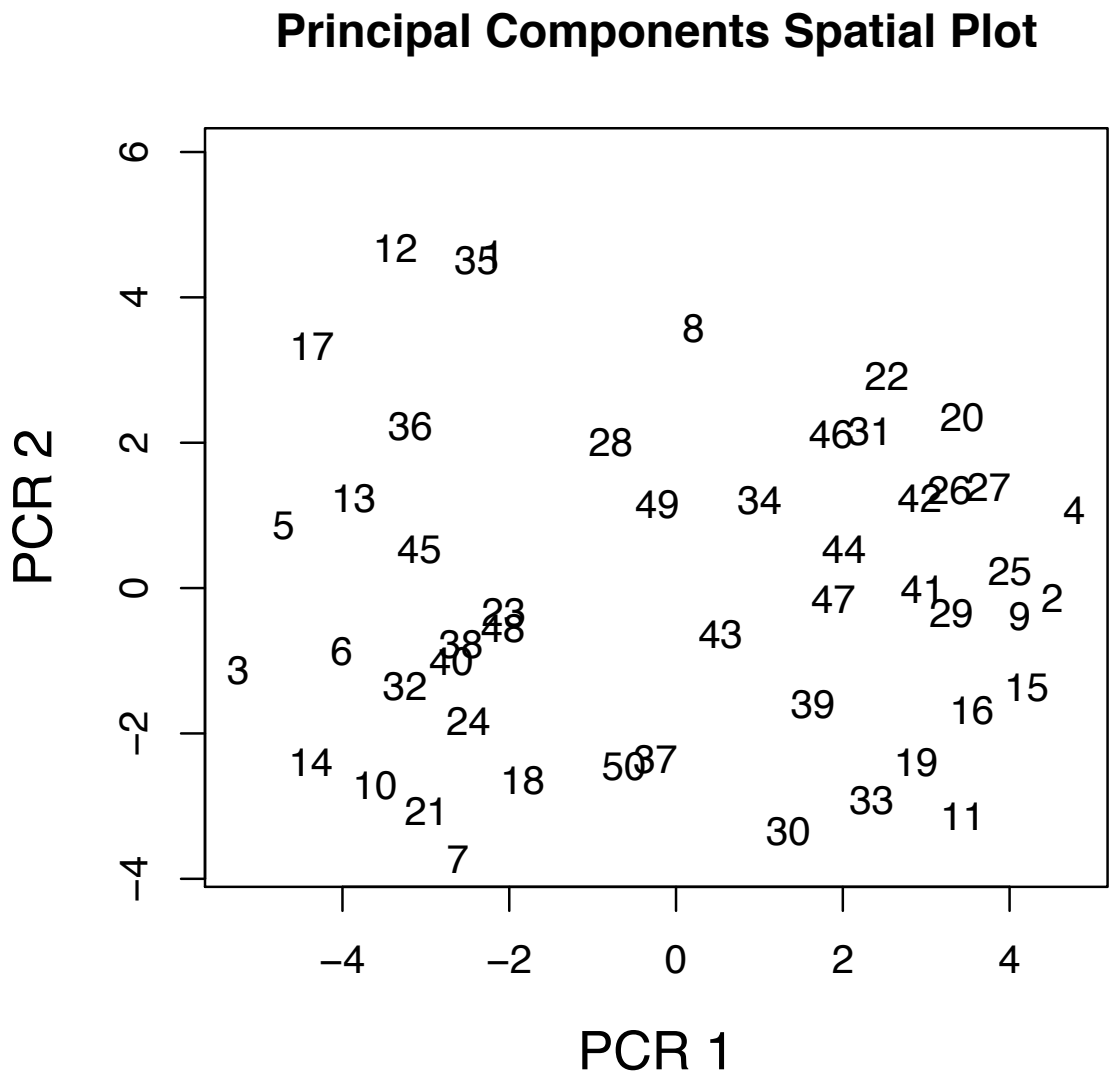
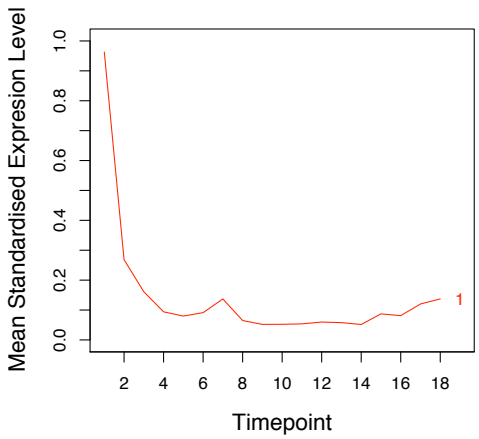
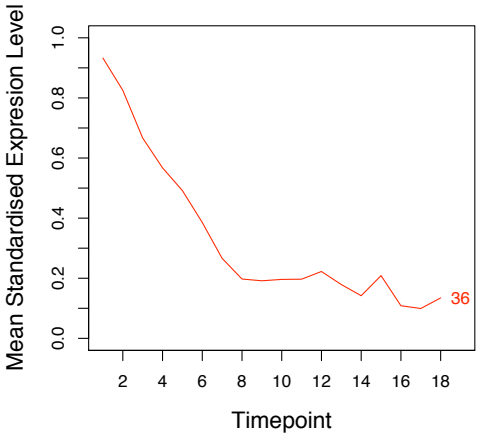
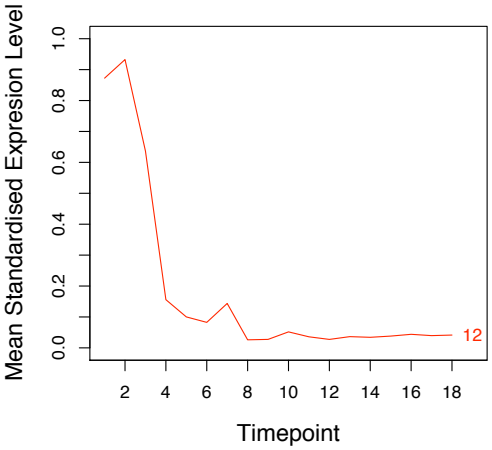
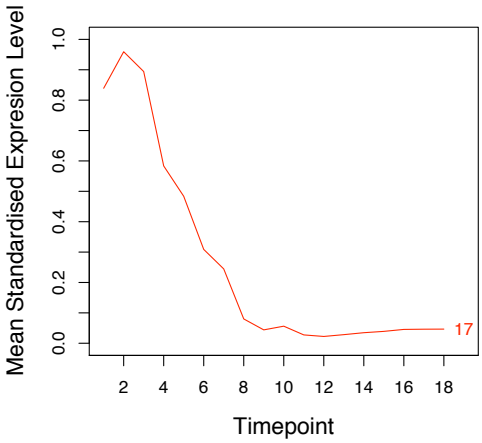


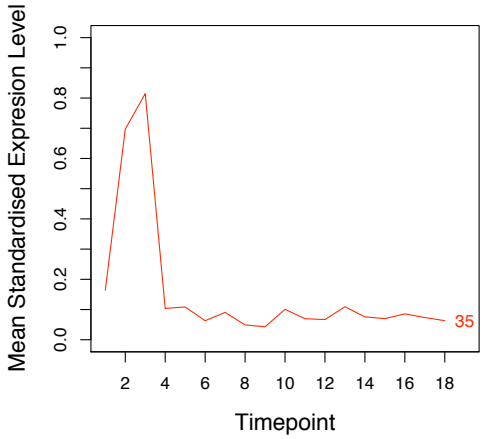
Figure 22a. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

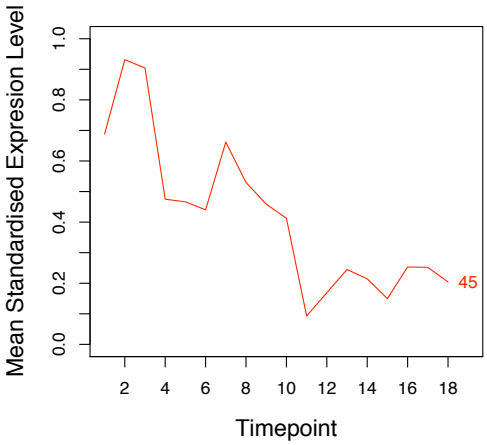


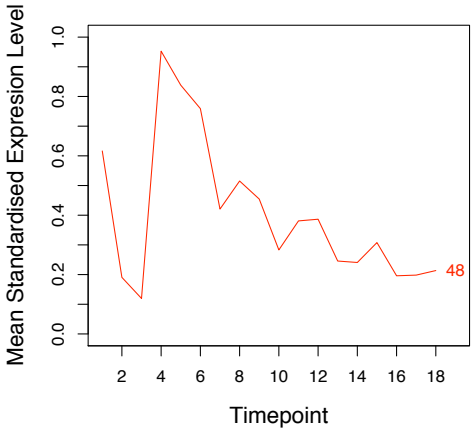












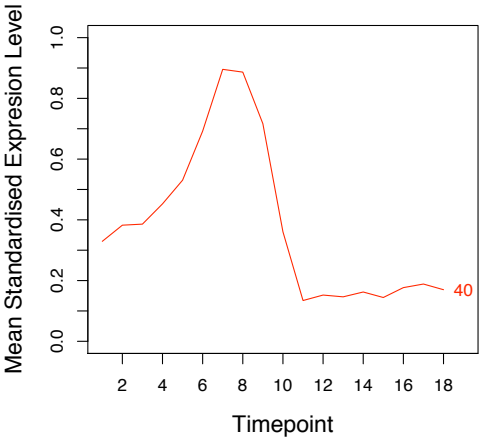
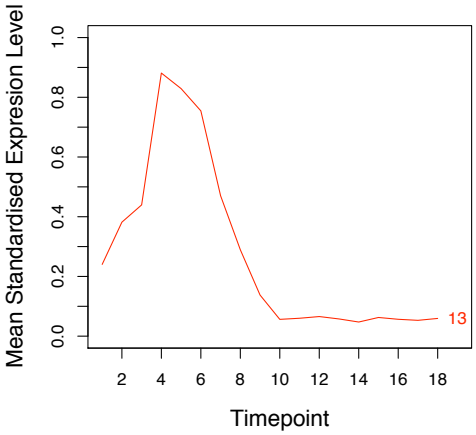
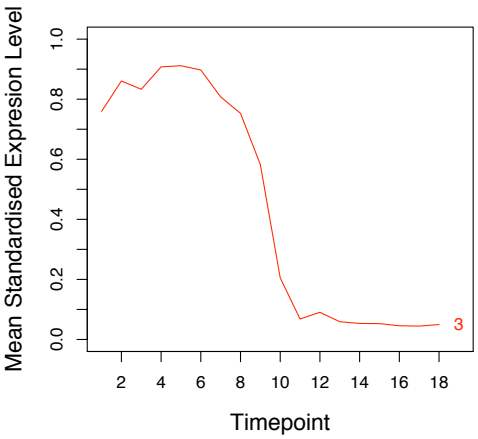
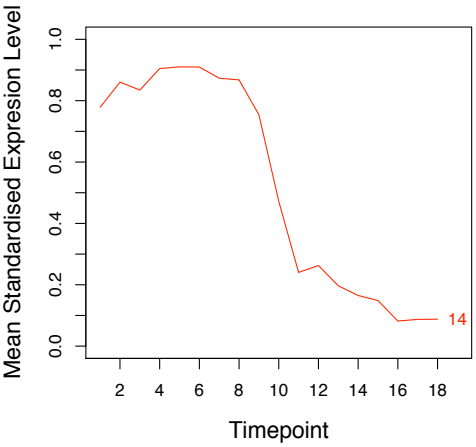
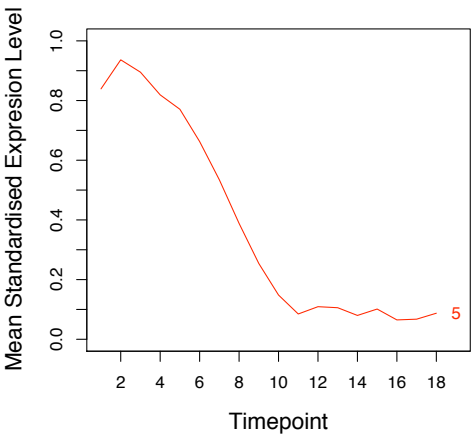


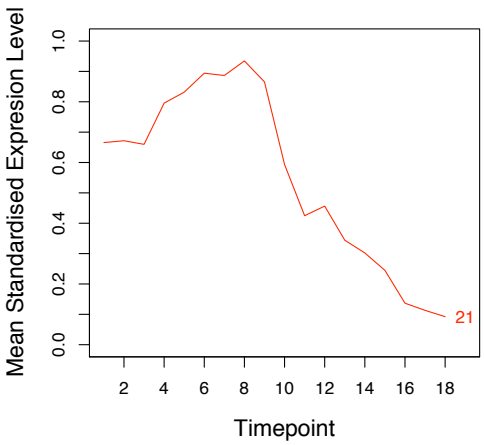
Figure 22b. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

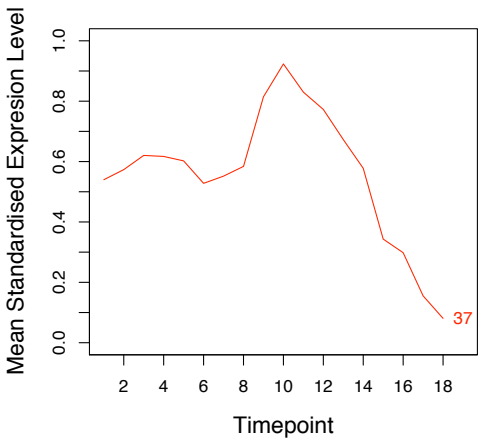


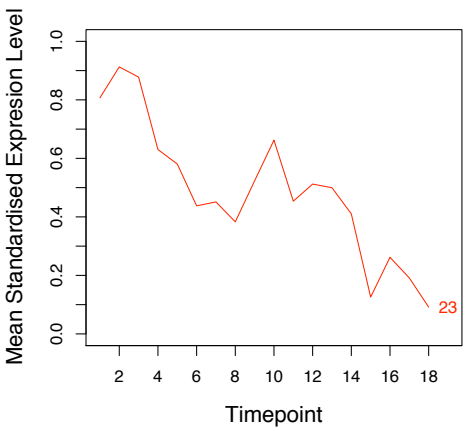












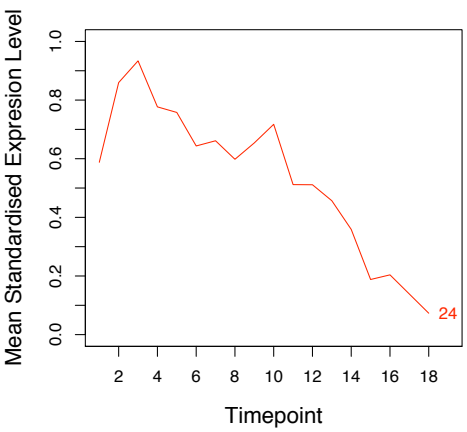
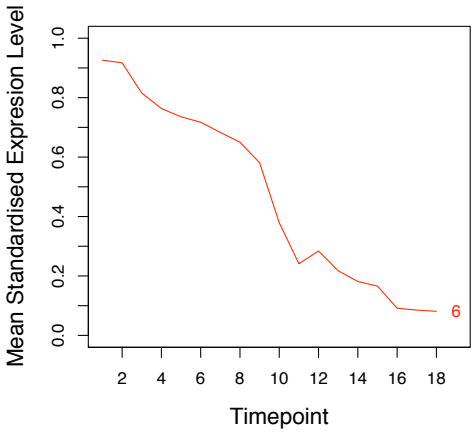
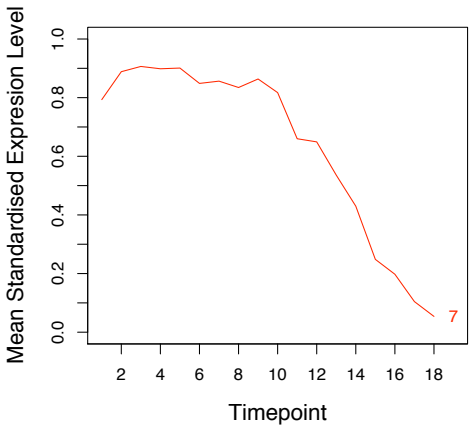
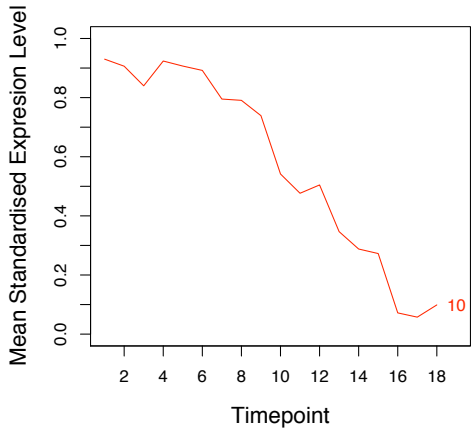
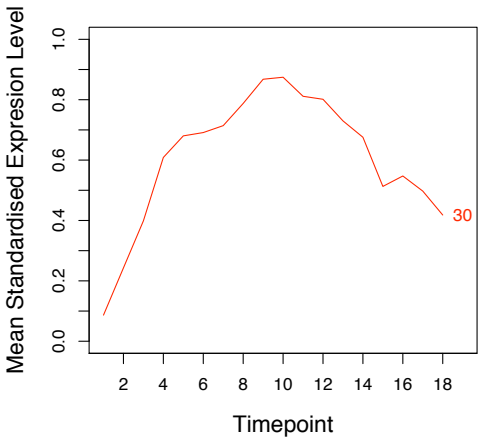


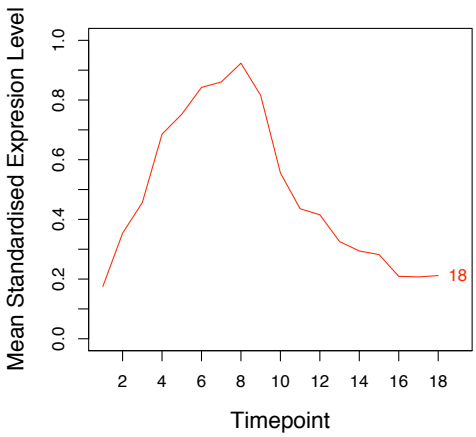
Figure 22c. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

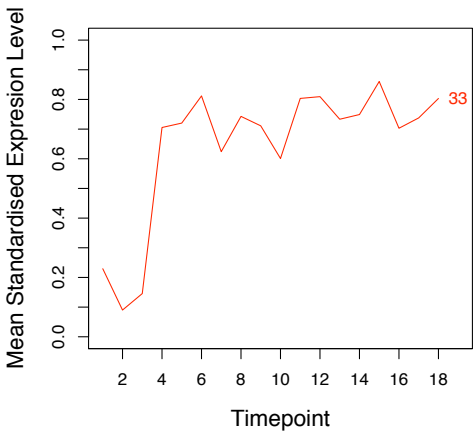


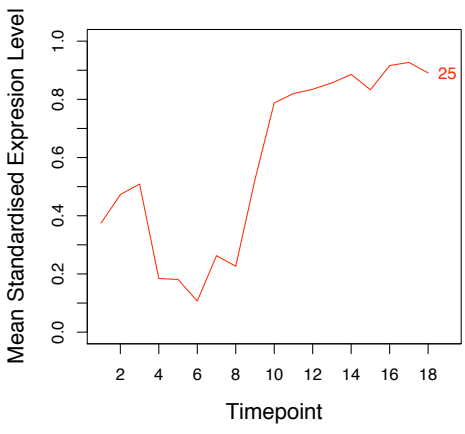












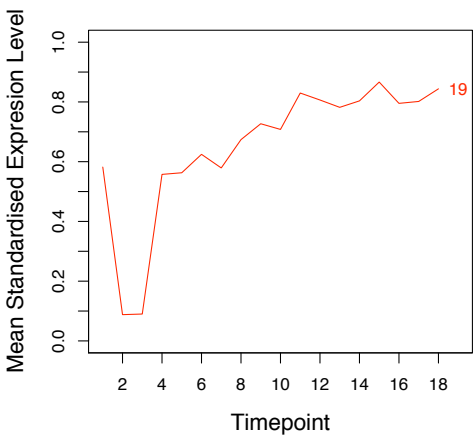


Figure 22d. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

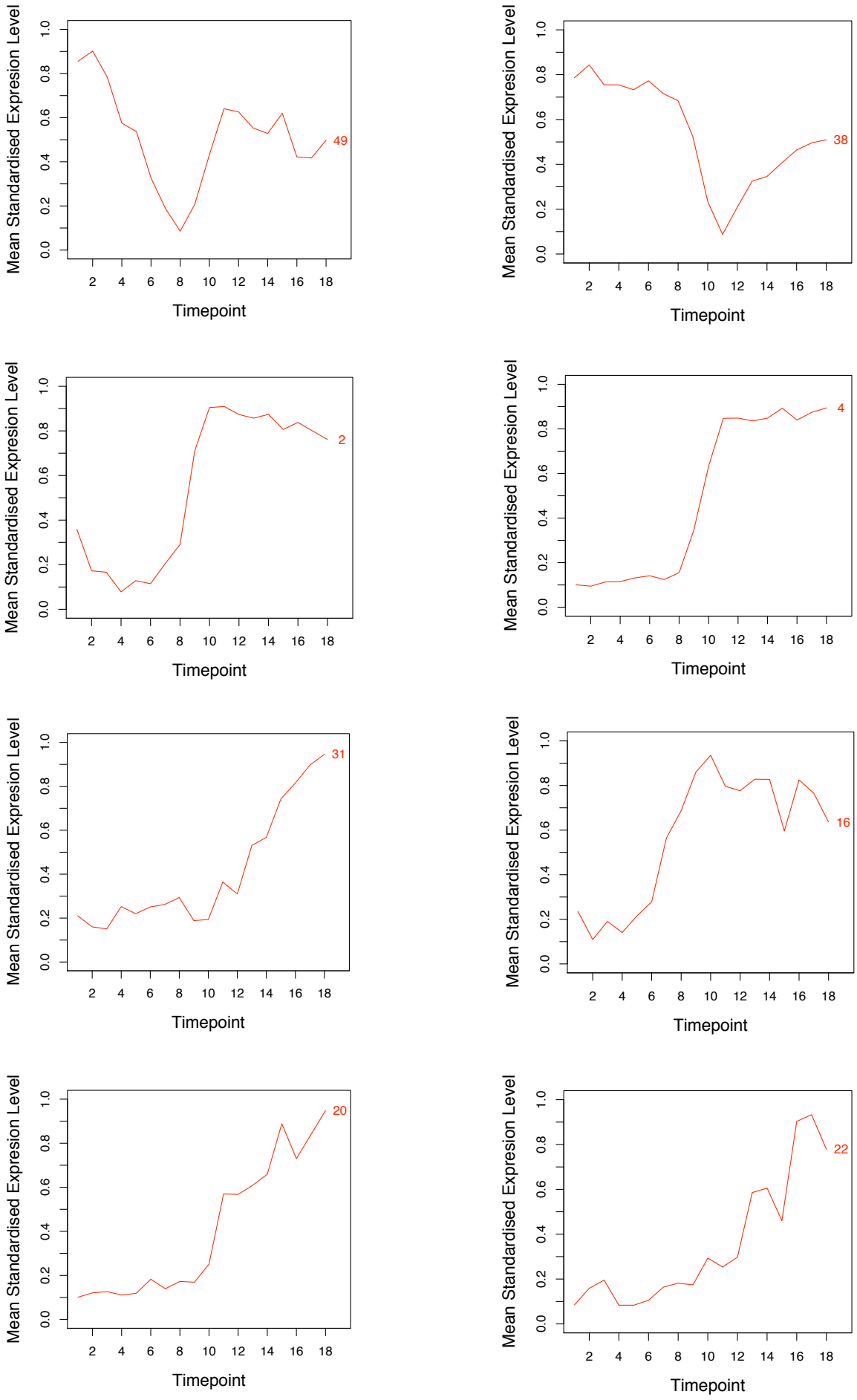
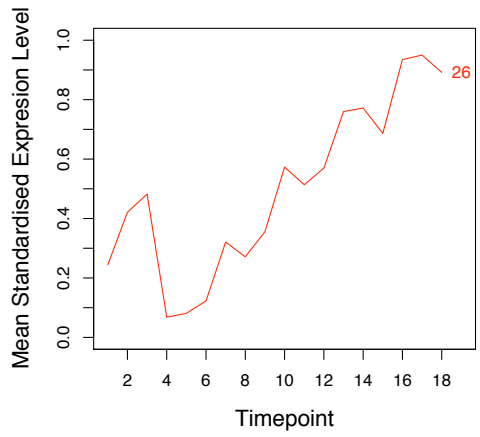
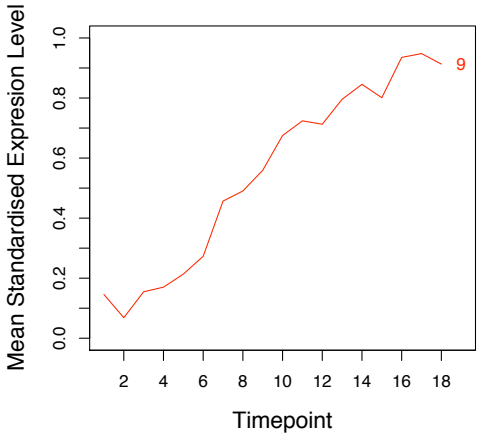
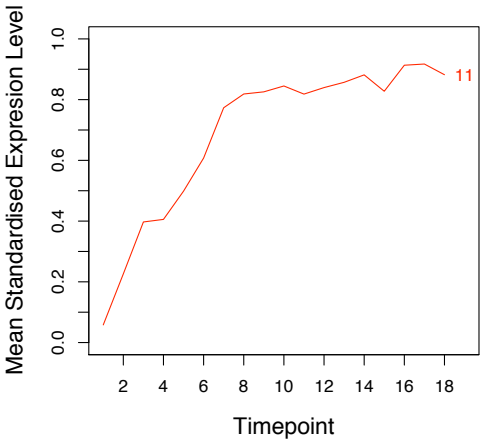
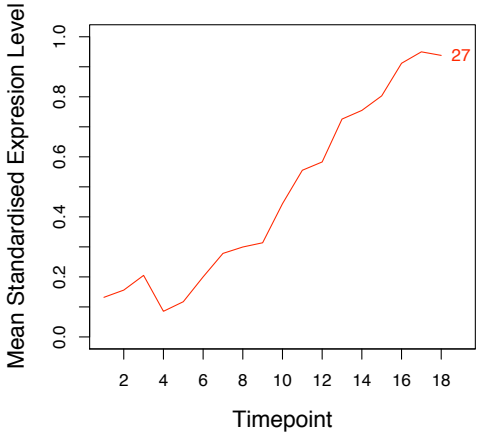


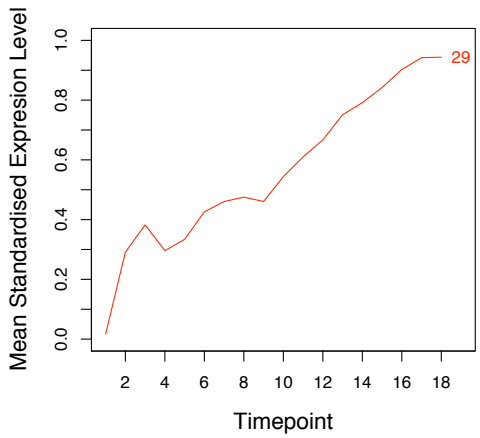
Figure 22e. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

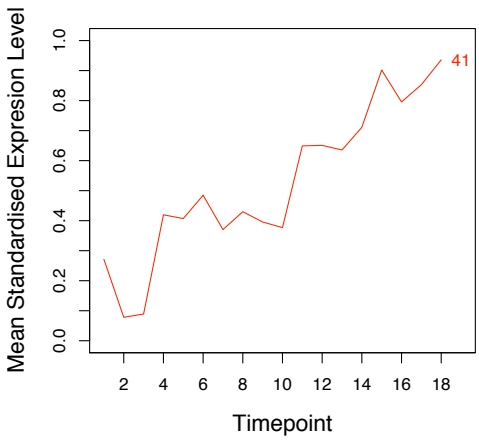


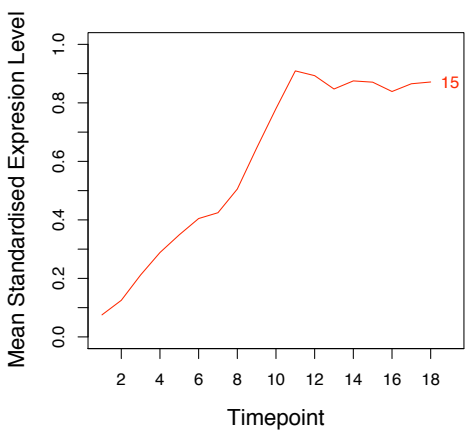












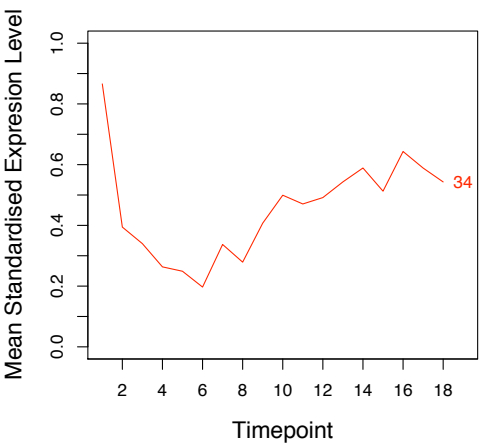


Figure 22f. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.

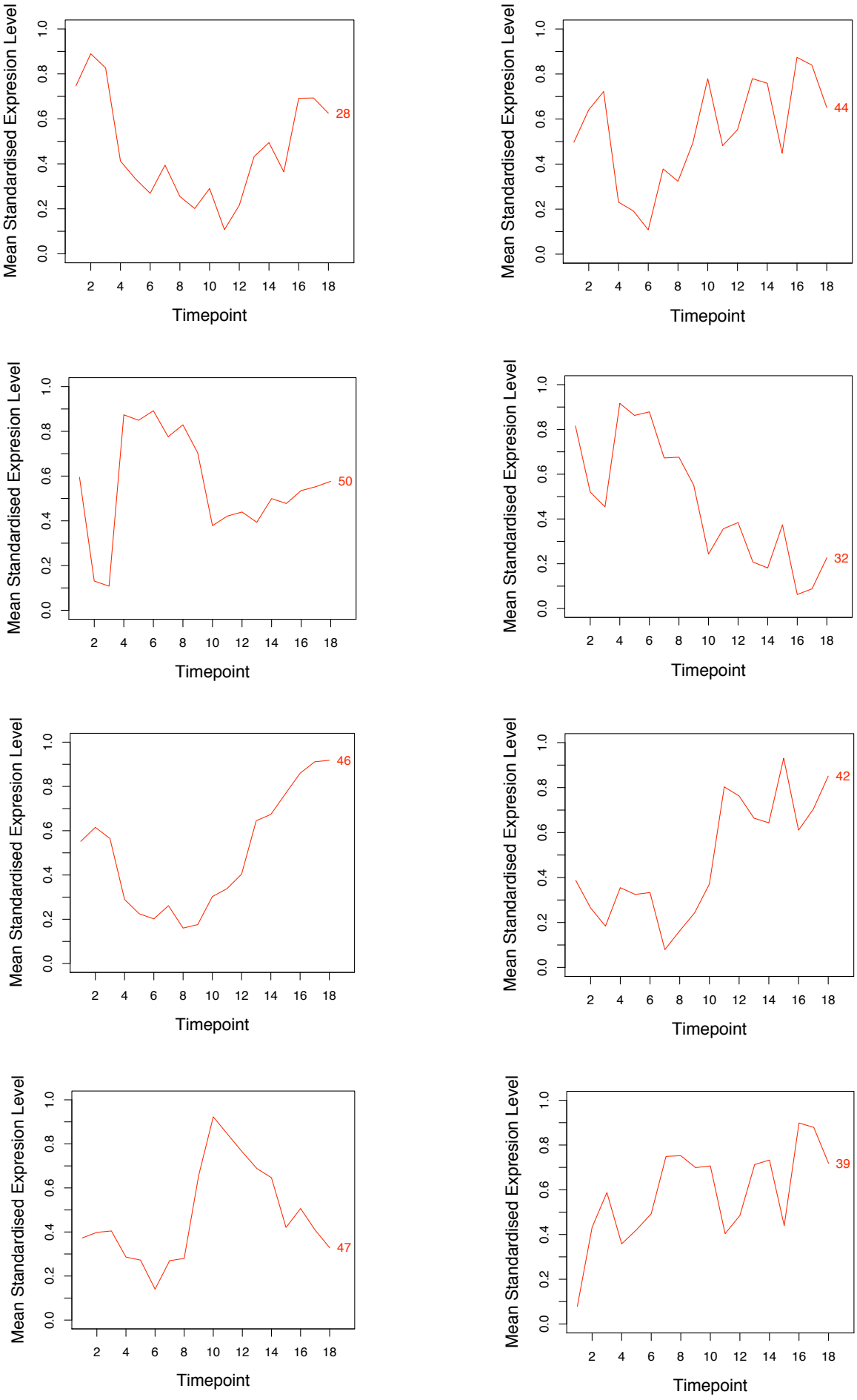
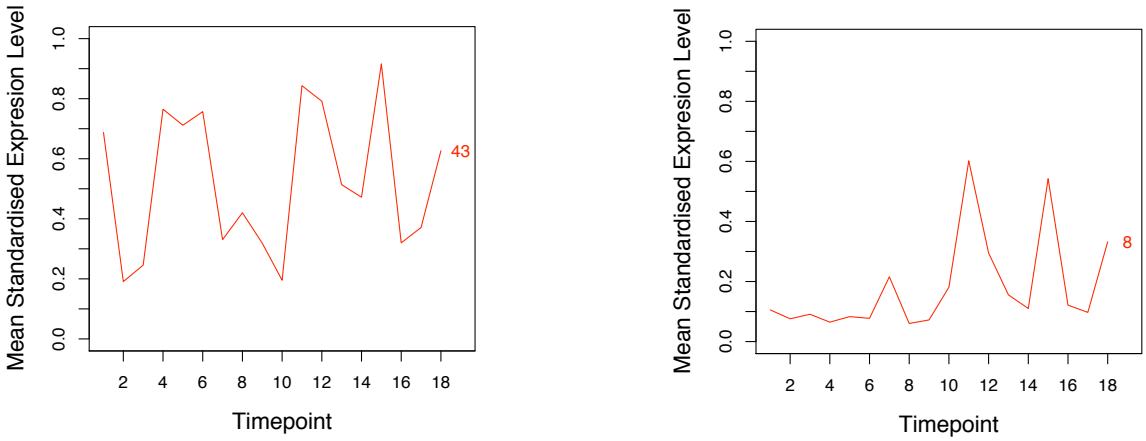


Figure 22g. Mean expression profiles during berry development. Data from the Willunga (WIL04) berry developmental series.



Hardy Wine Company, Clare 2003/2004 (CLA-04), 2004/2005 (CLA-05)

A trial established by the Hardy Wine company to investigate the effect of three different pruning regimes (Rod, Spur and Machine) on vine growth and berry quality was sampled over two seasons. Two treatments were sampled i.e. Spur pruned v Machine pruned vines. Flower and fruit samples were collected on a weekly (Spur pruned) and fortnightly (Machine pruned) basis during the 2003/2004 season commencing on 24/11/2003. The last sample from which usable RNA was extracted was taken on the 9/2/2004, shortly after veraison (Table 2). Analysis of a full developmental series was not possible as a very hot spell of weather severely damaged the vines and fruit and RNA could not be extracted from the fruit harvested after 9/2/2004. During the 2004/2005 season a full developmental series from flowering to commercial harvest was sampled (from 23/11/2004 to 16/03/2005). Both the Spur and Machine pruned vines were sampled at one week intervals.

Measurements of Berry Development & Composition

The progress of berry development was followed by measuring berry weight and the levels of total soluble solids (Fig. C1). Again the pattern of berry size increase was bisigmoidal although the lag phase appears longer than that observed for the Willunga series. The berry weight of the fruit from Machine pruned vines was less than that from Spur pruned vines at harvest (and throughout the majority of berry growth). Previous measurements on these vines over successive years has shown that machine pruning tends to encourage the production of more bunches having smaller fruit (Warren Birchmore pers. comm.). For the 2004/2005 season the yield difference between the two treatments was 5.85 t/ha for Machine pruned vines and 3.58 t/ha for Spur pruned vines. This could be related to the difference between Machine and Spur pruned vines in bud node numbers at the start of the season (85 versus 50) and the resulting bunch number (59.40 versus 46.08) per vine (see Materials & Methods). Interestingly the Brix levels for fruit from the machine pruned vines were lower than that for the fruit from spur pruned vines (Fig. C1). This difference is also reflected in the total hexose levels at harvest with the Spur treatment at 236.13 g/L and the Machine at 199.91 g/L (Fig. C2). This difference might be explained by crop load differences between the two pruning treatments and availability of sugar from photosynthesis being available for import into the berry from the leaves. As for the Willunga series the accumulation of glucose is lower than that of fructose during the later parts of ripening. This may reflect the preference for glucose as a metabolic substrate as it might be expected that the levels of fructose and glucose might be equal as most of the hexoses present in the berry after veraison are thought to arise from the cleavage of the disaccharide sucrose into its constituent monosaccharides.

Organic Acid Accumulation

The pattern of accumulation of both tartrate and malate in the Clare berry developmental series was similar to that for the Willunga series and the final levels at harvest were also similar. Tartrate concentrations remained fairly stable during the pre-veraison period but declined rapidly after veraison (Fig. C3). Malic acid levels increased between fruit set and veraison after which they declined rapidly. Although there were minor differences in acid levels between malate and tartrate between the two pruning

treatments at some timepoints the levels during the later stages of ripening were similar. As observed in the Willunga berry series the tartrate/malate ratio varied considerably during berry development with tartrate dominating in both early and late stages (Fig. C4). Like the other acids the levels of citric acid declined towards veraison and only minor differences in levels were observed between the fruit from the two treatments (Spur and Machine pruned) (Fig. C5). Total acids increased until veraison after which they decreased rapidly, the levels at harvest were similar between treatments and similar to the levels found in the berries from the Willunga series (Fig. C6).

Anthocyanin Accumulation

HPLC analysis was used to assay the accumulation of the various anthocyanins throughout berry development. In general the variation between replicates was higher than that observed for the data collected for the Willunga series (see above) which meant that the standard errors are greater for this series (Fig. C7). Malvidin glucoside was the most abundant single anthocyanin species, delphinidin glucoside followed a similar curve but at a lower level. Interestingly, there seemed to be a difference in the pattern of accumulation between the two treatments which was consistent across different anthocyanin species. Final total anthocyanin levels were higher in the fruit from Spur pruned vines (Fig. C7).

Amino Acid Accumulation

The amino acid determinations for the Clare series were somewhat compromised as the quality of the analyses for the Machine replicates was poor, therefore, only the data from the fruit of Spur pruned vines is presented and discussed at this stage. The patterns of amino acid accumulation for the samples from Clare Spur pruned fruit were very similar in most cases to those described for the spur pruned fruit from Willunga and not all are presented in this report. Some minor differences were observed in several of the amino acids that were at very low levels (for example tryptophan and tyrosine) and it is likely that these differences may be due to the technical problems associated with measuring low amounts of amino acids in these tissues. As was noticed in the previous series glutamine has the distinctive pattern where levels increased from fruit set until part way through the pre-veraison period after which they decreased to be low at about the beginning of the lag phase and remained low throughout the rest of development. Again proline was the amino acid that increased to the highest levels at harvest from fairly low levels pre-veraison (Fig. C8).

Elemental Analysis

The patterns of elemental accumulation for ten elements in the fruit from the Clare trial were similar to those found for the Willunga series although in some cases the levels at harvest were different (Table C1). The most notable differences are that the levels of sodium and sulphur appeared higher in the fruit from the Willunga series. The difference in sodium levels could arise from differences in soil types between sites and/or the salinity of the irrigation water used. Differences in sulphur levels may arise from sulphur sprays used to control fungal diseases. As for Willunga the element with the highest concentration was potassium (Fig. C9a). The level of potassium was higher in fruit from Spur pruned vines at harvest than in fruit from Machine pruned vines (Fig.

C9a, Table C1). Again potassium levels decreased from fruit set to about 4 wpf after which they rose steadily. Boron accumulation followed a similar pattern but at lower levels (Fig.C9b). Interestingly, the levels of both potassium and boron were lower in the fruit form Machine pruned vines than that from Spur pruned vines during the post-veraison period and at harvest. This correlates with the lower levels of hexoses observed in fruit for Machine pruned vines. The remainder of the elements assayed followed a similar trend of accumulation to that seen for the previous series i.e. higher levels at fruit set after which the levels decreased and stayed low (calcium and magnesium are given as examples Figs. C9c, C9d).

Table C1. Summary of concentration (mg/Kg) of elements in berries from different developmental series at harvest. The numbers in brackets represent standard errors.

	K	B	Ca	Cu	Mg	Mn	Na	P	S	Zn
Willunga	2576.0 (48.00)	7.2 (0.01)	115.7 (5.80)	1.2 (0.10)	88.8 (3.10)	0.3 (0.01)	41.9 (1.80)	174.4 (9.50)	166.1 (3.30)	1.1 (0.1)
Wingara Control	2551.0 (12.22)	7.3 (0.22)	124.0 (1.15)	1.2 (0.10)	86.8 (3.32)	1.0 (0.05)	10.5 (3.31)	167.3 (7.55)	92.5 (2.35)	1.63 (0.29)
Wingara PD	2455.7 (145.88)	6.5 (0.39)	123 (5.69)	1.0 (0.11)	82.6 (6.56)	1.0 (0.15)	18.2 (4.06)	152.8 (4.79)	93.1 (2.62)	2.6 (1.02)
Clare Spur	2846.0 (63.00)	9.1 (0.83)	120.0 (8.19)	1.4 (0.09)	93.0 (4.34)	0.6 (0.11)	9.0 (1.44)	230.0 (6.46)	122.0 (2.90)	0.8 (0.19)
Clare Mach	2162.0 (153.0)	7.5 (0.53)	132.0 (17.37)	1.5 (0.11)	85.0 (5.80)	0.8 (0.10)	17.0 (1.29)	185.0 (12.68)	107.0 (4.66)	1.4 (0.27)

Wine Sensory Analysis

The fruit used for winemaking from the Spur pruned vines seemed slightly riper than that used from the Machine pruned vines as the Brix and pH values were a little higher and the total acidity was a little lower in the fruit from Spur pruned vines (See above & Appendix 5b). The levels of summed malate/tartrate/citrate at harvest were similar (Fig. C3) but the tartrate/malate ratio was lower in fruit from Spur pruned vines (Fig. C4) which may explain the pH difference as tartaric acid is a stronger acid than malic acid. This trend followed through into the finished wine as wine from fruit from Spur pruned vines tended to have higher alcohol levels (Appendix 5b).

Wine sensory analysis was carried out under contract by Provisor Pty Ltd using a trained sensory panel. Initial sensory assessment showed that the replicates within each treatment were generally consistent, with no faults, and that there appeared to be subtle differences between the wines from the different treatments (Appendix 5b). Difference testing and descriptive analysis were carried out on all replicates from both treatments. Some minor differences in aroma were detected between the wines made from Spur pruned fruit replicates while the wine from replicates from the Machine pruned fruit could not be differentiated from each other (Appendix 5b, Table 3.1). Descriptive analysis also showed some minor differences between some wines with wines from Machine fruit having slightly more floral aromas that than from the Spur pruned

treatment. There was little difference between the wines on the palate and the conclusion was that Spur and Machine pruned treatments appeared to have little effect on aroma and flavour of the wines produced.

Measurements of Gene Expression by Microarray Analysis

Gene Expression Changes during Berry Development

To investigate gene expression changes that occurred during berry development and ripening, berry samples were collected at weekly intervals until commercial harvest and the RNA extracted from each sample used for microarray analysis. Data from the nearly 16,000 genes on the microarray GeneChip were then analysed to identify genes that showed significant expression changes. Out of the nearly 16000 genes on the chip, 7608 and 7425 genes were identified as having a significant ($p<0.0000001$) differential expression pattern for the Spur pruned vines (CLA05_sp) and Machine pruned vines (CLA05_ma) respectively. This result is similar to the previous Willunga results where 7215 genes were differential expressed during berry development. The gene clustering results obtained for the Clare Spur and Machine pruned vines were similar to that obtained for the Willunga dataset and are not presented here for the sake of brevity.

Berry Gene Expression Differences between Spur and Machined Pruned Vines.

The Clare trial provided the opportunity to apply microarray technology to investigate differences between viticultural treatments. Only minor differences were recorded for sensory analysis of the wine and for hexose, potassium, boron and anthocyanin levels between fruit from Spur and Machine pruned vines. The gene expression pattern for each of the nearly 16000 genes were statistically compared between treatments across all time points and ranked according to significance (p-value). There were only eight genes with expression differences between treatments that had p-values below the threshold of $p<0.0000001$. If a threshold of $p<0.001$ was adopted then this number jumped to 97 genes showing expression differences between pruning treatments. The gene expression profiles of the 8 top significant genes are shown in Fig. C10. Depending on the specific gene the expression difference between Spur and Machine pruned vines was seen pre-veraison (Fig. C10a,b,c,d), post-veraison (Fig. C10e,f) or during both developmental periods (Fig. C10g,h). The pre-veraison differences either occurred in berries 1 week (Fig. C10a,b) or 4-5 weeks (Fig. C10c,d) after anthesis. Post-veraison, the differences were mostly seen 10-13 weeks (Fig. C10e,f,g) or 15-17 weeks (Fig. C10h) after anthesis. An examination of the putative function of these top 8 genes indicated that function for most were unknown. For those that were assigned a putative function one was involved in amino acid metabolism (Fig. C10c), two were involved in nucleic acid metabolism (Fig. C10a,g) and one was associated with photosynthesis (Fig. C10f). The expression of one gene (Fig. C10d) identified as a putative hexose transporter, was especially interesting. Although the difference in expression in the fruit from the two treatments was detected during the pre-veraison phase there may be a link between its expression and the total Brix and hexose differences detected in berries between Spur and Machine pruned vines (Figs. C1, C2). Higher levels of expression of this gene were detected in the berries of Spur pruned vines which correlated with the higher final Brix and hexose levels found in these fruit. How this may relate to crop load requires further investigation.

The low number of genes that showed gene expression differences at a threshold p-value of $p<0.0000001$ between the two pruning treatments suggests that the pruning treatments at this single site had only minor effects on berry composition and quality

and this was confirmed with the analysis of berry composition and sensory analysis of the wine.

Figure C1.

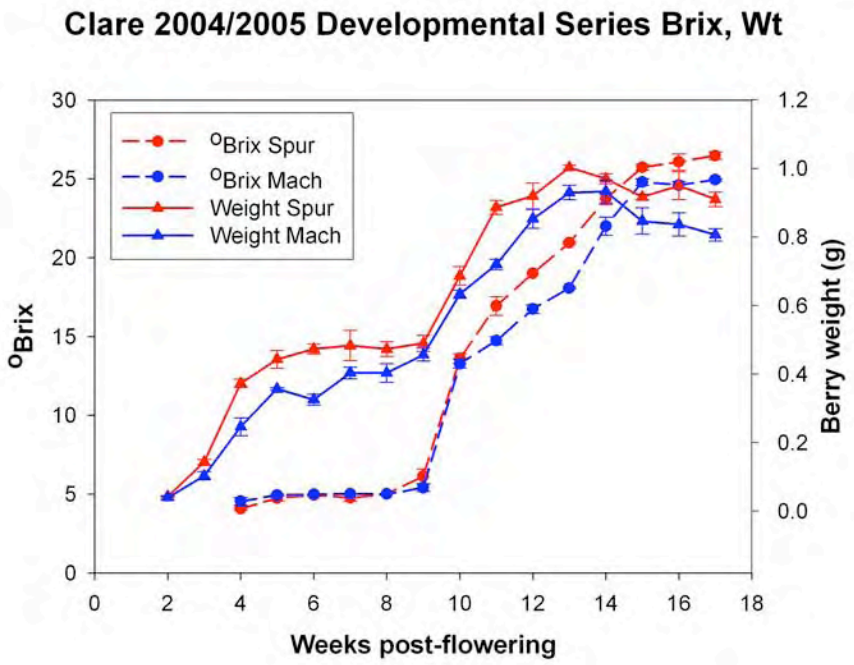


Figure C2.

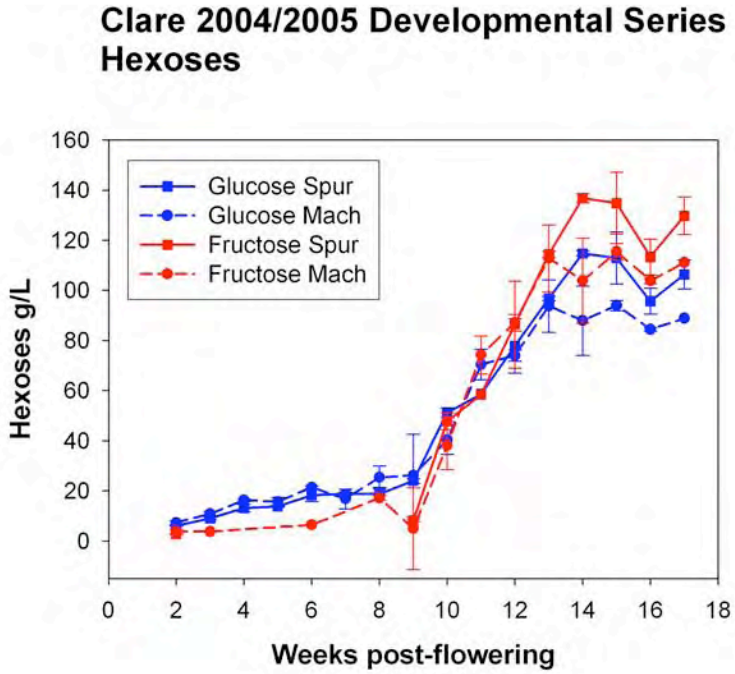


Figure C3.

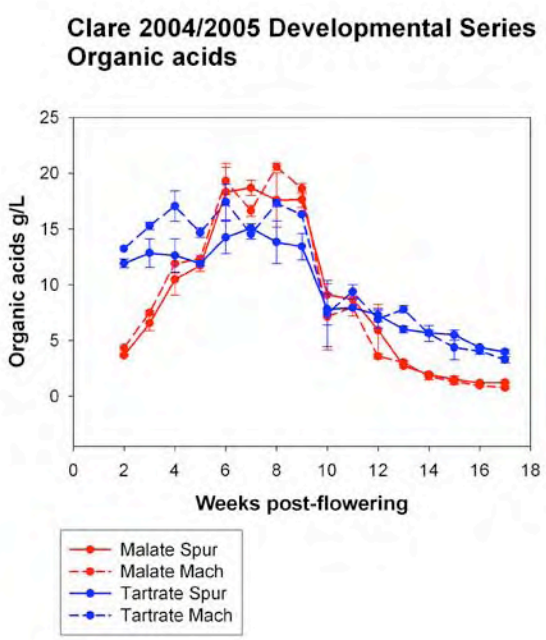


Figure C4.

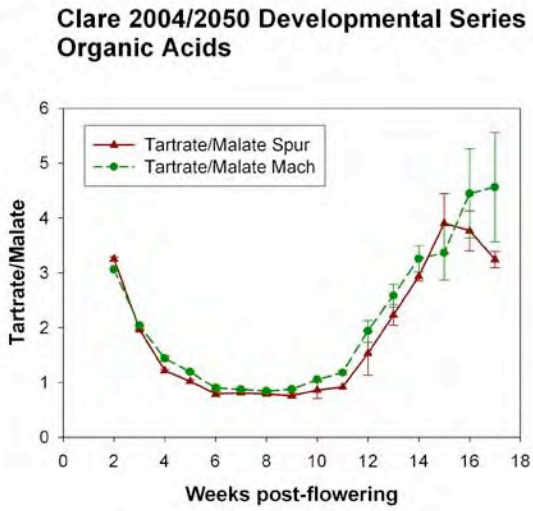


Figure C5.

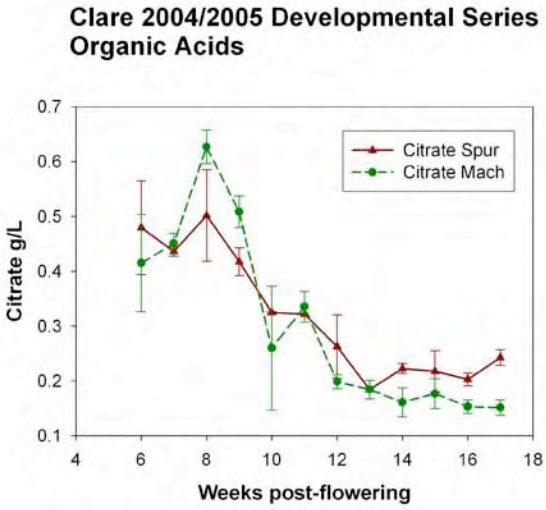


Figure C6.

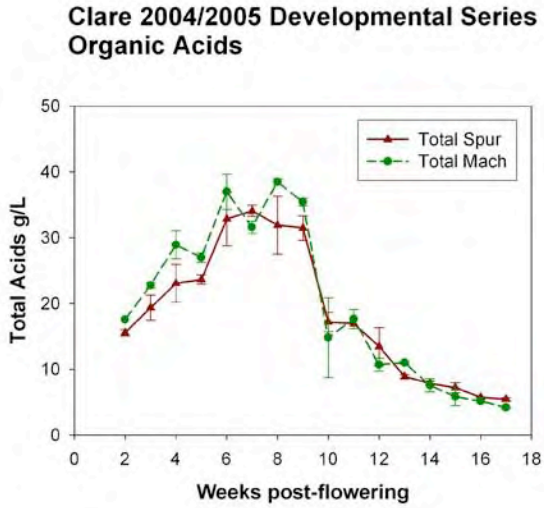


Figure C7.

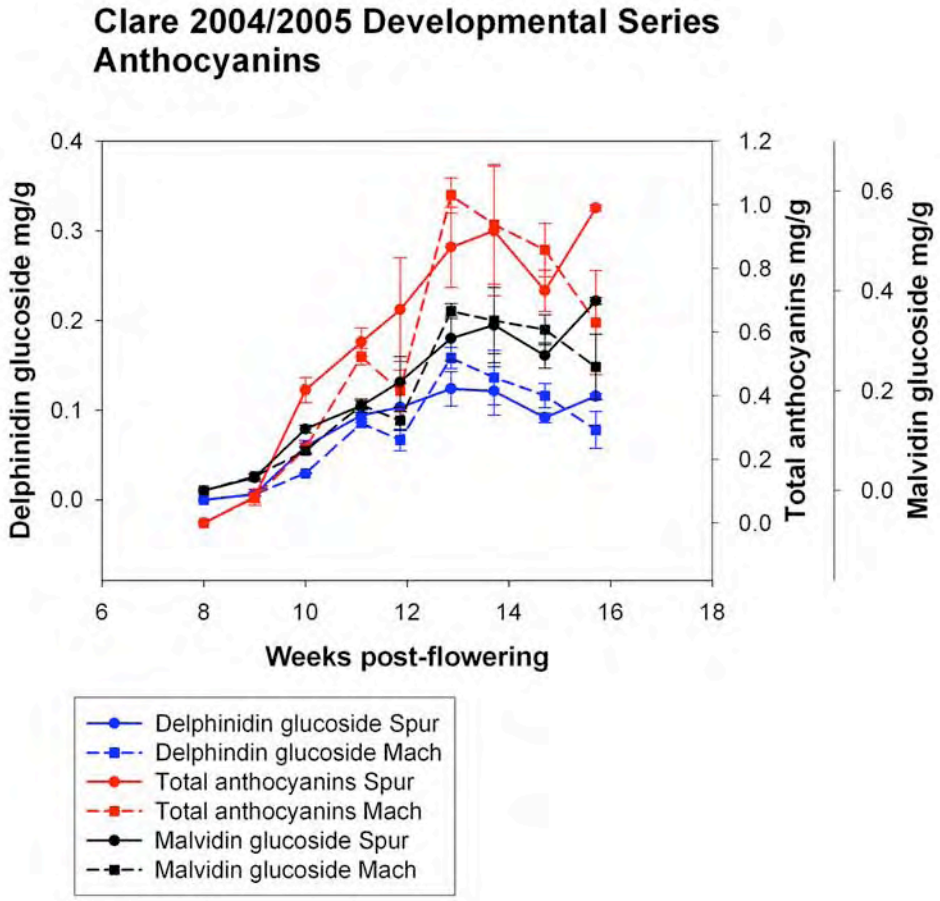


Figure C8.

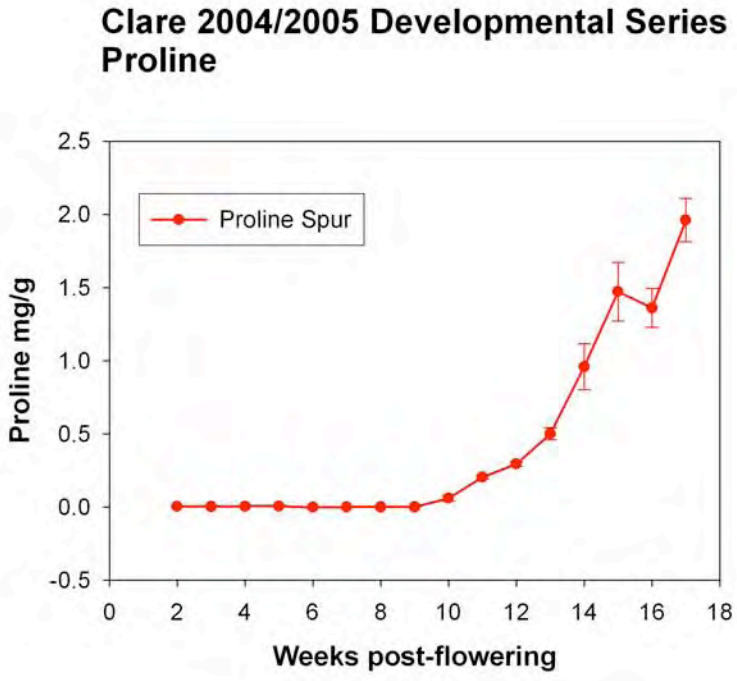


Figure C9a.

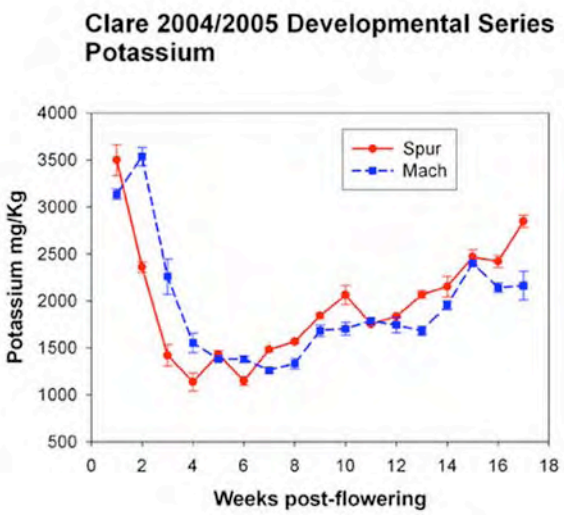


Figure C9b.

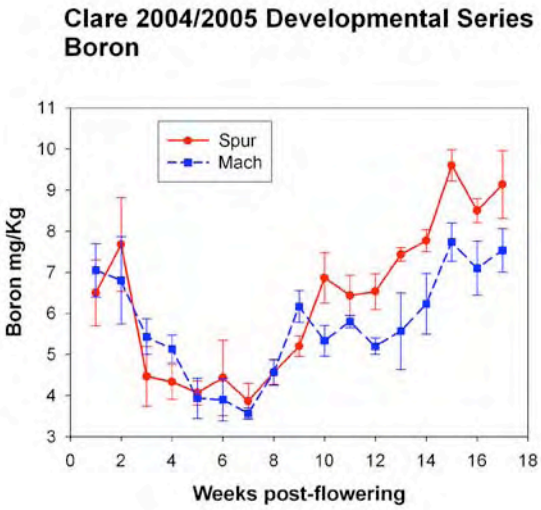


Figure C9c.

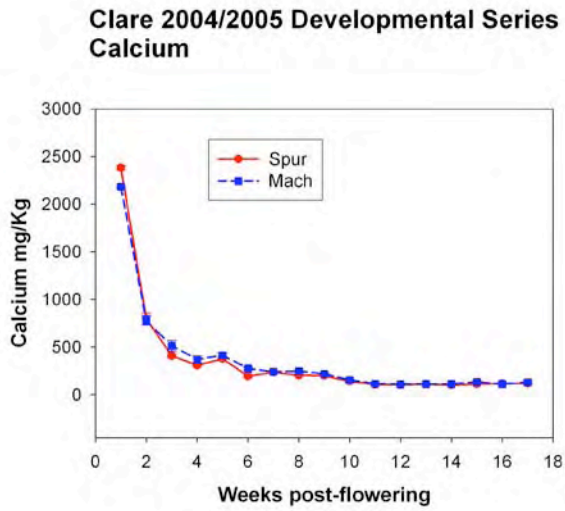


Figure C9d.

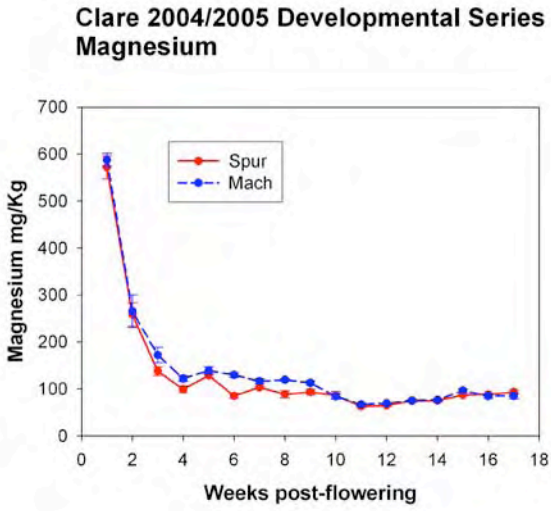
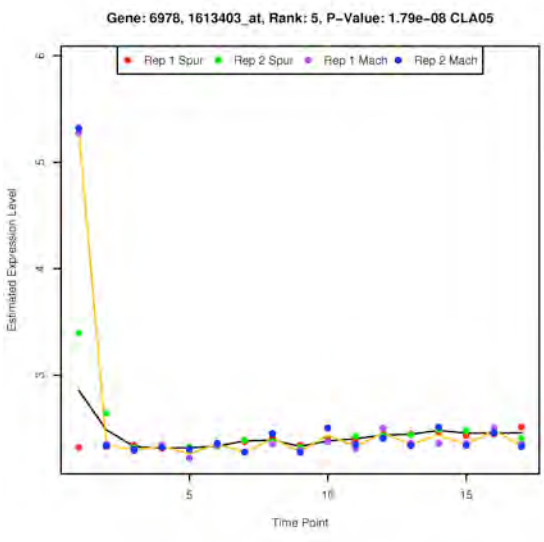
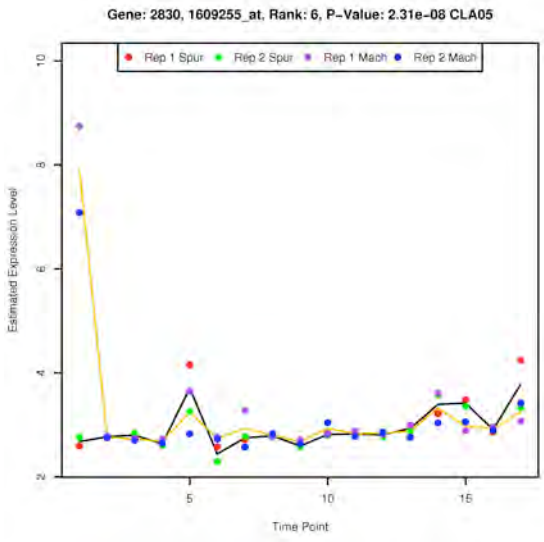


Figure C10. Genes showing significant expression differences during berry development between Spur prined and Machined pruned Caberent Sauvignon vines. Threshould p-value p<0.0000001.

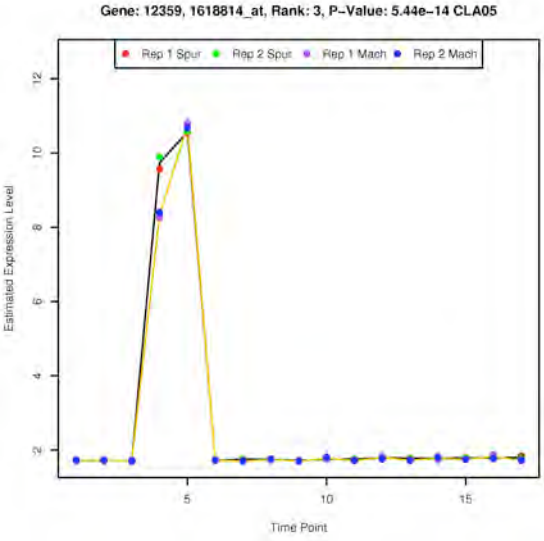
(a)



(b)



(c)



(d)

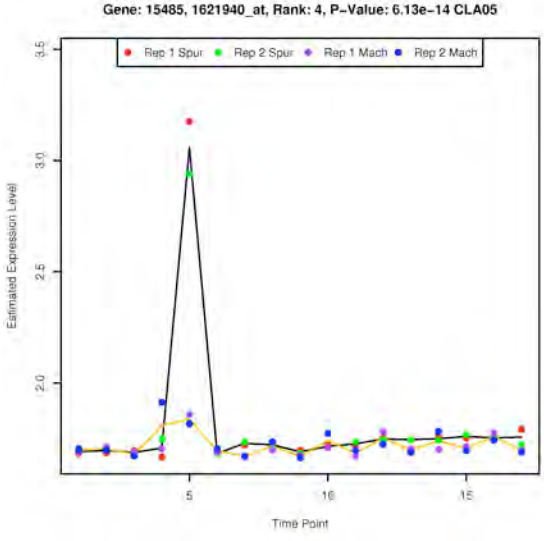
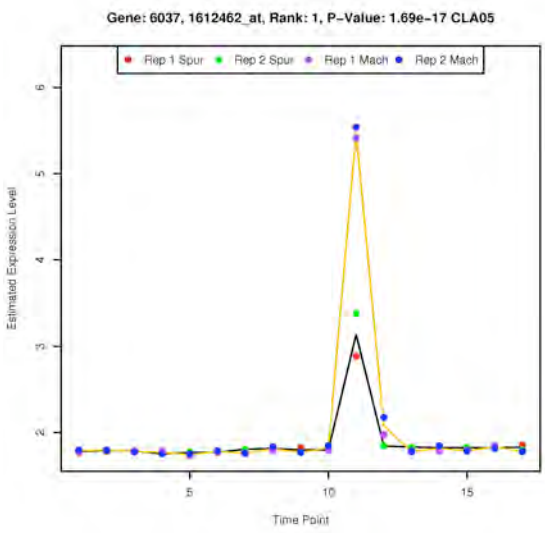
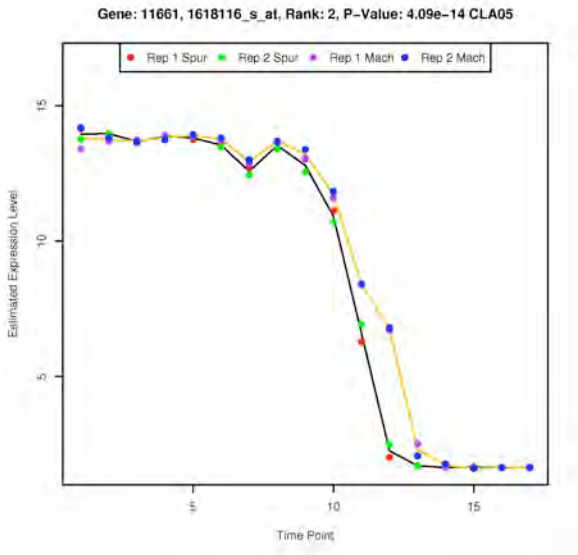


Figure C10. Genes showing significant expression differences during berry development between Spur prined and Machined pruned Caberent Sauvignon vines. Threshould p-value p<0.0000001.

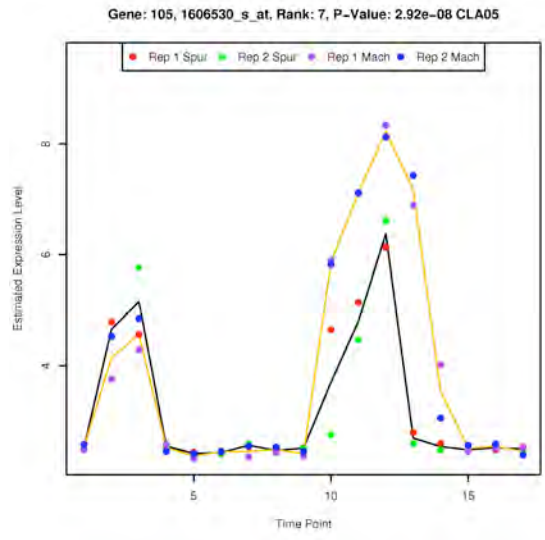
(e)



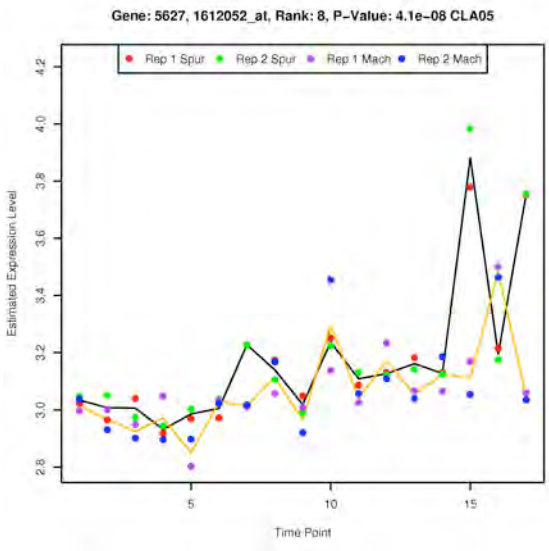
(f)



(g)



(h)



Wingara Wine Group, Deakin Estate, Wingara 2004/2005 (WIN-05)
The GWRDC project CRV 01/01 "Integrated strategies to manage seasonal variation in winegrape maturation" established irrigation trial at Deakin Estate, Wingara was used to investigate the effect of different watering regimes on vine and berry development (Tables 3, 4). Full berry developmental series were harvested and assayed for gene expression from two watering regime treatments i.e. 'normal' levels of irrigation and PD (Prolonged Deficit). During the 2004/2005 season a full developmental series from flowering to commercial harvest was sampled (10/11/2004 to 02/03/2005). Both the normal and PD vines were sampled at near one week intervals (Table 4).

Measurements of Berry Development & Composition
The progress of berry development was followed by measuring berry weight and the levels of total soluble solids (Fig. W1). The differing irrigation levels seemed to have little effect on berry Brix levels. As for the other two series the levels of hexoses rose sharply after veraison and the levels of glucose were lower than that of fructose (Fig. W2). As might be expected from the similar Brix levels the hexose levels were similar across the treatments. Berry weight was in general lower in PD treated fruit after veraison but the difference observed was minor at harvest (Fig. W1).

Organic Acid Accumulation
Organic acid accumulation in the fruit from the Wingara trial followed the same general pattern as for the other two series presented above with malic and tartaric acid levels increasing before veraison and then decreasing rapidly after veraison (Fig. W3). However, there was a large decrease in both malate and tartrate levels in fruit from both the treatments at 8 weeks post-flowering. The imposed irrigation treatment appeared not to have a significant effect on organic acid accumulation compared to the control (Fig. W3).

Interestingly the tartrate to malate ratio was much higher in the fruit from the Wingara trial than for the other two series examined in detail (compare Fig. W4 with Figs. 3, C4). This may be due to the warmer temperature at this site which may result in more rapid organic acid catabolism after veraison as the levels at veraison were similar at all sites. Although only a minor component of the organic acid makeup of the berry the pattern of citrate accumulation was different to that seen at the other two sites. Although levels were low in the later stages of development (as in the fruit from Willunga and Clare) the maximal citrate level was achieved post-veraison (11 wpf timepoint, Fig. W5) whereas the levels peaked before veraison in the other two series. Total acid levels at harvest were the same for the two irrigation treatments (Fig. W6) and about the same across all three sites.

Anthocyanin Accumulation
There was very little difference in the pattern of accumulation for the various anthocyanins between the two treatments (Control, Prolonged Deficit) or the final levels at harvest (Fig. W7). However, the accumulation pattern of the individual anthocyanins showed an interesting trend. Again malvidin glucoside was the greatest contributor to the total anthocyanin levels. The level of malvidin glucoside was still increasing at harvest (or at worst levelling off) however, the amounts of all the other anthocyanins

(delphinidin glucoside is shown as an example here) were on a downward trend at harvest (Fig. W7). This may reflect the lower stability of these anthocyanins under the hotter temperature conditions that prevail at the Wingara site, in the Riverland, compared to the other two sites.

Amino Acid Accumulation

The water regimes imposed during this trial, Prolonged Deficit (PD) and the Control treatment that had adequate watering, appeared to have little effect on amino acid metabolism as both the levels and patterns of amino acid accumulation were similar between treatments (data not shown). Whilst the patterns of amino acid accumulation in the Willunga and Clare Spur pruned fruit were very similar to each other the patterns of accumulation for a number of amino acids were different in the fruit from the warmer climate site at Wingara. Alanine, arginine, isoleucine, phenylalanine, proline and valine all increased after veraison. Proline was again the amino acid at the highest level at harvest (Fig. W8a). The concentrations of aspartic acid, glycine and histidine which increased after veraison in the Clare and Willunga fruit didn't alter greatly throughout development in the Wingara fruit. Although glutamic acid levels did increase after veraison, as occurred in fruit from the other sites, the increase was nowhere near as large in the Wingara fruit. Serine and threonine did exhibit the post-veraison increase in their levels as seen in the other series examined for Willunga and Clare, however, they showed an additional peak of accumulation in early berry development. Again glutamine exhibited the most distinctive pattern of accumulation with a large increase in its levels early in berry development followed by a sharp decrease so that its levels were low from approximately two weeks pre-veraison and remained low for the rest of berry development (Fig. W8b). This reflects the pattern found in fruit from the other sites. When plotted on a per berry basis it is clear that there is a burst of synthesis or import during the first four weeks of berry development followed by a period of catabolism (Fig. W8c).

In summary, there are distinct differences in the accumulation patterns of a number of amino acids when fruit from the hot, Wingara site are compared to fruit from the other two cooler regions. This would seem to suggest a difference in the basic metabolism in fruit from Wingara, perhaps due to temperature effects. As amino acids are fundamental to protein synthesis and many other synthetic reactions their metabolism may reflect the status of berry metabolism. The imposed water treatment seemed to have had little influence on amino acid accumulation.

Elemental Analysis

As observed with the fruit from the other sites most of the ten elements assayed accumulated in the same way, i.e. they were at higher levels in the flower sample and decreased rapidly to be at low levels in berries prior to veraison (as before calcium, magnesium, are given as examples (Fig. W9a,b). Again it was potassium and boron that had distinctly different accumulation patterns to those for the other ions as their levels were higher early in berry development then decreased until a few weeks before veraison after which they steadily increased until harvest (Figs. W9c,d). As distinct from the fruit from the pruning treatments (Clare series, see above) there were no obvious

differences in potassium and boron levels between the irrigation treatments imposed in this study.

Wine Sensory Analysis

Sensory analysis was conducted on wines from the Wingara site by Provisor Pty Ltd using a trained sensory panel. Initial assessment indicated that some of the wines had wine making faults (burnt rubbery characters) and were not used for further analysis. Difference testing showed no significant difference between wines from the Control and PD treatments (Appendix 5c). The only different attribute detected by descriptive analysis was that the PD wines had lower acidity than the Control. In summary, there were no large differences between the wines arising from the different irrigation treatments.

Measurements of Gene Expression by Microarray Analysis

Gene Expression Changes during Berry Development

To investigate gene expression changes that occurred during berry development and ripening, berry samples were collected at approximately weekly intervals until commercial harvest and the RNA extracted from each sample used for microarray analysis. Data from the nearly 16,000 genes on the microarray GeneChip were then analysed to identify genes that showed significant expression changes as previously described for the Willunga site. Out of the nearly 16000 genes on the chip, 6263 and 8046 genes were identified as having a significant ($p<0.0000001$) differential expression pattern for the control irrigated vines (WIN05_cn) and the Prolonged Deficit treated vines (WIN05_pd) respectively. This result is similar to the previous results for the Willunga and Clare studies where 7215, 7608 and 7425 genes were significantly differentially expressed during berry development. The gene clustering results obtained for the Wingara irrigated treatments were similar to that obtained for the Willunga and Clare datasets and are not presented here for the sake of brevity.

Berry Gene Expression Differences between Control and Prolonged Deficit Irrigated Vines.

The Wingara trial provided the opportunity to apply microarray technology to investigate differences between viticultural irrigation treatments. No major differences were recorded for sensory analysis of the wine and for hexose, anthocyanin, organic acid and elemental levels between fruit from both irrigation treatments. The gene expression pattern for each of the nearly 16000 genes were statistically compared between treatments across all time points and ranked according to significance (p-value). There were only eight genes with expression differences between treatments that had adjusted p-values below the threshold of $p<0.0000001$. If a threshold of $p<0.001$ was adopted this number increased to 51 genes showing expression differences between irrigation treatments. The gene expression profiles of the eight most significantly differentially expressed genes are shown in Fig. W10. There were two genes that showed significant expression differences at the very early pre-veraison stages of time points 1 & 2 with the Prolonged Deficit (PD) irrigation treatment having lower levels of gene expression compared to the control (Fig. W10d,e). These genes have not yet been assigned a known function. One gene had a significantly higher expression level in the fruit from the Control treatment at time point 17 just before harvest and was identified as having weak homology to a putative diphenol oxidase (laccase) enzyme (Fig. W10f). There were five genes that showed significant gene expression differences at time point

6. The fruit at time point 6 were pre-veraison and were collected on 12th December 2004 (Table 4). Three genes had higher expression levels at time point 6 in the fruit from the Prolonged Deficit treatment (Fig. W10b,c,h) while the remaining two genes showed a lower level of expression for the Prolonged Deficit treatment (Fig. W10a,g). The function of these genes are mostly unknown with one having weak homology to a flavonol induced gene (Fig. W10g). An examination of the irrigation treatment durations (Table 3) showed that the Prolonged Deficit treatment involved an RDI (regulated deficit irrigation) treatment from the 22nd November until the 9th December 2004. During the period between the completion of the RDI treatment and start of the Prolonged Deficit treatment the soil water profile was restored by three irrigation treatments (10th December 2004, 8 hours applying 12.48 mm; 13th December 2004, 8 hours applying 12.48 mm; 17th December 2004, 8 hours applying 12.48 mm). It is possible that the gene expression differences observed at time point 6 for the PD treatment may be in response to the change in water status of the vine and berry as a result of the change over from an RDI strategy to full irrigation that started on December 10.

The low number of genes that showed gene expression differences at a threshold p-value of $p<0.0000001$ between the irrigation treatments suggests that the irrigation treatments for the 2004/2005 season at this single site had only very minor, if any, effects on berry composition and quality and this was confirmed with the analysis of berry composition and sensory analysis of the wine.

Figure W1.

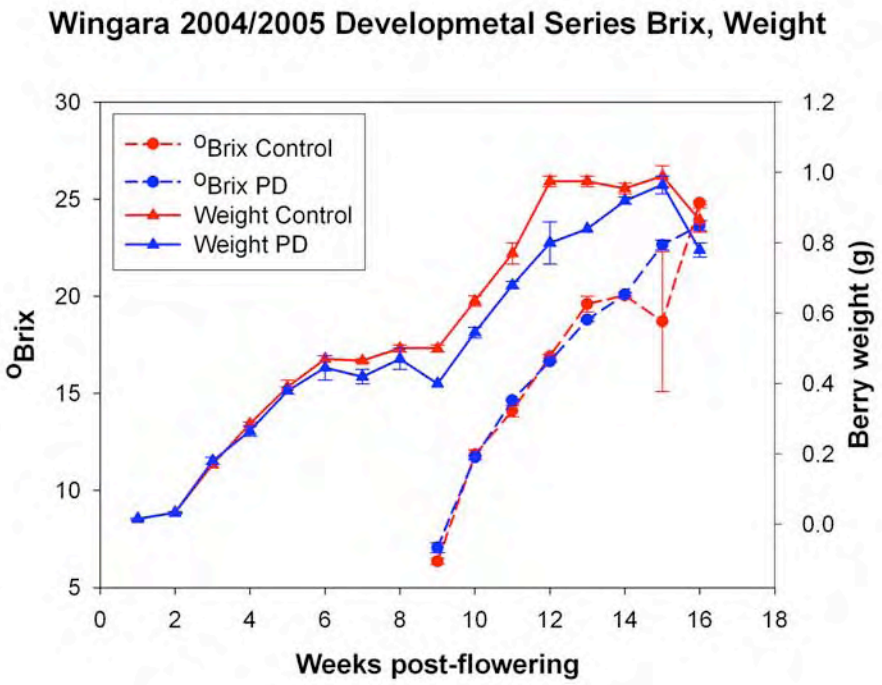


Figure W2.

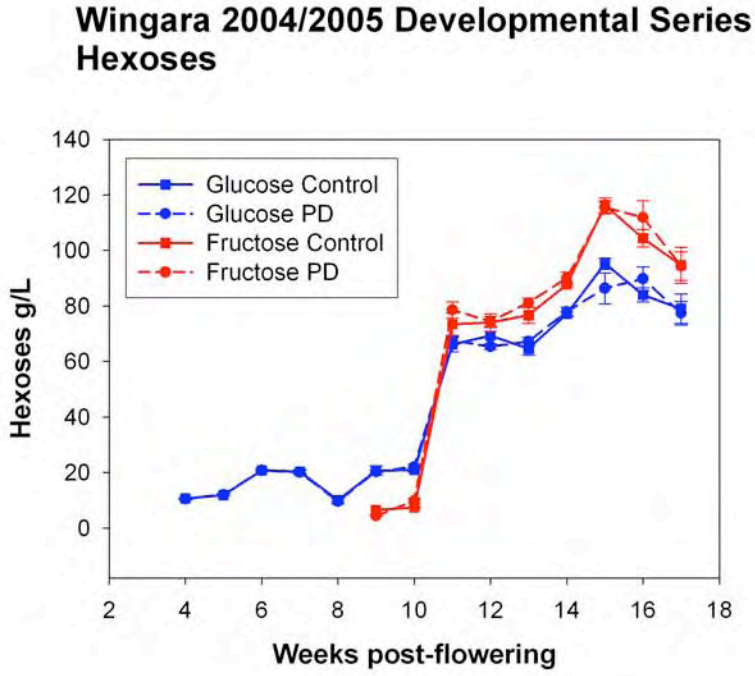


Figure W3.

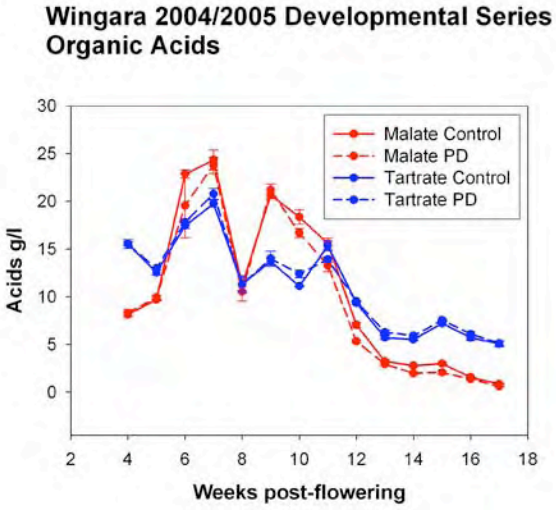


Figure W4.

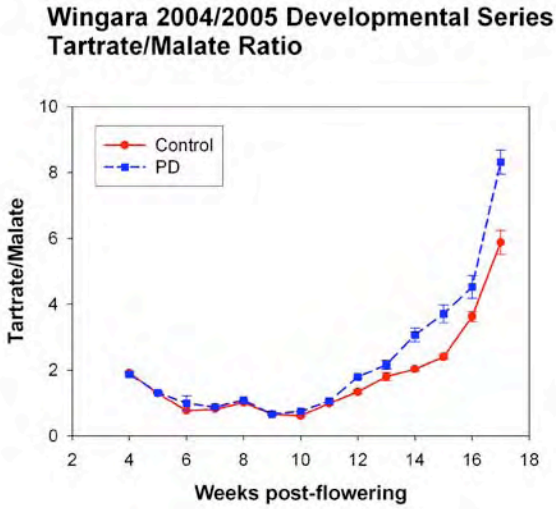


Figure W5.

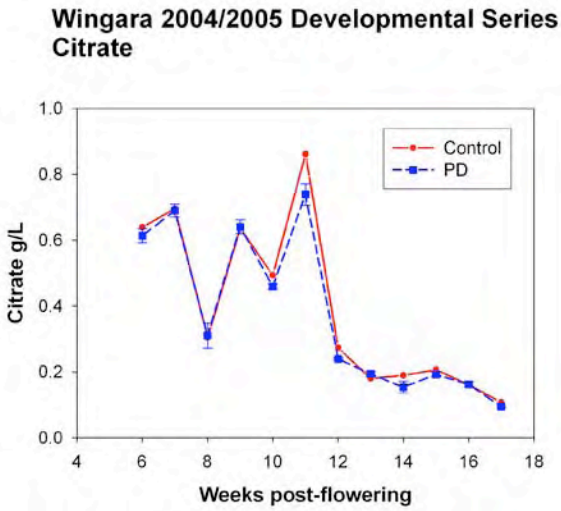


Figure W6.

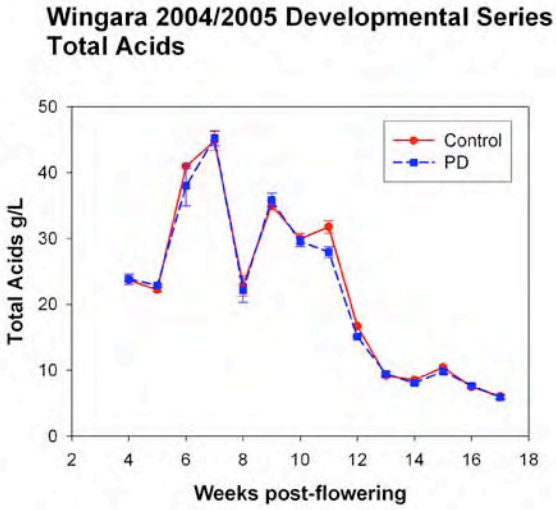


Figure W7.

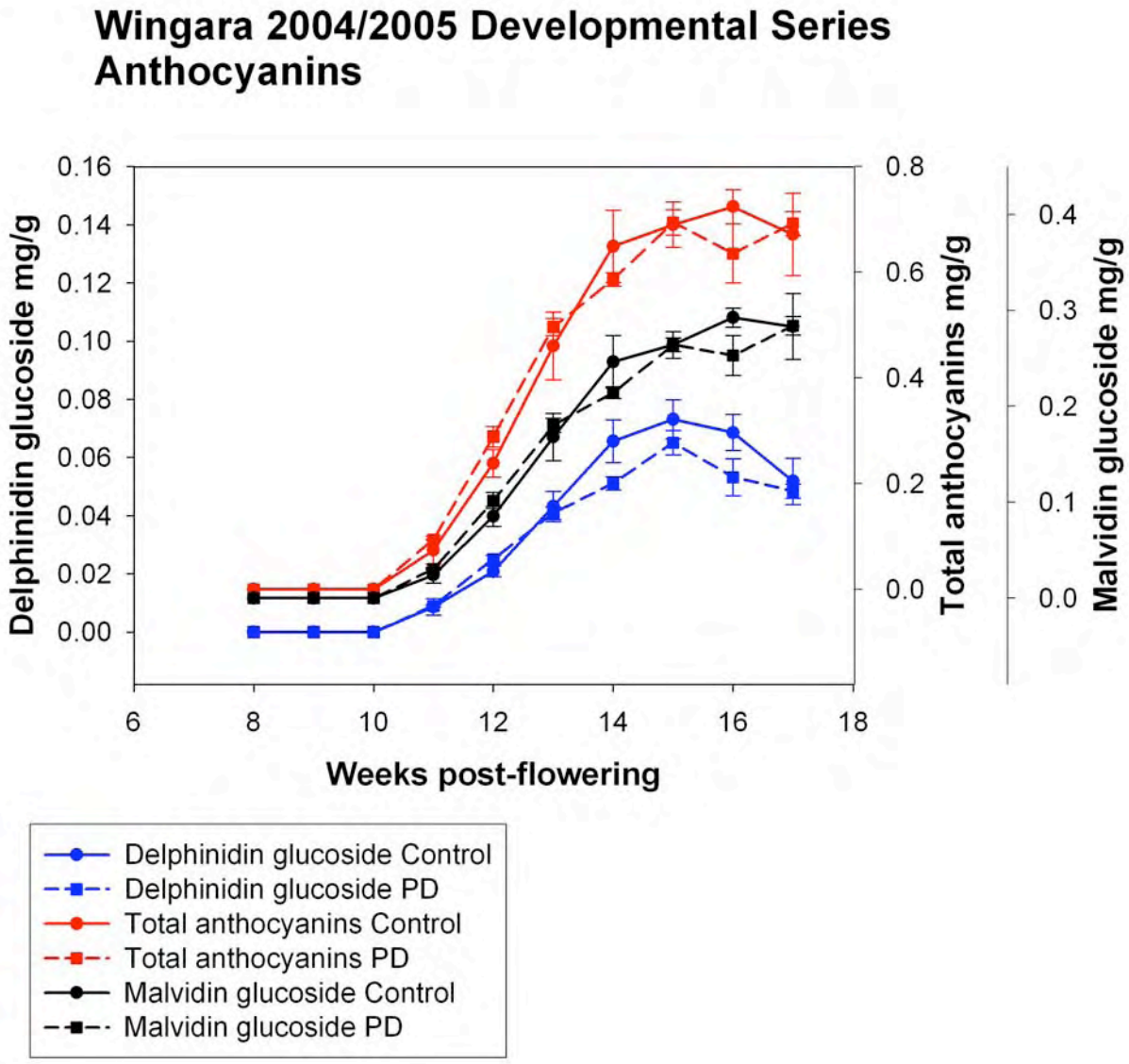

Wingara 2004/2005 Developmental Series
Anthocyanins
Delphinidin glucoside mg/g
Total anthocyanins mg/g
Malvidin glucoside mg/g
Weeks post-flowering
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.8
0.6
0.4
0.2
0.0
0.4
0.3
0.2
0.1
0.0
6
8
10
12
14
16
18
Delphinidin glucoside Control
Delphinidin glucoside PD
Total anthocyanins Control
Total anthocyanins PD
Malvidin glucoside Control
Malvidin glucoside PD

Figure W8a.

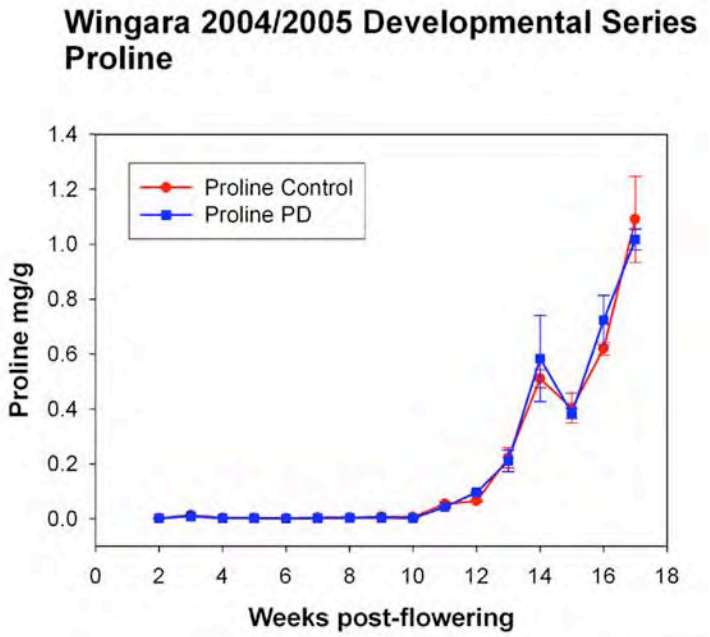


Figure W8b.

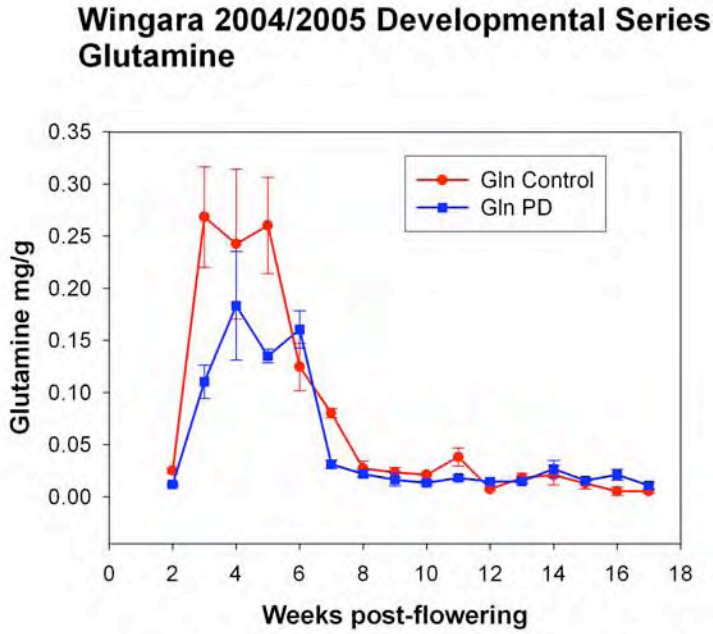


Figure W8c.

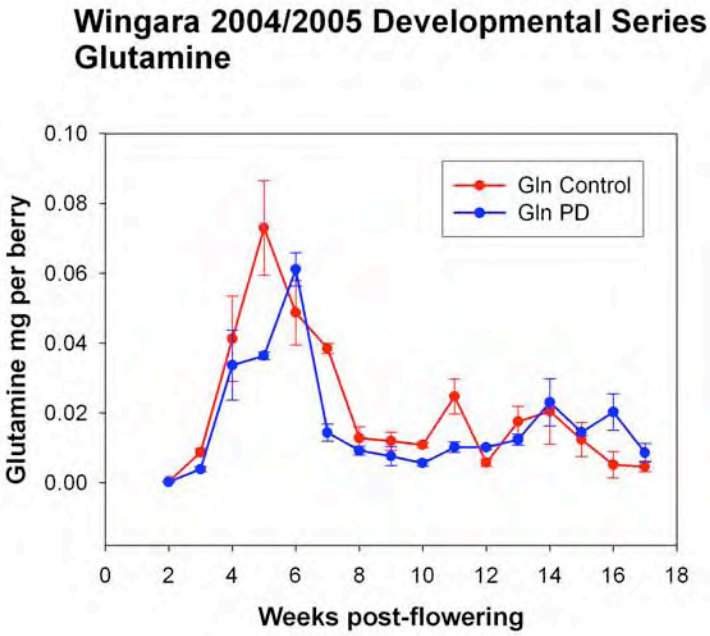


Figure W9a.

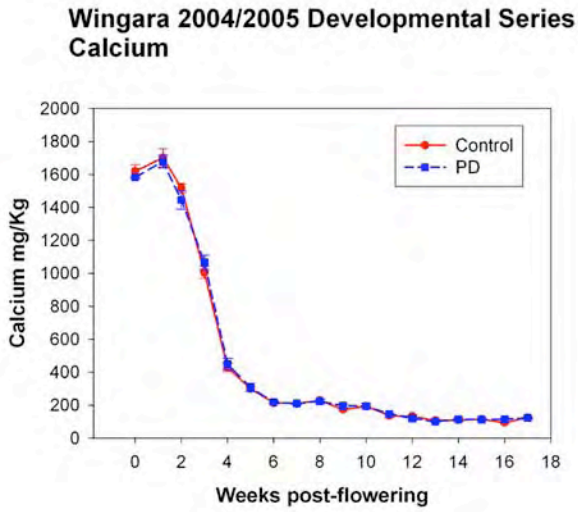


Figure W9b.

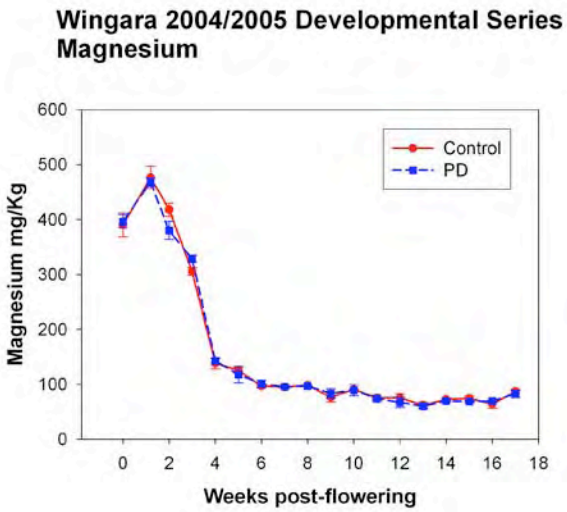


Figure W9c.

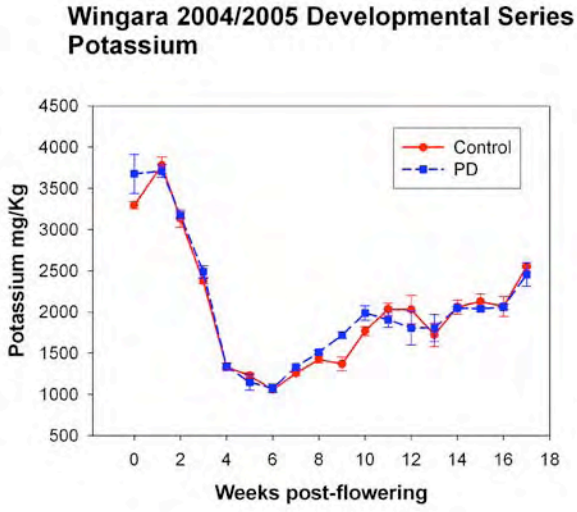


Figure W9d.

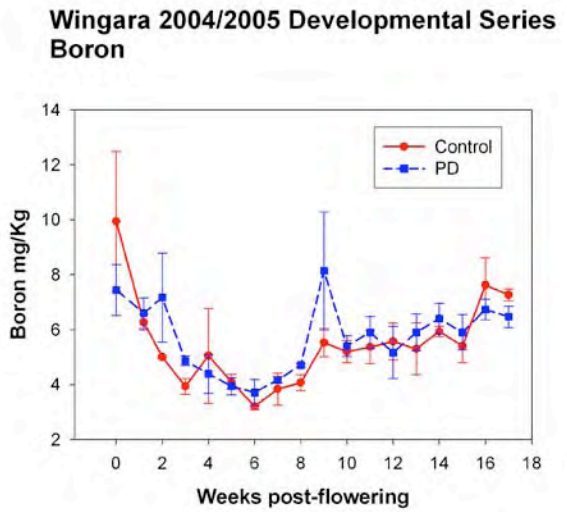
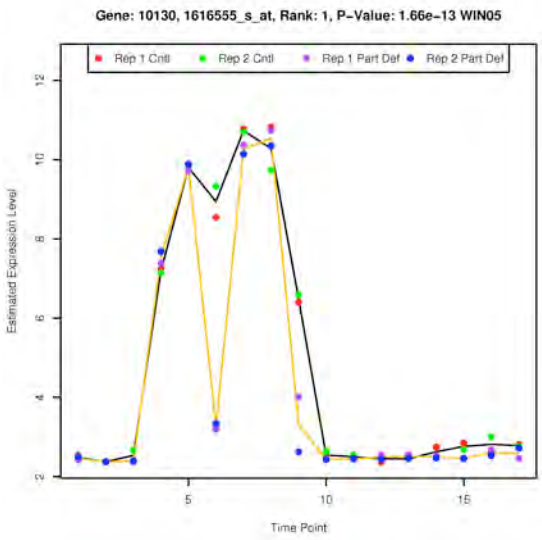
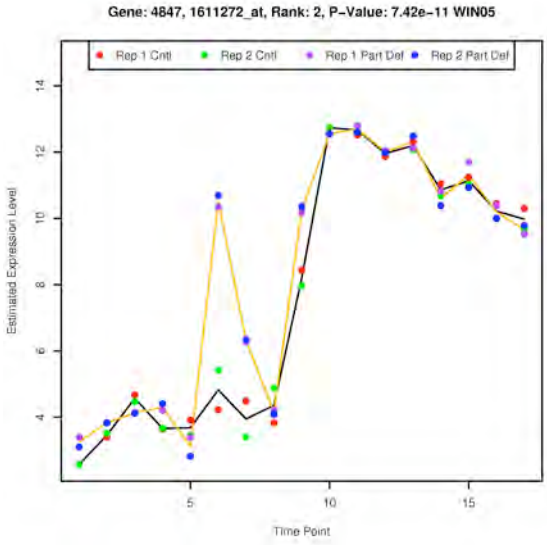


Figure W10. Genes showing significant expression differences during berry development between Control and Prolonged Deficit irrigated Caberent Sauvignon vines. Threshould p-value p<0.0000001.

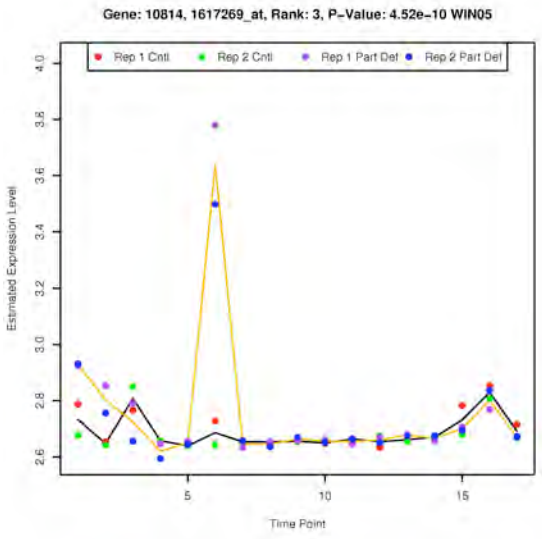
(a)



(b)



(c)



(d)

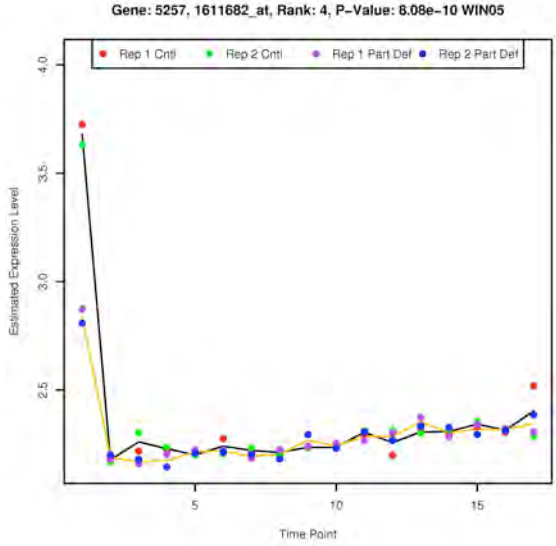
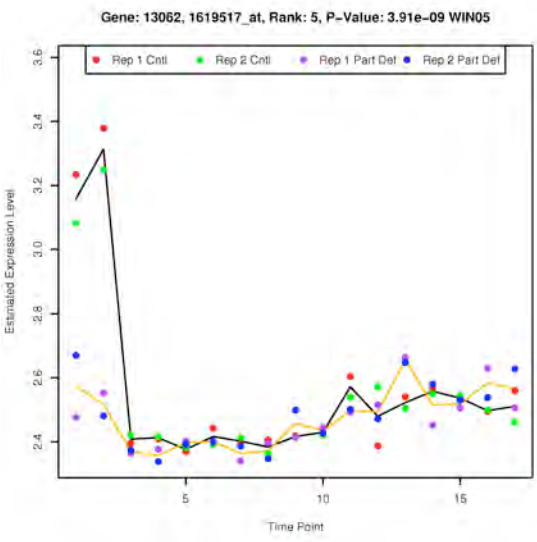
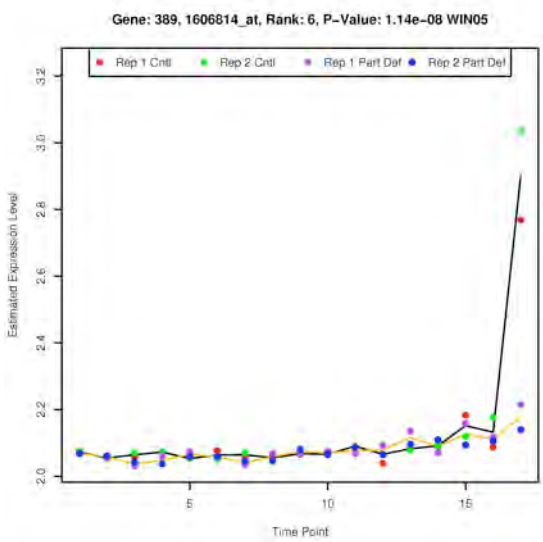


Figure W10. Genes showing significant expression differences during berry development between Control and Prolonged Deficit irrigated Caberent Sauvignon vines. Threshould p-value p<0.0000001.

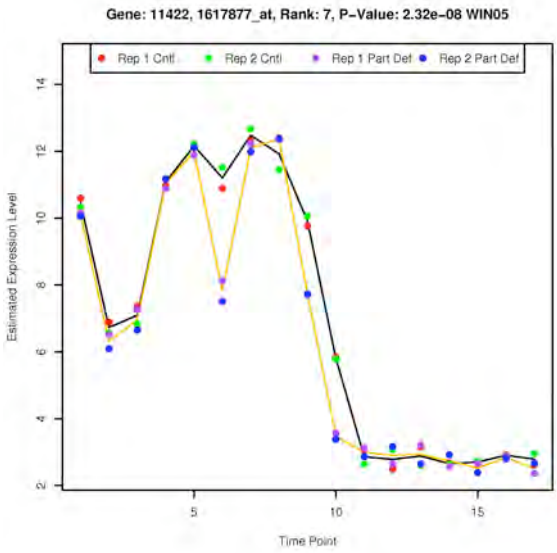
(e)



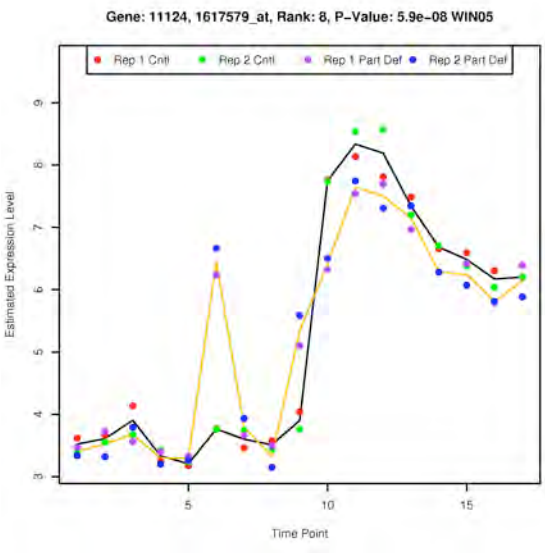
(f)



(g)



(h)



Linking Gene Expression Changes to Measurements of Berry Development & Composition/Quality Across Three Sites

The overall pattern of berry development across the three different sites was fairly similar, however, there were some differences in the timing and rate of development as measured by parameters such as the flowering date, veraison date and the rate of sugar accumulation (Table T1).

Table T1. Berry developmental stages across 3 sites.

	Willunga	Clare		Wingara	
		Spur pruned	Machine pruned	Control	PD
Flowering date	20/11/2003	23/11/2004	23/11/2004	10/11/2004	10/11/2004
Veraison date	22/1/2004	20/1/2005	20/1/2005	10/1/2005	10/1/2005
Days from flowering to veraison	63	54	54	61	61
Days from flowering at which 20^{o}Brix reached*	98.0	79.6	88.3	98.0	98.0
Days from veraison to 20^{o}Brix*	35.0	25.6	34.3	37.0	37.0

*Calculated from graphs in Figures 1, C1, W1 assuming a linear rate of sugar accumulation between the two sample time points on either side of 20 ^{o}Brix.

Some of the differences between the data from the Willunga site may be due to seasonal differences. The Clare and Wingara data comes from the same year and any differences would be due to site effects (soil, aspect, viticultural treatment etc.) and local weather patterns. Flowering was approximately two weeks earlier at the Wingara site compared to the Clare site. This is likely to be a temperature effect but the timing of pruning can also affect flowering date. Interestingly, the pre-veraison period was a little shorter in the Clare fruit than the fruit from the other two sites. The fruit that ripened earliest was from Clare Spur pruned vines, which in addition to having a shortened pre-veraison period, also appeared to store sugar (up to 20 ^{o}Brix) more rapidly (Table T1). Rate and accumulation of sugar is not only a berry function as other factors involved in source-sink relationships such as vine health and crop load may play a role.

It should be noted that the determination of flowering date (50% anthesis) is rather subjective when applied across a vineyard and some error may arise in its determination. Also, while the sampling regime adopted was the best available (see Material & Methods) ^{o}Brix measurements on a subpopulation of berries may result in minor imprecision in relation to the whole vineyard or treatment.

Although, as discussed above, there were minor differences in the timing and rate of berry development between treatments and sites, a comparison of biochemical and microarray data between sites was considered useful and valid. Data for the three sites detailing the changes in levels of a range of berry constituents have been detailed above.

Here we wish to use some of this data as examples to show that correlations can be made between gene expression as determined by microarray analysis and the accumulation of various compounds that are important for berry and wine composition/quality/style.

When the microarray data was analysed statistically it was found that in general there were only a relatively small number of genes that differed in expression between the two different viticultural treatments at the Clare and Wingara sites. Because of this it was considered more statistically powerful to combine the data from the pairs of treatments in the first instance for the purpose of developmental gene expression analysis and expression pattern clustering. Even allowing for some leeway for the possible differences in the rate of development noted above it was found that some genes had similar gene expression profiles across all three sites while others showed variation in gene expression. The degree of similarity between the profiles of expression for a particular gene at each of the sites appeared to depend on the identity of the gene and its putative function; examples are given below.

Sugar accumulation

Most of the sugars that are utilised during berry metabolism and in storage are imported. Not only are sugars vital in the winemaking process for the production of alcohol but they are essential for berry metabolism and play a role in signalling within cells and tissues. Sugar made in the leaves as a result of photosynthesis is transported in the phloem as the disaccharide sucrose (Fig. T1). How sucrose is unloaded from the phloem, transported within the berry, cleaved into its monosaccharide components (glucose and fructose) and used for metabolism or eventually stored in the vacuole is a complex process that has been the subject of much research and debate. In grape berries there are relatively low levels of sugars stored prior to veraison but after veraison there is a large increase in the levels of glucose and fructose that are stored in the berry vacuole (Figs. 2, C2, W2). We know that sucrose is cleaved in the berry to form glucose and fructose and there are two main types of enzyme that can do this. Sucrose synthase, despite its name, usually acts to cleave sucrose not make it. Figure T2 shows the expression patterns of two grape sucrose synthases genes that are expressed during berry ripening. The patterns of gene expression are fairly similar between the three sites but interestingly the two genes show opposite trends in expression. This may reflect the fact that berries appear to be superficially very simple structures but in fact are considerably complex. The tissues used for these experiments were deseeded berries (i.e. skin and flesh) and it could be suggested that this may explain the two differing patterns i.e. that each gene is expressed with a particular pattern in a distinct tissue. However, this is unlikely as both skin and flesh store sugars at roughly the same rate. It is more likely that there is cell specialisation within the tissues, for example the conductive tissues and/or their surrounding cells may be specialised in a particular aspect of sugar metabolism. Higher levels of sucrose synthase activity are thought to be involved in fruit set i.e. in the setting up of a sink for carbohydrates. The pattern shown by Gene 12768 (Fig. T2b) suggests that it may have a role in this function. In addition to specialisation at the tissue level there is evidence for cellular and intra-cellular specialisation in regard to sucrose synthase activity.

The other enzyme that is important in sucrose cleavage is invertase. Three types of invertases are commonly found in plants, an extracellular form, one located in the cytoplasm and one that is located in the vacuole. One mode of sugar accumulation that

could occur in the berry is for sucrose to be imported into the cell via plasma membrane located active transporters (Fig. T1). As grape vacuoles store hexoses this mode of sugar import requires both a sucrose transporter and a sucrose cleavage enzyme. It is postulated that sucrose imported into the vacuole is then cleaved by a vacuolar invertase. Figure T3 shows that sucrose transporters are indeed expressed in grape berries. Gene 13801 encodes a sucrose transporter that is more highly expressed after veraison in berries from all three sites. Another transporter (data not shown) has the opposite expression pattern and is more highly expressed in younger berries. Again this exemplifies the complexity of the control of gene expression during normal berry development.

Figure T4 shows the expression of two vacuolar-located invertase genes. The transcript level of both decrease at veraison, however, we know that the enzyme itself is stable throughout development (Davies and Robinson, 1996) and that the level of activity of this enzyme may be controlled by processes other than transcription. These expression patterns suggest both the components required to take up sucrose directly and cleave it to hexoses in the vacuole are present in grape cells.

Another way in which sugars can be imported into berry cells is through cleavage of sucrose in the apoplastic space into hexoses by a cell wall located invertase (Fig. T1). In this case the cells would then have to import glucose and fructose rather than sucrose. There are a number of different, plasma membrane located, hexose transporter genes that are expressed during berry development. In Figure T5 the patterns of expression of two grape hexose transporters are shown. The expression of one gene, Gene 2566, is highly up-regulated at veraison and stays elevated throughout the ripening phase in fruit from all three sites (Fig. T5a). The expression of another, gene 13236, is also up-regulated at veraison but subsequently decreases later during development (Fig. T5b). This helps validate some recent evidence that hexose transporters and perhaps sucrose transporters may be vital to the storage of large amounts of hexoses during ripening (Zhang *et al*., 2006, Hayes, Davies, Dry in press). This is because an apoplastic route of accumulation, facilitated by energy driven transporters, may be required to accumulate sugars against a concentration gradient.

Anthocyanin accumulation

The pathway for anthocyanin synthesis in grapes is now well understood. The first step in flavonoid synthesis is conducted by the enzyme phenylalanine ammonia lyase (PAL) (Fig. T6). Following this numerous sequential steps are performed by a range of enzymes. This results in the production of the coloured pigments found in berry skin that we know as anthocyanins. There are also a number of branches from this pathway that feed off into other pathways which produce a range of other secondary metabolites such as tannins and stilbenes. Figure T7 shows the expression of five genes of the anthocyanin pathway, four biosynthesis genes and one transcription factor gene. The changes in gene expression levels are shown for berries from the three different sites. The three genes in the upper part of the pathway PAL, chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H) have similar patterns in each of the three sites i.e. they all decrease in expression after being higher in flowers and very young berries and then increase rapidly in expression again at around the time of veraison. This confirms the earlier work of Boss *et al*. (1996). Interestingly, when the expression of the PAL, CHS and F3H genes are viewed from a single site each of the sites appears to have a constant and unique signature. The PAL, CHI and F3H genes show a particularly interesting

pattern in the Wingara samples, where there seems to be a peak (at 6 and 7 weeks post-anthesis) during the time when gene expression should be in a 'trough'. This pattern is not reflected throughout the expression of all genes (e.g. Fig. 7d,e) and is represented by two sampling time points. It would appear that these genes are quite responsive to their environment. The fact that there are branch points to other products (e.g. tannins) after the steps catalysed by these enzymes means that the different patterns in expression between the sites may not be reflected, or may only be partly reflected, in changes in the production of anthocyanins. This shows that although many genes involved in berry development have similar patterns in the fruit from all three sites, there are some that seem to vary in expression depending on the conditions at the site and this may also be reflected in differences in berry composition.

The fact that PAL, CHI and F3H genes have similar patterns of expression suggests that their expression is under the control of a common transcription factor. A good example of how genes with specific expression patterns can be matched to the corresponding transcription factor which controls their expression is the control of one of the last steps in the anthocyanin pathway. The final step in the synthesis of anthocyanins is conducted by UDP glucose-flavonoid 3-*0*-glucosyl transferase (UFGT). In Figure T7d it can be seen that UFGT expression is ripening-related as it is 'turned on' at veraison, this is in agreement with previous reports (e.g. Boss *et al*., 1996). The expression pattern is similar between all three sites. The gene encoding the transcription factor that controls UFGT expression, MybA has recently been identified (Kobayashi *et al*., 2002) and has the same expression pattern as UFGT (Fig. T7e).

It is interesting to note that the expression of all of the five genes in the Clare samples showed increases a week before the expression in the fruit from the other two sites (Fig. T7). This is in agreement with the estimation of the time from flowering to veraison shown in the above table (Table T1).

Stilbene accumulation

Stilbenes are phytoalexins that are low molecular weight compounds that have antifungal properties. Stilbenic compounds such as resveratrol have also been suggested to have human health benefits. Stilbene synthesis is induced by a number of factors including UV light and pathogen attack (Bais *et al*., 2000). The level of their synthesis is also under developmental control in the berry. Like anthocyanins, stilbenes are also derived from phenylalanine and share some early steps with the anthocyanin synthesis pathway (Fig. T8). Figure T9 shows the expression patterns of two stilbene synthase (STS) genes. Again the overall pattern from all sites for both genes is similar with some expression in flowers and very young berries and varying amounts of expression later in post-veraison development.

Proline accumulation

The amino acid proline has an important role in plant metabolism and in winemaking. In plants it is a basic building block in protein synthesis and in a range of other synthesis reactions. Its levels are also elevated by stress far above the normal levels required for general metabolism. Under hyperosmotic stress (drought, salinity), higher levels of proline are thought to act as a compatible solute helping the cell to successfully control changes in osmolarity (Delauney and Verma, 1993). High proline levels in wine musts can cause problems with stuck ferments as yeast can't metabolise proline as a nitrogen source and fruit high in proline can therefore contain insufficient

nitrogen to sustain fermentation. Understanding the accumulation of proline in berries may allow proline levels in fruit to be better controlled.

The level of proline increases considerably after veraison (Figs. 6j, C8, W8a). Pyrroline-5-carboxylate synthetase (P5CS) is a crucial step in the synthesis of proline from glutamic acid (Fig. T10). The levels of glutamic acid also rise after veraison suggestive of coordinated control (Fig. 6d). Microarray analysis showed that the transcript level of P5CS increases roughly 4 fold (the y axis is on a log2 scale) in the berries at about the time of the post-veraison increase in proline levels (Fig. T11). This suggests that at least some of the control over proline accumulation may be exerted through modulation of the transcript level of P5CS. This is in contrast to earlier findings that suggested that the transcriptional control of P5CS was not significant (Stines *et al*., 1990).

Tartrate accumulation

Organic acids are important in wine production to maintain low wine pH, which increases colour and microbiological stability and is important for taste. Two main organic acids exist in grape berries i.e. malic and tartaric. The metabolism of malate is complex but it accumulates pre-veraison then is quite rapidly degraded post-veraison. Tartrate appears to be a dead-end metabolite and although it accumulates, like malate, early in berry development it is not readily degraded during ripening. In warmer climates the degradation of malate is more extensive than in cooler climates and so the levels of tartrate are especially important. Recently, an enzyme, L-idonate dehydrogenase (L-IdnDH), which catalyses the proposed rate-limiting step in tartrate synthesis (Fig. T12) was identified (DeBolt *et al*., 2006). This enzyme converts L-idonic acid to 5-ketogluconic acid, a key step in the synthesis of tartaric acid from ascorbic acid. Our microarray analysis showed that L-IdnDH transcript levels are high during the pre-veraison period then decrease rapidly as the fruit enters ripening (Fig. T13). The patterns vary slightly between the three sites but the trend is the same. The gene expression pattern correlates well to the pre-veraison increase in tartrate levels (Figs. 3, C3, W3). The decrease in tartrate concentration after veraison is due mainly to the recommencement of rapid berry expansion (Figs. 1, C1, W1).

Potassium accumulation

The importance of tartaric acid has been mentioned above. Potassium levels are also important to final must pH. This is because tartaric acid can bind potassium ions to form the largely insoluble salt potassium tartrate, which results in the removal of tartaric acid from the must causing an undesirable increase in pH. Therefore, the level of potassium in the fruit at crushing is of considerable importance to industry. Potassium taken up by the roots is transported throughout the plant. Crucial transport steps in the berry involve the transport of potassium across the plasma membrane by energy requiring potassium transporters. Two grape potassium transporters have previously been functionally characterised (Davies *et al*., 2006) but there are additional, as yet, uncharacterised, sequences in the grape EST database which have homology to potassium transporters (data not shown). The expression of two of these are shown in Figure T14. The gene expression for Gene 816 (Fig. T14a) in fruit from all three sites increases from about 4 WPF and peaks at about veraison after which time the levels slowly decrease (Fig. T14). The expression pattern of Gene 3698 (Fig. T14b) is more striking as it increases at veraison and remains high throughout the ripening period. This pattern of expression tallies closely with the increase in potassium levels which occurs from about four weeks

post-flowering (Figs. 7g, C9a, W9c). The fold change in induction between pre and post-veraison is much greater for Gene 3698 than for Gene 816 and so it might be expected to be the more dominant factor in post-veraison potassium accumulation. This indicates that further analysis of these genes and the corresponding protein may be useful in determining the mechanism of potassium uptake in grape berries.

Indicators of developmental stage across all three sites

It is possible by looking at the clustering patterns and the data comparing three sites that reliable markers for the different stages of development can be identified. Obviously, some events, such as veraison, do not need molecular diagnostic markers as the physical changes are obvious, especially for varieties with black fruit. If molecular diagnostic markers were required for veraison the UFGT and MybA genes (Fig. T7 d,e) would be ideal for black fruit but not for white fruit. There are many genes whose expression is also up-regulated at veraison and this pattern can be seen from the clustering of expression profiles (Fig. 22) and may represent common veraison diagnostic markers for both white and black fruit. Some of these have been identified previously (e.g. Davies and Robinson, 2000). Many others have not previously been identified and their putative function is unknown as they have no close homologues on databases currently available. These represent a large number of genes that may potentially control and effect the development and composition of the berry during the ripening phase. Three examples of genes whose expression is up-regulated at veraison, and as such represent potential ripening-related genes, are presented in Fig. T15 (a, a gene encoding a thaumatin-like protein; b, a gene encoding a Grip 61 gene; c, a gene encoding Grip 4 a proline-rich putative cell wall protein). It is noticeable that the expression pattern for all three genes is similar between sites. Interestingly, the expression of all genes rises at the same time (8 wpf) in the Willunga and Wingara samples but the increase is a week earlier in the Clare samples. This agrees with the time of veraison being one week earlier in the Clare fruit (Table T1). This indicates that despite any seasonal and site differences the expression of genes such as this is always tightly correlated to the berry developmental pattern. A similar pattern of expression where the rise in the level of mRNA is a week earlier in the Clare samples also occurs in other genes associated with ripening, such as hexose transporters (Fig. T5), genes involved in the anthocyanin pathway (Fig. T7) and a gene involved in proline synthesis, P5CS (Fig. T11).

As mentioned above the clustered patterns demonstrate that there is a wide variety of gene expression patterns and a close inspection of the patterns of expression of the individual genes at three sites may lead to the identification of molecular diagnostic markers for many of the important events in berry development. At the present time a standardised way of determining berry growth stages is the modified E-L system (Coombe 1995) however this can be subjective. A more objective measure of berry growth stages, especially for subdividing the pre-veraison stage might be obtained using gene indicators identified in this present study. A few examples of potential markers for particular events and processes during berry development are given here.

Apart from the changes at veraison, events both early and late in development may be crucial for berry development. One of the most important events in berry development occurs at fruit set when fertilisation is followed by rapid cell division and growth. Events at this time can determine the fate of the berry and the amount of cell division can affect berry size. As cell division occurs early in development (Ojeda *et al*., 1999) genes involved in chromatin assembly (Fig. T16a) and cell cycle control (Fig. T16b) are

good diagnostic markers for early development. There are also many other genes with the same patterns, some of which are associated with different early events. These include genes involved in secondary metabolism such as phenolic metabolism (e.g. caffeic acid o-methyltransferase Fig. T17a) and shikimate pathway metabolism (e.g. shikimate dehydrogenase Fig. T17b).

In contrast to the genes discussed above there are a number of genes whose expression increases later in development and so may be useful markers of late events in post-veraison. Figure T18 shows an example where the expression of one of these genes, whose function is unknown, increases during the later part of berry development. Interestingly, this is one of the genes whose expression varies between the sites. Expression increases from 12 wpf in the Clare fruit (which reached higher sugar levels earlier) and from 14 wpf in the fruit from Willunga and Wingara.

Patterns of transcription factor expression across three sites

The clustering analysis demonstrates that there are numerous expression patterns (see Fig. 22). As these patterns must be dictated by changes in the transcription factors that control the expression of many of these genes, it follows that there must also be many corresponding expression patterns of transcription factor genes. We have already seen (Fig. T7) that the expression pattern of a gene involved in anthocyanin synthesis can be matched to its controlling transcription factor via gene expression profile. This is a good demonstration of how this array data might be used to discover the transcription factors that control particular genes in specific processes. These transcription factors may then be used to control whole or parts of metabolic pathways. There are many transcription factors whose expression varies around the time of veraison and there are also transcription factors with expression patterns similar to the 'early' and 'late' genes described above (data not shown). The MybA transcription factor that controls UFGT transcription is an example of a transcription factor that controls the expression of a limited number of genes. Homeobox genes encode a type of transcription factor that control large gene cascades and are important for controlling significant events in plant organ development. One of these transcription factor genes has an 'early' expression pattern (Fig. T19) which suggests it may be involved in the control of fruit set and early fruit development including such processes as DNA replication. This is suggested by the similarity of its expression profile with that shown in Fig. T16a for a gene involved in chromatid formation.

Some genes are expressed differently across the three different sites and may represent genes that are environment responsive

The above examples demonstrate that the expression of a number of developmentally controlled genes is fairly stable despite seasonal and site differences. There are also many genes whose expression varies in response to the vines environmental conditions. As such these differences in gene expression profile between the fruit from the different sites may at least in part explain the differences seen in berry development and fruit composition. The expression of the stilbene synthases (Fig. T9) is a good example of genes that show differences in expression due to environmental factors. Although there seems to be some evidence of developmental control, the expression patterns from the three sites do show distinct features. The peaks of expression (which are not coincidental between sites) in the fruit from the Clare site are the highest, with the expression from the Wingara fruit being the lowest in level. It could be speculated that these differences may be due to differential induction by fungal pathogens. Fungal

attack is generally more frequent in cooler, wetter environments such as the Clare Valley and so the warmer, more arid climate at Wingara may explain why the levels of STS are lowest in these fruit. The fact that the profiles are saw-toothed is unlikely to be an artefact of the sampling or analysis but more likely to result from periodic episodes of disease pressure. Stilbene synthesis is tightly controlled and not constitutive as they are also toxic to plant cells and can cause severe damage if present at higher, sustained levels. This, as well as the efficacy of the viticultural fungal control methods used, would explain the oscillating profiles. However, it is also possible that the STS expression differences observed between the 3 sites are caused by a factor(s) other than disease. There are many other examples where differences in gene expression can be related to environmental effects (data not shown).

Other genes showed more dramatic differences in their expression patterns in fruit from the three different sites, two representative examples are given in Figure T20. The expression of these genes does not seem to follow a distinct developmental pattern as has been seen for the examples described above. Their expression is highly variant between sites with many of the peaks and troughs of expression not aligning. The expression with a single developmental series is also erratic with large variations in gene expression levels between different time points. For example the data from Clare showed that there were over 440 genes whose gene expression pattern differed significantly ($p<0.0000001$) when compared to the other two sites. Statistical analysis of these profiles indicates that the data is reliable and that the patterns of expression are not due to problems with berry sampling of with the microarray analysis of gene expression. Therefore, it appears that these genes represent molecular diagnostic indicators of a berries varying response to differing environmental conditions.

Figure T1. Genes involved in sugar transport and metabolism in the berry.

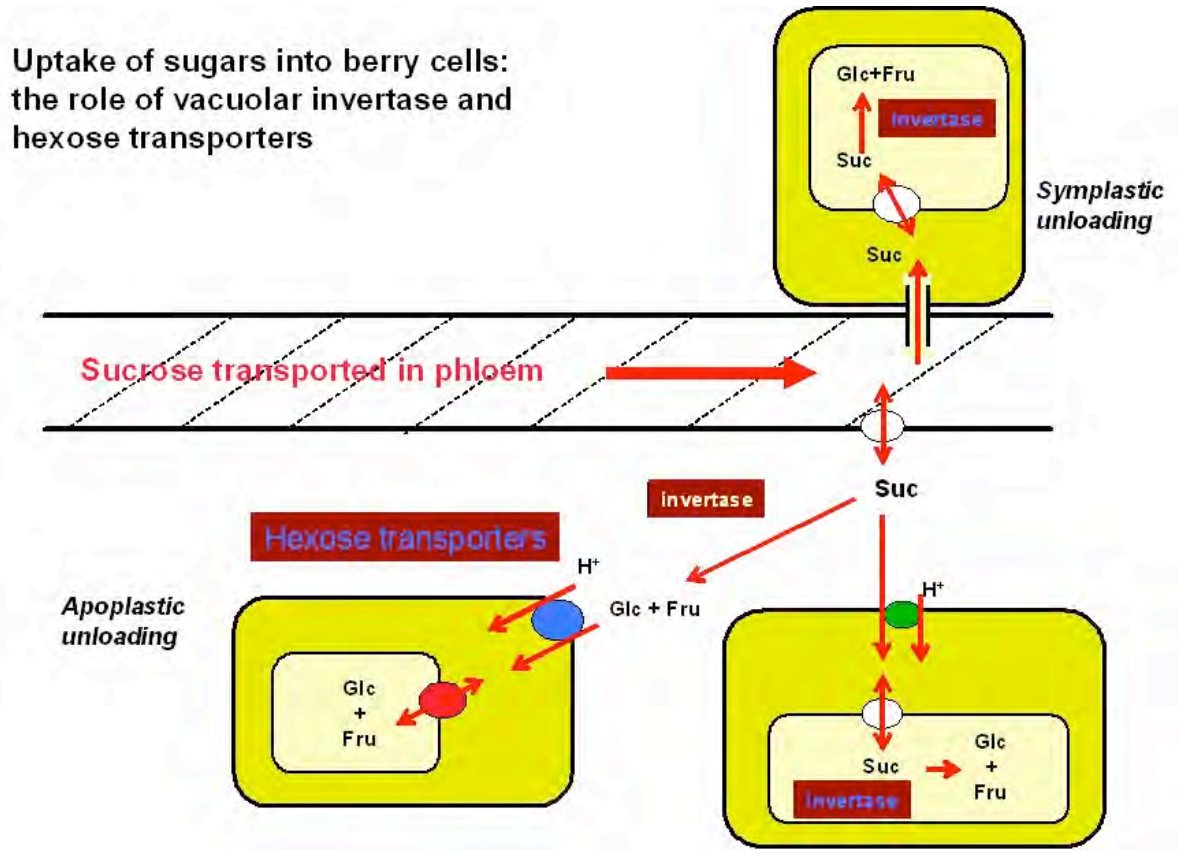
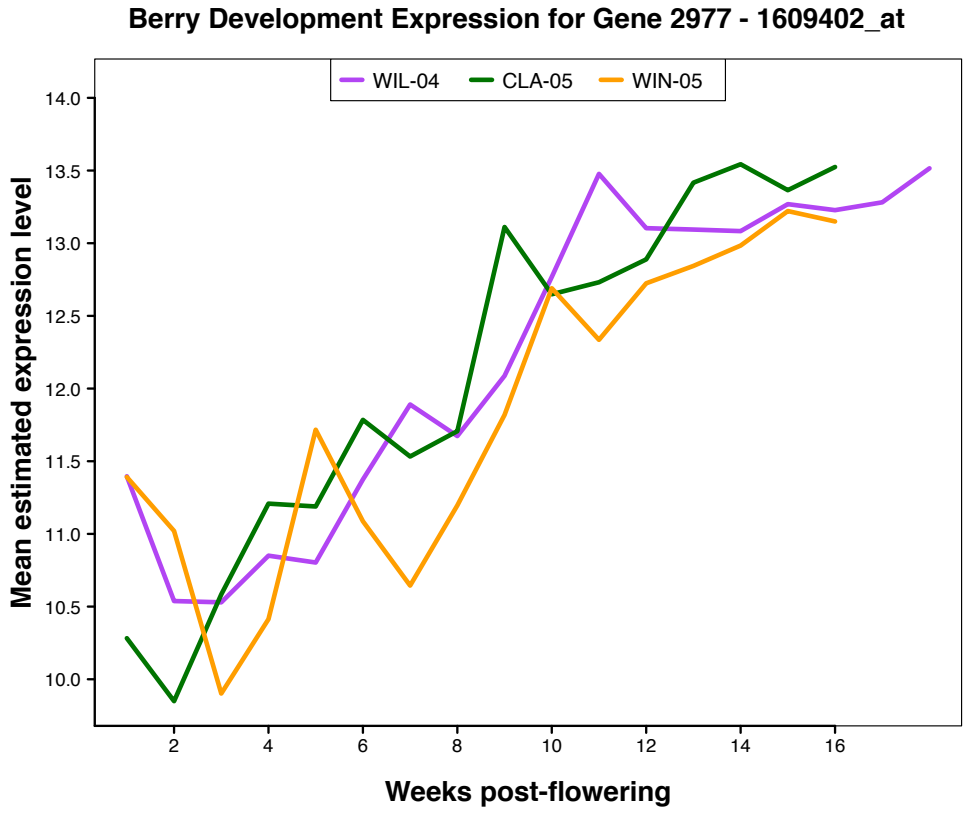


Figure T2. Expression profiles of genes involvd in sucrose cleavage - sucrose synthase.

(a)



(b)

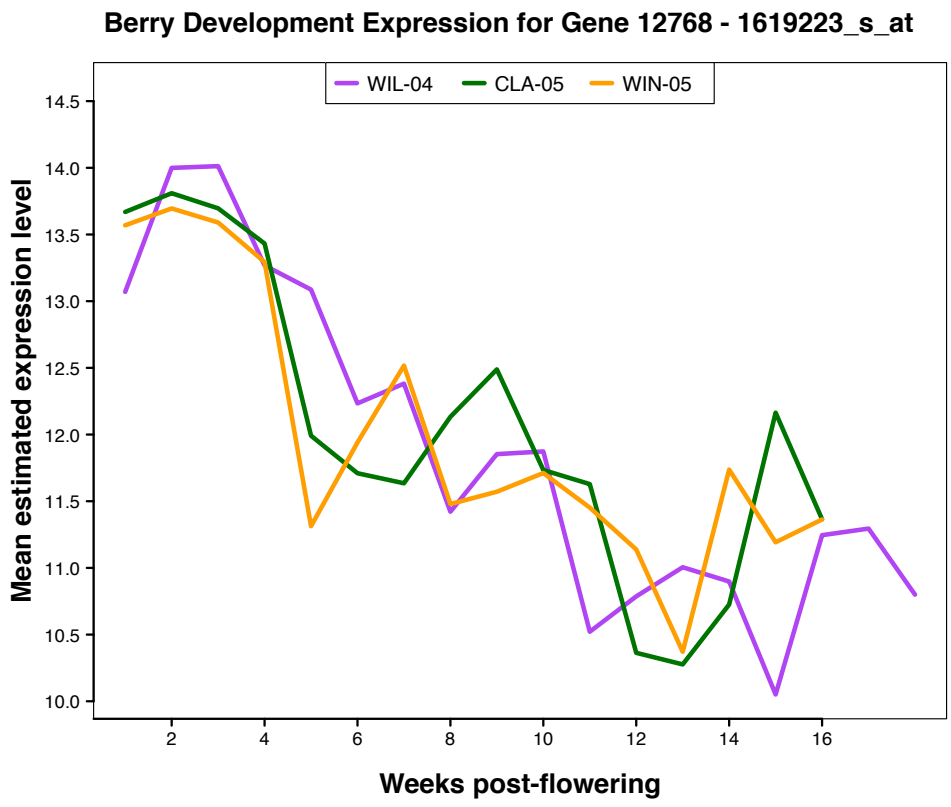


Figure T3. Expression profiles of a gene involvd in sucrose transport across the plasma membrane.

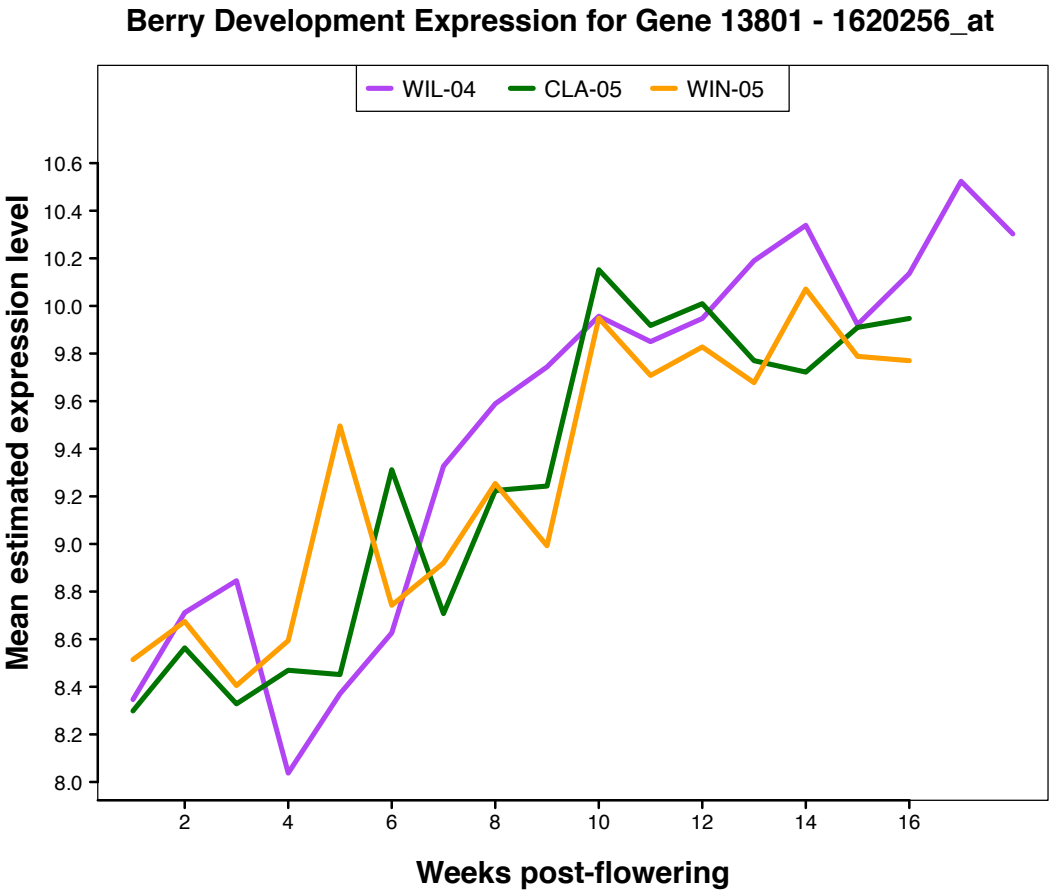
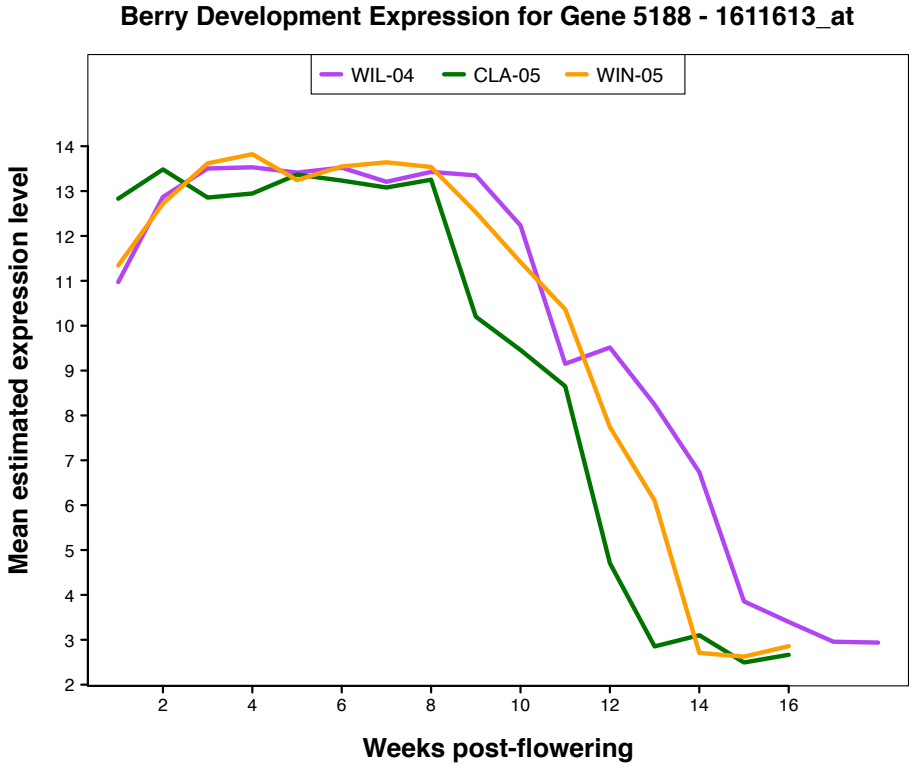


Figure T4. Expression profiles of genes involvd in sucrose cleavage - vacuolar invertases.

(a)



(b)

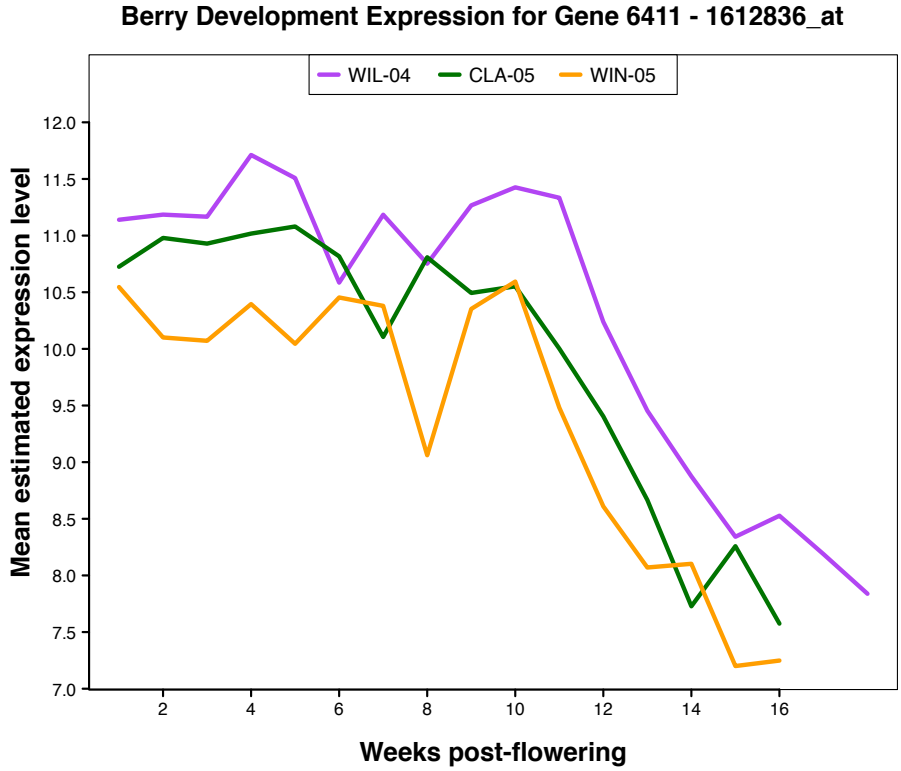
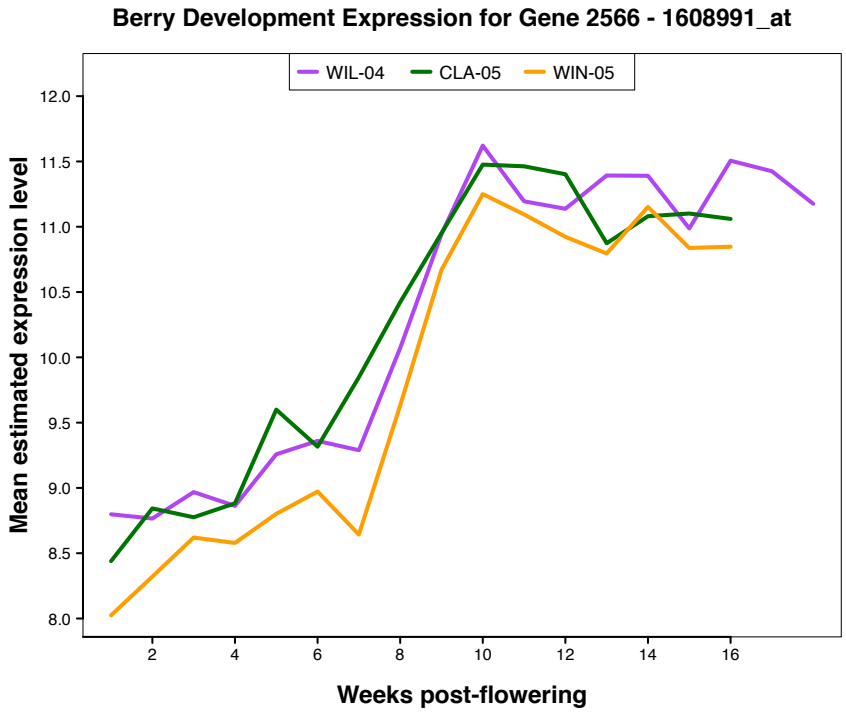


Figure T5. Expression profiles of genes involvd in hexose transport across the plasma membrane.

(a)



(b)

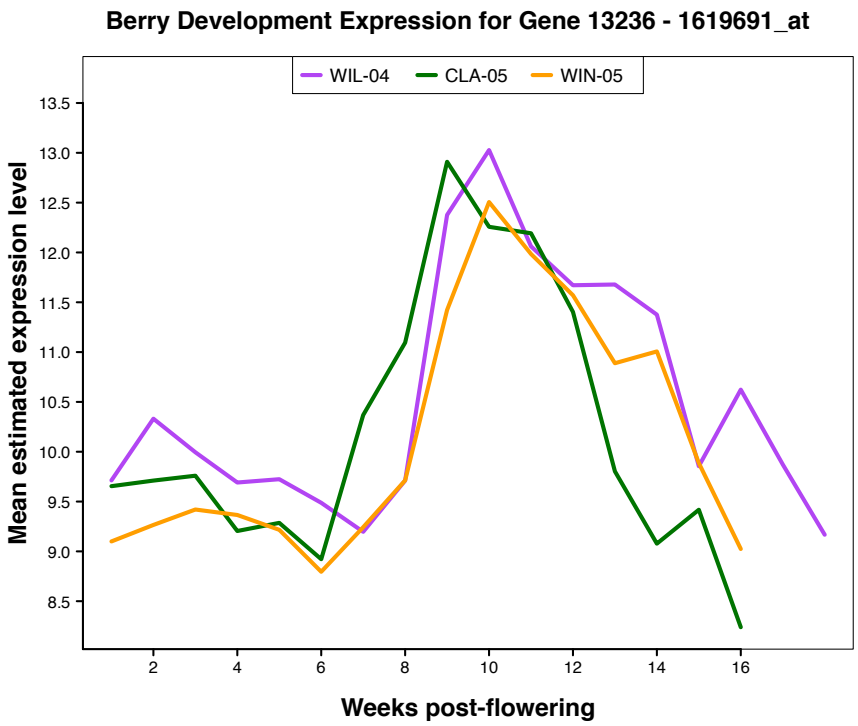


Figure T6. Anthocyanin biosynthesis pathway. From Boss *et al*., (1996).

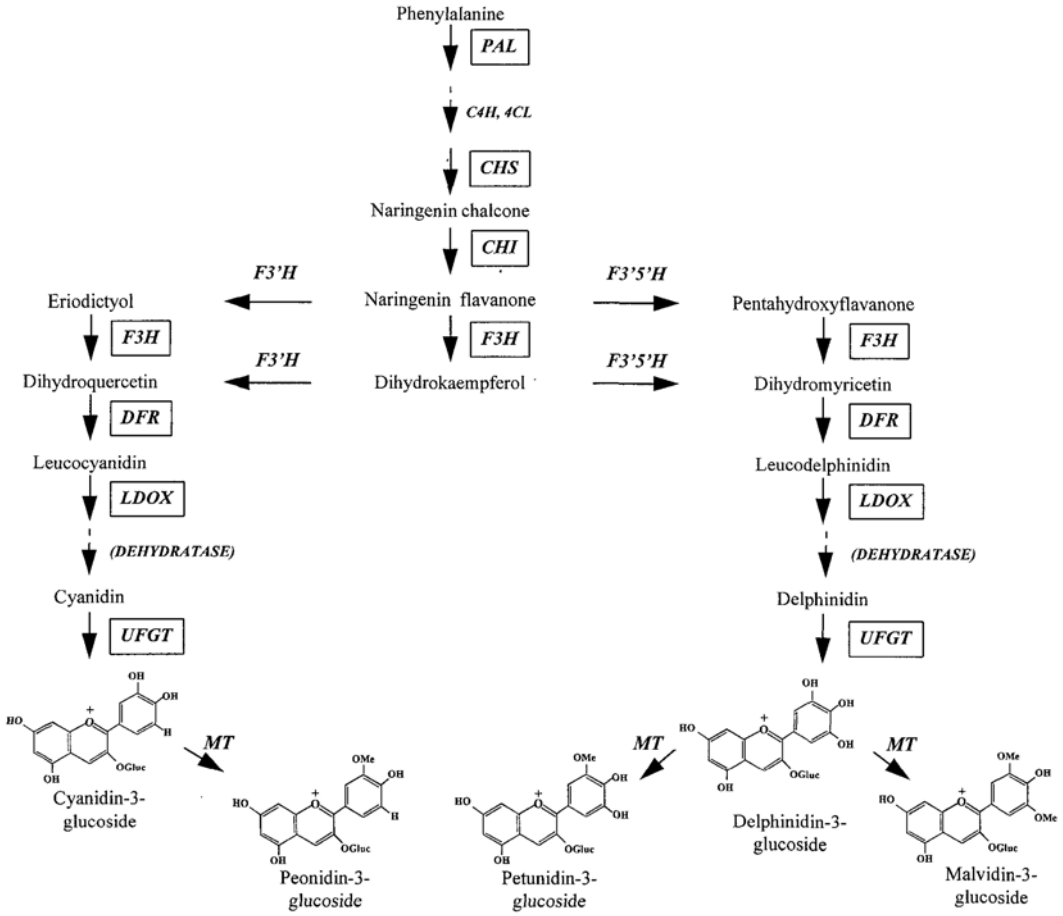
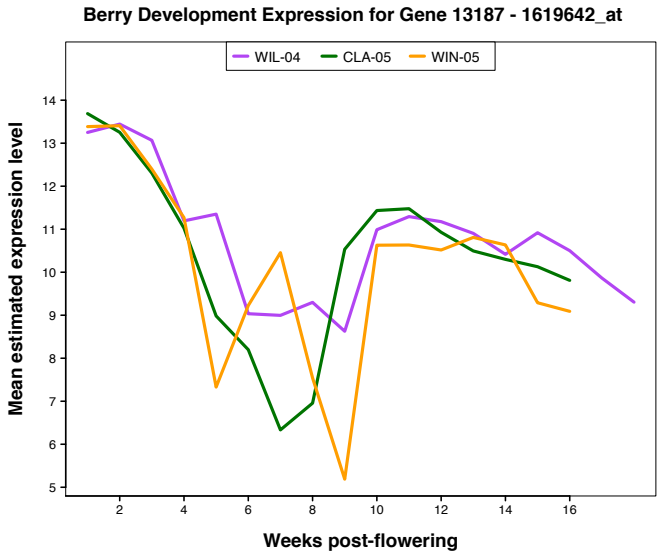
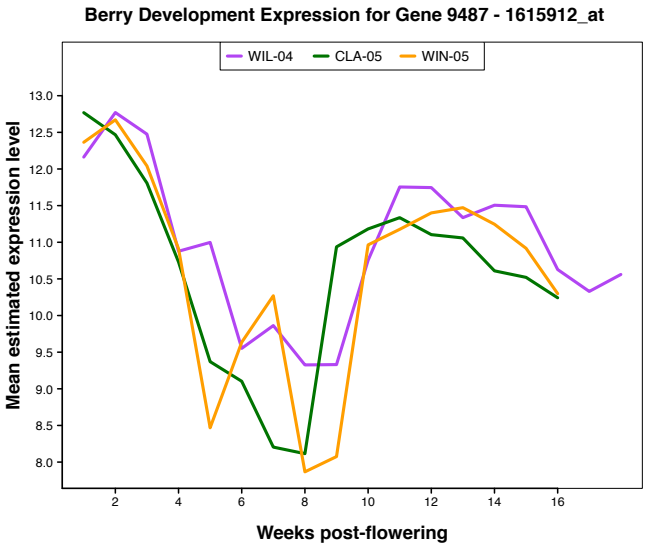


Figure T7. Profiles of genes involved in the anthocyanin pathway. PAL (a), CHI (b), F3H (c), UFGT (d) MYBA (e).

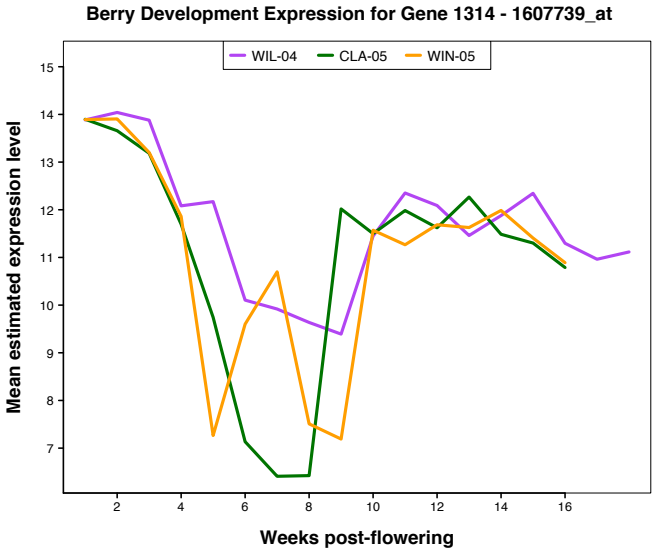
(a)



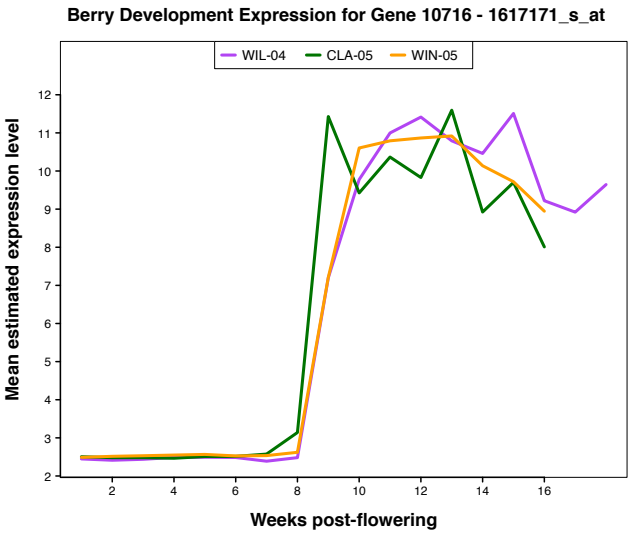
(b)



(c)



(d)



(e)

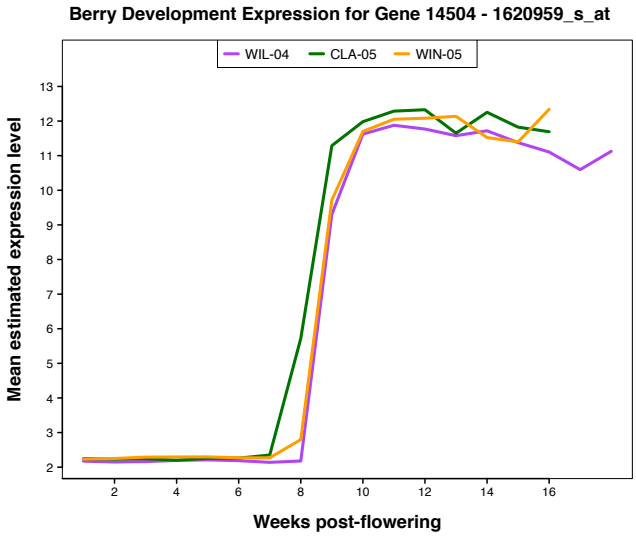


Figure T8. Stilbene biosynthesis pathway. From Bais *et al*., (2000).

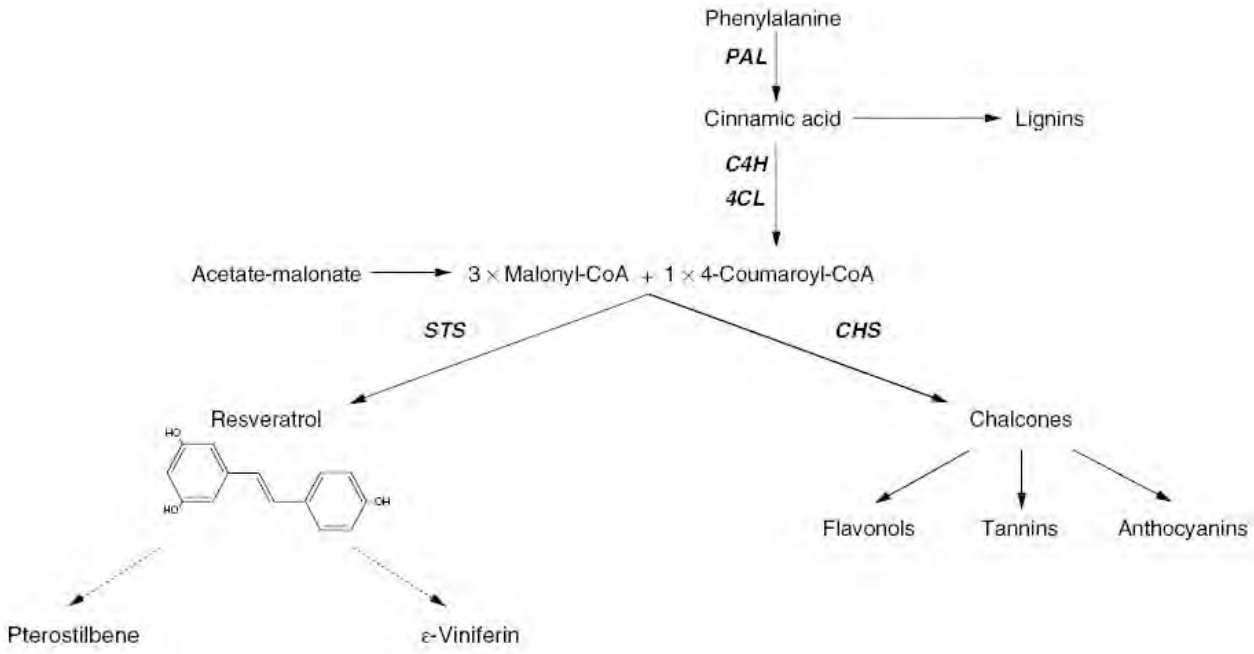
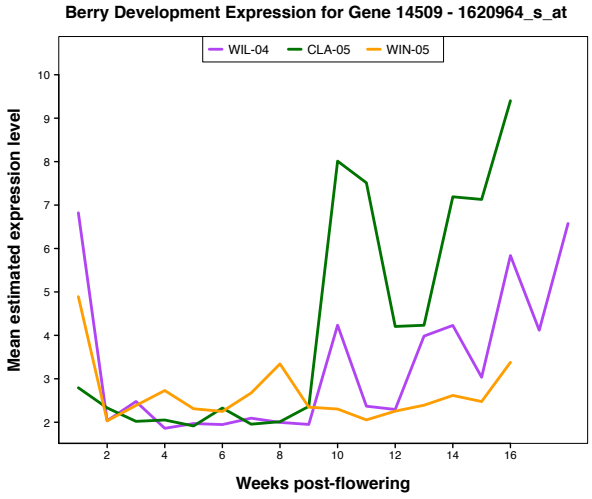


Figure T9. Gene profiles of stilbene synthase (STS) genes.



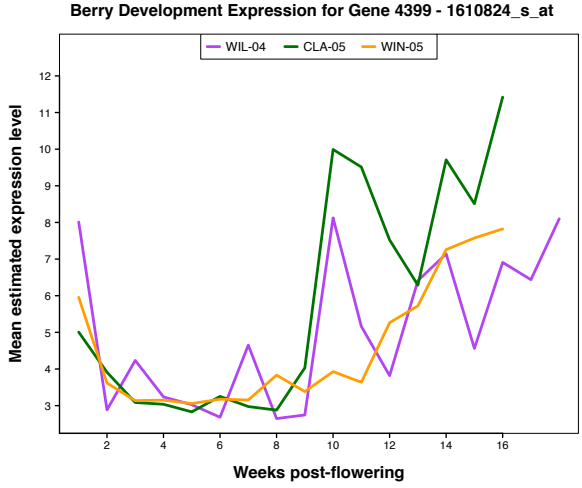


Figure T10. Pathways of proline biosynthesis in plants . From Delauney and Verma (1993).

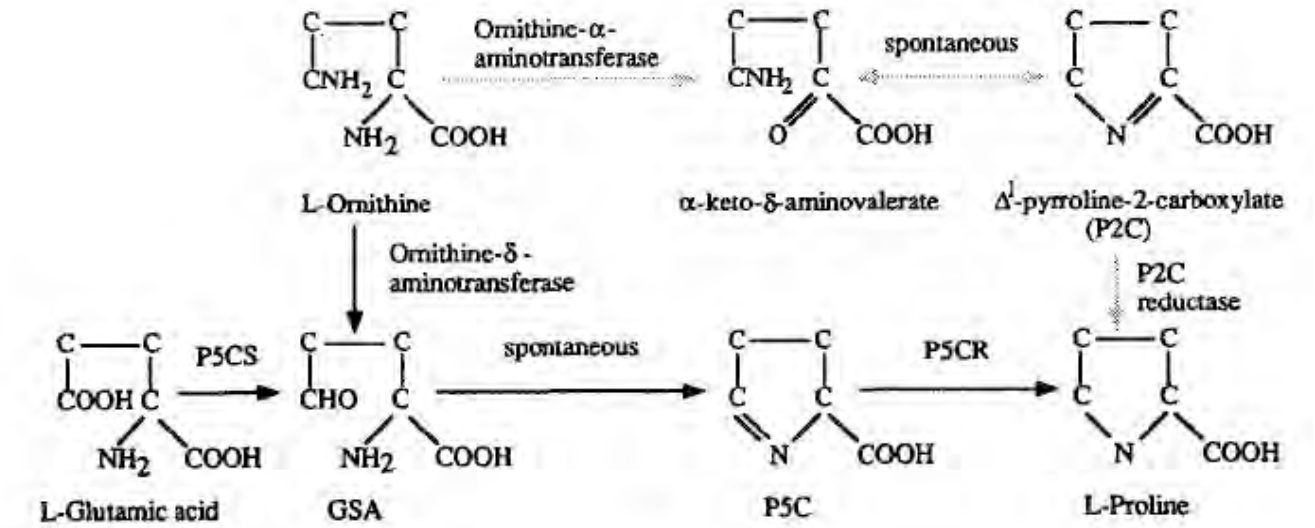


Figure T11. Gene expression profile of pyrroline-5-carboxylate synthetase (P5CS).

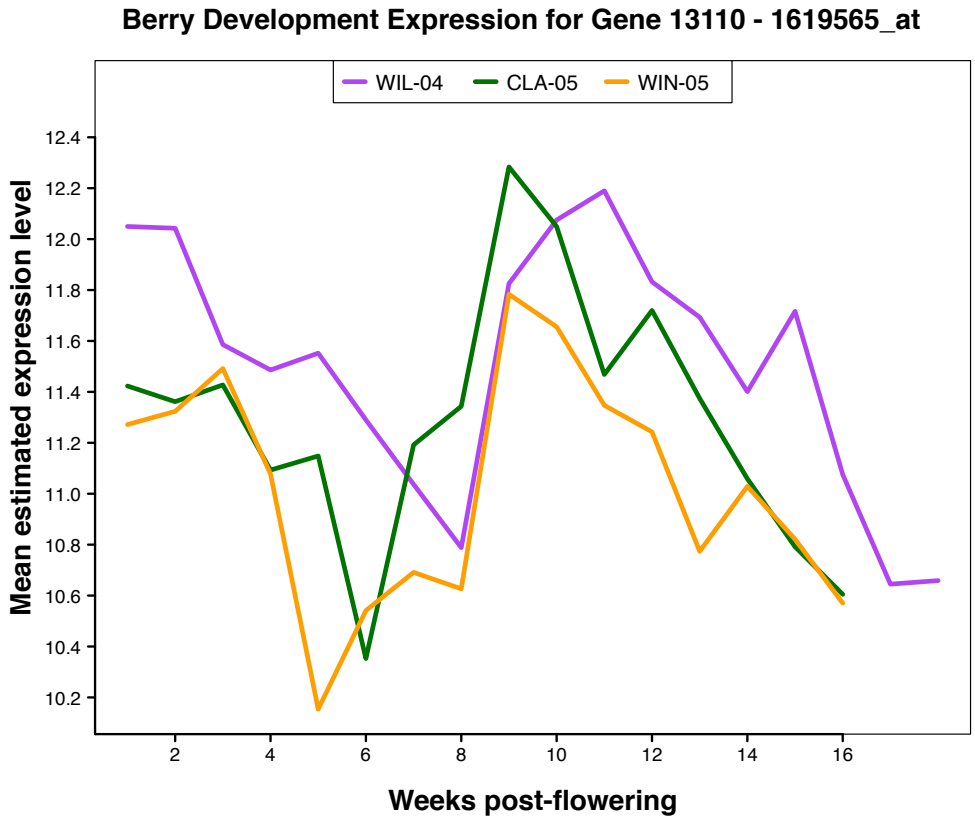


Figure T12. Proposed pathway of tartrate synthesis. From DeBolt *et al*. (2006).

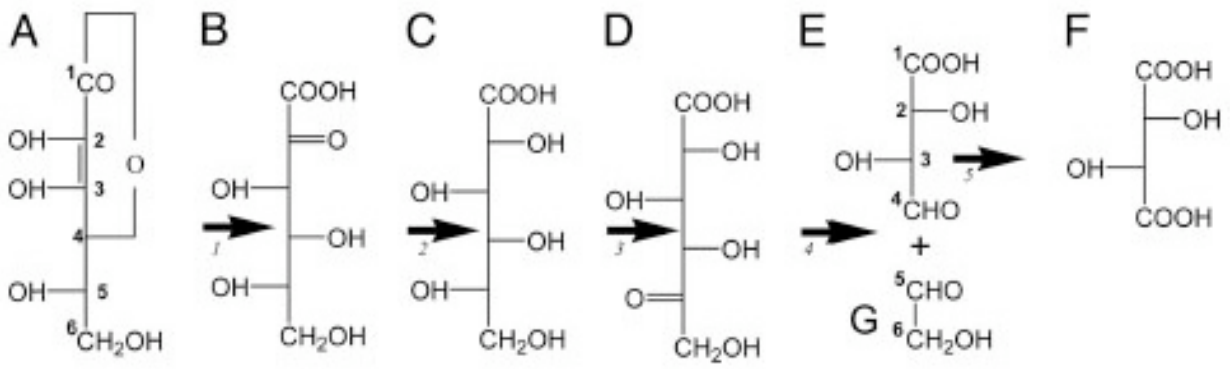


Figure T13. Gene Expression profile of L-idonate dehydrogenase involved in the conversion of C to D in Fig. T12.

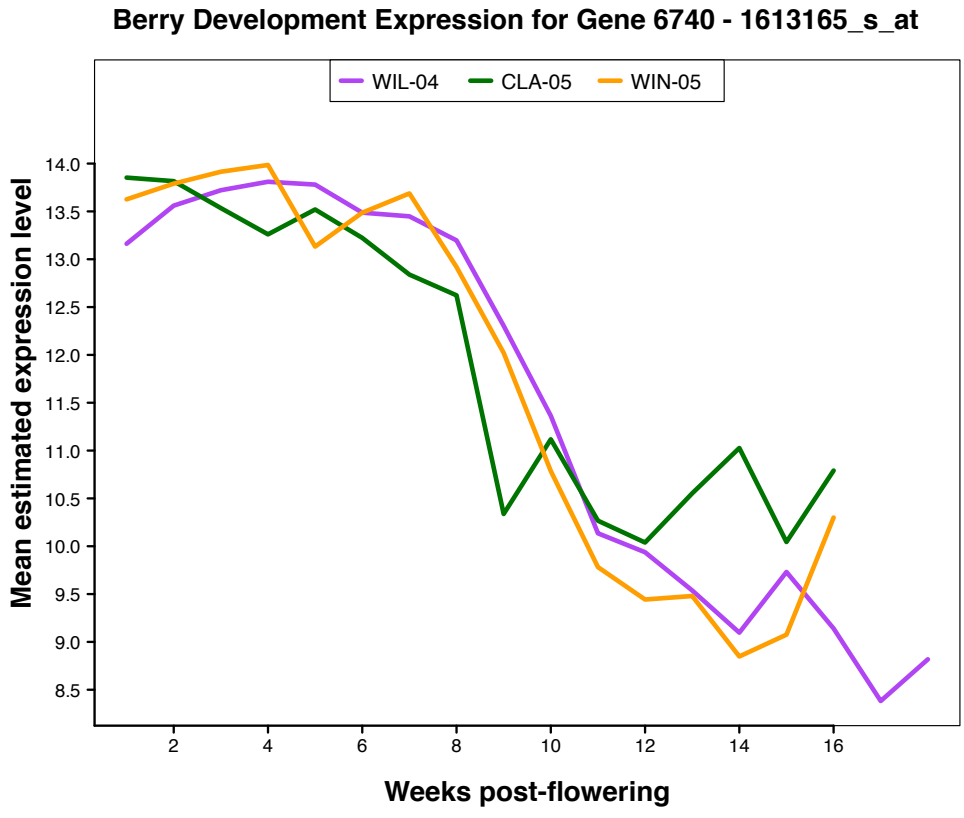
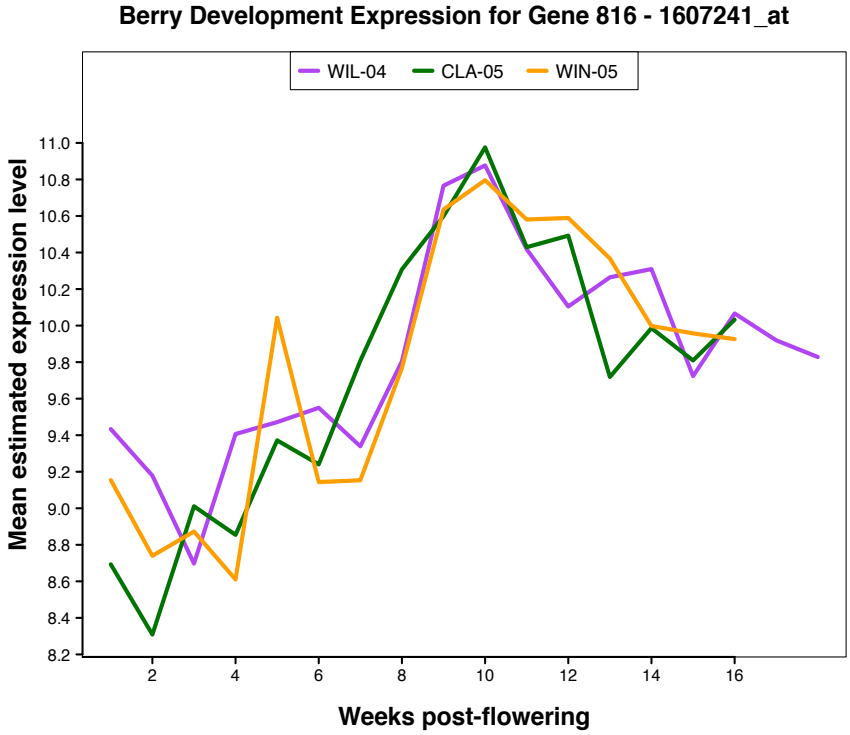


Figure T14. Expression profiles of genes involved in potassium transport across the plasma membrane.

(a)



(b)

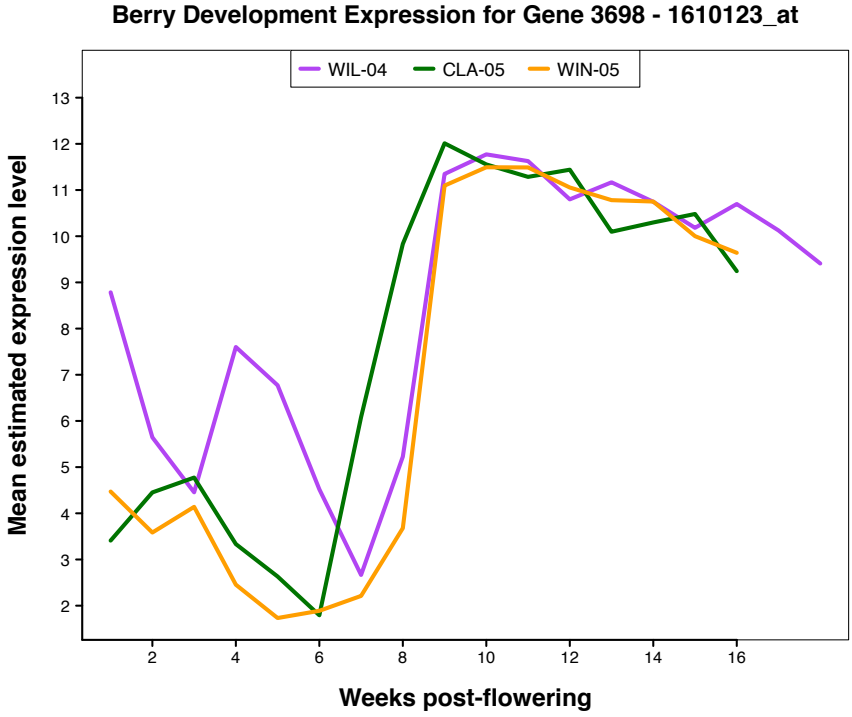


Figure T15a. Ripening-related expression of a gene encoding a putative thaumatin-like protein.

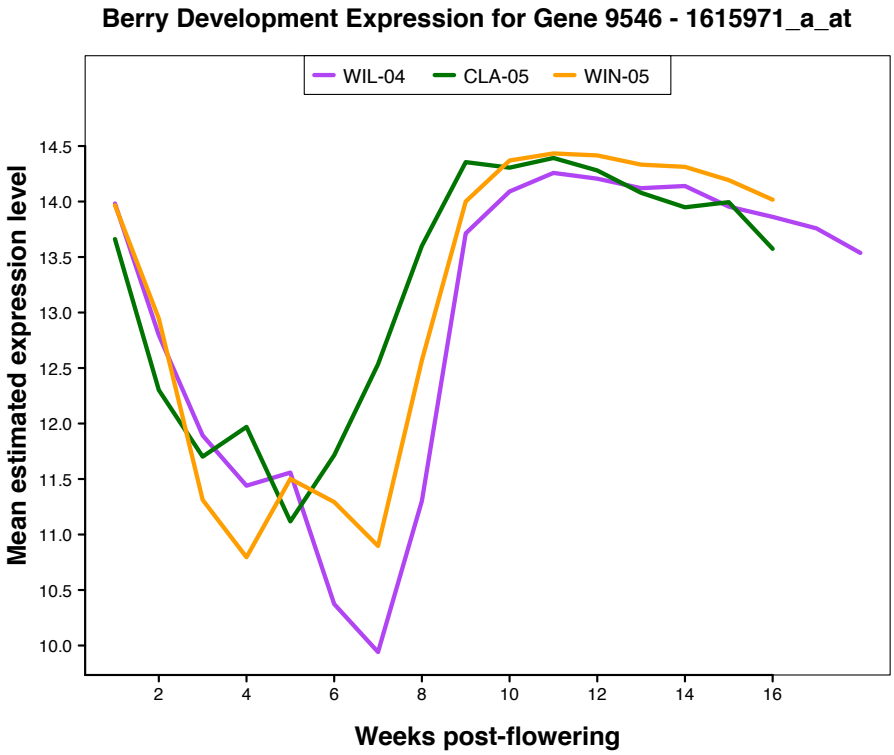


Figure T15a. Ripening-related expression of a gene encoding Grip 61.

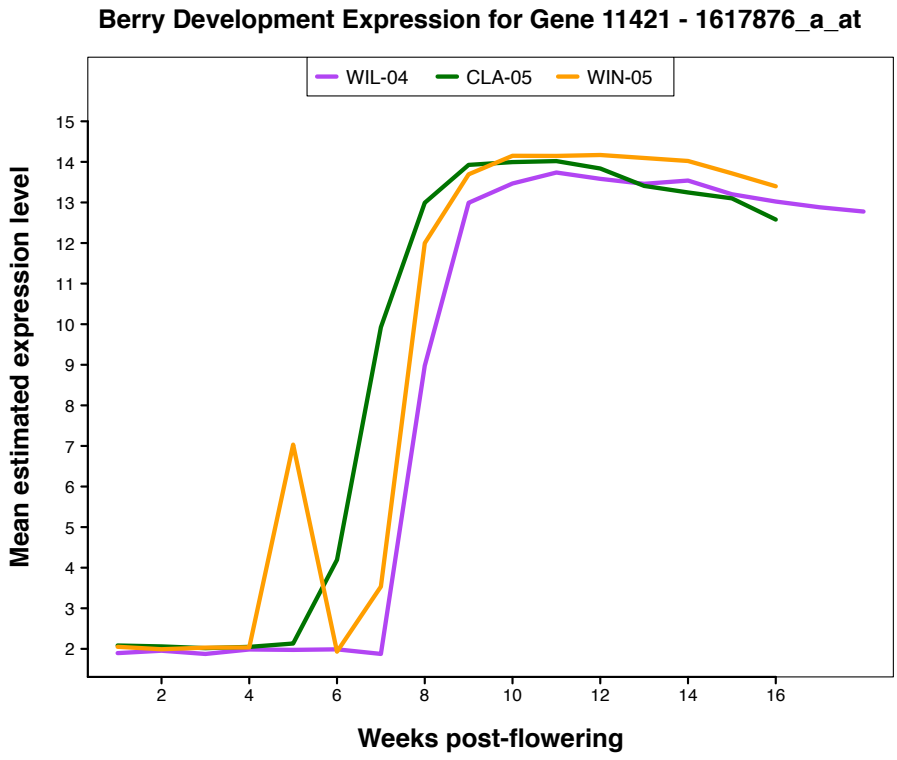


Figure T15c. Ripening-related expression of a gene encoding a putative proline rich cell wall protein (Grip4).

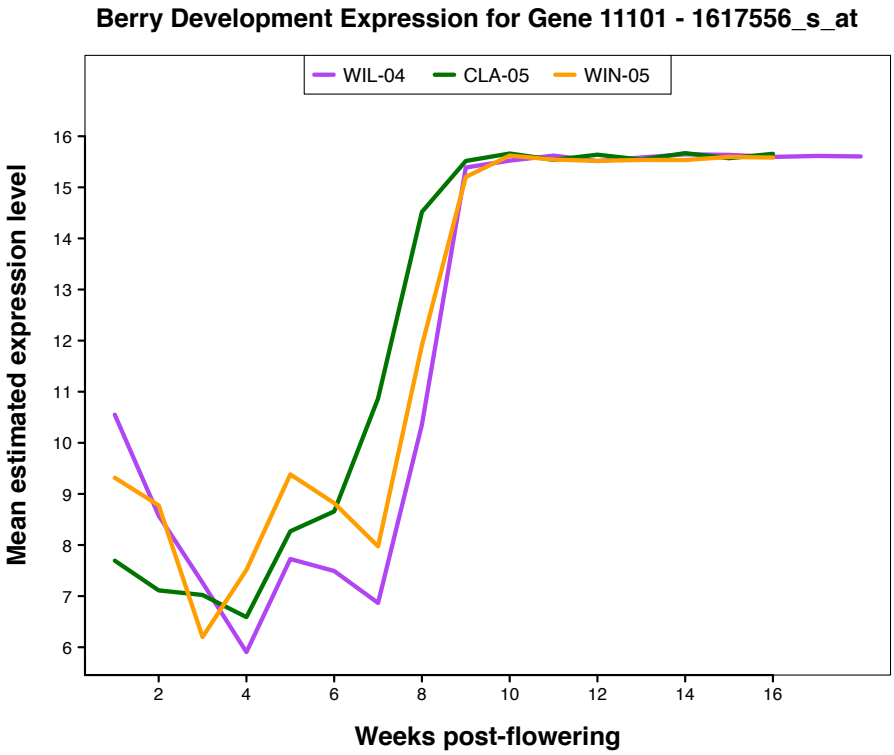


Figure T16a. Expression profiles of a putative chromatin assembly factor.

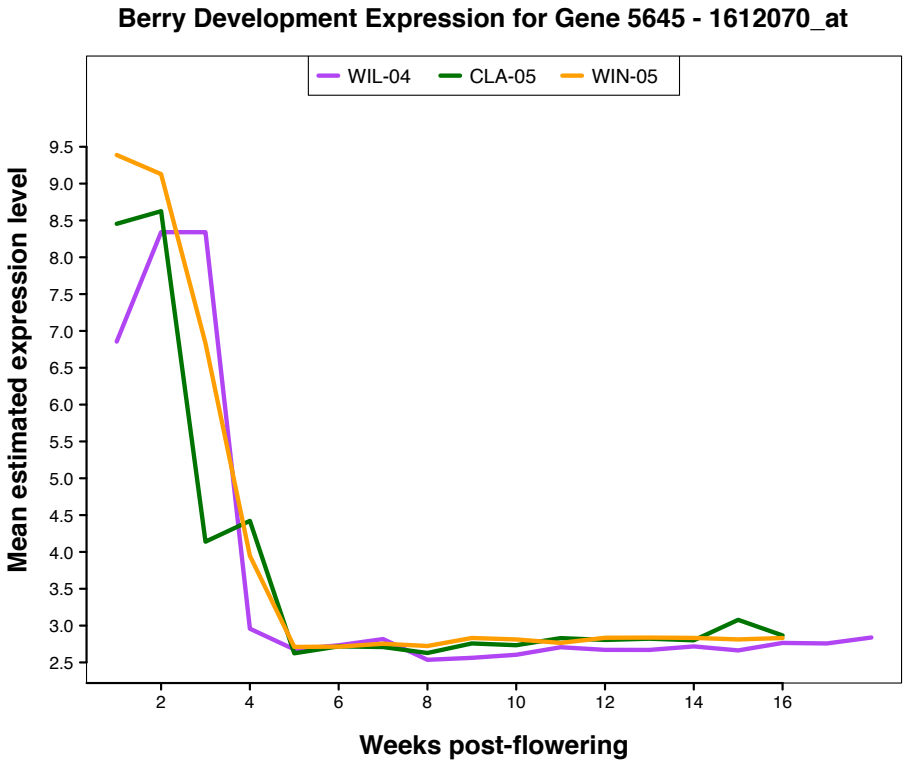


Figure T16b. Expression patterns of a putative cyclin D-3 gene.

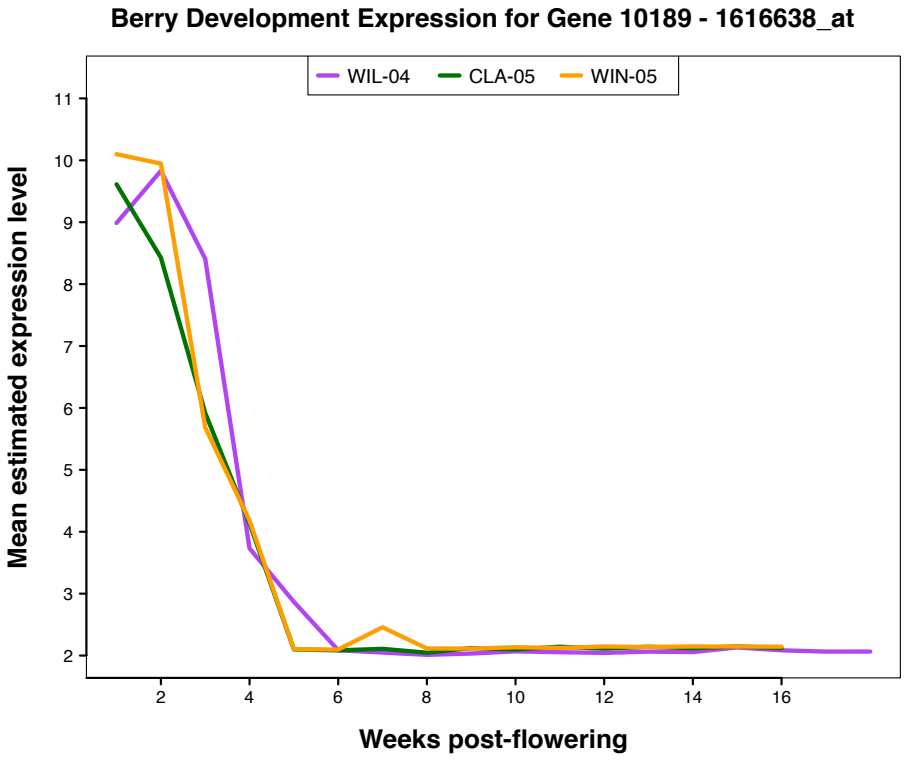


Figure T17a. Expression profiles of a gene encoding a putative caffeic acid o-methyl transferase emzyme.

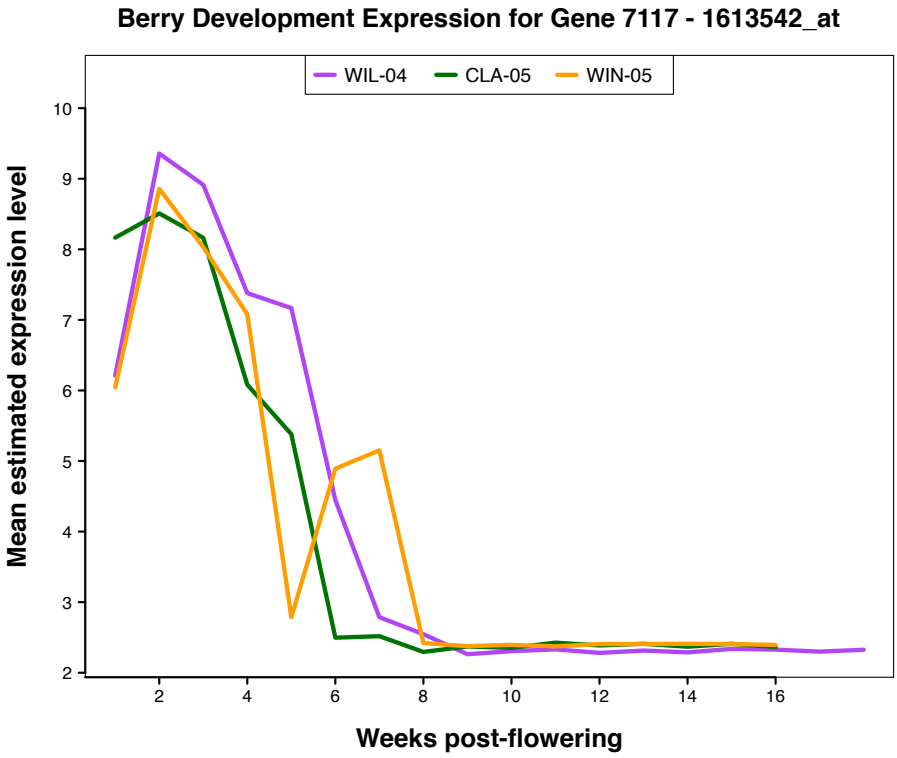


Figure T17b. Expression profiles of a gene encoding a putative shikimate dehydrogenase enzyme.

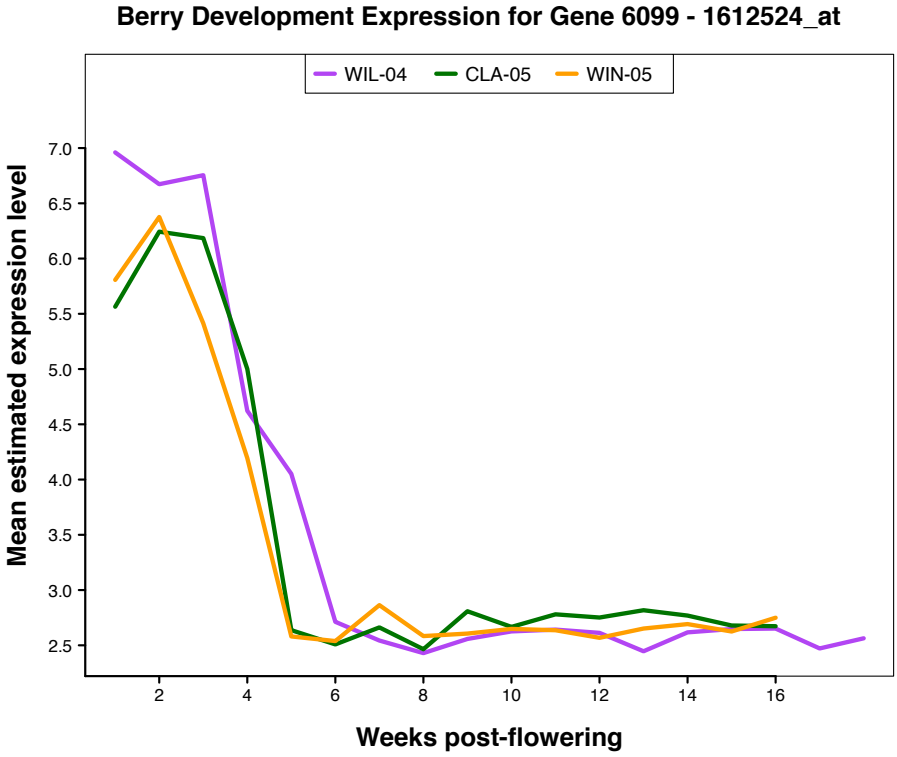


Figure T18. Expression profiles of a gene of unknown function showing late post-veraison expression.

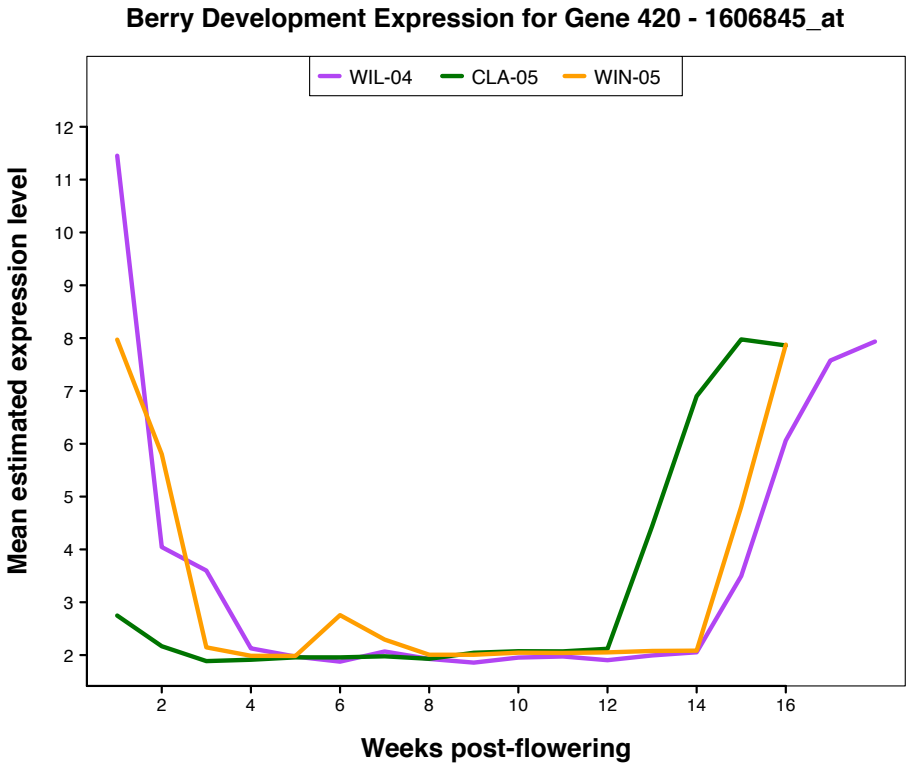


Figure T19. Expression profiles of a gene encoding a putative homeobox transcription factor.

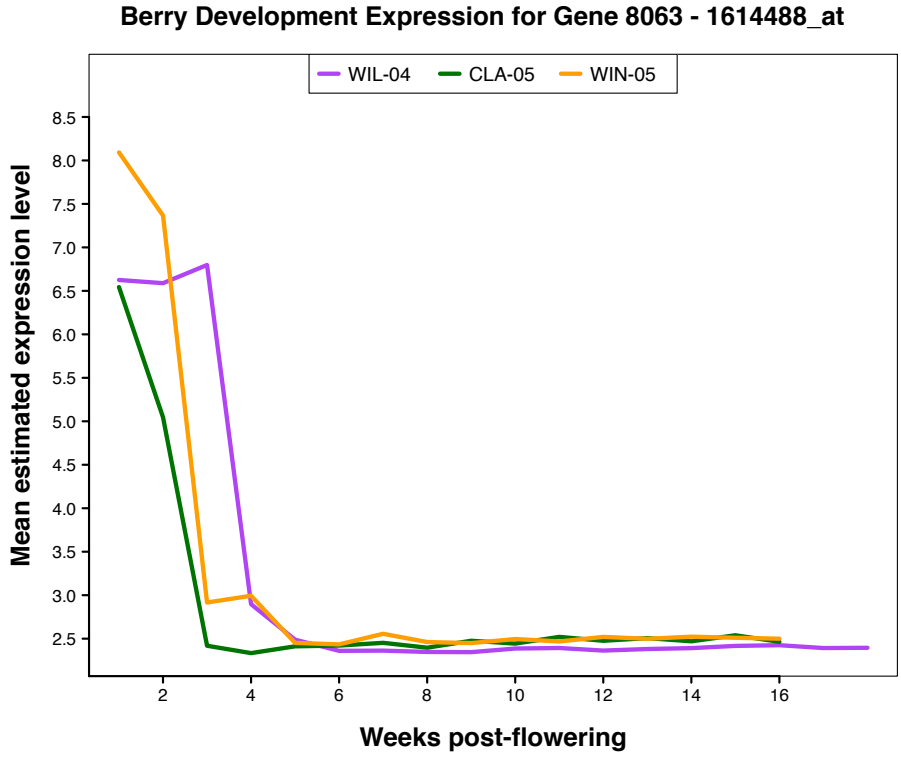
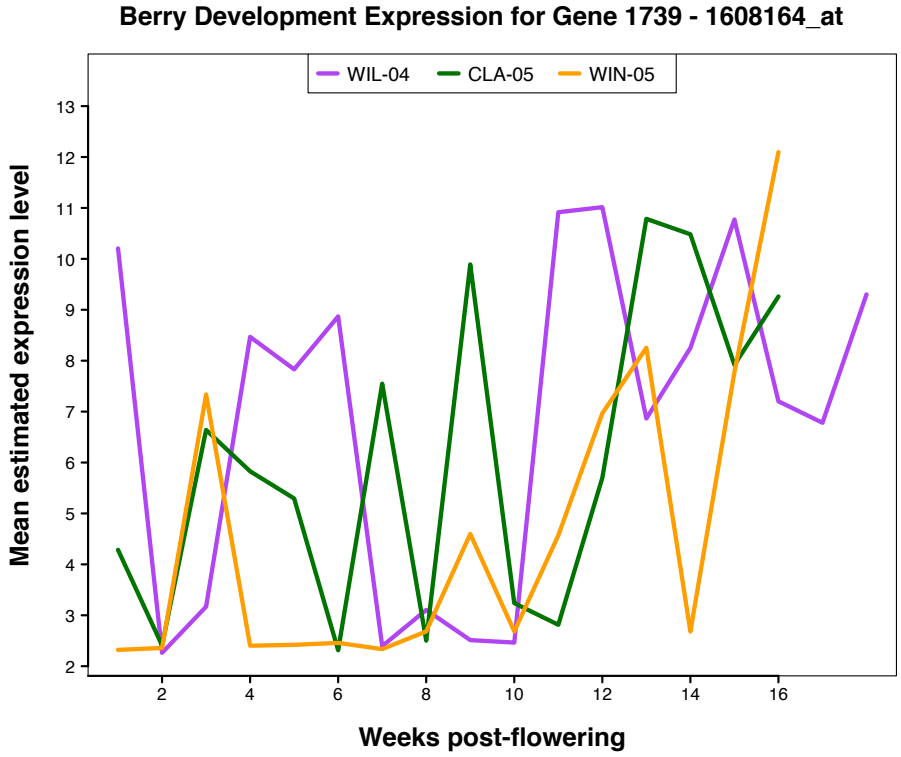
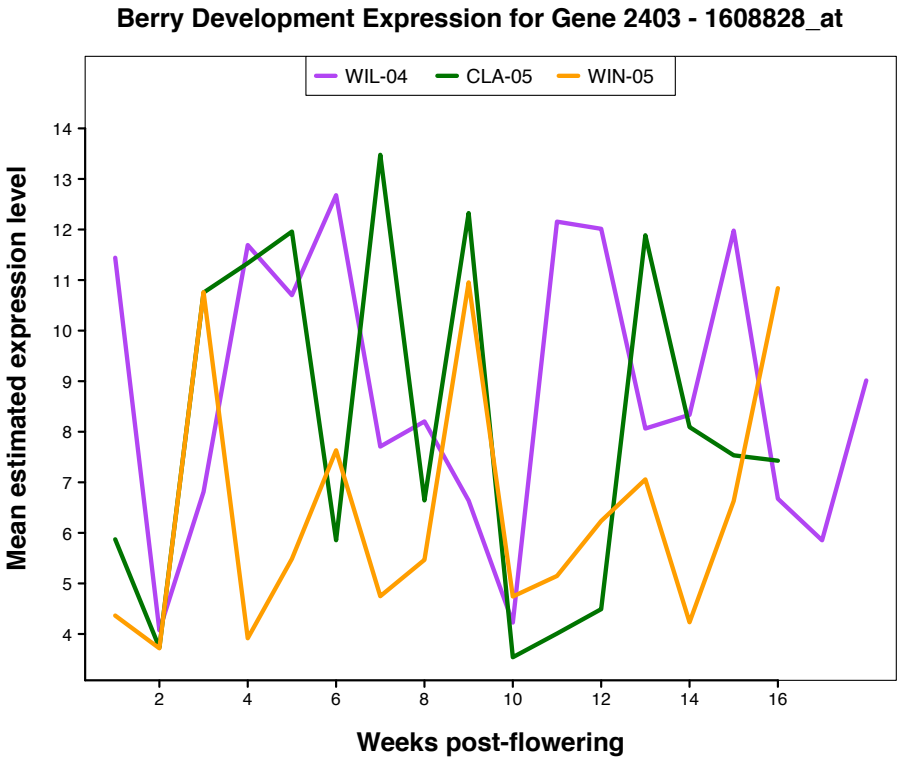


Figure T20. Expression profiles of two genes showing showing very different patterns between the 3 sites suggesting that these genes are regulated by external factors such as the environment.





Outcomes / Conclusions:
This project investigated the application of microarray technology for the study of berry development and berry composition. The measurement of gene expression changes during berry development could provide diagnostic molecular indicators of processes involved in berry growth, ripening and composition that ultimately have a role in determining final wine quality. The microarray grape GeneChip used in these studies contained nearly 16000 gene features that represented between one third to one half of all the protein coding genes expected to be present in the grapevine genome. This coverage is sufficient to give a detailed global analysis of grape berry gene expression and represents the most thorough coverage currently available.

The project was designed to investigate gene expression similarities and differences during berry development of Cabernet Sauvignon on own roots between three sites (Willunga, Wingara, Clare) and two viticultural treatments; one at Clare, spur versus machine pruned vines, and one at Wingara, control irrigation versus prolonged deficit irrigation.

To relate gene expression data to berry development and composition as well as wine quality a holistic approach was taken with sensory analysis performed on the wine, and berry development and composition parameters measured throughout development including; berry weight, brix, sugars, anthocyanins, acids, inorganic elements and amino acids. This is the first time such a study has been attempted.

The sampling, RNA preparation and microarray methods used in this study resulted in high quality datasets with high reproducibility between replicates and indicated that the microarray protocols and technology used in the study represented a relative robust and objective measure of gene expression changes that occur during berry development. The datasets were suitable for high level mathematical and statistical analysis. To interrogate the large microarray datasets the R statistical environment was used and involved statistical and Bioconductor packages as described in the Method section.

The transition from flower to berry was characterised by more than 4,960 genes which, in at least in one of the developmental series, showed a significant expression difference between anthesis and one week of berry development. This one week period from anthesis was targeted for investigation because this is a critical period for fruit set and initiation of berry development. Further investigation of these genes may lead to a better understanding of the processes and key genes involved in fruit set and growth.

It was found that in the berry a group of genes displayed changing expression levels during a 24 hour day/night cycle. This group of genes were classified, using information from a field-based experiment, into those that responded to light or those that were regulated by an internal circadian clock within the berry. In addition, a gene's response to diurnal rhythms was influenced by berry development stage. This information assists in the assigning of likely function to genes whose function has not previously been determined. As a result of these findings it is clear that the time of berry sampling in the vineyard is important for biochemical, physiological and gene studies to reduce errors and variation as a result of diurnal effects.

To investigate changes throughout berry development from 1 week post-anthesis until harvest in more detail a nested F-test was used to identify differential expression over

time. Genes were selected for further analysis using FDR adjusted p-values. Out of the nearly 16000 genes on the chip it was found that for all three sites and treatments a similar number of genes were identified as having a significant ($p<0.0000001$) expression pattern; 6263 (Wingara control irrigated vines), 8046 (Wingara prolonged deficit treatment), 7215 (Willunga), 7608 (Clare spur pruned vines) and 7425 (Clare machine pruned vines). This means that gene expression patterns are quite fluid during berry development as a large percentage of the genes assayed change significantly during development. To group these significant genes into similar patterns during berry development the clustering algorithm CLARA was used. Cluster patterns of expression were identified with many unique gene profiles occurring during the pre-veraison period as well as the post-veraison period. These patterns may represent indicators of important processes occurring in the berry during these stages. Up until now it has been difficult to accurately measure key changes that occur during the pre-veraison stage and the microarray results have provided new gene markers for further subdividing both the pre and post-veraison stages of berry development. Further investigation of these gene patterns, as well as those of the gene regulators (transcription factors) controlling the expression patterns of these genes, is required.

The expression patterns of genes involved in key berry quality traits were investigated to see if there was any correlation with the expression patterns and metabolite presence and accumulation. The processes of sugar, anthocyanins, stilbenes, proline and potassium accumulation were investigated. It was found that specific genes associated with these processes had expression patterns which were in some cases correlated with the presence and accumulation of the metabolites. Future investigation of the role of these genes through functional analysis may provide a better understanding of these processes and may indicate how accumulation of these compounds might be manipulated.

The results from the microarray studies of viticultural treatments were surprising. Very few significant gene expression changes were identified when pruning treatments at Clare, or irrigation treatments at Wingara, were compared. These results were confirmed by the biochemical analysis of berry composition and wine sensory analysis where in both cases there were only minor, or no, differences detected. This suggests that for the years studied the viticultural treatments had very minor effects on the expression of genes that determined berry composition. However, what was of interest was the large number of genes whose expression pattern varied between the three sites suggesting that some of these genes may represent molecular diagnostic indicators of a berries varying response to differing environmental conditions. Further investigation of these genes, especially how the changes in expression relate to particular environmental factors, is required.

The Affymetrix-based technology and microarray datasets have proved to have had wider use outside of this current project and have been utilised by groups at the University of Adelaide and CSIRO for investigating specific berry processes and the genes involved in these processes. It is expected that the microarray datasets produced within this project will be a valuable resource for many years to come and is already being used in another GWRDC-funded student project investigating “Bioinformatic analysis of berry development to identify gene and environment interaction”.

Recommendations:
The unique results and outcomes of the project have led to the following recommendations:

- Microarray technology has proved to be a powerful technology for objectively measuring gene expression changes of thousands of genes in grapevine. However, the large datasets produced requires suitable computational, mathematical and statistical treatment. It is suggested that future studies involve multi-disciplinary teams that combine viticultural, molecular and bioinformatic expertise to maximise the benefit of the large datasets produced.

- It is expected that a 2nd generation microarray grape GeneChip will be produced in 2008-9 that will contain all of the grapevine genes present in the genome. The use of this technology in new studies that involve well designed experiments will allow a complete understanding of berry development and composition to be developed at the level of gene expression. As the GeneChip will contain all the grapevine genes the same technology could also be used to investigate any tissue (flowers, roots, leaves, etc) and process (e.g. fruitfulness, rootstock-scion interactions).

- The microarray technology is suitable for investigating differences between viticultural treatments as well as site differences where environmental factors may influence gene expression and ultimately berry development and composition. It is recommended that further studies be carried out in this area given the difficulties with other methods for objectively measuring differences between sites and the increasing importance of climate change. Understanding what genes and processes are influenced by environmental effects, and what ones are not, will be key to deciding how best to respond to seasonal effects and climate change. The objectivity and statistical treatment of the data will also help researchers and viticulturalists in their decision making when comparing differences between viticultural treatments.

- The microarray technology is suitable for investigating differences between varieties and will form a key role in identifying important genes controlling berry development and composition. This knowledge can be applied in functional genomic studies and in the breeding of new varieties better suited to Australian conditions, regions and the changing climate.

- This study adopted a holistic approach to investigate berry development and quality/composition by measuring gene expression changes, berry growth, berry metabolites and finally wine quality as determined by sensory analysis. While such studies requires a team with a mix of skills it is suggested that there are significant benefits to this approach and that future studies consider adopting a more holistic approach to processes being studied.

Appendix 1: Communication:

The project aims, methodology and progress have been discussed a number of times with representatives from the major wine companies and with a range of producers and wine makers. The companies, Hardy Wine Company, Chalk Hill Wines, Nepenthe Wines and Wingara Wine Group have been very supportive of the work and have been instrumental in the conduct of our extensive field sampling program. More formal oral presentations have also been presented to some of these parties and similar, but less formal, discussions have been held with other small. Presentations to, and discussions with, the CRCV2 Program 3 Industry Reference Group have occurred each year. Information regarding this project was published in the CRCV information pamphlets 'Gene Technologies and the Australian Industry' numbers 3 and 4 (http://www.crcv.com.au/resources/Gene%20Technology/). A number of articles regarding this work have been published in both industry and scientific journals and many talks have been presented regarding this work both locally and overseas (see below).

Publication List:

Thomas MR, Davies C, Iocco P, Dry IB, Barker C, Jermakow A, Boss PK, Koltunow A. Genomics: the foundation for vine improvement. Australian Wine Industry Technical Conference, Melbourne, Australia, 25-28 July, 2004

Davies C, Iocco P, Hua CT, Thomas MR. Advances in Grapevine Genomics
SBBIOTEC -Brazilian Society of Biotechnology Congress: Frontiers of Biology, August 16- 20, 2004 Salvador / Bahia

Davies C, Iocco P, Hua CT, Thomas MR. Advances in Grapevine Genomics
ARSIA (Regional Agency for Development of innovation in agriculture) Second Symposium on Sangiovese, Firenze, Italy, 17 – 19 Nov 2004

Davies C, Hua CT, Iocco P, Thomas MR. Grapevine ESTs and microarrays – A spaghetti western. International Grapevine Bioinformatics Meeting. Reno, USA, June 6-7th 2003

Iocco P, Hua C, Davies C, Thomas MR (2003) Use of microarray analysis to study genes controlling grapevine berry ripening and development
Abstract for 3rd Microarray Meeting 23 – 26 of July 2003 (South Stradbroke Island, QLD)

Davies C, Hua CT, Iocco P, Thomas MR. New insights into berry growth and quality. The Australian and New Zealand Grapegrower and Winemaker. 32nd Technical issue, p117-118

Thomas, MR, Hua CT, Iocco P, Davies C. Grapevine ESTs and their use in microarray analysis to study genes controlling grapevine berry ripening and development. Proceedings 7th International Symposium on Grapevine Physiology and Biotechnology (California, 21-25 June, 2004)

Davies C, Iocco P, Shavrukov Y, Symons GM, Dry IB, Reid JB, Thomas MR. Microarray analysis of berry development identifies a role for brassinosteroids in grape berry ripening. (Udine Italy 2006)

Iocco P, Davies C, Thomas, MR The use of the affymetrix *V. vinifera* genechip for the analysis of temporal gene expression in grapevine. Proceedings 5th Australian Microarray Conference (Barossa Valley, 29 September-1 October, 2005).

Iocco P, Davies C, Symons GM, Reid JB, Shavrukov Y, Dry IB, Thomas MR The use of the Affymetrix *V. vinifera* GeneChip to identify key genes involved in berry development. (ISPMB Adelaide Australia, August 21–25, 2006).

Appendix 2: Intellectual Property:
As determined by the CRCV project agreement.

Appendix 3: References:

Aharoni A, Keizer LCP, Bouwmeester HJ, Sun ZK, Alvarez-Huerta M, Verhoeven HA, Blaas J, van Houwelingen AMML, De Vos RCH, van der Voet H, Jansen RC, Guis M, Mol J, Davis RW, Schena M, van Tunen AJ, O'Connell AP *et al*. (2000) Identification of the SAAT gene involved in strawberry flavour biogenesis by use of microarrays. Plant Cell **12:** 647-661

Bais AJ, Murphy PJ, Dry IB (2000) The molecular regulation of stilbene phytoalexin biosynthesis in *Vitis vinifera* during grape berry development. Aust J Plant Physiol **27:** 425-433

Bolstad BM (2004) in Biostatistics (University of California, Berkeley), pp. 156

Boss PK, Davies C, Robinson SP (1996) Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol **111**: 1059-1066

Coombe BC (1995) Adoption of a system for identifying grapevine growth stages. Australian Journal Grape Wine Research **1**: 104-110

Davies C, Robinson SP (1996) Sugar accumulation in grape berries: Cloning of two putative vacuolar invertases and their expression in grapevine tissues. Plant Physiol. **111**: 275-283

Davies C, Robinson SP (2000) Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress proteins. Plant Physiol. **122**: 803-812

Davies C, Shin R, Liu W, Thomas MR, Schachtman DP (2006) Transporters expressed during grape berry (*Vitis vinifera L.*) development are associated with an increase in berry size and berry potassium accumulation. J Exp Bot **57:** 3209-3216

DeBolt S, Cook DR, Ford CM (2006) L-Tartaric acid synthesis from vitamin C in higher plants. PNAS **103:** 5608-5613

Delauney and Verma (1993) Proline biosynthesis and osmoregulation in plants. Plant J. **4:** 215-223

Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) Affy - analysis of Affymetrix GeneChip data at the probe level. Bioinformatics **20**: 307-15

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol **5**: R80

Hayes MA, Davies C, Dry IB (2007) Isolation, functional characterisation and expression analysis of grapevine (*Vitis vinifera* L.) hexose transporters: differential roles in sink and source tissues. (In Press).

Hubble GD, Crocker RL (1941) A soil survey of the Red Cliffs irrigation district, Victoria.

Kaufman L, Rousseeuw P (2005) Clustering Large Applications (Program CLARA). In Finding Groups in Data: An Introduction to Cluster Analysis. New York: John Wiley & Sons; pp. 126-146

Kobayashi S, Ishimaru M, Hiraoka K, Honda C (2002) Myb-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta **215**: 924-933

Krstic M, Mould G, Panagiotopoulos, West S (2003) Growing quality grapes to winery specification. Ed. S. Collings, Winetitles, Adelaide, South Australia, Australia.

Ojeda H, Deloire A, Carbonneau A, Ageorges A, Romieu C (1999) Berry development of grapevines: Relations between the growth of berries and their DNA content indicate cell multiplication and enlargement. Vitis **38**: 145-150

Reiner A, Yekutieli D, Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368-75

Ruffner HP (1982) Metabolism of tartaric and malic acids in *Vitis*: A review – Part A. Vitis **21:** 247-259

Schaffer R, Landgraf J, Perez-Amador M, Wisman E (2000) Monitoring genome-wide expression in plants. Curr Op Biotech **11:** 162-167

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 13000 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell. **13:** 61-72

Shoemaker R, Keim P, Vodkin L, Retzel E, Clifton SW, Waterston R, Smoller D, Coryell V, Khanna A, Erpelding J, Gai XW, Brendel V, Raph-Schmidt C, Shoop EG, Vielweber CJ, Schmatz M, Pape D, Bowers Y, Theising B, Martin J, Dante M, Wylie T, Granger C (2002) A compilation of soybean ESTs: generation and analysis. Genome **45:** 329-338

Stines AP, Naylor DJ, Høj PB, van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ^1-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiol **120:** 923-931

Smyth GK (2004) Statistical Applications in Genetics and Molecular Biology 3, Art 3.

Wu Z, Irizarry RA, Gentleman R, Martinez-Murillo F, Spencer F (2004) A Model-Based Background Adjustment for Oligonucleotide Expression Arrays The Journal of the American Statistical Association **99:** 909–917

Zarcinas BA, Cartwright B, Spouncer LR (1983) Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. Communication in Soil Science and Plant Analysis **18**: 131-146

Zhang XY, Wang XL, Wang XF, Xia GH, Pan QH, Fan RC, Wu FQ, Yu XC, Zhang DP (2006) A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. Plant Physiol **142:** 220-232

Appendix 4: Staff

Project Staff & People who assisted with the project

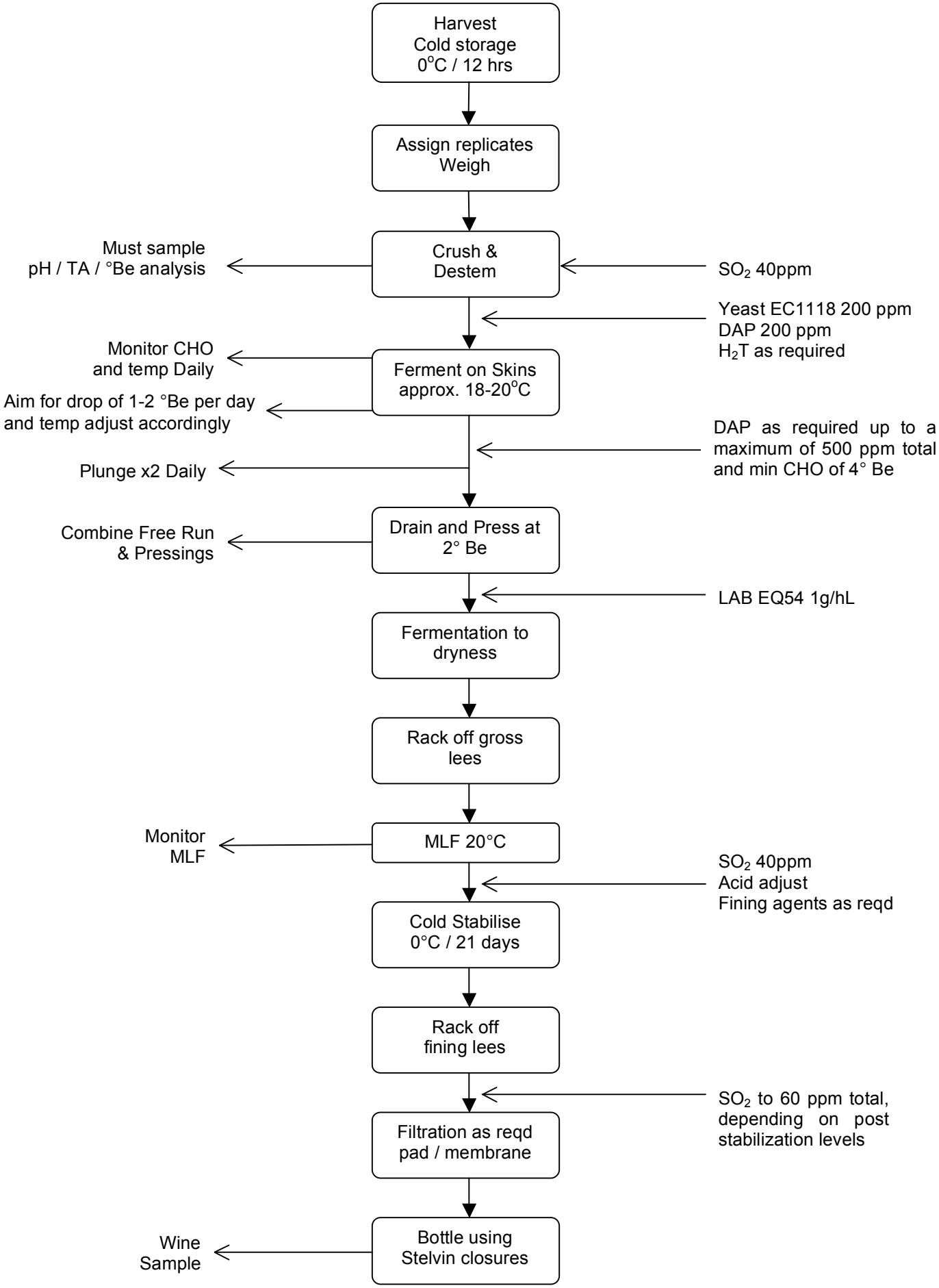
Dr Mark Thomas, CSIRO Plant Industry
Dr Christopher Davies, CSIRO Plant Industry
Dr. Jim Spiers, CSIRO Plant Industry
Dr. Anna Koltunow, Plant Industry
Ms Pat Iocco, CRCV
Mr Peter Clingeleffer, CSIRO Plant Industry
Dr Nicky Cooley, CSIRO Plant Industry
Ms Rachael Hanlin, CSIRO Plant Industry
Dr. Gary Glonek, School of Mathematical Sciences, University of Adelaide
Dr. Chris Wilkinson, School of Mathematical Sciences, University of Adelaide
Mr Jason Ellul, School of Mathematical Sciences, University of Adelaide

Appendix 5: Additional Data

Appendix 5a

PROVISOR PTY LTD
UPDATED 12TH FEB 2003

SMALL LOT RED WINEMAKING PROCESS



Appendix 5b: Sensory analysis of wines from Clare pruning treatments.

Report #:	**PR06104**
Name:	**CSIRO Clare Cabernet Sauvignon Pruning Trial November 2005**
Author:	**Briony Liebich**
Position:	**Sensory Analyst**

Approved by,

Dr. Darren Oemcke
CEO

Date: 10/02/06

– GENERAL DISCLAIMER AND LIMITATION OF LIABILITY–

This document has been prepared by Provisor Pty Ltd ("Provisor") for a specific purpose and is intended to be used solely for that purpose and unless expressly provided otherwise does not constitute professional, expert or other advice.

The information contained within this document ("Information") is based upon sources, experimentation and methodology which at the time of preparing this document Provisor believed to be reasonably reliable and Provisor takes no responsibility for ensuring the accuracy of the Information subsequent to this date. No representation, warranty or undertaking is given or made by Provisor as to the accuracy or reliability of any opinions, conclusions, recommendations or other information contained herein except as expressly provided within this document. No person should act or fail to act on the basis of the Information alone without prior assessment and verification of the accuracy of the Information.

To the extent permitted by law and except as expressly provided to the contrary in this document all warranties whether express, implied, statutory or otherwise, relating in any way to the Information are expressly excluded and Provisor, its officer, employees and contractors shall not be liable (whether in contract, tort, under any statute or otherwise) for loss or damage of any kind (including direct, indirect and consequential loss and damage of business revenue, loss or profits, failure to realise expected profits or savings or other commercial or economic loss of any kind), however arising out of or in any way related to the Information, or the act, failure, omission or delay in the completion or delivery of the Information. In the event that any legislation or rule of law implies any condition, warranty or liability with respect to Provisor or the Information, Provisor's liability for breach of any condition, warranty or liability shall be limited, at the option of Provisor, to the re-supply of that Information; the cost of acquiring equivalent Information or the payment of the cost of having the Information re-supplied.

The Information is confidential and may be legally privileged. If you are not the intended recipient of the Information, please immediately notify Provisor and destroy the Information. Unless expressly provided in this document, Provisor retains ownership of the copyright in the Information and no part of the Information may be reproduced or copied in any form or by any means without the prior written consent of Provisor.

The Information must not be used in a misleading, deceptive, defamatory or inaccurate manner or in any way that may otherwise be prejudicial to Provisor, including without limitation, in order to imply that Provisor has endorsed a particular product or service.

1 Introduction

CSIRO engaged Provisor to assist in the evaluation of the key sensory properties of Cabernet Sauvignon wines to evaluate the influence of vineyard pruning effects. There were two types of pruning undertaken in a Clare vineyard prior to the 2005 vintage; spur pruning (SP) and machine pruning (MP), giving a total of two treatments. Each treatment was made in fermentation triplicates, giving a total of six wines for analysis. The wines were made and bottled by Provisor at the Hickinbotham Wine Science Laboratory.

2 Winemaking and Bottling

Provisor made six wines from 50kg fruit batches using our small-scale winemaking and bottling facilities at the Hickinbotham Roseworthy Wine Science Laboratory adhering to standard protocols.

The following reports were issued by Provisor for this stage of the project (refer Appendix 1 and 2):

- Fruit receival report; and
- Bottling report detailing chemical analysis results of all wines.

3 Sensory Analysis Methods

Sensory analysis was conducted using Provisor's trained sensory panels to measure the sensory properties of each trial wine. Generation of these results consisted of the following stages:

1. Informal assessment;
2. Difference testing; and
3. Descriptive analysis.

2.1 Informal Assessment

Provisor's internal panel (6 panellists) met to evaluate each of the six project wines independently followed by a group discussion. The wines were assessed after a minimum of four weeks storage following bottling as per our standard protocol.

2.2 Difference Testing

Difference testing was used to determine whether the three fermentation replicates of each treatment were perceptibly different overall, giving a total of six comparisons. The triangle test method was used to evaluate samples by aroma only. In each test, three coded samples were presented simultaneously to the panellists; two the same and one different. The panellists were instructed to pick the different sample. There were 18 responses in total. Samples were presented at room temperature under orange lights in three-digit coded covered ISO standard tasting glasses (25mL) using individual tasting booths.

2.3 Descriptive Analysis

Provisor's descriptive analysis panel (12 assessors) developed an attribute list for 12 aromas and 10 palate (taste, flavour and mouthfeel) characteristics (Table 2.3). Panellists were trained to recognise these sensory attributes in the samples that were presented to them. Each of these terms had an agreed definition and a suitable reference standard which was presented at each sensory session. Samples were presented in duplicate in random order across the panellists in three-digit coded, covered ISO standard tasting

glasses (25mL). The panellists rated each of the attributes on an unstructured 10 cm line scale, with anchors of 'low' and 'high' placed at 1 cm and 9 cm respectively. FIZZ software (Version 2.10, Biosystemes, Couternon, France) was used for the collection of all data. Analyses of variance (ANOVA) were performed on each attribute rated by the panel. All statistical analyses were performed using FIZZ and XLStat (Version 7.5.2, Addinsoft, USA).

4 Sensory Analysis Results

4.1 Informal Assessment

This assessment concluded that there were subtle but detectable differences among the treatment wines and all fermentation replicates were quite similar to each other. There were no faults or off-flavours noted. The discrete differences apparent between wines suggests that performing descriptive analysis on all six wines would provide more meaningful information and it would not be necessary to blend fermentation replicates.

4.2 Difference Testing

Difference testing results are summarised in Table 3.1.

Table 3.1. Results of triangle difference tests for aroma of wines made in fermentation triplicates.

Test Comparison	**No. correct responses/ No. responses**	**Significance**[1]
Wines from machine pruning method		
MP1 vs MP2	7/18	ns
MP1 vs MP3	8/18	ns
MP2 vs MP3	5/18	ns
Wines from spur pruning method		
SP1 vs SP2	10/18	*
SP1 vs SP3	14/18	***
SP2 vs SP3	11/18	*

[1] ns = no significant difference, and *, *** denote significance $p<0.05$, 0.001, respectively

4.3 Descriptive Analysis

The results of ANOVAs are presented in tabular form listing the significant attributes (Table 3.3), and graphically, as radar plots (Figures 3.1 and 3.2). These profile plots present an overview of the mean aroma and palate sensory scores for each attribute rated. Principal component analysis (PCA) simplifies examination of data by showing a sensory map of the relationships between the attributes and wine treatments (Figure 3.3). The PCs are derived to explain the maximum variation in the data and the orientation of each vector indicates their correlation with each PC and with each other. Vectors with small angles are directly correlated. Vectors lying opposite each other are inversely correlated.

5 Discussion

The objectives of this evaluation were two-fold:

1. To characterise the sensory properties of 2005 Cabernet Sauvignon wines made from a vineyard in the Clare wine region; and

2. To evaluate the sensory effects of two different pruning methods undertaken prior to harvest, namely spur and machine pruning.

5.1 Difference Testing

Sensory difference testing showed that the fermentation replicates of the machine pruning method could not be differentiated from each other. Panellists who gave correct responses rated any differences as quite small. The wines made from the spur pruning method showed that differences in aroma could be perceived. There was a significant difference perceived between replicates 1 and 2 and also replicates 2 and 3. Panellists who gave correct responses rated any differences as quite small. There was a highly significant difference perceived between replicate 1 and 3. Comments by panellists who gave correct responses in this test noted the size of differences as small to medium.

Although the chemical analysis results show that all wines were similar (Appendix 2), sensory analysis has shown that differences that could be perceived. Whether these differences were directly related to the pruning effect was the next stage to be addressed by sensory profiling.

5.2 Descriptive analysis

The sensory profiling of the Cabernet sauvignon wines showed that descriptors significantly differentiating the wines were floral, mint and licorice aromas and prickly heat (Table 3.3). Table 3.4 shows how wines were differentiated among these significant attributes. There we no statistically significant differences perceived among the wines in any other attributes rated for aroma or palate.

When comparing all 6 Cabernet sauvignon wines, the following trends were noticed:

- More differences were perceived by aroma than by palate attributes.
- The MP1 wine had significantly more intense floral, mint and licorice aromas.
- The MP2 wine was also quite intense in mint aroma.
- The MP1 and SP2 wines were highest in the aromas of fresh berry fruit and pepper and also had a more peppery flavour.
- SP3 was highest in prickly heat which correlates to the higher alcohol recorded (Appendix 2), however the other wines were also quite high in alcohol so this effect cannot be assumed to be an effect of the pruning treatment.

The differences between the spur pruned treatment replicates meant that the descriptive analysis data could not be pooled. Therefore a comparison of the machine and spur pruning treatments using mean values of the attributes rated for three fermentation replicates would not be valid.

The first two components from PCA accounted for 57% of the variance. The first PC differentiated the samples according to the relative intensity of the stalky, unripe, drying attributes as opposed to the level of fresh berries, fruit weight and peppery attributes. The SP replicates were not grouped together at all, which was supported by difference tests. SP1 showed common characteristics to MP3 in mint, sweet spice and licorice aromas. SP1 had lower intensity berry fruit, pepper and mint while SP2 showed more berry fruit and fruit weight generally. The MP replicates showed more characteristics in common with each other.

6 Closing Comments

We can conclude that there were some overall differences in sensory properties between the wines. However, these differences showed no clear relationship to the effect of pruning method used. The wines with the machine pruning effect appeared to have more aroma intensity overall. The palate profile of all wines was quite similar. The variance associated with the pruning treatments was not significantly larger than the variance perceived between the fermentation replicates of the spur pruned method.

We trust that you find this evaluation useful. Please do not hesitate to contact us if you have any questions.

Attachments:	Emailed 'Raw sensory DA data for 2005 Clare Cabernet'

Table 2.3. Attributes and definitions for the descriptive analysis of Cabernet Sauvignon wines from 2005 vintage.

Attribute	Definition
Aroma	
Fresh berry fruit	Blackberry, blackcurrant, raspberry, cherry, plum
Jammy	Berry jam, plum jam, stewed red fruit
Floral	Violet, linalool
Mint	Menthol, eucalyptus
Stalky/grassy	Grape marc, stalks, stems, grassy
Herbaceous	Leafy, tomato leaf and stalk, oregano
Savoury	Beef stock, gravy-like aromas
Sweet Spice	Includes cinnamon, clove, mixed spice
Licorice	Includes aniseed
Chocolate	Milk or dark chocolate
Pepper	Black and white pepper
Smoky tobacco	Includes fresh pipe tobacco, smoky aromas
Earthy	Dampness, beetroot earthiness
Other	Any other aroma term
Palate	
Acidity	Sourness, tart, sharp sour taste
Ripe fruit	Includes ripe berries, jam, sweet fruit
Unripe fruit	Presence of green, unripe characteristic of fruit not fully ripened
Grassy/stems	Flavours like grape marc, fresh cut grass
Sweet spices	Fruitcake, mixed sweet spice flavours, licorice
Peppery	Hot spice, peppery flavours
Overall fruit weight	Overall intensity or fullness of fruity flavours, body
Drying	Puckering, grippy sensation, feeling of dehydration
Viscosity	Force required to move wine in mouth, consistency, low=thin, watery, high=thick, syrupy
Flavour persistence	Length of perception of fruity flavours 10 to 15 seconds after spitting
Prickly heat	Warm feeling due to presence of alcohol
Bitterness	A harsh taste like caffeine, usually noticed at back of palate
Other	Any other palate term

Table 3.3. Results of ANOVA from the descriptive analysis data of all 6 x 2005 Cabernet Sauvignon wines (12 assessors, duplicate) with Fisher's least significant difference (LSD, 0.05) values.

Attribute	Wine p-values[1]	Significance[2] LSD
Aroma		
Fresh berries	0.118	ns
Jammy	0.631	ns
Floral	**0.004**	** 0.38
Mint	**0.011**	* 0.49
Stalky/grassy	0.765	ns
Herbaceous	0.786	ns
Savoury	0.247	ns
Sweet Spice	0.615	ns
Licorice	**0.025**	* 0.38
Chocolate	0.554	ns
Pepper	0.192	ns
Smoky tobacco	0.439	ns
Earthy	0.703	ns
Palate		
Acidity	0.714	ns
Ripe fruit	0.597	ns
Unripe fruit	0.544	ns
Grassy/stems	0.615	ns
Sweet spice	0.758	ns
Peppery	0.134	ns
Fruit weight	0.284	ns
Drying	0.787	ns
Viscosity	0.688	ns
Flavour persistence	0.480	ns
Prickly heat	**0.027**	* 0.33
Bitterness	0.776	ns

[1] Bold values are significant at the 95% level ($p<0.05$), which signifies that one or more wines was perceived as different to the others in that particular attribute.
[2] ns = no significant difference, and *, ** denote significance $p<0.05$, 0.01, respectively

Table 3.4. Wines showing differences in the significant attributes, where ns= no significant difference, and L, M, H denote significant mean intensity ratings of low, medium, and high based on LSD scores.

	Wine Treatment					
Attribute	**MP1**	**MP2**	**MP3**	**SP1**	**SP2**	**SP3**
Floral	H	ns	ns	ns	ns	ns
Mint	H	ns	L	L	ns	ns
Licorice	H	ns	ns	ns	ns	M
Prickly heat	ns	ns	L	ns	ns	H

Figure 3.1. Aroma profile plot of 2005 Cabernet Sauvignon wines from an identical vineyard using 2 pruning treatments made in triplicate ferments; machine (MP) and spur (SP). Assessed by 12 panellists in duplicate (*, ** = significance $p<0.05$, $p<0.01$).

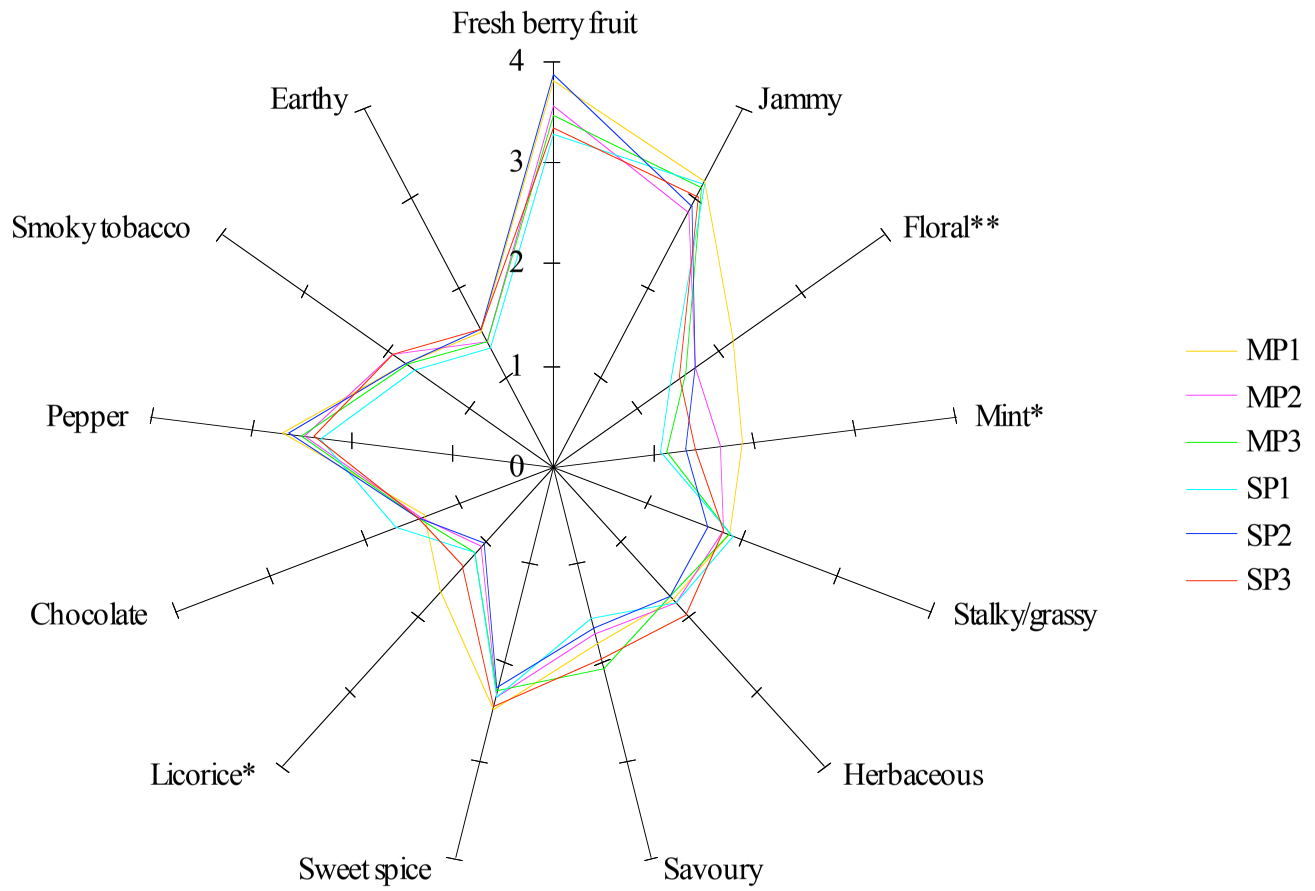


Figure 3.2. Palate profile plot of 2005 Cabernet Sauvignon wines from an identical vineyard using 2 pruning treatments made in triplicate ferments; machine (MP) and spur (SP). Assessed by 12 panellists in duplicate (* = significance $p<0.05$).

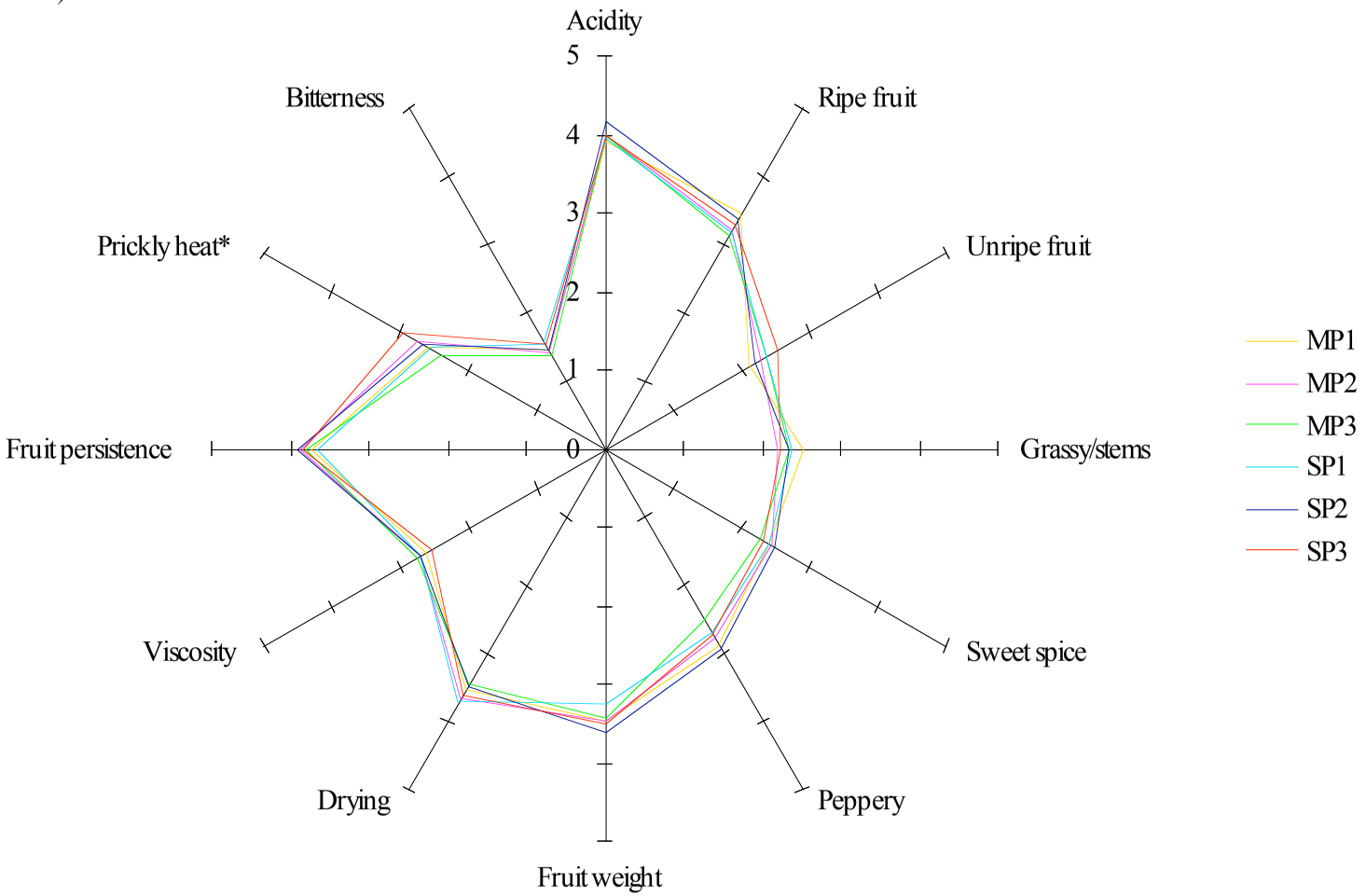
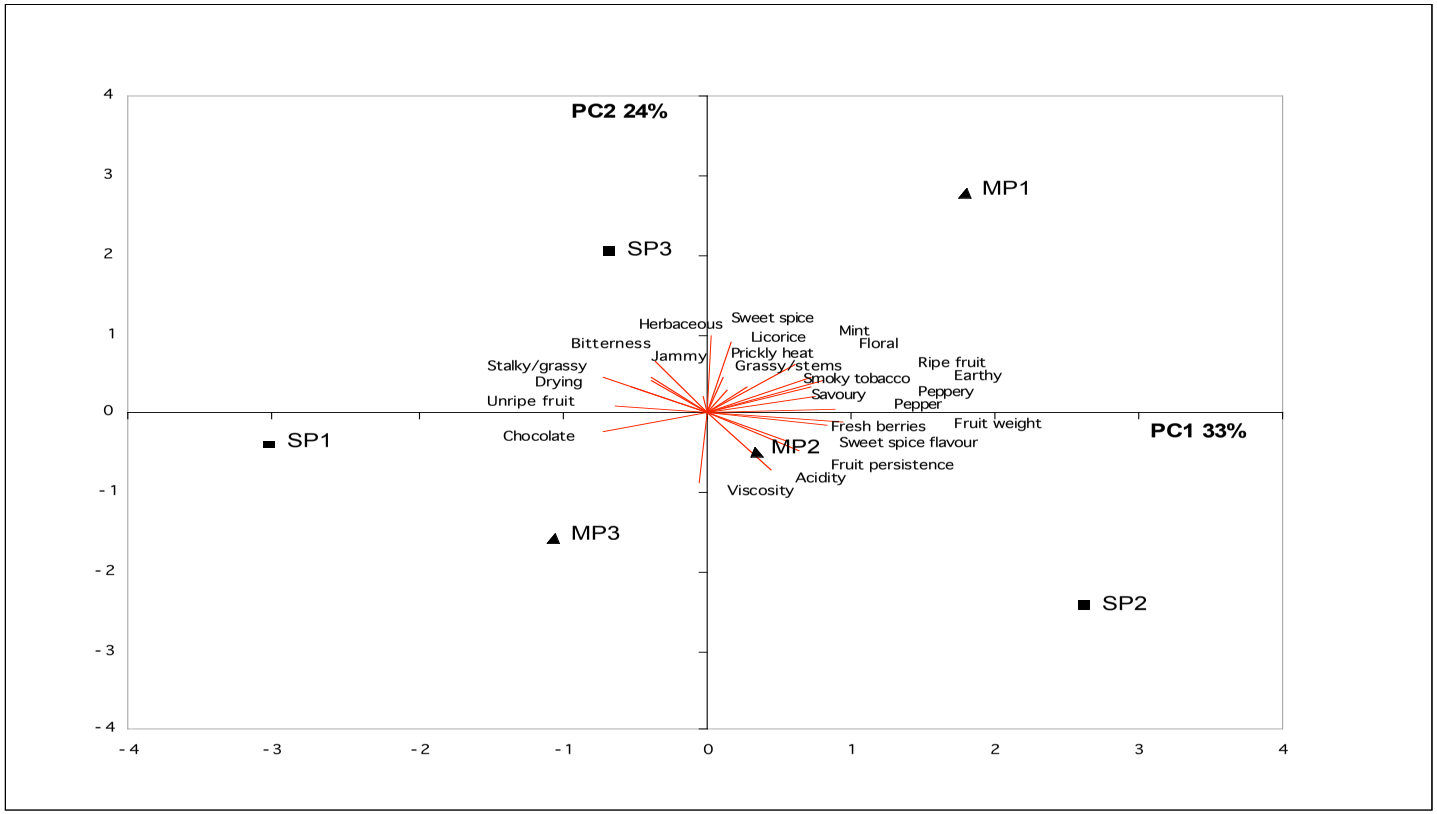


Figure 3.3. Projection of sensory descriptive analysis data of Cabernet Sauvignon wines on principal components 1 and 2. Six wines were assessed by 12 panellists in duplicate.



March 21, 2005

provisor
for grape and wine research

Provisor Pty Ltd
ABN 41 101 149 482
Hartley Grove
Urrbrae SA 5064

PO Box 219
Glen Osmond SA 5064

Telephone +61 8 8303 8700
Facsimile +61 8 8303 8698
www.provisor.com.au

Chris Davies
CSIRO

Dear Chris

Re: Pruning trial small-scale winemaking

Fruit Receival Report

Thank you for delivering the fruit for the above project. The fruit was received on March 18 in sound condition. Our analysis at receival and following crushing was as follows:

Treatment	Fruit Weight (kg)	Brix / Baume	pH	T.A. (g/L H_2T)
Spur pruned Rep 1	50	28.0 / 15.5	3.73	4.4
Spur pruned Rep 2	50	28.1 / 15.8	3.73	4.4
Spur pruned Rep 3	50	28.2 / 15.9	3.76	4.3
Machine pruned Rep 1	50	27.0 / 15.4	3.58	4.7
Machine pruned Rep 2	50	26.9 / 15.3	3.56	4.6
Machine pruned Rep 3	50	26.8 / 15.2	3.55	4.7

We will inform you when all of this project's wines have finished primary fermentation, which is anticipated to be in approximately 2 weeks time.

Kind regards,

Chris Day

Winemaker

October 18, 2005

Provisor Bottling Report

Project	2005 CSIRO (CD) Clare Cabernet Sauvignon Pruning Trial
Bottling Date	October 12, 2005

I am pleased to inform you that the wines for the above project have been bottled and are ready for collection from the Wine Science Laboratory. Final analysis of the wines was as follows:

Treatment	pH	T.A. (g/L tartaric)	Total SO_2 (ppm)	% Alcohol	V.A. (g/L acetic)	Glucose + Fructose (g/L)	Malic Acid (g/L)	Number of bottles (750mL + 375mL)
Spur Pruned Replicate 1	3.28	7.6	Adjusted to 75	14.7	0.23	0.04	0.05	15 + 10
Spur Pruned Replicate 2	3.30	7.5	Adjusted to 75	15.3	0.26	0.44	0.03	16 + 10
Spur Pruned Replicate 3	3.29	7.6	Adjusted to 75	15.4	0.24	0.01	0.19	17 + 10
Machine Pruned Replicate 1	3.29	6.8	Adjusted to 75	15.0	0.20	0.13	0.01	16 + 10
Machine Pruned Replicate 2	3.24	7.1	Adjusted to 75	14.8	0.18	0.20	0.03	16 + 10
Machine Pruned Replicate 3	3.22	7.3	Adjusted to 75	14.7	0.20	0.24	0.04	16 + 10
						Total number of boxes		12

T.A. = Titratable Acidity, V.A. = Volatile Acidity

Comments: All wines sound and completed as per client specifications. Spur pruned replicates 2 and 3 took some time to complete malolactic fermentation, although it is not considered that the quality of the wines were affected. Please note that spur pruned replicate 3 has a small amount of malic acid remaining, this represents approximately 90% malolactic fermentation completion. It was considered prudent to sulphur this wine so that the trial could be completed, and not risk spoilage of this batch.

Filtration Level	Z6 grade pad (polishing, non-sterile)
Closure	Screw-cap
Bottle Size	750mL and 375mL (10 of each replicate)

Please note that the first two 750mL bottles of each batch are marked accordingly – although for each run approximately twice the void volume of the filtration setup is discarded prior to filling the first bottle, you may wish to avoid using these bottles for sensory purposes.

Please note that the wines may be currently suffering from 'bottle-shock' commonly associated with the bottling process, and consequently may currently be sensorially subdued. Accordingly, we recommend delaying thorough sensory evaluation of the wines for at least 4 weeks following bottling.

Thank you for the opportunity to work with you on this project, we look forward to collaborating again in the future.

Kind regards,

Chris Day

Winemaker

Attachments	Nil

Appendix 5c: Sensory wines from Wingara irrigation treatments.

Report No.:	PR05108
Date:	3/11/05
Name:	CSIRO Wingara Cabernet Descriptive Analysis 2005
Author:	Briony Liebich
Position:	Sensory Analyst
Approved:	Dr. Darren Oemcke

Approved by,

Dr. Darren Oemcke
CEO

– GENERAL DISCLAIMER AND LIMITATION OF LIABILITY–

This document has been prepared by Provisor Pty Ltd ("**Provisor**") for a specific purpose and is intended to be used solely for that purpose and unless expressly provided otherwise does not constitute professional, expert or other advice.

The information contained within this document ("**Information**") is based upon sources, experimentation and methodology which at the time of preparing this document Provisor believed to be reasonably reliable and Provisor takes no responsibility for ensuring the accuracy of the Information subsequent to this date. No representation, warranty or undertaking is given or made by Provisor as to the accuracy or reliability of any opinions, conclusions, recommendations or other information contained herein except as expressly provided within this document. No person should act or fail to act on the basis of the Information alone without prior assessment and verification of the accuracy of the Information.

To the extent permitted by law and except as expressly provided to the contrary in this document all warranties whether express, implied, statutory or otherwise, relating in any way to the Information are expressly excluded and Provisor, its officer, employees and contractors shall not be liable (whether in contract, tort, under any statute or otherwise) for loss or damage of any kind (including direct, indirect and consequential loss and damage of business revenue, loss or profits, failure to realise expected profits or savings or other commercial or economic loss of any kind), however arising out of or in any way related to the Information, or the act, failure, omission or delay in the completion or delivery of the Information. In the event that any legislation or rule of law implies any condition, warranty or liability with respect to Provisor or the Information, Provisor's liability for breach of any condition, warranty or liability shall be limited, at the option of Provisor, to the re-supply of that Information; the cost of acquiring equivalent Information or the payment of the cost of having the Information re-supplied.

The Information is confidential and may be legally privileged. If you are not the intended recipient of the Information, please immediately notify Provisor and destroy the Information. Unless expressly provided in this document, Provisor retains ownership of the copyright in the Information and no part of the Information may be reproduced or copied in any form or by any means without the prior written consent of Provisor.

The Information must not be used in a misleading, deceptive, defamatory or inaccurate manner or in any way that may otherwise be prejudicial to Provisor, including without limitation, in order to imply that Provisor has endorsed a particular product or service.

1 Introduction

CSIRO engaged Provisor to assist in the evaluation of the key sensory properties of Cabernet Sauvignon wines to assess the contribution of two irrigation effects in the vineyard, labelled RDI and PD. There was also a Control wine, giving a total of three treatments.

The wines were made and bottled at Wingara winery where they were analysed for routine chemical data, except for alcohol content.

2 Analysis of Wines

2.1 Chemical Analysis

Harvest and Bottling data was performed at Merbein and supplied by CSIRO.

Additional analyses of alcohol (NIR) were conducted at the Hickinbotham Wine Science Laboratory.

2.2 Sensory Analysis

Sensory analysis was conducted using Provisor's sensory panels. Generation of these results consisted of the following stages:

1. Informal assessment (internal panel);
2. Difference testing (external panel); and
3. Descriptive analysis of two samples (external panel).

Results included in this report were those generated by the external panel.

1. Informal assessment

The internal panel (6 assessors) met for one hour to perform informal rating assessments on all nine wines followed by a group discussion. This assessment provided guidelines for our sensory analyst and gave an indication of any major differences among the treatments and replicate wines, and the presence of any faults or off-flavours. The wines were assessed after a minimum of four weeks storage following bottling as per our standard protocol.

2. Difference testing

The objective of difference testing is to determine whether the two treatments are perceptibly different overall. The duo-trio method (balanced reference mode) was used to evaluate samples by aroma and palate. Two coded samples and a reference are presented simultaneously to the panellists. The panellists are instructed to pick the sample that is the same as the reference sample. These tests were held on September 21 and November 2, 2005. There were 24-25 responses in total.

Sample preparation involved blending the two replicates of the PD and RDI treatments. The control used was fermentation replicate 2. All wines were presented at room temperature under orange lights in controlled conditions.

3. Descriptive Analysis

The external descriptive panel (9-13 assessors) met once weekly for five weeks and participated in training sessions to develop an attribute list for 7 aromas and 9 by mouth (taste, flavour and mouthfeel) characteristics (Table 3.3.1). Panellists were trained to recognise these sensory attributes in the samples that have been presented to them. Each of these terms had an agreed definition and suitable reference standard which was presented at each sensory session.

Samples were served at room temperature under orange lights to mask the appearance of the wines. Pouring of wines occurred at least one hour prior to assessing to allow the reductive characters to dissipate as much as possible.

Wines were presented in triplicate in random order across the panellists in coded, covered ISO tasting glasses (25mL). The panellists rated each of the attributes on an unstructured 10 cm line scale, with anchors of 'low' and 'high' placed at 1 cm and 9 cm respectively. FIZZ software (Version 2.10, Biosystemes, Couternon, France) was used for the collection and analysis of all data.

3 Results

3.1 Chemical analysis

The compositional characteristics and phenolic profile results of the wines are shown in Tables 3.1.1 (see attached) and 3.1.2.

Table 3.1.2 Mean Alcohol data of wines assessed in triplicate.

Treatment	Alcohol (% v/v)
Control	14.6
RDI	14.6
PD	14.3

3.2 Informal Assessment

After panel discussion it was concluded that four wines had quite reduced characters of sulphide compounds with aromas like burnt rubber and cooked cabbage. These wines were considered too reductive to enable other characters to be rated consistently. Therefore no further sensory analysis was carried out on the following wines:

- Control replicates 1 and 3;
- RDI replicate 4; and
- PD replicate 3.

3.3 Difference testing

The results for duo-trios testing are summarised in Table 3.3.1. There were three separate comparisons made; the control wine with each of the two irrigation treatments and also comparing any overall difference between the two irrigation treatments.

Table 3.3.1. Results of three duo-trio test comparisons

Test Comparison	No. Assessors	No. correct responses	Significance[1]	
Control vs PD	25	14	0.345	ns
Control vs RDI	25	19	0.0073	**
RDI vs PD	24	12	0.581	ns

[1] ns = not significant (α=0.05), ** = highly significant (α=0.01)

3.4 Descriptive Analysis

An ANOVA of the descriptive analysis data was performed to confirm any significant differences among wines in 19 attributes. The data is presented in tabular form listing the significant attributes (Table 3.3.2), and graphically, as radar plots (Figures 3.3.1 and 3.3.2). The radar plots present an overview of the average aroma and by mouth sensory scores for each wine treatment.

Table 3.4.1 Aroma and my mouth attribute list with agreed panel definitions

Attribute	**Definition**
Aroma	
Fresh dark fruit	Includes blackcurrant, raspberry, mulberry, cherry, plum
Jam/dried fruit	Cooked/stewed fruit, plum jam, dried fig, raisin
Fresh vegetal	Includes green bean, asparagus
Mixed sweet spice	Includes clove, cinnamon, star anise
Pepper	Black and white pepper
Sulphide/rubbery	Struck match, burnt rubber
Sulphide/cabbagey	Canned vegetable brine, cooked cabbage
Other	Any other aroma term
By Mouth	
Acidity	Degree of sourness, tart or sharp taste
Ripe fruit	Includes ripe berries, jam, sweet fruit
Unripe fruit	Presence of unripe characteristics of fruit not fully ripened
Metallic	A hard finish and flavour of metal or like blood
Fullness of flavour	Impression of weight of wine flavours in mouth, low = light, high =heavy/full
Viscosity	How heavy wine feels in mouth, consistency, Low= watery, High= thick like honey
Drying	Puckering grippy sensation, feeling of dehydration
Prickly Heat	perceived feeling of warmth from alcohol, tingly sensation
Bitterness	A taste like caffeine, usually noticed at back of palate
Other	Any flavour or mouthfeel term

Table 3.4.2 Significant attributes with ANOVA p-values for three wines.

Attribute	**Wine p-values**[1]	**Significance**[2]
Fresh dark fruit	*0.145*	ns
Jam/dried fruit	0.253	ns
Fresh vegetal	0.619	ns
Mixed sweet spice	0.821	ns
Pepper	0.831	ns
Sulphide/rubbery	0.643	ns
Sulphide/cabbagey	0.810	ns
Acidity	**0.010**	*
Ripe fruit	0.430	ns
Unripe fruit	*0.087*	ns
Metallic	*0.085*	ns
Fullness of flavour	0.395	ns
Viscosity	0.386	ns
Drying	0.914	ns
Prickly Heat	0.240	ns
Bitterness	0.635	ns

[1] Bold values are significant at the 95% level (α=0.05), values in italics are on the edge of statistical significance (α=0.2)
[2] ns = not significant, * = significant difference (α=0.05),

4 Discussion

The objective of this evaluation was to compare the sensory properties of three Cabernet Sauvignon wines, a control and two irrigation treatments. This report concludes that there were some subtle differences between these three wines.

Chemical data will not be discussed in detail in this report. However, the three treatments and their fermentation triplicates showed relatively similar results. It was noted that four of the nine wines had copper sulphate additions of 0.3-0.5ppm to counteract their reduced aromas. These reduced characters were perceived across the wines in different intensities at different time points during the project. Therefore it was difficult to consistently rate sulphide/rubbery and sulphide/cabbagey aromas.

Sensory difference testing showed that the control wine could not be differentiated from the PD wine. Comments made by tasters who gave correct responses included the PD wine having lower intensity fruit aromas with higher acidity and slightly more drying.

The RDI and PD wines were also not perceived to be different by the panel. Panellists who gave correct responses rated any differences as quite small.

There was a significant difference perceived between the control and RDI wines. Comments made by tasters who gave correct responses included the

RDI wine having different fruity aromas, higher reduced characters with more acidity and a slightly bitter taste,

Descriptive analysis showed that at the accepted 95% level ($\alpha = 0.5$) of statistical significance, the only significant attribute was acidity. At the wider level of significance ($\alpha = 0.2$) there were significant differences perceived in fresh dark fruit aroma, unripe fruit and metallic flavours.

The PD wine was significantly lower in acidity than the control and RDI wines. This correlates with the unripe fruit flavour of the PD wine when compared with the other two wines, which were both higher in ripe fruit flavours and acidity.

Overall, the control wine was perceived higher in fresh dark fruit and dried fruit aromas. Fresh vegetal, sweet spice, pepper and reduced aromas were similar in all three wines.

Of the palate attributes, only acidity and metallic showed some differences. The RDI wine had a higher metallic taste than the control and PD wines. Fullness of flavour, viscosity, drying, prickly heat and bitterness were similar in all three wines.

We trust that you find this evaluation useful. Please do not hesitate to contact us if you have any questions.

Attachments:	1. Table 3.1.1 Chemical data as supplied by CSIRO
	2. Figure 3.3.1 Sensory radar plot for aroma attributes
	3. Figure 3.3.2 Sensory radar plot for flavour attributes

Table 3.3.1 Chemical data as supplied by CSIRO

Merbein Cabernet Sauvignon 2005 Winemaking Data for Sensory

WINE NUMBER	Treatment	Replicate	Yeast Type	Harvest Date	Grape Processing Date	Harvest Brix	Harvest Baume	Harvest pH	Harvest Acid (g/L)	No of Bottles (ml)	Bottling pH	Bottling TA (g/L)	Bottling FSO2 (ppm)	Residual Sugar (g/L)	TOTAL TA ADDED (g/L)	TOTAL CuSO4 ADDED (ppm)
164	Control	1	254D	3/3/05	4/3/05	24.3	13.5	3.43	6.37	19 x 750	3.58	8.20	14.4	2.5	1.3	0.3
165	Control	2	254D	3/3/05	4/3/05	24.4	13.6	3.43	6.58	13 x 750	3.54	8.22	12.8	1.3	1.3	0.0
166	Control	3	254D	3/3/05	4/3/05	23.0	12.8	3.47	6.29	17 x 750	3.55	8.22	12.8	1.3	0.5	0.5
169	RDI	2	254D	3/3/05	4/3/05	23.8	13.2	3.47	6.12	20 x 750	3.50	8.78	12.8	1.3	0.9	0.0
170	RDI	3	254D	3/3/05	4/3/05	24.0	13.3	3.44	6.16	18 x 750	3.47	8.48	12.8	1.3	0.5	0.0
171	RDI	4	254D	3/3/05	4/3/05	23.9	13.3	3.42	6.30	18 x 750	3.49	8.33	17.6	1.3	0.5	0.0
173	PD	2	254D	3/3/05	4/3/05	22.7	12.6	3.44	5.93	12 x 750	3.44	8.88	11.2	1.3	0.4	0.5
174	PD	3	254D	3/3/05	4/3/05	24.2	13.4	3.44	6.09	11 x 750	3.50	8.23	17.6	1.3	0.2	0.0
175	PD	4	254D	3/3/05	4/3/05	23.8	13.2	3.39	5.99	12 x 750	3.41	8.58	16.0	1.3	0.4	0.3

Figure 3.3.1 Aroma Profile of three Wingara Cabernet Sauvignon wines

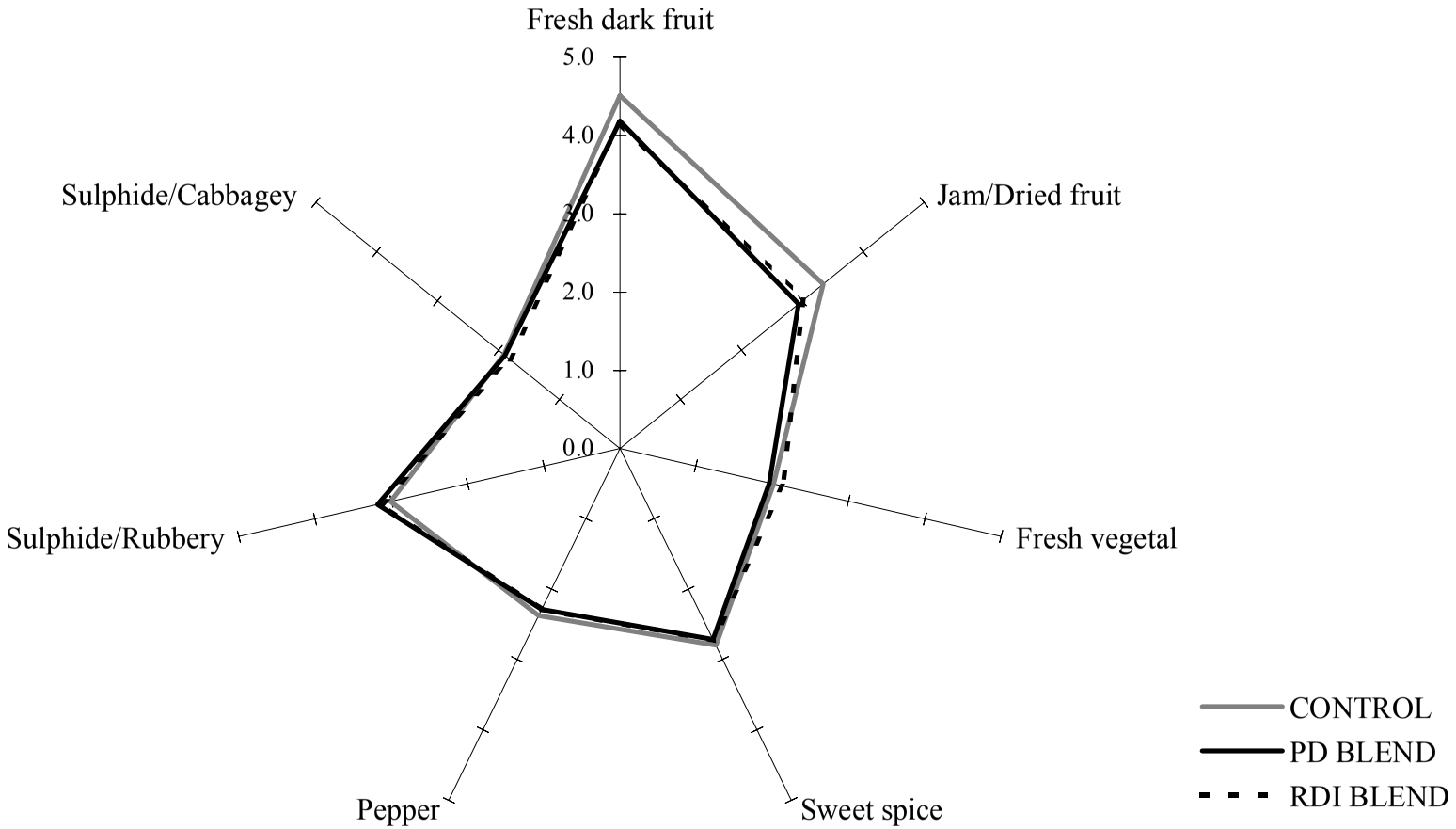


Figure 3.3.2 Flavour Profile of three Wingara Cabernet Sauvignon wines

