# Understanding and managing the timing of berry ripening and the flavour-ripe/sugar-ripe nexus

## FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSP09/05**

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Research Organisation: **CSIRO**

Date: **30/6/2014**

**Project title:** Understanding and managing the timing of berry ripening and the flavour-ripe/sugar-ripe nexus

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**Date report completed:** 30/06/2014

**Publisher:** CSIRO

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## 1. Abstract

The primary aim of this project was to further our understanding of berry ripening and how its onset and progression are controlled by endogenous plant growth regulators (PGRs). The knowledge gained was used to develop and test methods for manipulating the timing of veraison/harvest using exogenous PGRs in both laboratory and field settings. We have demonstrated the ability to delay ripening and harvest time and increase ripening synchronicity through PGR application while maintaining similar wine sensory properties. In addition, experiments were conducted to get a better understanding of the influence of harvest time versus sugar levels on wine flavour/aroma volatiles.

## 2. Executive Summary

Changes in market requirements and climate have generated new challenges for the grape and wine industries. Warmer, drier growing conditions and higher $CO_2$ levels appear to be the causes of altered grapevine growth and berry ripening. Grapes of all varieties are ripening earlier in the season and one consequence of this is 'seasonal compression' where the length of the harvesting season is considerably shortened. A consequence of this is that bottlenecks occur in grape intake and processing as the ability to process the fruit in a timely manner is swamped by large intakes during a restricted period. This can result in wastage, increased costs and reduced wine quality. These issues have been exacerbated by the apparent disconnect between sugar accumulation and the accumulation of flavour and aroma compounds and their precursors in the berry. More rapid sugar accumulation has led to fruit with adequate sugar levels but under-ripe flavour potential. In an effort to reduce 'green characters' in grapes they are frequently left on the vine to reach higher sugar levels which has resulted in a steady increase in wine alcohol levels.

A better understanding of ripening and tools to alter it are required to alleviate some of the challenges discussed above. Plant growth regulators (PGRs) are key signalling molecules that control and coordinate plant development, including grape berry ripening. This makes them ideal targets for altering ripening in a global manner. This is not without challenges as their effects are wide ranging and the interactions between pathways are complex. In this project considerable progress has been made in understanding the metabolism and action of a number of key PGRs. Improved and detailed knowledge of the control of the initiation of grape berry ripening, which is a major determining factor in harvest timing, has been developed, which is vital to any attempts to usefully manipulate ripening.

We have concentrated our efforts on PGRs that are thought to be involved in, and can alter, the timing of ripening initiation. As many significant issues are due to earlier ripening much of the work has focused on auxins. Endogenous auxins delay ripening and can also be applied to fruit to delay its ripening. Although the pathways targeted by auxins are yet to be determined a detailed knowledge of auxin metabolism, both biosynthesis and breakdown, has been developed. We now know much about how auxin levels are controlled and why some auxins are more effective than others in controlling the timing of veraison. New tools have been developed to study the effects of auxins, which have led to the possibility of novel fit for purpose synthetic versions. Another important PGR is ethylene, which is involved in the control of ripening in a range of fruit. Our understanding of its role in grape ripening has been greatly advanced and we have shown that it can be used to delay ripening if applied at the appropriate time. Ethylene also interacts with auxins and so knowledge of its action is required to better tailor any manipulation of ripening.

PGRs that advance ripening have also been studied. Aminoethoxyvinylglycine (AVG) inhibits both ethylene and auxin production and has the potential to significantly advance ripening. Other PGRs that do not affect the timing of veraison, but affect other aspects of berry development, have also been investigated. At least one of these appears to be involved in controlling some aspect of the mid/late stages of berry ripening with the potential to be important to wine flavour and aroma.

Scientific evidence regarding the relative influence of berry harvest date and sugar content on wine volatile composition has been produced. This is important to our understanding of the effects of higher sugar content and delayed harvest times on wine composition. The results

indicate that while many flavour/aroma metabolites with a positive influence on wine style are related to sugar content some with negative effects require time to decrease. Therefore, extending hang time may not improve the positive aspects of wine flavour but will reduce the concentration of negative flavours.

The scientific outcomes described above have been put to use under commercial vineyard conditions to develop new ways of managing berry growth and ripening to alleviate the problems associated with altered berry development. Substantial and predictable delays in ripening, and therefore harvest, have been demonstrated in both white and red wine cultivars in different vineyards, under different climates during a number of seasons using a range of different auxins. Much knowledge on the timing and manner of application has been gained. Apart from delaying harvest other benefits have included an increase in the synchronicity of berry ripening. Wine metabolite levels, and in some cases sensory properties, have been significantly altered but these changes were not dramatic and may simply reflect the delayed fruit ripening later in the year under slightly different climatic conditions.

This work has considerably enhanced our knowledge of berry ripening, has demonstrated some of the potential for PGRs in controlling ripening and has made progress towards a practical application of new techniques to usefully manipulate it.

## 3. Background

The Australian grape and wine industries need to remain competitive in the face of changing environmental conditions and changing markets with preferences for different wine styles. The changing growth conditions have had, and will continue to have, significant effects on vine and berry growth with important implications for wine alcohol levels and flavour. In addition, the changes in ripening patterns and harvest dates result in problems in harvesting fruit and in wine production. New and emerging markets also require the adaptation of wine styles to suit market preferences in highly competitive domestic and world markets.

A particular example of the issues outlined above is the market requirement for red wine styles without 'green flavours'. The response to these requirements have been to extend the 'hang times' of fruit on the vine, a consequence of which is that the fruit has higher sugar levels and thus, there has been a steady increase in the alcoholic content of many Australian wines. Higher alcohol levels raise issues regarding public health, consumer wine style preference and higher taxation levels in some markets. Late picked, highly sugared grapes can also lead to production problems due to stuck primary and secondary fermentations. There are post-harvest approaches to reduce the impact of high sugars, but they involve considerable extra cost and can adversely affect wine flavour. Higher temperatures resulting in faster vine and berry growth and maturation rates may be the root cause of the 'sugar-ripe before flavour-ripe' syndrome. Higher sugar concentration may also be related to changes in management practices and environmental influences, causing grapes to accumulate sugar more rapidly and earlier than previously. This suggests that the altered conditions have resulted in a miss-coordination of sugar and flavour development.

Another important issue resulting from more rapid growth and faster sugar accumulation in berries is what can be called 'seasonal compression'. It is clear that more rapid berry development has shortened the growing season of many varieties in many production regions. In the past there was a predictable flow during the harvest season from early and later white grapes through to early and later red grapes. In recent seasons this pattern has been disrupted with many more varieties being sugar-ripe at about the same time. This puts pressure on the workforce and infrastructure for harvesting and processing. One consequence is that some producers harvest some grapes earlier, or later, than they would like on the basis of berry composition, simply to try to relieve bottlenecks in processing. This can affect wine style in an undesired and unplanned way and reduce profitability.

It is clear from the above discussion that the process of berry ripening and its timing, and hence harvest timing, is very important to the timely production of fruit of the desired style. Despite this there is still a great deal about berry development and metabolism and their control that we do not know. We have an incomplete understanding of what triggers ripening, how sugar accumulation is controlled and what controls the production of the wide array of flavour and aroma metabolites and their precursors. One example is the lack of knowledge regarding the possible metabolic linkage between sugar accumulation and the accumulation of aroma and flavour compounds. This understanding is crucial to determining and controlling the balance between flavour and sugar ripeness.

The process of berry ripening is similar in some ways to the ripening of other fruit and is a complex process that is best thought of as a series of sub-processes that are coordinated by a control system that signals when these processes are required to change as part of the fruit developmental program or in response to environmental cues. Grape berry ripening involves

the coordination of a large number of events. The pre-veraison period can be seen as a phase of berry growth, during which fertile seeds develop. The post-veraison period, also known as the ripening phase, is when the accumulation of sugar and flavour/aroma metabolites and flavour/aroma precursors occurs. Although there are continuous changes in gene expression, protein levels and hence metabolism throughout berry development, veraison is a watershed event (Conde *et al.*, 2007, Davies and Robinson, 2000, Deluc *et al.*, 2007, Giribaldi *et al.*, 2007, Ollat *et al.*, 2002, Pilati *et al.*, 2007, Terrier *et al.*, 2005). The timing of veraison is a critical factor in the timing of harvest and the change into the sugar accumulation phase is heralded by a general, coordinated change in gene expression that results in large changes in many metabolic processes.

The molecules that control the coordination of berry ripening are known as plant growth regulators (PGRs) and have long been known to control plant development through coordinating change, they are critical to the ripening of all fruit. PGRs act to coordinate global changes in gene expression during crucial events of plant development. Not only do they control flowering, berry growth and the initiation of ripening but they may also have more subtle effects on development and metabolism that affect berry composition and also respond to external cues such as water stress or infection and act as intermediates in the interaction between the plant and its environment. As the ripening process is so complex, it requires a complex system of control interactions. For this reason, although individual PGRs may be the significant players in the control of a particular process, there are always interactions with one, or more, of the other PGR pathways. The increasing number of reports of crosstalk between PGR networks (Gazzarrini and McCourt, 2003, Vandenbussche and Van Der Straeten, 2007) show just how intricate these systems are.

Plant growth regulators range in size, structure and metabolic origin from the gas ethylene to the complex brassinolides. They have different, but not infrequently overlapping functions and as described above their signalling pathways frequently interact. For example, auxins are critical to tissue/organ growth while ethylene is known to be involved in the response to wounding but, as will be demonstrated in this report, they interact in the control of fruit ripening. The presence and concentration of PGR molecules are recognised by the plant cell through receptor proteins that bind them with high specificity. The binding of PGRs to their respective receptors then evokes a signal that is rapidly amplified and transduced through a complex signalling pathway. Ultimately, this signal results in changes in the activity of transcription factors which in turn alters gene transcription and hence protein levels and cellular metabolism. The structure of signalling pathways explains the power of PGRs in coordinating changes in berry structure and metabolism.

In general, all fleshy fruit go through similar changes as they ripen in that they store some form of carbon (starch, sugars, oils etc.), lose unpleasant flavours (e.g. excessive organic acids) and accumulate additional compounds, such as aromatic substances, that are attractive to the vectors of their seed. Despite such similarities there are significant differences in the way the ripening process is controlled. Traditionally fruit have been divided into two broad groups described as either climacteric or non-climacteric (Tucker, 1993). Climacteric fruit, such as tomato and banana, undergo a peak in respiratory activity during the ripening process (Seymour, 1993). In many climacteric fruit a burst of production of the PGR ethylene is associated with the respiratory peak. In contrast, non-climacteric fruit tend to show a slow decline in respiratory activity during ripening with no major peak in ethylene levels (Seymour, 1993) although as we shall see ethylene may still play a role in the ripening of non-climacteric fruit. The divide between climacteric and non-climacteric fruit may not be as

distinct as first proposed and could be thought of as more of a spectrum between the two extremes of 'fully climacteric' and 'fully non-climacteric'. Grapes are regarded as non-climacteric (Coombe and Hale, 1973) along with such fruit as strawberries, olives and capsicums. While the role of ethylene in climacteric fruit ripening, especially in tomato, is well established, the role of ethylene in affecting grape berry ripening remains the subject of debate (Davies and Böttcher, 2009a).

As PGRs exert such powerful influence over many wide ranging processes they are ideal as targets for manipulation of berry development. However, because of their ability to change fruit development to such a significant extent we need to develop a very good understanding of the way in which they work and the processes they affect to make best use of their powers and avoid any negative effects.

Research on the role of PGRs in grape berry development over a number of years has demonstrated the role that some of them play, but due to the complexity of their biosynthetic, signalling and perception pathways and the interactions between them there is still much to uncover (Böttcher and Davies, 2012). Evidence for the role of various PGRs during berry development comes from a range of sources including application studies, the use of specific inhibitors, the levels of PGRs measured during berry development and the changes that occur in gene expression during development and upon treatment with PGRs. In over-simplified terms PGRs can be divided into those that promote berry ripening and those that retard it. Promoters of ripening include abscisic acid (ABA) and brassinosteroids (BRs) that can advance the initiation of ripening when applied at a particular, pre-veraison time (Symons *et al.*, 2006, Wheeler *et al.*, 2009). Conversely, there is considerable data to show that some PGRs, for example, auxins retard the initiation of ripening (e.g. Böttcher *et al.*, 2010, Davies *et al.*, 1997).

PGRs are widely used in agriculture and horticulture to control such processes as flowering, fruit set and fruit size. Despite some progress, and indication of potential, much of the mechanisms by which PGRs effect changes and how they influence berry development, composition and wine quality is unknown. Continued studies of grape berry development are required to define the interactions between the proposed promoters and inhibitors of grape ripening and to understand how they might be best used to manipulate berry development for the benefit of industry, for example, to alter the timing of berry ripening and hence harvest. As PGRs directly affect the ripening process their application either singly or in combination, offers an opportunity to affect wine style and quality through manipulating berry composition. PGRs offer a means of manipulating berry timing and composition without having to develop new planting material through either classical breeding or transgenesis, both of which are useful but are longer term and less rapidly adaptable approaches. Many of the reagents used are synthetic copies of naturally occurring molecules which can be delivered by spray application. Through further research we are likely to expand the options available to us using this approach.

Sugars have also been shown to have signalling properties that influence plant development (Rolland *et al.*, 2002) and they have been shown to interact with other PGRs (Rook *et al.*, 2006). This complicates the situation in grapes as they store large amounts of sugars during ripening and so the influence of sucrose, glucose and fructose cannot be ignored. Not only may sugars have a direct role in cellular signalling, but their higher levels during berry ripening alter the osmotic potential that could also be responsible for changes in metabolism. It is possible that the level of sugars could influence changes in the levels of various flavour

and aroma compounds and their precursors. This concerns the connection between primary and secondary metabolism and how flexible the nexus between the two is. Anecdotal evidence of sugar and flavour ripeness varying between seasons suggests that it should be possible to affect aspects of secondary metabolism without major effects on sugar accumulation, and vice versa. The relationship between sugar, time on the vine and wine style is of considerable importance to the grape and wine industries. This nexus is also examined in this study by altering one or both of the hang time or sugar level and determining what effect this has on the levels of the volatile flavour constituents of wine produced from these grapes.

Some of the PGRs important to ripening are also involved in the response of plants to stress. Moderate levels of stress are believed by some viticulturists to improve the flavour potential of berries. Manipulation of PGR levels may allow the desired response in quality without risking stress-induced damage to the vine. Although the application of certain PGRs pre-veraison may promote ripening initiation, altering levels nearer harvest time may also have benefits for colour and flavour accumulation. Endogenous PGRs may also be involved during these later stages of development and an improved understanding may allow for manipulation nearer harvest.

As noted above, some of the PGRs present in berries have been shown to be involved in berry development and in particular in ripening (Böttcher and Davies, 2012). The levels of a number of PGRs have been determined throughout berry development but the levels of other known PGRs still remain to be analysed. In addition, some of the previous studies of PGR levels have been shown to be erroneous due to the techniques used at the time. In this project we have made significant progress in quantifying the levels of a range of PGRs during berry development using accurate, state of the art technology. This has resulted in the identification of putative new PGRs and a revision of the thinking about the levels and potential roles of previously identified PGRs.

Much of the work in this project concentrates on auxins. This is because many of the problems with berry ripening related to climate change are due to earlier harvest times, compressed seasons and accelerated sugar accumulation. These can all be related to ripening commencing earlier and proceeding more rapidly. In cool climates faster ripening may be an advantage but as most grapes grown in Australia are grown in temperate or warmer regions it seems that delaying/slowing ripening will be more useful to the grape and wine industries. In this light our previous studies on auxins, showing that they can significantly delay ripening (Böttcher *et al.*, 2010, Davies *et al.*, 1997), offer an opportunity to develop tools to alleviate some of the problems experienced with ripening. In addition, it seems that presence of significant amounts of auxins, the most common one in grape berries is indole-3-acetic acid (IAA), prevents ripening. Therefore, even if the aim was to advance ripening the auxin levels would have to be reduced first and so an understanding of auxin metabolism would still be crucial.

Two very powerful scientific tools to better understand the effects of PGRs are to apply compounds that are inhibitors of PGR perception, signalling or metabolism or to alter the levels of key proteins in grapevines through transgenesis. Both of these approaches have been used in this project and the advances made using laboratory, glasshouse and field studies are described in this report. This includes the generation of a novel inhibitor of IAA-amido synthetase enzymes.

As stated above the role of ethylene during grape berry ripening is controversial (reviewed by Böttcher and Davies, 2012). In this project we describe work that investigates the role of ethylene in berry development with a focus on its effect on ripening. This work has resolved a seeming paradox first reported by Hale *et al*. (1970), who showed that ethylene can both delay and promote ripening depending on the timing of application. It also shows how IAA is involved in this through a direct effect of ethylene on IAA biosynthesis.

Clearly, a better understanding of the factors that control ripening and the means to manipulate various aspects of ripening, e.g. the timing of veraison, is of great scientific interest and will provide the means for viticulturists and wine makers to have greater control over berry and wine composition and assist in winery scheduling. This report describes progress we have made in developing this better understanding and how it has been used to develop methods to manipulate berry ripening with relatively low cost methods.

## 4. Project Aims and Performance Targets

The original project aims were:
This project is aimed at developing a better understanding and providing solutions to two inter-related, emerging problems. One issue is the challenge of producing fruit-driven wine styles with ripe characters but without excessive alcohol levels. The other relates to the logistical and berry/wine composition problems associated with earlier and compressed harvest seasons that are being experienced. Both the relationship between sugar and flavour ripeness and the triggers that control the initiation of berry ripening are poorly understood. Our first objective is to better understand the processes of sugar and flavour metabolite accumulation, and their relationship with each other, the second is to understand how berry ripening is controlled. This knowledge will be used to develop strategies for managing the balance between sugar and flavour in a predictable manner and for manipulating the timing of harvest. An ability to produce fruit with the desired flavours but with more moderate alcohol levels in a predictable way would 1) minimize the need for costly intervention at winery to reduce alcohol levels 2) reduce the likelihood of stuck/problem ferments and 3) result in wines with improved market acceptance. The ability to manipulate the timing of veraison and hence harvest date will enable harvest to be better planned to improve winery logistics and reduce the need for further investment in winery infrastructure. It will also reduce the intake of over-ripe fruit, resulting in lower sugared musts and delaying veraison would allow ripening to occur during cooler parts of the season with concomitant expected positive changes to wine style.

| Year 1 July 2010-June 2011 | Output | Due Date mm/yy | Activities |
|---|---|---|---|
| A | Provision of additional technical support | 30/09/2010 | Hire new Technical Officer |
| B | Knowledge of the effects of PGRs on berry and wine composition, synchronicity and effects on key gene expression levels under different environmental conditions | 30/11/2010 | Analyse data from PGR field and *in vitro* studies, berry samples, and small scale wine lots to determine effects of treatments on berry and wine composition, analysis of *in vitro* samples |
| C | Understanding of how treatments to reduce the rate of sugar accumulation affect berry development and ripening | 30/06/2011 | Design and conduct field and glasshouse experiments to test a range of methods for reducing rate of berry sugar accumulation, samples taken, data for berry growth rates, and ripening markers analysed |
| D | Initiation of production of transgenic vines | 30/06/2011 | Make constructs to manipulate endogenous PGR levels in berries to further determine their role in ripening control, commence transformation/regeneration process |
| E | Berry and wine samples for determining effects of delayed veraison, by PGR treatments, in different varieties | 30/06/2011 | Test PGR treatments aimed at delaying ripening and harvest times in different varieties, collect berry developmental data, sample fruit for further studies, small scale ferments |
| F | Improved understanding of the metabolism of a factor crucial to the timing of veraison | 30/06/2011 | Study mechanism of auxin sequestration into an inactive conjugate during berry development, prepare paper. |

| Year 2 July 2011-June 2012 | Output | Due Date mm/yy | Activities |
|---|---|---|---|
| A | Knowledge of how treatments from Year 1c reduce rate of berry sugar accumulation and how this alters metabolite accumulation | 30/11/2011 | Analyse berry samples from field/glasshouse studies for sugar and secondary metabolite levels |
| B | Knowledge of the effects of PGRs on berry and wine composition and effects on key gene expression levels in different varieties | 30/11/2011 | Analyse PGR study field data, berry samples, and small scale wine lots to determine effects of treatments on berry and wine composition |

| | | | |
|---|---|---|---|
| C | Berry and wine samples from treatments to slow berry sugar accumulation, for further testing of best viticultural approach | 30/06/2012 | Further test in field and glasshouse experiments two methods for reducing rate of sugar accumulation chosen from previous years experiments, make small scale wine lots for analysis |
| D | Methods for flavour metabolite measurement | 30/06/2012 | Develop additional methods for flavour metabolite detection |
| E | Fruit from trials using viticultural treatments to mimic delayed veraison as caused by PGR applications | 30/06/2012 | Field test viticultural treatments aimed at delaying ripening and harvest times, collect berry developmental data, sample berries for further studies |
| F | Determination of strength of linkage between sugar and flavour accumulation | 30/06/2012 | Analyse/compare all project data to determine nature of linkage between flavour and sugar accumulation |

| Year 3 July 2012-June 2013 | Output | Due Date mm/yy | Activities |
|---|---|---|---|
| A | Knowledge of how above treatments Year3 c) reduce rate of berry sugar accumulation, how this alters metabolite accumulation, and which metabolic pathways are affected | 30/11/2012 | Analyse berry samples for differences in accumulation of sugars and flavour metabolites, determine relationship between them |
| B | Knowledge of effect of reduced sugar accumulation on wine composition/style | 30/11/2012 | Analyse small scale wine lots made from field experiments with reduced sugar accumulation, determine differences in outcome of the two treatments to reduce sugar accumulation |
| C | Knowledge of the effects of viticultural treatments to delay veraison on berry composition and effects on key gene expression levels | 30/11/2012 | Analyse berry samples derived from experiment to delay ripening using viticultural means, analyse berry development parameters, berry composition |
| D | Berry and wine samples for studies aimed at manipulating berry and wine composition through combing techniques to delay veraison and delay sugar accumulation | 30/06/2013 | Test methods for reducing rate of sugar accumulation and delaying veraison in combination, collect berry growth data, samples for later analysis and make small scale wine lots for analysis |
| E | Determine which metabolic/flavour pathways are affected by sugar accumulation, paper prepared, information disseminated to industry | 30/06/2013 | Analyse all project data to determine which metabolic pathways are affected by sugar accumulation and make predictions regarding the influence of sugar accumulation rate on flavour outcomes |

| Year 4 July | Output | Due Date mm/yy | Activities |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 2013-June 2014 | | | |
| A | Knowledge of how combined treatments in Year 3 reduce rate of berry sugar accumulation and delay veraison affect berry composition | 31/01/2014 | Analyse berry samples for differences in accumulation of sugars and flavour metabolites and changes in gene expression of the relevant biosynthesis pathways |
| B | Knowledge of how above treatments affect wine composition | 28/02/2014 | Analyse small scale wine lots made from fruit from field experiments |
| C | Knowledge of the role of PGRs in the control of berry ripening through the use of transgenic grapevines with altered PGR metabolism | 30/04/2014 | Analyse transgenic plants with altered PGR metabolism for growth and ripening characteristics and changes in gene expression, develop models for PGR involvement in control of ripening |
| D | Findings regarding methods to alter flavour through delaying harvest date by a) delaying veraison, b) slowing the rate of ripening/sugar accumulation | 30/04/2014 | Analyse and compare data from experiments over all seasons and conditions |
| E | Communication of project results and recommendations to interested parties | 30/6/2014 | Prepare final report, prepare papers for publication, disseminate final project outcomes to interested parties, investigate avenues for application investigated |

# 5. Method

A wide range of methods have been used during this study as the aims of the project were diverse and broad in scope. New tools/techniques for the study of berry development and its control have also been developed. In addition, a better understanding of the process of berry development and, in particular ripening, was sought. To achieve this better basic understanding of these processes research was carried out at the whole plant level to the molecular level. The methods have been divided into two main sections, General Methods and Experiment Specific Methods, the latter with subsections that relate to particular experiments.

Field studies A – grape berries were sampled throughout development to provide material for the determination of endogenous PGR levels and for gene expression studies (including the expression of PGR biosynthesis pathway and breakdown genes).

Field studies B – various PGRs and control treatments were applied and the effects on berry development, ripening and key gene expression were studied. In some cases small scale wine lots were made and metabolite and sensory analysis was conducted.

*Ex planta* experiments – fruit was removed from plants and placed on agar plates containing various PGRs and different sugars. Changes in gene expression levels and PGR levels were determined to assess the interaction between sugars and PGRs.

Laboratory experiments – various chemistry, biochemistry, molecular and protein-based experiments were conducted, to gain further insight into the mechanisms which control ripening.

Transgenic experiments – transgenic plants were used to determine the role of various PGRs in grape berry and grapevine development.

As much of the work from this project has been published in scientific (and in some cases industry) journals it is under copyright restrictions. To this end where the work has been published reference is made to the relevant publications to prevent breaking copyright. Work that has not been published is presented within this report.

## 5.1. General Methods

### 5.1.1. Determination of Berry Anthocyanin and Total Soluble Solid Levels

The analysis of anthocyanin and total soluble solid levels in berries was carried out as described by Böttcher *et al*. (2011a).

### 5.1.2. Preparation of Grape RNA and cDNA Synthesis
Grape RNA was isolated and first-strand cDNA was synthesised essentially as described by Böttcher *et al.* (2010).

### 5.1.3. Chemical Synthesis of Labelled Auxin Amino Acid Conjugates
IAA-amino acid, NAA-Asp and BTOA-Asp conjugates were synthesised according to the protocol of Ilić *et al*. (1997) as described by Böttcher *et al.* (2010). The conjugates were purified as described by Böttcher *et al.* (2010).

### 5.1.4. *Ex Planta* Berry Induction Assay
In general sampled bunches were sterilized in 0.05% (v/v) Tween 20 containing one Milton antibacterial tablet $L^{-1}$ (Milton Australia, Laverton North, Victoria) for 10-60 mins and washed 3 times with sterile nanopure water. All following procedures were carried out in a laminar flow under sterile conditions. A thin slice was cut off horizontally around the brush area of each berry to facilitate compound absorption and 20 berries were placed on petri dishes filled with 25 ml of Gamborg's media, 0.025% (w/v) Casein hydrosylate, 0.8% (w/v) agar, pH 5.7-5.8 and other additives as required (see details in sections below).

Berries were placed on the plates with the brush facing the agar, the plates were sealed with Parafilm and kept in the dark at room temperature. After the required period had elapsed the berries were harvested, deseeded and frozen in liquid nitrogen.

### 5.1.5. Statistical Data Analysis
The significance of any differences between samples was tested by Student's *t*-test (unpaired) or ANOVA with Duncan's *post hoc* test, using IBM SPSS Statistics ver. 20 (IBM Australia, Sydney, NSW, Australia).

## 5.2. Experiment Specific Methods

### 5.2.1. The Effect of NAA in Delaying Veraison and Harvest in Riesling
#### 5.2.1.1. NAA Treatment of Field-grown Riesling Berries
The control and NAA treatments were carried out as described by Böttcher *et al*. (2012a).

#### 5.2.1.2. Berry Analysis
Berry weight and malic acid content analyses of berries were carried out as described by Böttcher *et al*. (2012a).

#### 5.2.1.3. Small Scale Wine Making - Riesling
Small scale wine making from conrol and NAA-treated Riesling fruit was conducted by the Wine Industry Cluster winemaking services (Adelaide, South Australia) in triplicate as described by Böttcher *et al*. (2012a).

#### 5.2.1.4. Headspace Volatile Compound Analysis
The sample preparation and analytical methods were identical to that reported by Böttcher *et al.* (2010).Solid phase microextraction – gas chromatography – mass spectrometry (SPME-GC-MS) was used to analyze the volatile constituents of the small scale wines produced from the Control and NAA-treated berries as described by Böttcher *et al*. (2012a).

Identification and quantification of volatile compounds was carried out as described by Böttcher *et al*. (2012a).

#### 5.2.1.5. Sensory Analysis of Wine – Duo-trio Test

Difference testing was conducted as described by Böttcher *et al*. (2012a) to determine if the wine made from NAA-treated grapes had different sensory characteristics to that made from Control fruit.

### 5.2.2. The Different Effects of Different Auxins on Grape Berry Development and the Role of a Family of IAA-amido Synthetases (see Results 6.2)

#### 5.2.2.1. Plant Material

Treatments with different auxins were conducted with *Vitis vinifera* L. cv Shiraz fruit as described by Böttcher *et al.* (2011a).

Flower and berry tissues for the analysis of developmental changes in gene expression in *Vitis vinifera* L. cv Cabernet Sauvignon were collected as described by Böttcher *et al*. (2012a). Grape berries (*Vitis vinifera* L. cv. Shiraz) for the ex planta experiment were sampled as described by Böttcher *et al*. (2011a) .

#### 5.2.2.2. Phylogenetic Analysis

GH3-related plant sequences were identified by BLASTP and the sequences were aligned using the ClustalX (version 2.0.10) program as described by Böttcher *et al*. (2011a).

#### 5.2.2.3. cDNA Synthesis and qRT-PCR

Quantitative RT-PCR was conducted as described by Symons *et al*. (2006) using the gene-specific primer pairs as described by Böttcher *et al*. (2011a). The data was analysed as described by Böttcher *et al*. (2011a).

#### 5.2.2.4. *Ex Planta* Berry Induction Assay

*Ex planta* berry induction assays using IAA (0.5 μM), NAA (0.5 μM), BTOA (0.5 μM), 12% (w/v) sucrose were carried out as described by Böttcher *et al*. (2011a).

#### 5.2.2.5. Protein Purification and Enzyme Assay

The coding region of *GH3-2* was amplified by PCR from a Cabernet Sauvignon berry cDNA template as described by Böttcher *et al*. (2011a). Cloning, heterologous expression and purification of GH3-2-His (C-terminal fusion) as well as assays for IAA-amino acid conjugate formation were performed as described by Böttcher *et al*. (2010) for GH3-1.

#### 5.2.2.6. Determination of Kinetic Parameters

Steady-state Michaelis-Menten kinetic parameters for the GH3-1 and GH3-2 enzymes were determined as described by Böttcher *et al*. (2011a).

#### 5.2.2.7. LC-ESI-MS/MS Analysis of Auxins and Conjugates

For the quantification of IAA and IAA-Asp in grapes, auxins were extracted from 100 mg of grape berry tissue, purified and quantified as described by Böttcher *et al*. (2011a). The analysis of conjugates produced by the *in vitro* reactions with recombinant GH3-1 and GH3-2 were also performed as described by Böttcher *et al*. (2011a).

### 5.2.3. A Novel Tool for Studying Auxin Metabolism: the Inhibition of Grapevine Indole-3-acetic Acid-amido Synthetases by a Reaction Intermediate Analogue

#### 5.2.3.1. Synthesis of AIEP

The novel novel IAA-amido synthetase inhibitor, adenosine-5´-[2-(1H-indol-3-yl)ethyl]phosphate (AIEP), was synthesised, purified and its structure was confirmed as described by Böttcher *et al*. (2012b).

#### 5.2.3.2. Plant Material

For the *ex planta* berry experiment, grape berries (*Vitis vinifera* L. cv Shiraz) were sampled from a vineyard in the Adelaide Hills, South Australia (35.018223, 138.838220) 5 weeks before the initiation of ripening (10 January 2011) between 0900-1000 hrs and kept on ice until used.

#### 5.2.3.3. *Ex planta* Berry Assay

*Ex planta* berry assays using the grapes described above were as conducted as described by Böttcher *et al*. (2012b).

#### 5.2.3.4. Protein Purification and GH3 Enzyme Assay

The coding region of *GH3-6* was amplified by PCR from a Cabernet Sauvignon berry cDNA template as described by Böttcher *et al*. (2012b). Cloning, heterologous expression and purification of GH3-6-His (C-terminal fusion) were essentially performed as described by Böttcher *et al*. (2010), with modifications as described by Böttcher *et al*. (2012b). The TLC-based assays for IAA-amino acid conjugate formation were performed as described by Böttcher *et al*. (2010).

#### 5.2.3.5. Determination of Kinetic and Inhibition Parameters

The determination of kinetic and inhibition parameters for AIEP using GH3-1 and GH3-6 enzymes was as described by Böttcher *et al*. (2012b).

#### 5.2.3.6. LC-ESI-MS/MS Analysis of Auxins and Conjugates

The quantification of IAA, IAA-Asp and NAA-Asp in grapes was performed as described by Böttcher *et al*. (2012b). The analysis of conjugates produced by the *in vitro* reactions with recombinant GH3-1 and GH3-6 was performed as described in Böttcher *et al.* (2011a) using the solvent conditions described by Böttcher *et al*. (2012b).

### 5.2.4. Crystal Structure of an Indole-3-Acetic Acid Amido Synthetase from *Vitis vinifera* Involved in Auxin Homeostasis

#### 5.2.4.1. Protein Purification and GH3 Enzyme Assays

Heterologous expression and purification of GH3-1 containing a His6-tag fused to the C-terminus were performed as described by Böttcher *et al.* (2012a, 2010). The cloning, expression and purification of GH3-3, GH3-4 and GH3-5 were as described by Peat *et al*. (2012).

The N-terminal fusion of the His6-tag with the GH3-1 protein was as described by Peat *et al*. (2012). Protein expression and purification were performed as described above for the C-terminal fusion protein. Mutated versions of GH3-1 (N-terminal His6-tag) were expressed and purified under the same conditions as the wild-type protein. Spectrophotometric assays of GH3 activity, TLC-based assays for IAA-amino acid conjugate formation and confirmation of the identity of reaction products via LC-MS were performed as described by Peat *et al*. (2012).

#### 5.2.4.2. Site-directed Mutagenesis of GH3-1
Site-directed mutagenesis of GH3-1 was performed as described by Peat e*t al*. (2012).

#### 5.2.4.3. Determination of Kinetic Parameters
Enzymatic assays and the determination of kinetic parameters were performed as described by Peat *et al*. (2012).

#### 5.2.4.4. Metal-binding Assay
Enzymatic assays to test the role of Mg in the reaction mechanism parameters were performed as described by Peat *et al*. (2012).

#### 5.2.4.5. Crystallization and Data Collection
The crystallization of C-terminal His6-tagged GH3-1 protein (performed at the CSIRO Coolaborative Crystallization Centre) and the collection of X-ray diffraction data (at the Australian Synchrotron) were performed as described by Peat *et al*. (2012).

#### 5.2.4.6. Structure Solution and Refinement
The solution and refinement of the 3-D GH3-1 protein structure was as described by Peat *et al.* (2012).

#### 5.2.4.7. Small Angle X-ray Scattering (SAXS) Data Measurements
SAXS measurements were made at the SAXS-WAXS beamline of the Australian Synchrotron, Melbourne, Australia as described by Peat *et al.* (2012).

#### 5.2.4.8. SAXS Data Analysis
Data analysis was performed using version 2.3.1 of the ATSAS suite of software as described by Peat *et al.* (2012). The program CRYSOL (Svergun *et al.*, 1995) was used to calculate scatter from both monomeric and putative dimeric forms of GH3-1 from the x-ray crystal structures and to compare the calculated scatter with the measured data (see Peat *et al.*, 2012). As there was no discernable change in the SAXS data as a function of concentration (see Peat *et al.*, 2012). the highest protein concentration data was used for modelling (3 mg/mL) giving the highest signal to noise ratio.

#### 5.2.4.9. Modelling of Auxins into the Active Site
IAA, BTOA, and NAA were docked into the active site of GH3-1 using the molecular docking package FRED (OpenEye Scientific Software) as described by Peat *et al.* (2012). All crystallographic and modelling figures were produced with PyMol.

Coordinates and structure factors can be found in the Protein Data Bank under accession code 4B2G.

### 5.2.5. Auxin Treatment of Shiraz Berries Post-veraison does not Significantly Alter Berry Weight and Brix
The effect of post-veraison auxin treatments on *Vitis vinifera* L. cv Shiraz berry development was investigated by comparing the effects of Control (0.1% (v/v) Chemwet 1000, Nufarm, Laverton, Australia) and NAA (50 mg/L NAA, Gibco BRL Life Technologies, Grand Island, USA in 0.1% (v/v) Chemwet 1000) treatments. 10 bunches were sprayed for each of three replicates (randomised) at a commercial vineyard (Hahndorf, South Australia, -35.018223, 138.838220) on 10/2/2010 (16 days post-veraison). The progression of ripening was

measured by measuring the weight and Brix of 50 berries per replicate over the course of development.

### 5.2.6. Treatment of Entire Vines with NAA Delays Ripening as Effectively as Targeted Treatment of Bunches and does not Cause Notable Differences to Vine Growth

A comparison of the effects on ripening of treating entire *Vitis vinifera* L. cv Shiraz vines (including bunches) vs bunches only was conducted at a commercial vineyard (Willunga, South Australia-35$^{o}$.26, 138$^{o}$.55). Vines were treated during the pre-verasion period (4/11/2011) at a time predicted to result in a significant delay in veraison and harvest. Control and NAA treatments were compared. The NAA treatment was 50 mg/L NAA (Gibco BRL Life Technologies, Grand Island, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia), the Control was 0.1% (v/v) Chemwet 1000. Two nearby rows were used. In one row an entire vine was sprayed with the Control solution, a one vine buffer was left and the next vine was treated with the NAA solution. In the nearby row 10 bunches on one vine were treated with the Control solution, a one vine buffer was left and 10 bunches were NAA treated.These sprays were repeated on 11/11/2011.The progression of berry ripening was followed throughout the season by regular sampling of 50 berries per treatment, Brix levels were measured for individual berries (n=60).

### 5.2.7. Shiraz and Cabernet Sauvignon, Developmental Berry Series for 2010/2011

For the analysis of developmental changes in gene expression *Vitis vinifera* L. cv Cabernet Sauvignon and Shiraz berries from 3-16 weeks post flowering (wpf) were collected at weekly intervals, (22 November 2010 - 15 March 2011) from a commercial vineyard (Willunga, South Australia-35$^{o}$.26, 138$^{o}$.55) in the 2010/2011 season. Sampling was completed between 09:30 and 11:30, berries (60-200 berries sampled at each time point) were immediately deseeded and tissue frozen in liquid nitrogen and stored at -80°C until used. Real-time qPCR analysis of gene expression was conducted as described above.

### 5.2.8. Auxin Delay of Shiraz Berry Ripening by Different Auxins, Willunga, 2010/2011

A replicated (three replicates), randomized trial using Shiraz vines was undertaken at a commercial vineyard at Hahndorf, South Australia (-35.018223, 138.838220). Four treatments were applied by spraying bunches only (20/11/2011) until runoff with 80mg $L^{-1}$ IAA (Sigma-Aldrich, St. Louis, USA), 50 mg $L^{-1}$ NAA (Gibco BRL Life Technologies, Grand Island, USA) or 20 mg $L^{-1}$ BTOA (American Cyanamid Company, Princeton, USA) all in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia). The Control consisted of 0.1% (v/v) Chemwet 1000. 30 bunches were treated per replicate. 60 berries (2 berries per bunch) were harvested per replicate at various times throughout development and their Brix and weight measured as described above.

### 5.2.9. Interactions between Auxin and Ethylene are Crucial to the Control of Grape (*Vitis vinifera* L.) Berry Ripening

#### 5.2.9.1. Plant Material

Ethrel treatments and sampling to follow the progress on develeopment were performed using *Vitis vinifera* L. cv. Shiraz fruit in two consecutive years (Böttcher *et al.*, 2013c). Berries for the *ex planta* experiment were also collected as described by Böttcher *et al.* (2013c).

#### 5.2.9.2. Phylogenetic Analysis

TAR- and YUC-related grapevine sequences were identified by BLASTP searches and aligned as described by Böttcher *et al.* (2013c).

#### 5.2.9.3. qRT-PCR

qRT-PCR analyses were performed as described by Böttcher *et al.* (2013c).

#### 5.2.9.4. *Ex planta* Berry Induction Assay using AVG, Ethrel and sucrose

*Ex planta* experiments using AVG, Ethrel and sucrose were conducted as described by as described by Böttcher *et al.* (2013c).

### 5.2.10. Treatment of Shiraz with Various PGRs to test their effect on Ripening: 2012/2013 Season

This experiment was conducted using Shiraz vines in a commercial vineyard (Blewitt Springs SA, -35.169449, 138.567469). A randomised trial with three replicates per treatment was established. Bunches were sprayed with either 300 µL $L^{-1}$ Ethrel (144 mg $L^{-1}$ ethephon; Bayer CropScience, East Hawthorn, Australia) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia), solution pH 3.1 or 0.1% (v/v) Chemwet 1000 solution (Control) or with AVG (1.67g $L^{-1}$ Retain, Valent Biosciences) in 0.1% (v/v) Chemwet 1000 at two times (13/12/2012 and 21/2/2012).

Berries were sampled at weekly intervals (55-80 berries per treatment replicate) after treatment up until 49 days after the initial spray when the experiment was abandoned due to vine conditions. Berry weight and Brix were measured as described above.

### 5.2.11. Shiraz Transgenic Vines Over-expressing either *VvGH3-1* or *Atetr1-1*

The *etr1-1* mutant receptor gene from *Arabidopsis* was a kind gift from Prof. Harry Klee. The *GH3-1* gene from grapevine was as described above. Transformation procedures were performed basically as described previously by Iocco *et al*. (2001). Each binary construct in pART27UGFP, containing *VvGH3-1* or *Atetr1-1* driven by the CMV 35S promoter was transformed into the *V. vinifera* cultivar Shiraz and transgenic embryos were selected on 100 µg $mL^{-1}$ kanamycin using GFP as a visual marker. Seven and nine different lines were produced for the *GH3-1* and *etr1-1* over-expressing constructs respectively. These were then grown under PC2 conditions in a glasshouse.

qRT-PCR for expression of various genes including transgene and endogenous genes as was described above. The primer sequences are also given above.

Measurements of the levels of IAA and IAA-Asp in the leaves of Control and transformed plants were carried out as described above (5.2.2.7).

Southern blot analyses were carried out as described by Davies and Robinson (1996) as modified by Davies *et al*. (2006). Probes specific to the trangenes were prepared by random priming on isolated PCR-derived DNA fragments using random hexamers and Klenow fragment with $CTP^{32}$ as label.

### 5.2.12. Different Wine Flavour and Aroma Compounds are Variously Influenced by harvest Date and Fruit Sugar Content

#### 5.2.12.1. Grapes

Riesling grapes were grown and sampled as described by Boss *et al.* (2014). Berries were sampled at four dates, 04/03/2011 (1H), 08/03/ 2011 (2H), 17/03/2011 (3H) and 23/03/2011 (4H) and were sorted into four groups based on Brix levels, i.e. 10.1-12 $^{o}$Brix (12), 12.1-14 $^{o}$Brix (14), 14.1-16 $^{o}$Brix (16) and 16.1-18 $^{o}$Brix (18), or discarded if the berry had a Brix value that fell outside this range.

#### 5.2.12.2. Micro-fermentation Conditions (25 g Samples)

Micro-fermentations were carried out as described by Boss *et al.* (2014). Fermentations were allowed to proceed until no further mass loss was noted. Wines were harvested by removing yeast cells by centrifugation (615 x *g* for 2 min). Ethanol content and pH were measured in the wines using an Oenofoss instrument (Foss, Hillerød, Denmark) following the manufacturer's instructions.

#### 5.2.12.3. Headspace Volatile Analysis

SPME-GC-MS was used to analyze the volatile constituents of the wines produced from the fermentation of the experimental musts and the identity of detected volatiles was determined as described by Boss *et al.* (2014).

#### 5.2.12.4. Data Analysis

The data from the SPME-GC-MS analysis of headspace volatiles was analysed by a range of methods including general linear modeling, cluster analysis and PCA analysis as described by Boss *et al.* (2014).

### 5.2.13. A Study of PGR Levels in Developing *Vitis vinifera* cv. Shiraz Berries Reveals a Surprising Increase in Cytokinin Levels during Ripening

#### 5.2.13.1. Grape Berry Material

Shiraz berries were sampled at a commercial vineyard, processed and analysed as described by Böttcher *et al*. (2013b).

#### 5.2.13.2. Chemicals and Calibration Curves

All standards and calibration curves for PGR analysis were as described by Böttcher *et al*. (2013b).

#### 5.2.13.3. Instrumentation

PGR analysis was conducted by the Plant Biotechnology Institute, National Research Council of Canada (NRCC), Saskatoon, Saskatchewan, Canada using the instrumentation as described by Chiwocha *et al.* (2003, 2005).

#### 5.2.13.4. Extraction and Purification

PGRs were extracted from the lyophylised grape samples and purified as described by Böttcher *et al*. (2013b).

#### 5.2.13.5. PGR Quantification by UPLC-ESI-MS/MS

PGR quantification of multiple PGRs and their metabolites by UPLC-ESI-MS/MS has been described in detail by Chiwocha *et al.* (2003, 2005).

# 6. Results/Discussion
## 6.1. The Effect of NAA in Delaying Veraison and Harvest in Riesling

For a complete presentation of this work please see Böttcher, C., Boss, P. K. & Davies, C. (2012a) Delaying Riesling grape berry ripening with a synthetic auxin affects malic acid metabolism and sugar accumulation, and alters wine sensory characters, *Functional Plant Biology*, 39:(9), 745-753.

Due to the differences in timing of the funding cycle compared to the grape growing season and the time required for completion of the more complex assays some work commenced in the 2009/2010 season was continued during the period of the current project. This work was completed as part of the current project and as it is relevant to the continuing study it is included here.

As shown for the red variety Shiraz (Böttcher *et al.*, 2010 and in previous report, CSP06/06 Figures 16-19) the treatment of fruit with the auxin NAA also delayed veraison and harvest in the white-skinned variety Riesling (Figures 24-27 CSP06/06 report and see below). In addition, the application of NAA to pre-veraison Shiraz berries had previously been shown (Böttcher *et al.*, 2010 and in previous report, CSP06/06 Figure 23) to make ripening more synchronous as measured by Brix and malic acid measurements. A similar effect was observed when NAA was applied to pre-veraison Riesling berries (Böttcher *et al.*, 2010 and Figures 30, 31 previous report, CSP06/06). Some of the data representing the effects on Riesling berry development detailed in the previous report (Fig. 1c) is required to understand the further outcomes presented below and additional analysis of the data has revealed some previously unreported features.

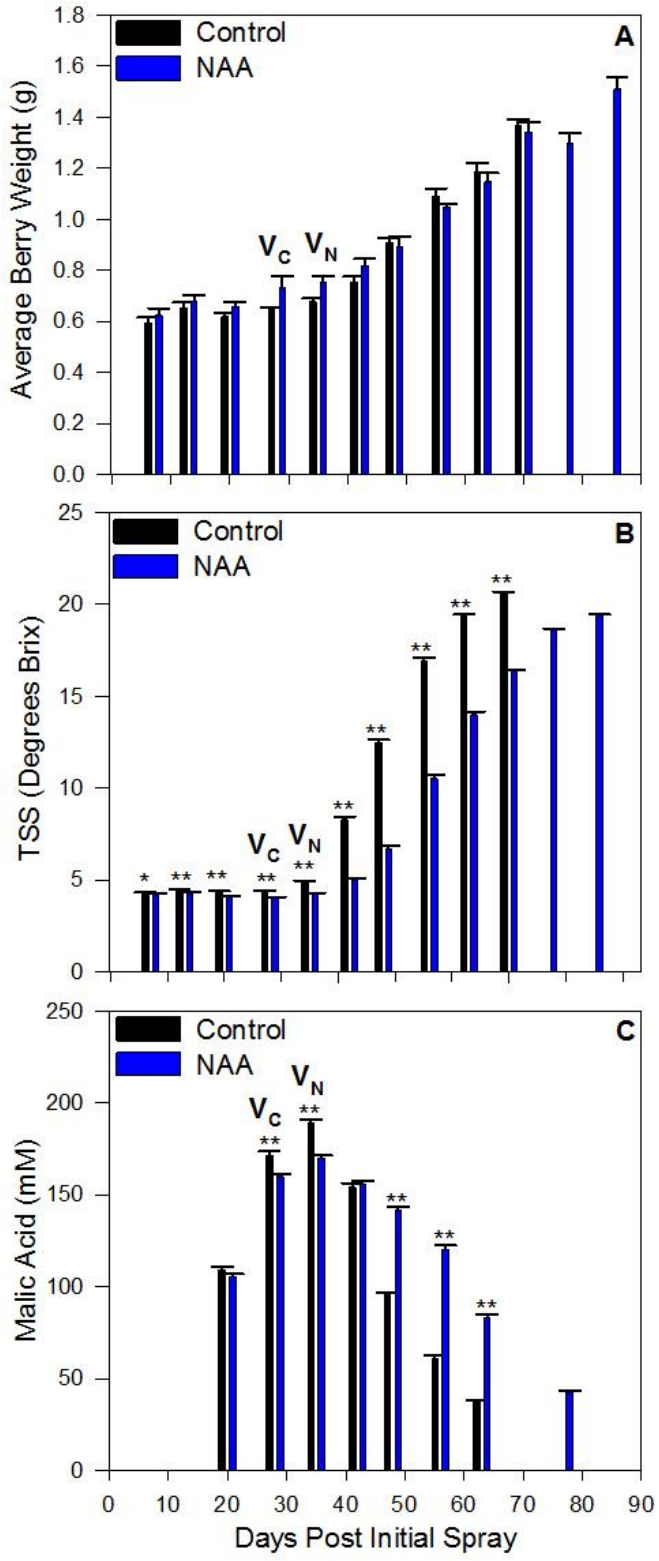



**Figure 1. Changes in berry development due to pre-veraison treatment of Riesling berries with NAA.** Control berries (black), were treated with 0.1% (v/v) Chemwet 1000, NAA-treated berries (blue), were treated with 50 mg/L NAA in 0.1% (v/v) Chemwet 1000. Treatments were done at 0, 22 and 37 dpis. (A) Changes in berry weight (B) Changes in TSS, (C) Changes in malic acid level. The asterisks denote those times when there was a statistical

difference, as determined by Student's t-test (* $p < 0.05$, ** $p < 0.01$), between treatments. $V_C$ and $V_N$ indicate the timing of veraison in Control and NAA-treated berries respectively.

### 6.1.1. NAA Applied to Pre-veraison Riesling Berries Significantly Delayed Ripening as Measured by Brix and Juice pH but did not Affect Berry Weight

The berry weight profiles for Control and NAA-treated fruit were similar throughout development (Figure 1a). The mean weight of NAA-treated berries was not significantly different to that of the Control berries at harvest. Berry Brix levels were significantly different between Control and NAA-treated fruit at all time-points (Figure 1b). Berry ripening was delayed by NAA treatment as measured by Brix (Figure 1b). Veraison was determined to be at 28 dpis for Control and 35 dpis for NAA-treated berries. The rate of Brix increase was lower in NAA-treated fruit. This is shown by the difference in slope between the two Brix curves (Figure 1b). The Control fruit was harvested 15 days earlier than the NAA-treated fruit and even then the NAA-treated fruit did not attain the same final Brix as the Control fruit (Table 1). The plot of Brix measurements against berry weights (Figure 2a) shows quite clearly that for any given Brix level the weight of the NAA-treated berries was higher than that for the Control.

**Table 1. Basic biochemical analysis of berries (at harvest) and wine from Control and NAA-treated Riesling bunches.**

| | Berry | | Wine | |
|---|---|---|---|---|
| | **Control** | **NAA-treated** | **Control** | **NAA-treated** |
| **TSS (Brix)** | 20.4 | 19.1* | | |
| **% Alcohol (v/v)** | | | 13.1 | 12.0 |
| **pH** | 3.17 | 3.27** | 3.13 | 3.26* |
| **Titratable acid ($g/LH_2T$)** | 8.8 | 8.5 | 8.6 | 8.4 |

* indicates mean for NAA-treated berries or wine significantly different to that of the Control at the $p < 0.05$ level as determined by t-test

** indicates mean for NAA-treated berries or wine significantly different to that of the Control at the $p < 0.01$ level as determined by t-test

Malic acid levels were significantly different at most time points (Fig. 1c). Malic acid levels for both treatments peaked on the same date and the peak level was significantly lower in NAA-treated fruit. Malic acid levels declined in both samples but the rate of decline was lower in NAA-treated berries (Figure 1c). At harvest the pH of juice from NAA-treated berries was significantly higher than the juice from the Control fruit, but the levels of titratable acid were not significantly different (Table 1).

A more detailed analysis of the effects of NAA on Riesling berry development, including effects on malic acid levels can be found in Böttcher *et al.* (2012a).

### 6.1.2. Biochemical Analysis of the Small Scale Wine Lots shows Differences in Volatile Composition between Control and Delayed Ripening Wine

Table 1 demonstrates that the Brix levels of the juice from Control and NAA-treated berries were significantly different but the % alcohol of the wines was statistically similar. The pH was slightly lower in wine made from Control fruit (as was the juice pH) but the titratable acidity was not significantly different.

A detailed analysis of wine volatile compounds was undertaken using headspace SPME-GC-MS analysis. 105 compounds were identified in the volatile profiles, 19 were found at higher concentrations in the headspace of the wines made from NAA-treated grapes and 16 were more abundant in the Control wines. Most of the differences were relatively small. The detailed results of this analysis can be found in Böttcher *et al.* (2012a).

### 6.1.3. Wines from Control and Delayed Ripening Fruit are Distinguishable by Sensory Analysis

Difference testing by sensory analysis showed that tasters could distinguish between wines made from NAA-treated (delayed ripening) and Control fruit. 25 of the 40 participants correctly identified the same wine as the control in a duo-trio test, ($p = 0.04$). However, the informal tasting comments did not suggest a particular liking for either wine.

A full discussion of the results is presented in Böttcher *et al.* (2012a) but a summary is given below.

1) The timing of veraison and harvest were delayed and there appeared to be some increase in the synchronicity of ripening during certain periods of development.

2) The long delay in harvest date for the NAA-treated fruit indicates that it is possible to alter harvest time in a manner that would be useful in commercial vineyards where controlling the fruit harvest times may be beneficial for winery intake scheduling. This would allow fruit to be harvested at an optimal time.

3) Another advantage in delaying ripening may be the ability to ripen berries during cooler periods of the year. Wines made from grapes grown in cooler climates frequently achieve a higher price than those made from fruit grown in warmer climates. Webb *et al.* (2008) have indicated that for a number of varieties there is a linear relationship between mean January temperature and winegrape price.

4) There was a difference in growth between Shiraz and Riesling berries in response to NAA treatment. The berry weight profiles were similar for Control and NAA-treated Riesling fruit (Figure 1a), in contrast to the work on Shiraz where the increase in berry weight was delayed in NAA-treated fruit but once they started enlarging the berries had a significantly higher mean weight (Böttcher *et al.*, 2011a, 2011b).

5) The rate of Brix increase during ripening was lower for NAA-treated fruit than Control fruit (Figure 1b). This suggests a change in the relationship between elapsed time and the increase in TSS. If the characters desired in Riesling wines are dependent on time rather than sugar levels it may be that the use of NAA to delay ripening will produce wine of a style usually associated with high Brix levels from grapes with lower Brix levels. This may be an advantage to the wine industry in regard to concerns about the production of overly alcoholic table wines and indicates that the rate of sugar increase can be controlled.

6) This study revealed some interesting effects on malic acid accumulation that are discussed in full in Böttcher *et al.* (2012a). Malic acid is a useful marker for berry development throughout its entirety as its levels are always changing whereas Brix levels only change significantly after veraison.

7) In contrast to wines from a Shiraz study (Böttcher *et al.*, 2011b) the Riesling wines were distinguishable by sensory analysis. The informal taster comments did not indicate the presence of any negative characters in the wines from the NAA-treated berries.

8) Significant differences in the concentration of volatile compounds in the headspace above the wines produced from the NAA-treated and Control berries were observed, a detailed discussion is given in Böttcher *et al.* (2012a).

9) The greater synchronisation of grape berry development resulting from pre-veraison treatment with NAA appears to be reproducible, having now been demonstrated in both Riesling (this work) and Shiraz (Böttcher *et al.*, 2011b). NAA could be used to ameliorate severe levels of asynchronous development.

We have previously shown that auxins can be used to delay ripening in the red wine variety Shiraz. This work shows that auxin treatment at a suitable time during the pre-veraison period can delay the ripening of white grapes very effectively. Ripening synchronicity was improved by the treatment. Measurement of wine volatiles in the headspace showed that there were some differences between the wine made from Control and NAA-treated fruit. However, these differences were rather small and in a limited number of compounds. Sensory analysis showed that the two sets of wines could be distinguished. The differences could be due to the delay in ripening and harvest rather than a direct effect of the treatment as the fruit delayed in ripening ripened under different, somewhat cooler conditions later in the season.

## 6.2. The Different Effects of Different Auxins on Grape Berry Development and the Role of a Family of IAA-amido Synthetases

For a complete presentation of this work please see Böttcher, C., Boss, P. K. & Davies, C. (2011a) Acyl substrate preferences of an IAA-amido synthetase account for variations in grape (*Vitis vinifera* L.) berry ripening caused by different auxinic compounds indicating the

importance of auxin conjugation in plant development, *Journal of Experimental Botany*, 62:(12), 4267-4280.

The varied effects resulting from the application of different auxins are associated with the properties of a family of grapevine enzymes that inactivate auxins *in planta*.

We have shown previously that the application of different auxins had different effects on berry ripening. Application of the auxin predominantly present in grapevines, IAA, had no significant effect on berry ripening as measured by the levels of sugars, anthocyanins (colour) and berry weight (previous report CSP06/06 Figures 33-35, a summary of this data, Figure 2, is presented below for information). In contrast, the synthetic auxin NAA, which is widely used in horticulture, significantly delayed ripening as measured by Brix, weight and colour and subsequently delayed harvest. Another synthetic auxin, BTOA, was even more effective in delaying ripening and harvest.

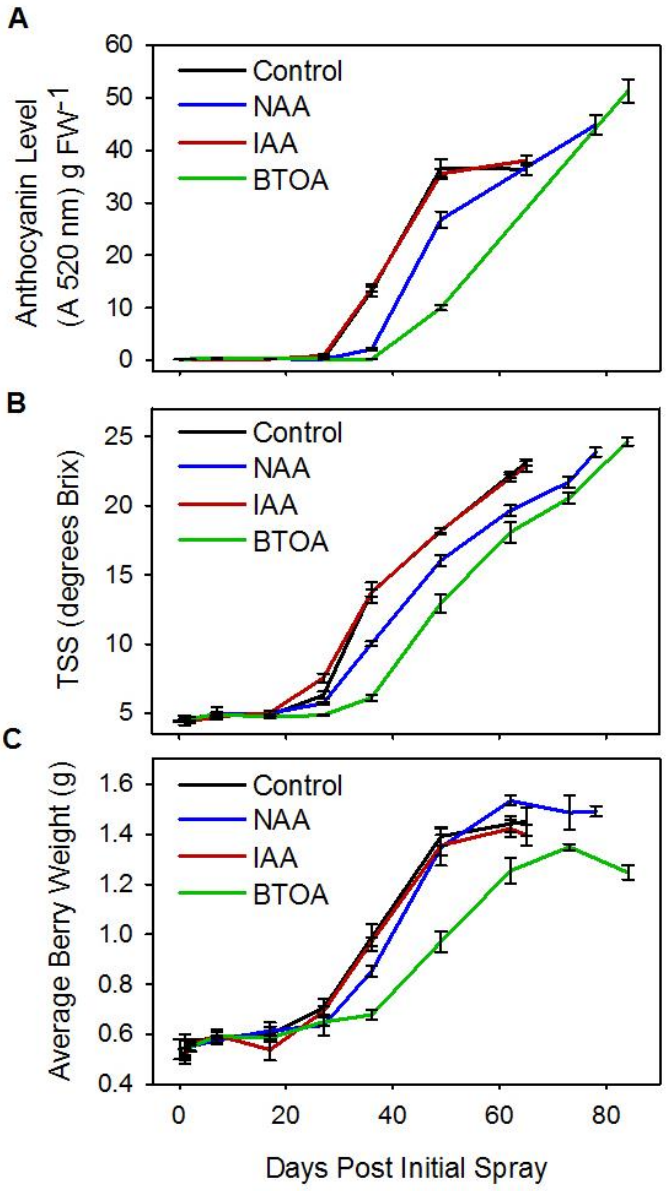



**Figure 2. Delayed ripening of Shiraz berries after treatment with different auxins.** (A) Anthocyanin levels (absorbance at 520 nm), in field-grown Shiraz berries treated twice (24 d and 9 d pre-veraison) with 80 mg $L^{-1}$ IAA, 50 mg/L NAA, 20 mg/L BTOA in 0.1% (v/v) Chemwet 1000 or 0.1% (v/v) Chemwet 1000 (Control) (B) changes in TSS, measured as degrees Brix and (C) changes in berry weight. All data represent means +/- STERR (n=3).

An understanding how this differential efficacy occurs is important if auxin-like PGRs are to be used effectively to control grape berry ripening. As described in the last report the key to understanding this process may lie in the mechanism of action of a group of enzymes that inactivate auxins in the plant.

### 6.2.1. A Family of Auxin Conjugating Enzymes are Crucial to the Control of Grape Berry Development Including Ripening

Nine genes in grapevine encode acyl-amido synthetase enzymes (also known as GH3 proteins). These enzymes conjugate acyl groups to a range of amino acids thus altering the properties of the conjugated acyl group. In this case the acyl group involved is the auxin IAA. In a previous report we described the activity of one of these enzymes, GH3-1. It has a biphasic expression pattern during berry development (previous report CSP06/06 Figure 37, Böttcher *et al.*, 2010) and its expression is induced by various auxins (previous report CSP06/06 Figure 39, Böttcher *et al.*, 2010). It conjugates IAA to both aspartic acid and tryptophan *in vitro* (previous report CSP06/06 Figure 40, Böttcher *et al.*, 2010) but only the IAA-Asp conjugate is found in berries (previous report CSP06/06 Figure 41, Böttcher *et al.*, 2010). This is probably due to the relatively low concentration of tryptophan in grape berries (data not shown). The IAA-Asp conjugate is thought to be inactive as a PGR and therefore, the GH3-1 enzyme is thought to play an important role in controlling plant growth and in particular grape berry development including ripening.

As these genes and their respective proteins are important for grapevine development, and in particular ripening, it is important to have a better understanding of the function of the other five IAA-amido synthetase (GH3) proteins.

### 6.2.2. Grape GH3 Genes Involved in the Response to Exogenous Auxins

There are three main families of early auxin response genes (Abel and Theologis, 1996), one of which is the *GH3* gene family. The IAA-amido synthetase encoding *GH3* genes are of particular interest due to the known selectivity of these enzymes for their auxin substrates (Staswick *et al.*, 2005) and the involvement of at least one grapevine GH3 protein (GH3-1) in the ripening process of berries (Böttcher *et al.*, 2010). The expression of *GH3-1* was induced in pre-veraison berries treated with IAA, NAA and BTOA (Böttcher *et al.*, 2010) suggesting that GH3 enzymes may be involved in the differences in ripening delay caused by exogenous application of IAA, NAA and BTOA (Figure 2).

All putative IAA-conjugating grapevine GH3 proteins that are likely to conjugate to auxins are detailed in Table 2.

**Table 2. GH3 family in grapevine.**

| Protein | Accession number [a] | Protein length (aa)[b] |
|---|---|---|
| GH3-1 | XP_002271252 | 598 |
| GH3-2 | XP_002283886 | 600 |
| GH3-3 | CAN83696 | 613 |
| GH3-4 | XP_002263353 | 596 |
| GH3-5 | XP_002276241 | 588 |
| GH3-6 | XP_002268278 | 593 |
| GH3-7 | XP_002272560 | 592 |
| GH3-8 | XP_002271002 | 578 |
| GH3-9 | CAN69491 | 579 |

[a]NCBI or Genbank accession number of predicted open-reading frames
[b]Length of the predicted polypeptide in amino acids

An unrooted phylogenetic tree was constructed from the alignment of 67 full-length GH3 protein sequences to demonstrate the relationship between grapevine GH3 proteins and those from other plant species (Böttcher *et al.*, 2011a).

GH3-1 has already been proposed to be involved in the deactivation of exogenous auxins in grapes (Böttcher *et al.*, 2010). In the current work we determined the expression of the remaining 5 genes during berry development and upon auxin treatment (see Böttcher *et al.*, 2011a for a more detailed presentation/discussion of these results). An example of the setup of a plate containing berries in the *ex planta* experiment is given in Figure 3. All five *GH3* genes displayed high transcript accumulation in Cabernet Sauvignon flowers and young berries. During this period of berry development auxin levels are high (Böttcher *et al.*, 2010, Cawthon and Morris, 1982, Deytieux-Belleau *et al.*, 2007, Inaba *et al.*, 1976, Zhang *et al.*, 2003), therefore the activity of these GH3 proteins in early stages of berry development might contribute to the control of auxin levels. *GH3-1*is different as it has a second peak of expression at veraison (Böttcher *et al.*, 2010), transcript levels of the other five *GH3* genes declined to be low at veraison and remained low for the rest of berry development (Böttcher *et al.*, 2011a).

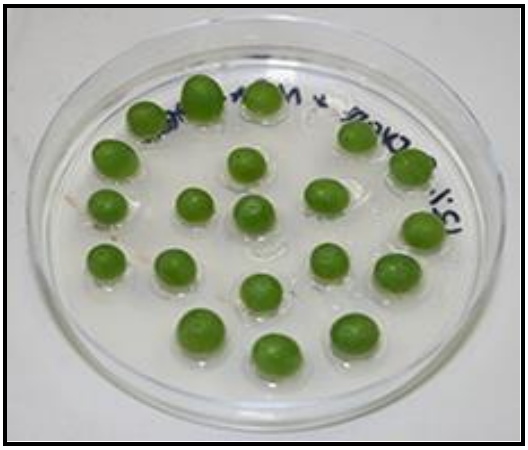


**Figure 3. Example of a plate used in the *ex planta* experiment.** Surface sterilised berries were trimmed and placed on agar containing different PGRs as described in General Methods.

*GH3-2* was the only gene out of the five tested that showed a significant induction of expression in response to exposure to 0.5 μM IAA, NAA, or BTOA for 24 h in an *ex planta* induction experiment using Shiraz berries at both 22 and 12 days pre-veraison. NAA and BTOA were stronger inducers than IAA. A similar response to the three auxins has also been reported for the closely related *GH3-1*gene (Böttcher *et al.*, 2010). A detailed discussion of the above experiments containing all the data relating to all auxin conjugating *GH3* genes can be found Böttcher *et al*. (2011a).

### 6.2.3. GH3-2 is an IAA-amido Synthetase with the Same Amino Acid Preferences as GH3-1

Since *GH3-1* and *GH3-2* were the only GH3 auxin conjugating genes that were induced by the application of IAA, NAA and BTOA all further experiments focused on those two genes and their corresponding proteins. To getinformation about the *in vitro* function of GH3-2, the purified recombinant protein was tested for its ability to conjugate IAA to 20 different amino acids. It was shown that this enzyme makes two conjugates that were confirmed to be IAA-Asp and IAA-Trp using liquid chromatography-mass spectrometry (LC-MS) (data not shown).

The high degree of similarity between the protein sequences of GH3-2 and GH3-1 seems to translate into identical substrate specificities since IAA-Asp and IAA-Trp have also been reported as the sole reaction products of GH3-1 (Böttcher *et al.*, 2010).

### 6.2.4. Recombinant GH3-1 and GH3-2 are Highly Selective for their Amino Acid and Acyl Substrates

The Michaelis-Menton kinetics were determined for the GH3-1 and GH3-2 enzymes. GH3-1 and GH3-2 showed very similar trends in their substrate affinities ($K_m$) and turnover rates ($k_{cat}$), having higher affinities for IAA than for ATP or the amino acids. The grapevine GH3s conjugated Asp 100-fold (GH3-1) and 80-fold (GH3-2) more efficiently than Trp, making Asp the preferred amino acid substrate. Also the physiological concentrations of Trp in grapes are approximately 100-fold lower than the $K_m^{Trp}$ values determined for GH3-1 and GH3-2, whereas Asp concentrations are about 10-fold higher than the respective $K_m^{Asp}$ values (Böttcher *et al.*, 2011a and pers. comm., C. Davies, M.R. Thomas, P. Corena). This is in

agreement with the previous finding that IAA-Asp accumulated in considerable amounts in grapes whereas IAA-Trp could not be detected in berries of various developmental stages (Böttcher *et al.*, 2010).

For GH3-1 and GH3-2 a change of the acyl substrate from IAA to NAA or BTOA with Asp as the amino acid substrate led to dramatic reductions in affinities, turnover rates and resulting efficiencies. With NAA as the auxin, substrate efficiencies were 50-fold (GH3-1) and 21-fold (GH3-2) lower compared to IAA and the formation of the BTOA-Asp conjugate was either not detected (GH3-1) or 47000-fold less efficient than the formation of IAA-Asp (GH3-2).

The decrease in catalytic efficiencies observed for GH3-1 and GH3-2 with NAA may be because the naphthalene ring of NAA might not be a perfect steric fit for the active site of GH3 proteins (Chen *et al.*, 2010). BTOA, with a sulphur atom in the indole ring and an oxyacetic acid side chain is even less closely related structurally to IAA than NAA which could explain why it was such a poor substrate for the grapevine GH3 proteins. This could lead to the berries being exposed to high levels of free auxinic compounds (BTOA, NAA) for extended periods which in turn could be the reason for the observed delay in ripening (Figure 2). The efficiency of conjugation by the GH3 enzymes is inversely proportional to a compound's ability to act as an auxin and explains the different abilities of IAA, NAA and BTOA to delay ripening.

A detailed discussion of the above experiments containing all the data relating to auxin conjugating *GH3* genes can be found Böttcher *et al*. (2011a).

### 6.2.5. In IAA-treated Berries Changes in IAA and IAA-Asp Levels are Associated with the Expression of GH3-2

If a delay in berry ripening after treatment with NAA and BTOA was caused by a lack of GH3-catalysed conjugation of these compounds the opposite would be true for exogenous IAA which had little or no effect on the timing of ripening. It was therefore determined if the application of IAA to pre-veraison berries led to the formation of IAA-Asp. One day after berries were sprayed with IAA a 7.5-fold increase in IAA levels was found that was matched by a 200-fold increase in IAA-Asp levelsThe concentration of IAA returned to Control levels two days after the application, whereas IAA-Asp levels decreased more slowly and were still elevated one week after the treatment. The reduction of the IAA-Asp levels is probably due to oxidation of the bound IAA (Östin *et al.*, 1998, Staswick *et al.*, 2005), a process that links this conjugate to the irreversible inactivation of IAA.

The increased levels of IAA-Asp in IAA-treated berries could indicate GH3-1/GH3-2 activity. To further investigate this possibility the gene expression of *GH3-1* and *GH3-2* was analysed in the same berry tissues. Transcript levels of *GH3-2* were increased 12-fold one day after the IAA treatment followed by a decrease to Control levels one week after auxin application. From these results it can be deduced that GH3-2 might be linked with the accumulation of IAA-Asp and therefore the decrease of free IAA levels in IAA-treated pre-veraison berries.

In conclusion, acyl substrate preferences of an auxin-inducible IAA-amido synthetase expressed in pre-veraison berries might be responsible for the variations in ripening delay caused by the application of different auxinic compounds.

Numerous studies on their function in Arabidopsis, rice and the moss *Physcomitrella patens* have established GH3-catalysed auxin conjugation as an important factor in plant growth, organ development and response to light and stress (Ludwig-Müller, 2011, Woodward and Bartel, 2005). The findings presented in this study support the addition of fruit ripening (Böttcher *et al.*, 2010, Liu *et al.*, 2005) to the list of GH3-regulated processes.

## 6.3. A Novel Tool for Studying Auxin Metabolism: the Inhibition of Grapevine Indole-3-acetic Acid-amido Synthetases by a Reaction Intermediate Analogue

For a complete presentation of this work please see Böttcher, C., Dennis, E. G., Booker, G. W., Polyak, S. W., Boss, P. K. & Davies, C. (2012b) A novel tool for studying auxin-metabolism: The inhibition of grapevine indole-3-acetic acid-amido synthetases by a reaction intermediate analogue, *PLoS One*, 7:(5), e37632.

The auxin class of plant PGRs, plays essential roles in many critical aspects of plant growth and development including embryogenesis, vascular tissue differentiation, photo- and gravitropisms, lateral branching of shoots and roots and fruit development (Davies, 2010, Gillaspy *et al.*, 1993, Østergaard, 2009, Simon and Petrášek, 2011, Tanaka *et al.*, 2006, Vanneste and Friml, 2009). Auxin-regulated processes depend on the tight control of the cellular auxin concentration, which is controlled through the coordination of biosynthesis, sequestration, degradation and transport (Delker *et al.*, 2008, Woodward and Bartel, 2005, Zhao, 2010). Compounds that disturb this highly complex system are invaluable for the field of research. The identification and functional characterisation of the membrane proteins involved in polar auxin transport has been enabled through the use of auxin transport inhibitors such as naphthylphthalamic acid, 2,3,5-triiodobenzoic acid and gravacin (De Rybel *et al.*, 2009). Chemical inhibition has also been used to assist in the study of auxin biosynthesis (He *et al.*, 2011).

The metabolic fate of auxins is poorly understood with the notable exception of the conjugation of IAA to amino acids catalysed by the family of GH3 proteins discussed earlier in this report. The biological function of GH3 proteins in higher plants has proven difficult to study due to large gene numbers and functional redundancy. Consequently, knockout mutants of single IAA-amido synthetase genes in Arabidopsis (Khan and Stone, 2007, Staswick *et al.*, 2005, Takase *et al.*, 2004) and rice (Ding *et al.*, 2008) were undistinguishable from the wildtype or displayed very subtle phenotypes. However, the overexpression of *GH3* genes has demonstrated a complex involvement of IAA-conjugating GH3 proteins in plant development. In Arabidopsis, GH3-5 seems to be involved in light signal transduction pathways and stress responses (Park *et al.*, 2007, Zhang *et al.*, 2007) and for GH3-9 a role in auxin redistribution in roots has been suggested (Khan and Stone, 2007). Rice overexpression mutants provided evidence for a link of GH3 activities with pathogen resistance (Ding *et al.*, 2008, Domingo *et al.*, 2009, Fu *et al.*, 2011) and in drought adaptation (Zhang *et al.*, 2009a). Based on correlative evidence the activity of GH3 proteins has also been associated with fruit ripening in the pungent pepper fruit (*Capsicum chinense* Jacq.) (Liu *et al.*, 2005) and in grape berries (as described earlier in this report). A chemical approach targeted to inhibit the activity of IAA-amido synthetases could be used to overcome redundancy issues and the dependence on overexpression mutants provided that the inhibiting compound was efficacious with all members of this protein family.

The conjugation process is a multistep reaction consisting of two partial reactions (Chen *et al.*, 2010). Initially IAA is adenylated in a reaction requiring MgATP, followed by the transfer of IAA onto the amino acid substrate with the liberation of AMP. Thus, IAA-amido synthetases are members of a family of enzymes that is characterised by the employment of an adenylated reaction intermediate to attach organic acids onto substrates (Artymiuk *et al.*, 1994, Gottlieb *et al.*, 2011). Hence, one approach towards designing a small molecule inhibitor is to create a mimic of the adenylated intermediate where the labile linker is replaced with a more stable one. Non-hydrolysable bi-substrate analogues have been reported as inhibitors of tRNA amino-acyl synthetase, biotin protein ligase and cysteine ligase (Bernier *et al.*, 2000, Brown *et al.*, 2004, Fan and Blanchard, 2009, Forrest *et al.*, 2000, Soares da Costa *et al.*, 2012, Ueda *et al.*, 1991).

The design and synthesis of a stable bi-substrate analogue of the GH3 reaction intermediate, adenosine-5´-[2-(1H-indol-3-yl)ethyl]phosphate (AIEP), and the characterisation of its inhibitory effects on two grapevine IAA-amido synthetases, namely GH3-1 and GH3-6 are described in brief below. This work provides the basis for the study of IAA-amido synthetase function through chemical inhibition. For a more detailed exposition of this work please read Böttcher *et al*. (2012b).

### 6.3.1. Recombinant Expression and Kinetic Analysis of the Grapevine GH3-6 Protein

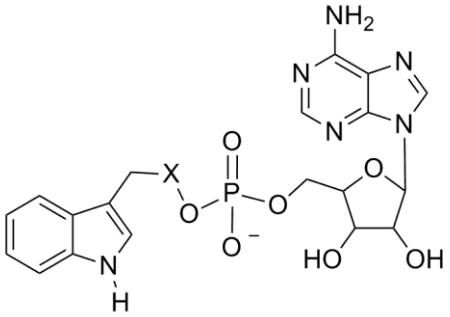
The gene encoding the putative grapevine IAA-amido synthetase GH3-6 (Böttcher *et al.*, 2011a) was isolated and cloned for recombinant expression in *Escherichia coli*. SDS-PAGE analysis of the protein eluted from the column revealed the enzyme preparation to be >90% pure. A thin layer chromatogram of the reaction mixtures with IAA and 20 amino acids demonstrated the possible conjugation of Gln, Met, Tyr, Val, Phe, Gly, Asn, Ile, Trp, Asp, Glu, Ala and His. A number of the reaction products (Glu, Met, Tyr and Ala) were tested against their corresponding IAA-amino acid standards and were found to have matching $R_f$-values. Glu, His and Trp were the preferred amino acid substrates as judged by the intensity of the product bands. The broad specificity of GH3-6 contrasted with the previously characterised grapevine IAA-amido synthetase GH3-1, which has a strict requirement for just two amino acid substrates, namely Asp and Trp (described earlier in this report and see Böttcher *et al.*, 2010). Next the kinetic parameters for GH3-6 were measured and compared to those of GH3-1. The $K_m$-values of 13.2 µM for MgATP and 21.1 µM for IAA were similar to those previously observed for GH3-1 (see above and Böttcher *et al.*, 2011a) and the $K_m$-values for GH3-6 were found to be 2.3 µM for MgATP and 44.7 µM for IAA. The turnover rates for GH3-6 were approximately 10-fold lower than those measured for GH3-1, indicating that GH3-6 had a reduced catalytic efficiency when compared to GH3-1. GH3-6 was therefore useful in the studies described below to ascertain the effectiveness of the novel inhibitor as it allowed the inhibitor to be tested against an enzyme with different properties to the GH3-1 enzyme.

### 6.3.2. AIEP is a Competitive Inhibitor of Two Grapevine IAA-amido Synthetases *in Vitro*

To address the role of IAA-amido synthetases in auxin-regulated processes an inhibitor with broad activity across all members of this enzyme family is required. Therefore, AIEP was designed to be a chemical analogue of the reaction intermediate (Figure 4) shared by all IAA-amido synthetases. The chemical structure of AIEP was confirmed by NMR analysis.

The bi-substrate inhibitor was then assayed for inhibitory activity against both GH3-1 and GH3-6. Michaelis-Menten plots illustrated that AIEP reduced the activity of both grapevine

GH3 proteins in a concentration-dependent manner. Consistent with a competitive inhibitor that occupies the same binding sites as ATP and IAA $V_{max}$-values were unchanged with increasing inhibitor concentrations but the $K_m$-values progressively increased. Dixon plots were performed to confirm the mechanism of inhibitor action using a selection of this data. This analysis revealed that the inhibitor was indeed competitive with both IAA and MgATP. Together these data were in agreement with the initial proposal of AIEP being a mimic of the IAA-amido synthetase reaction intermediate.




**A:** X = $CH_2$, Adenosine-5'-[2-(1H-indol-3-yl)ethyl]phoshate (AIEP).
**B:** X = CO, Adenosine-5'-[2-(1H-indol-3-yl)acetyl]phosphate.

**Figure 4. Design of AIEP.** (A) is the structure of AIEP and (B) is the structure of the proposed reaction intermediate.

**6.3.3. Treatment with AIEP Reduced Auxin-Asp Conjugate Formation in Grape Berries**
In order to analyse the *in vivo* effects of AIEP, Shiraz berries at about five weeks prior to veraison were treated with 0.5 µM NAA, 20 µM inhibitor or combinations of 0.5 µM NAA and three different inhibitor concentrations (5 µM, 10 µM and 20 µM) for either 6 h or 24 h in an *ex planta* experiment. Changes in the levels of the free endogenous auxin IAA, the *in vitro* GH3 reaction product IAA-Asp as well as the conjugate of Asp with the synthetic auxin NAA were analysed using LC-ESI-MS/MS. A synthetic auxin was chosen to judge the inhibitory effect of AIEP on the conjugation of an exogenous auxin of a known concentration. NAA has previously been reported as an *in vitro* acyl substrate of two grapevine IAA-amido synthetases including GH3-1 (Böttcher *et al.*, 2011a). None of the treatments had a significant effect on the concentration of IAA in the berry tissue which was found to be in a comparable range to previous studies (Böttcher *et al.*, 2010, Inaba *et al.*, 1976). The concentration of IAA-Asp in the Control berries was also in good agreement with previously published data (Böttcher *et al.*, 2010) and was not affected by the exposure to NAA for 6 h or 24 h. Treatment with 20 µM inhibitor resulted in a 2-fold decrease in the IAA-Asp concentration at both time points independent of an additional treatment with NAA and indicated that AIEP inhibited GH3 activities *in vivo*. Lower inhibitor concentrations (10 µM and 5 µM) led to a comparable reduction in IAA-Asp levels. A NAA-Asp concentration of 400 ± 30 pmol/g fresh weight (FW) was measured 6 h after berries were exposed to NAA. NAA-Asp levels were about 11-fold higher after 24 h. The additional treatment with 20 µM inhibitor resulted in a 2.2-fold reduction in the accumulation of NAA-Asp after 6 h which matched the observed decrease in IAA-Asp levels. At the later time point (24 h) no significant effect of AIEP on the concentration of NAA-Asp in the berry tissue was detected.

Exposure to the lower inhibitor concentration of 10 μM and 5 μM did not lead to significant changes in NAA-Asp conjugate levels at either of the two time points.

As discussed earlier the conjugation of IAA to amino acids, catalysed by a group of GH3 proteins (Staswick *et al.*, 2005), has been recognised as an important aspect in the control of auxin levels in plants (Delker *et al.*, 2008, Wang *et al.*, 2008, Woodward and Bartel, 2005), which is implicated in a diverse range of processes including fruit ripening. A high degree of functional redundancy and large gene numbers have made the elucidation of the *in planta* function of GH3 proteins difficult. A specific, dose-dependent, chemical inhibition of GH3 activities would circumvent this problem and allow a broader analysis of the IAA-amido synthetase family without the time consuming generation of multiple and potentially lethal mutants. To this end, we designed and synthesised a chemical inhibitor proposed to have broad activity towards auxin-conjugating members of the GH3 enzyme family. This compound, a non-hydrolysable analogue of the adenylated reaction intermediate, was shown to be an effective bi-substrate inhibitor with activity against both GH3-1 and GH3-6 *in vitro*.

One potential use of AIEP would be to dissect the possible roles of GH3 enzymes in grape berry development. GH3-1 has been associated with the control of grape berry ripening, possibly by inactivating endogenous IAA through the formation of the non-cleavable IAA-Asp conjugate (described earlier in this report and also see Böttcher *et al.*, 2010). The expression of *GH3-6* in flowers and young berries (Böttcher *et al.*, 2011a) also indicates a role for this GH3 protein in berry development.

In order to determine the *in vivo* inhibitory properties of AIEP, IAA and auxin-conjugate levels were measured in young berries that had been exposed to the synthetic auxin NAA, the inhibitor and different concentrations of the inhibitor in combination with NAA. The test was performed on agar plates to facilitate the uptake of the compounds within a short period of time. A 2-fold decrease in IAA-Asp levels after 6 h and 24 h exposure to 20 μM of AIEP was suggestive of an inhibition of GH3 activities. It was not possible to judge the degree of inhibition since the detected IAA-Asp could either have been derived from *de novo* synthesis or it could have been residual conjugate that had been synthesised before commencement of the experiment. Not much is known about the metabolic fate of IAA-Asp, but there is evidence that oxidation of the bound IAA can occur (Östin *et al.*, 1998, Staswick *et al.*, 2005) which leads to the irreversible inactivation of the auxin.

A significant inhibition of the formation of NAA-Asp that occurred in response to the incubation on 0.5 μM NAA medium could only be detected after 6 h at the highest inhibitor concentration (20 μM) tested. The reduced NAA-Asp levels demonstrated the *in vivo* inhibition of auxin-conjugate formation by AIEP and indicated that the constant uptake of NAA from the medium possibly resulted in a high NAA concentration within the cells, which consequently reduced inhibition over time and at low inhibitor concentrations.

In conclusion, this study presents the synthesis and initial characterisation of the most potent inhibitor of the GH3 family of enzymes so far described. For the first time an inhibitor of GH3 activities has been shown to be effective *in vivo*. AIEP is expected to be a useful tool in the study of GH3 protein function, either as a complement to knockout and overexpression mutants in model species like Arabidopsis, or as a means to manipulate GH3 activities in non-model species such as grapevine where long generation times and/or transformation restrictions limit experimental manipulation.

## 6.4. Crystal Structure of an Indole-3-Acetic Acid Amido Synthetase from *Vitis vinifera* Involved in Auxin Homeostasis

For a complete presentation of this work please see Peat TS, Böttcher C, Newman J, Lucent D, Cowieson N, Davies C (2012) Crystal structure of an indole-3-acetic acid amido synthetase from grapevine involved in auxin homeostasis. *Plant Cell,* 24:(11), 4525-4538.

The crystallographic work in this section was made possible through the generous time, expertise and facilities provided by Drs Tom Peat, Janet Newman and Del Lucent, CSIRO Materials, Science and Engineering, Parkville, Victoria and Nathan Cowieson, Australian Synchrotron, Clayton, Victoria.

As described above auxins such as IAA are powerful affectors of plant development and as such their levels and activitiy are tightly controlled by a range of mechanisms. Acyl-amido synthetases of the GH3 family comprise a group of plant-specific proteins with a role in PGR conjugation acting on auxins, jasmonic acid (JA), salicylic acid (SA) and benzoates (Okrent *et al.*, 2009, Staswick *et al.*, 2005, Staswick *et al.*, 2002, Westfall *et al.*, 2010). The importance of GH3 proteins in plant development is indicated by the presence of families of these proteins throughout the plant kingdom (Okrent and Wildermuth, 2011).

GH3 proteins display differing preferences for both amino acid and acyl substrates (Böttcher *et al.*, 2011a, Böttcher *et al.*, 2012b, Böttcher *et al.*, 2010, Chen *et al.*, 2010, Ludwig-Müller *et al.*, 2009, Okrent *et al.*, 2009, Staswick *et al.*, 2005).

A reaction mechanism based on kinetic data has been developed for the auxin-conjugating Os GH3-8 enzyme from rice (Chen *et al.*, 2010) and recently, three-dimensional structures have been determined for two Arabidopsis acyl-amido synthetases: At GH3-12/PBS3, which conjugates benzoate substrates and the JA-specific At GH3-11/JAR1 (Westfall *et al.*, 2012). Apart from detailing the amino acids in the active site important for substrate binding and catalysis, Westfall *et al.* (2012) described a large conformational change in protein structure. The smaller, C-terminal domain, which acts as a cap over the active site, is seen to move significantly in the different crystal forms, changing between open (ATP-bound) and closed (AMP-bound) forms.

Below we describe the elucidation of the three-dimensional structure of the grapevine IAA-amido synthetase GH3-1. Crystallization was achieved through the use of the inhibitor (adenosine-5´-[2-(1H-indol-3-yl)ethyl]phosphate (AIEP)) described above which mimics the adenylated intermediate of the IAA-conjugation reaction (see also Böttcher *et al.*, 2012b). Whilst the grapevine GH3-1 structure is similar in many ways to that reported for the benzoate and JA-conjugating enzymes from Arabidopsis, the active site has unique features related to it using auxin as the acyl substrate. It is the first structure ever developed for an IAA conjugating enzyme. Mutational analysis showed that amino acid specificity can be altered by a single, conservative mutation in the active site. Experimental evidence confirmed that these proteins act as monomers and further details on the reaction mechanism with respect to ATP and magnesium were determined. Modelling of IAA and synthetic auxins into the active site provides an explanation for the relative abilities of these auxins to act as substrates and this selectivity may explain observations regarding the effectiveness of these molecules as auxins in plants. This structure opens the way for a better understanding of the activity of IAA-amido synthetases and their roles in plant development. It will also assist in

the development of more potent inhibitors to characterize and manipulate auxin-related processes and offers the opportunity to develop new auxin-based herbicides and growth regulators.

The resolution of the 3-D structure is a very complicated process as is the detailed analysis of the implications of the structure for the activity and specificity of this enzyme. We therefore, only present a summary of the data here and refer readers who wish for more detail to Peat *et al.* (2012).

### 6.4.1. Crystallization and Structure of Grapevine GH3-1

Crystals of the grapevine GH3-1 protein (Figure 5) were obtained in the presence of a tightly binding inhibitor (AIEP), which mimics the transition state of the auxin-amino acid conjugation reaction (Böttcher *et al.*, 2012b). All attempts to crystallize the uninhibited form of the protein were unsuccessful.

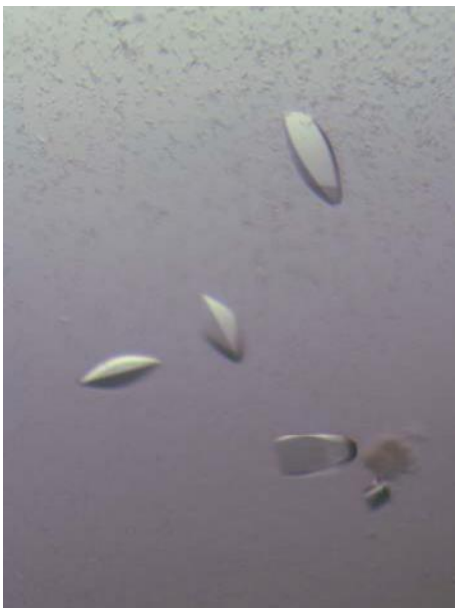


**Figure 5. Micrograph of GH3-1 protein crystals as used for structure determination by X-ray crystallography.**

The protein structure (Figure 6) consists of two domains; a large, well-ordered N-terminal domain of almost 440 residues that includes most of the inhibitor binding site and a smaller C-terminal domain, less well defined by electron density, which includes residues 438- 598.

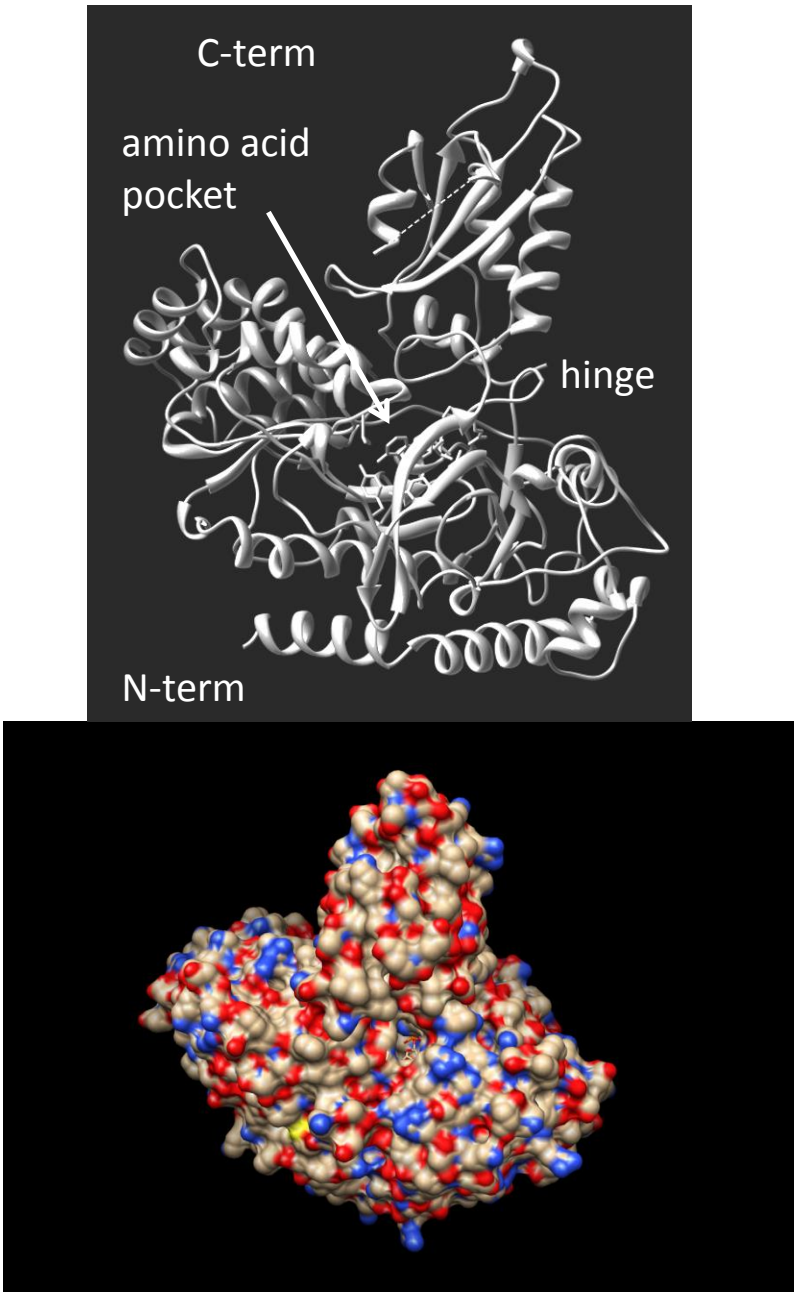



**Figure 6. GH3-1 protein structure**
Upper panel, cartoon diagram of GH3-1 monomer showing the two domains joined by a flexible hinge with the active site indicated by the arrow. Lower panel, surface representation of GH3-1.

The overall fold of the structure is similar to that of the At GH3-11 and At GH3-12 structures reported recently (Westfall *et al.*, 2012), and of the adenylate-forming enzymes in general (reviewed in Gulick, 2009, reviewed in Schmelz and Naismith, 2009). The N-terminal

domain is an α/β fold with two separate β-structures (on either side of the inhibitor/catalytic site) each surrounded by α-helices (Figure 6). The smaller C-terminal domain has a four-strand β-sheet which is sandwiched between four α-helices (Figure 6). Additionally, the C-terminal domain has a small beta loop structure between residues 438-448 and this structure forms a cap over the inhibitor.

### 6.4.2. Quaternary Structure of GH3-1 and Metal Binding in the GH3-1 Reaction

The crystal packing of GH3-1 suggested a possible biologically relevant dimer. To investigate this, Small Angle X-ray Scattering (SAXS) was used to determine the solution state of the protein. SAXS data were measured at varying protein concentrations between 3 and 0.1 mg/mL to determine if there was any concentration dependent oligomerization. The molecular weight and radius of gyration of the species in solution were calculated from the SAXS data at each concentration. These parameters did not vary across the range and were therefore consistent with a monomeric species (see Peat *et al.*, 2012).

This was further validated byback-calculating the SAXS data from both the monomeric and putative dimeric crystal structure forms and comparing with the experimental data. The measured data was found to fit well with the monomeric species (chi 1.101) and poorly with the dimeric form (chi 6.442) (Peat *et al.*, 2012). This result strongly supports a monomeric solution state of GH3-1.

Although magnesium was present in the protein formulations used in crystallization, it was not seen in the resultant electron density maps. This suggested that the metal was only required for stabilization of the binding of ATP, and was not required for the subsequent steps of the conjugation reaction. To test this hypothesis, activity tests with GH3-1 protein (C- and N-terminal tag) and its known substrates IAA and Asp (Böttcher *et al.*, 2010) were performed where magnesium was either, present for the entire reaction (Control), excluded from the entire reaction, or present just for the first part of the reaction. Without the metal during the first half of the reaction, essentially no product was obtained (~780-fold decrease when compared to Control). With metal present during the first half of the reaction only, IAA-Asp conjugate was still produced as each intermediate was turned over, but no subsequent reaction occurred (~40-fold decrease when compared to Control). With metal present during the entire reaction, further turnover events occurred and both proteins produced ~2300 nmol IAA-Asp/mg protein.

### 6.4.3. Substrate Binding Site of GH3-1

There is clear electron density for the substrate mimetic inhibitor in each of the protomers of the crystallographic asymmetric unit (see Peat *et al.*, 2012). Some of the main interactions with the inhibitor (Peat *et al.*, 2012) are with residues Met335-Ser339 (ATP/AMP binding Motif II; Chang *et al.*, 1997). A full description of the binding of the inhibitor and the substrates is given in Peat *et al.* (2012).

The protein was crystallized in the presence of malonate and this molecule is seen in the structure tightly bound to Arg115 which is part of ATP/AMP-binding Motif I. The interactions with malonate are described in detail in Peat *et al.* (2012). The guanidine group of Arg115 makes hydrogen bonds to the bound malonate carboxylate moiety. On the other side of the malonate, the Lys158 amino group is 2.8 to 2.9 Å to the carboxylate. This configuration suggests that an amino acid such as aspartic acid might bind in a similar fashion to the malonate and the Arg115 residue was subsequently mutated to Lys to explore this possibility (see below). The amino acid substrate pocket, as determined by the positions

of the bound malonate molecule and the indole ring of IAA, is defined by residues Arg115, Lys158, Ser108, Pro171, Leu173, and Ser444 (Peat *et al.*, 2012). Arg115 is held in place by Thr106, Val520 and Gln523, with the Gln523 head group within 3.5 Å of the epsilon nitrogen of the Arg115 sidechain.

### 6.4.4. Analyses of Mutated GH3-1 Proteins

As described above Thin-Layer-Chromatography TLC)-based *in vitro* activity assays with a C-terminal His6-tagged version of grapevine GH3-1 indicated a high selectivity for the amino acid substrate with only Asp and Trp being conjugated (see also Böttcher *et al.*, 2010). However, a quantitative spectrophotometric assay performed in this study demonstrated that while Asp and Trp were the preferred amino acid substrates of this enzyme, other amino acids could also be conjugated at a lower efficiency (see Peat *et al.*, 2012 for details). For all amino acids at least a low level of activity was detected but Met, Tyr, Phe, Gly and Glu in particular seemed to be conjugated in addition to Asp and Trp. For the C- and N-terminal tagged versions of GH3-1, the identity of the seven main reaction products was further confirmed using LC-MS (see Supplemental Figures in Peat *et al.*, 2012). Irrespective of the location of the tag, the preferred amino acid substrate for grapevine GH3-1 was found to be Asp (Böttcher *et al.*, 2011a, 2010, Peat *et al.*, 2012), as has been described for many of the auxin-conjugating GH3 enzymes characterized to date (Chen *et al.*, 2009, Fu *et al.*, 2011, Staswick *et al.*, 2005).

The N-terminal-tagged version of GH3-1 was further used for the generation of selected mutants via site-directed mutagenesis. The exchange of Ser108, suggested as essential for the binding of ATP in the first half of the reaction (see above), to Ala led to a mutant protein that showed the same expression and purification behaviour as the wild-type GH3-1 protein (Peat *et al.*, 2012) but showed a general reduction in specific activities in the spectrophotometric conjugation assay and accordingly produced no detectable IAA-conjugates in a TLC-based activity assay (see Supplemental Figures in Peat *et al.*, 2012).

Based on the binding of malonate in the GH3-1 crystals (see above) Arg115 was identified as being potentially involved in the selective binding of the preferred amino acid substrate Asp. An R115K mutant protein was expressed and purified similarly to the wild-type protein (see Peat *et al.*, 2012) and its specific activities determined in the spectrophotometric assay. The specific activity with Asp as the amino acid substrate was reduced by 12-fold whereas activities with the remaining 19 amino acids were in the range of the wild-type GH3-1 enzyme. To further confirm that the binding of Asp was affected in the mutant, a kinetic analysis of IAA-Asp formation by LC-ESI-MS/MS was performed for R115K and compared to wild-type GH3-1 protein. The 14-fold reduction in overall activity of the mutant compared to the wild-type was due to a 17-fold decrease in affinity for Asp, as indicated by Km-values of 323 µM for wild-type GH3-1 and 5543 µM for R115K, resulting in a 175-fold decrease in catalytic efficiency. The wild-type $\kappa_m$-values of 9.89 µM for ATP and 22.04 µM for IAA were in the range of previously published data for GH3-1 (C-terminal His6-tag; Böttcher *et al.*, 2011a, 2012b).

### 6.4.5. Amino Acid Specificities of IAA-Conjugating Grapevine GH3s

Details of the preference for amino acid substrates for GH3-1, GH3-2 and GH3-6 have been published (Böttcher *et al.*, 2011a, 2012b, 2010). This study completes this dataset by providing the amino acid preference for the GH3-3, GH3-4 and GH3-5 enzymes. The highest specific activity for GH3-3 was with Asp followed by Met and Trp. GH3-5 also preferred Asp, but with an 8-fold reduced activity when compared to GH3-3. In contrast, GH3-4 had its

highest specific activity with Glu, followed by Met and Gly. As for GH3-5, the highest specific activity observed for GH3-4 was 8-fold lower than the highest activity for GH3-3. The results of the spectrophotometric assay were confirmed by TLC screens (see Peat *et al.*, 2012). The conjugation of Trp is overestimated by the TLC assays since the indole ring of the amino acid enhances the staining of the reaction product.

Each of the six IAA-conjugating GH3 enzymes from grapevine displayed their highest *in vitro* activity with the acidic amino acids, Asp (GH3-1, GH3-2, GH3-3, GH3-5) or Glu (GH3-4, GH3-6). Interestingly the preference for either Asp or Glu is reflected in the protein sequences. The residue Arg115 in GH3-1 is conserved in the four Asp-conjugating grapevine GH3s, whereas Glu-conjugating GH3s have a Pro in that position. The same pattern can also be found in IAA-conjugating GH3 enzymes with reported amino acid substrate preferences from Arabidopsis (At GH3-2 to At GH3-6, At GH3-17) (Staswick *et al.*, 2005) and rice (Os GH3-8, GH3-13) (Chen *et al.*, 2010, Zhang *et al.*, 2009a). Regardless of their substrate preferences the Ser108 residue in Vv GH3-1 is conserved in all proteins analyzed, confirming the crucial role of this residue in the binding of ATP (see above).

### 6.4.6. Modelling of Three Auxins into the Active Site of GH3-1

It has been demonstrated previously that two grapevine GH3 proteins display a pronounced preference for IAA as the acyl substrate when compared to NAA and BTOA (Böttcher *et al.*, 2011a). To investigate the possible mechanistic reasons for this, IAA, BTOA, and NAA were docked into the active site of GH3-1 using the molecular docking package FRED (McGann, 2011).

In the case of the top binding conformations for these molecules, it was shown that despite binding in a similar fashion to IAA, NAA and BTOA can form competitive or non-productive interactions that may affect adenylation. For NAA, replacing indole with naphthalene eliminates the possibility for a hydrogen bond with Tyr342 (which in the case of IAA, helps keep the auxin in the bottom of the pocket). This difference manifests as a small but significant change in binding affinity that favours IAA over NAA (Peat *et al.*, 2012). Additionally, the added steric bulk of the extra carbon in the naphthalene ring causes the carboxylate group to point slightly upwards toward the purine-binding portion of the pocket. As a result, the most stable conformations of NAA may impinge on the binding of ATP.

It would seem that NAA can bind in a way that will allow adenylation, but the lack of a hydrogen bond acceptor as well as the added steric bulk leads to potentially non-productive binding. BTOA on the other hand binds quite strongly with residues that are necessary for positioning ATP for catalysis. It can therefore be deduced that NAA and BTOA are less preferred as substrates than IAA for GH3-1 and probably other GH3 proteins.

This demonstrates that quite small changes in the structures of auxin molecules can have large effects on their ability to act as substrates for GH3 proteins, as NAA is conjugated with a greatly reduced efficiency while BTOA is virtually not conjugated (Böttcher *et al.*, 2011a). Nevertheless, BTOA and NAA can still act as auxins and therefore must be recognized by auxin receptors which presumably have different structural limitations for binding. Indeed, modelling of IAA, NAA and 2,4-D into the TIR1 receptor binding site has shown that despite their different structures all three bind well due to the promiscuous nature of the PGR-binding site (Tan *et al.*, 2007). The observed order of efficacy as an auxin in grapes, i.e. in increasing order IAA, NAA, BTOA (Böttcher *et al.*, 2011a), may therefore be explained by the

differences in binding of the auxins in the GH3 protein active site which in turn influences their longevity *in planta*.

**6.4.7. A large change in GH3-1 enzyme structural conformation occurs during actalysis**
Comparison of the structure presented here for the grapevine GH3-1 enzyme with that published for the Arabidopsis GH3-12 protein (Westfall *et al.*, 2012) shows that their three-dimensional structures are highly conserved despite amino acid sequence divergence (Peat *et al.*, 2012) and differences in substrate specificities. These proteins have an N-terminal domain (437 amino acids in Vv GH3-1), that contains the bulk of the residues involved in catalysis, and a smaller C-terminal domain (161 amino acids in Vv GH3-1). The C-terminal domain of At GH3-12 undergoes a large change in conformation relative to the N-terminal domain during catalysis with a flexible region between the two domains acting as a hinge about which the two domains twist approximately $180^{o}$ relative to each other (Westfall *et al.*, 2012). Such changes in conformation have been observed in other members of the large family of adenylating enzymes (reviewed in Gulick, 2009, reviewed in Schmelz and Naismith, 2009). Due to the close structural similarities between the grapevine and Arabidopsis proteins, noted above, it can be predicted that the C-terminal domain of the grapevine GH3-1 enzyme also changes in conformation during catalysis.

The large conformational change during catalysis is confirmed by a comparison of the Vv GH3-1 structure to the At GH3-12 structure with an ATP analogue bound (PDB code 4EWV, Westfall *et al.*, 2012). The AMPCPP-bound At GH3-12 is in the 'active site open' form while the inhibitor-bound Vv GH3-1 protein is in the 'active site closed' conformation. The two domains in both proteins are similar in secondary structure, but the C-terminal domain is twisted relative to the N-terminal domain in Vv GH3-1 (Peat *et al.*, 2012). The observed twisting between the two structures shows the considerable movement of the C-terminal domain in relation to the N-terminal domain that occurs during catalysis.

Further evidence for functional divergence within the GH3 family comes from the expression data for the six grape GH3 gene family members that conjugate auxins, which shows variation in the transcription of these genes during berry development (Böttcher *et al.*, 2011a, 2010), in response to various inducers (Böttcher *et al.*, 2011a, 2010), and in different tissues (Böttcher *et al.*, 2011a, 2010). These data indicate that these genes are under a range of transcriptional controls. The reason for this may be the importance of auxins throughout plant development. The role of auxins in many developmental processes, including all those that involve growth, means that a range of systems has evolved to provide great flexibility in the control of auxin effects. Obviously, biosynthesis, transport and signalling are additional key points of control providing even further depth to the regulation of auxin-dependent processes.

## 6.5. Auxin Treatment of Shiraz Berries Post-veraison does not Significantly Alter Berry Weight and Brix

We have demonstrated previously, and in this report, that the application of auxins to grape berries during a one to two week window prior to veraison delays both veraison and harvest. If done earlier the treatments were ineffective. The most likely explanation for this is that the applied auxins are metabolised either, in part, or entirely, by the GH3 family of enzymes, by the time of the 'normal' initiation of ripening and therefore cannot delay ripening. Some data indicates that later treatments are ineffective in delaying ripening suggesting that once commenced ripening cannot be affected by the addition of auxins. It is important to confirm

the window of opportunity available to manipulate ripening and harvest timing using auxins. To test the effect of later applications a triplicate randomised trial was performed using Shiraz grapes at the Hahndorf site. The effect of a Control (wetting agent alone) treatment during the post-veraison period (10/2/2010) was compared to either NAA or BTOA treatments. To monitor berry development, berry weight and Brix were measured at regular intervals after spraying (2, 8, 14, 21 days after treatment). Neither BTOA nor NAA application had a significant effect on berry weight (Figure 7A) or Brix (Figure 7B). This result indicates that auxins cannot delay the ripening phase once it has commenced. Thus, although auxins can delay the onset of ripening the process appears unable to be reversed or even delayed by post-veraison treatments. It may be that some other processes that we have not measured might be affected by post-veraison auxin application, e.g. there might be changes to the amount and activity of proteins encoded by some auxin-responsive genes, but if these changes do occur they do not significantly affect two commonly used measures of berry development.

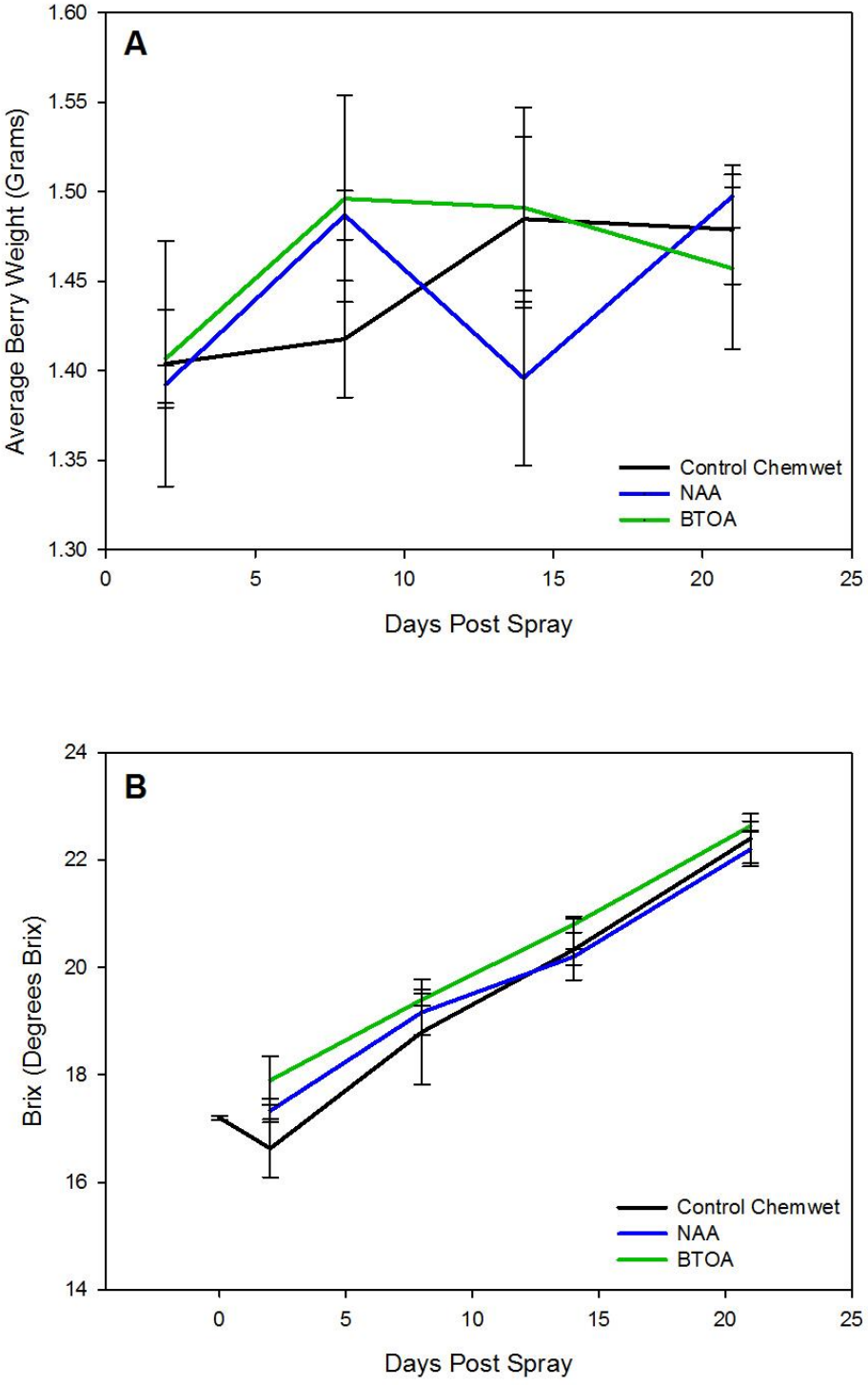



**Figure 7. Post-veraison treatment of Shiraz berries with synthetic auxins.** (A) Average berry weight of Shiraz berries (Hahndorf) treated post-veraison (10/2/2010, Brix 17.2) with either Chemwet alone (Control), NAA or BTOA. Each data point represents the mean of three biological replicates (40-50 berries per replicate), standard errors are given for each data point. (B) Average Brix of berries from Figure 7A.

## 6.6. Treatment of Entire Vines with NAA Delays Ripening as Effectively as Targeted Treatment of Bunches and does not Cause Notable Differences to Vine Growth

In the previously described field trials where auxins such as NAA, when applied during a window of effectiveness pre-veraison, delayed ripening, bunches alone were targeted for treatment rather than the entire vines. The reasons for this were two-fold. Initially we wanted to isolate the effects on bunches alone so that we could test the effects on berries without reference to any effects on the rest of the vine, i.e. to isolate cause and effect to the berry. Second, the trials are not yet at a commercial scale level as a number of different treatment factors are being tested (including such things as different PGRs, different timings, different application concentrations, different varieties etc.). Using small scale equipment is much more efficient to target bunches for spraying. This also has the benefit of reducing the cost of reagents to be used.

However, as the treatments with auxins in particular showed promise for controlling the timing of veraison and harvest it was determined that whole vines should be treated to see if there were any other effects on vine physiology and if such treatments were still effective in delaying ripening. Both of these outcomes are important if such treatments are to be used in a commercial setting.

The data in Figure 8 compares the effect of the treatment of whole vines vs bunches using Brix measurements as an indicator of berry development. Data for both Control (Chemwet alone) and NAA (NAA + Chemwet) treatments are given. It can be seen from this that NAA treatment of either bunches alone or the entire vines (including bunches) was effective in delaying ripening (also see Figure 28). There was no significant difference between the two NAA-treated samples; some small differences were noted between the Control samples. At the final sampling point the NAA-treated samples were 5.3 $^{o}$Brix lower than the mean Control Brix measurements (Control 24.0, NAA 18.7).

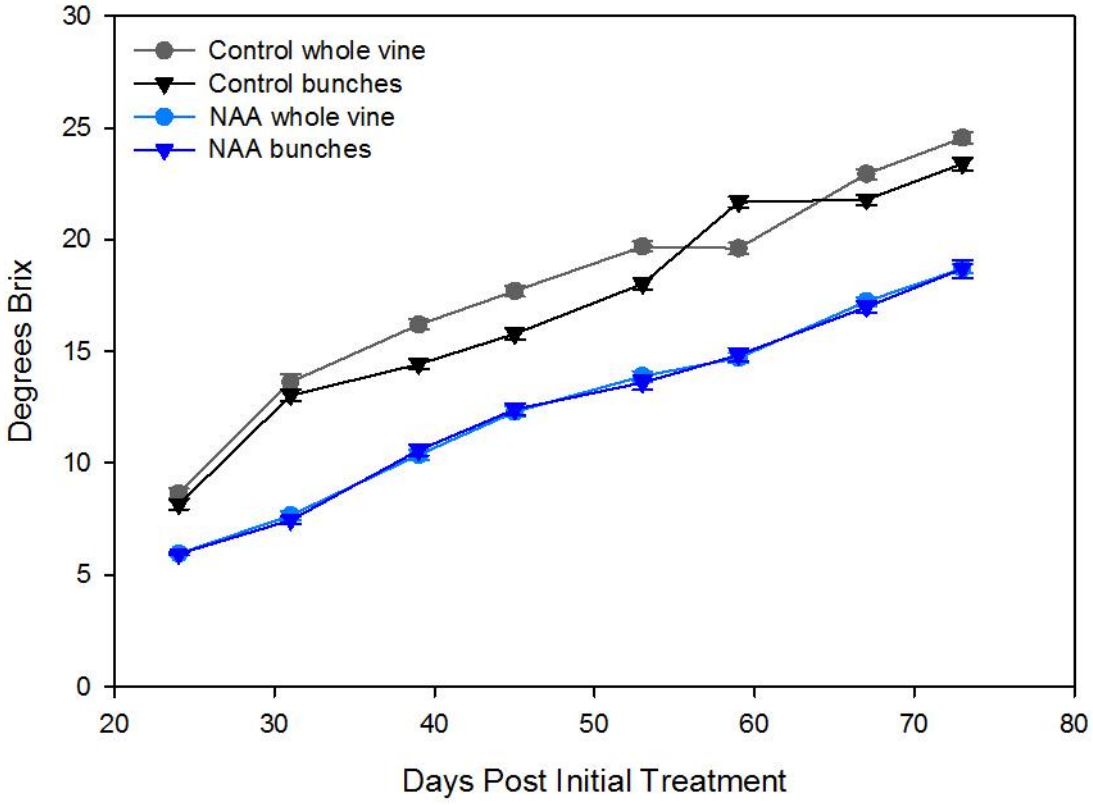



**Figure 8. Comparison of the effects of treating whole Shiraz vines vs grape bunches only with the auxin NAA, Willunga 2010/2011 season.**



**Figure 9. Effect of NAA on Shiraz berry ripening.** Individual bunch on right sprayed with NAA, Control bunch on left. Note: clear delay in ripening as evidenced by reduced colour and berry size. Photo taken 9/2/2011.

## 6.7. Shiraz and Cabernet Sauvignon Berry Developmental Series 2010/2011

During the 2010/2011 season sampling was completed for two berry developmental series from two common red varieties, Shiraz and Cabernet Sauvignon, at the Slate Creek Vineyard, Chalk Hill Wines, Willunga. The purpose of this sampling was to produce two complete series from flowering to berry maturity for a range of future experiments. Two different varieties were chosen to allow for comparisons to be made, as we would expect many aspects of the berry development of these two varieties to be similar, but there are also varietal differences that could provide valuable data regarding grape berry development and its control. Red varieties were chosen as they include a range of secondary metabolites, e.g. anthocyanins, not present in white varieties. The developmental data for these series is given in Figure 10. As might be expected, there are clear differences between the developmental patterns of these two varieties. The berry weight was much lower for Cabernet Sauvignon berries compared to Shiraz. At 16 wpf the fruit of the two varieties were at approximately the same Brix ($23^{o}$) and yet the concentration of anthocyanins (A520nm) was lower in Shiraz. Although the final harvest time and Brix at harvest were different, the Cabernet fruit was harvested later and with a higher Brix, the berries of both varieties had very similar Brix profiles up until 16 wpf when the Shiraz fruit was harvested. These series were collected in triplicate to allow statistical analysis. RNA was produced from all the replicates of both series and cDNA has been made from this RNA.

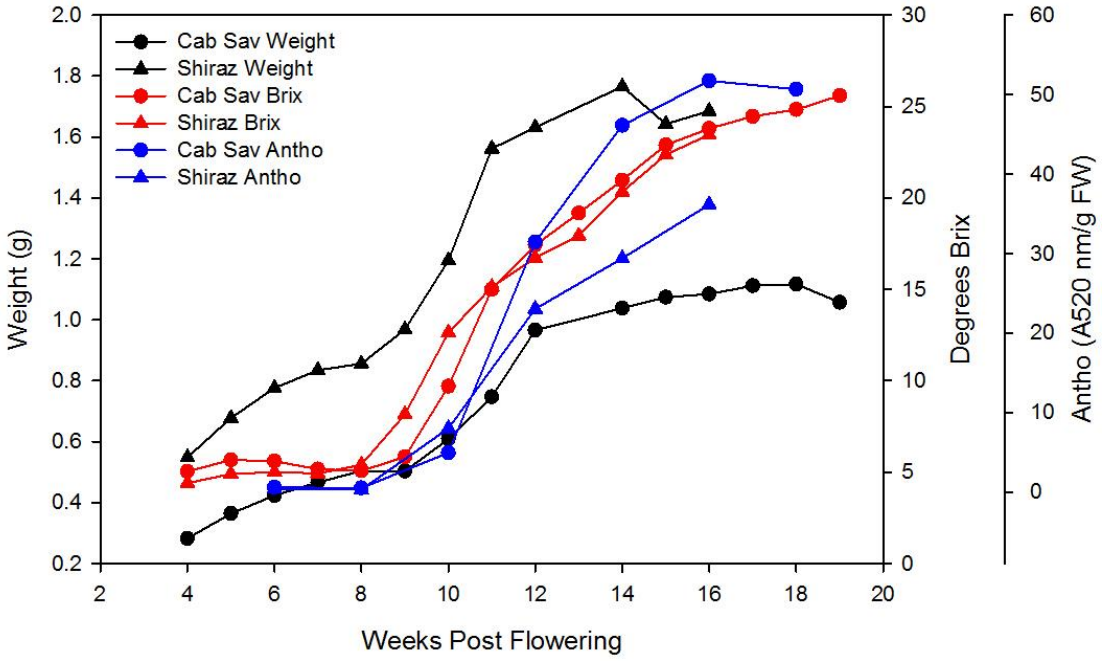



**Figure 10. Developmental data for Shiraz and Cabernet Sauvignon berry series, Willunga 2010-2011.** Each data point represents the mean of three biological replicates (60-200 berries per replicate); standard errors are not shown to aid clarity.

These developmental series have been used in preliminary investigations of gene expression during berry development and appear to be valuable in testing whether genes are

developmentally expressed or not and for determining differences in gene expression between these two cultivars. This work will be reported on in the next project report when it has been completed.

## 6.8. Auxin Delay of Shiraz Berry Ripening by Different Auxins, Willunga, 2010/2011

Although we had shown previously that treatment with an auxin could delay ripening under warm and cooler growing conditions (Davies *et al.*, 1997, Figures 1, 2) a comparison of the relative effectiveness of three auxins, one the endogenous IAA, the other two synthetic auxins, had not been tested under warm conditions. Therefore, an experiment was conducted to compare the effect of these three auxins on berry development when applied pre-veraison.

BTOA-treatment resulted in a delay in the berry weight increase during the earlier part of ripening (Figure 11) but these berries 'caught up' so that at harvest there was no significant difference between the berry weight of Control and BTOA-treated fruit. IAA and NAA had very similar effects on berry weight increase. Both delayed the increase in weight during the early ripening phase, but the delay was not as great as found with BTOA. However, the final weights at harvest were higher than for the Control. As stated above the mean weight of the BTOA-treated berries was the lowest. The cause of the small 'overshoot' in berry weight caused by NAA-, and in this case IAA-treatment, has not yet been determined. Similarly, it is not yet understood why IAA and NAA result in slightly larger berries at harvest while BTOA-treatment either reduces berry size at harvest (Figure 2) or results in no significant difference in berry weight (Figure 11). All three reagents are classified as auxins and biological and gene expression assays seem to confirm that they effect grape berry development in an auxin-related manner (data not shown).

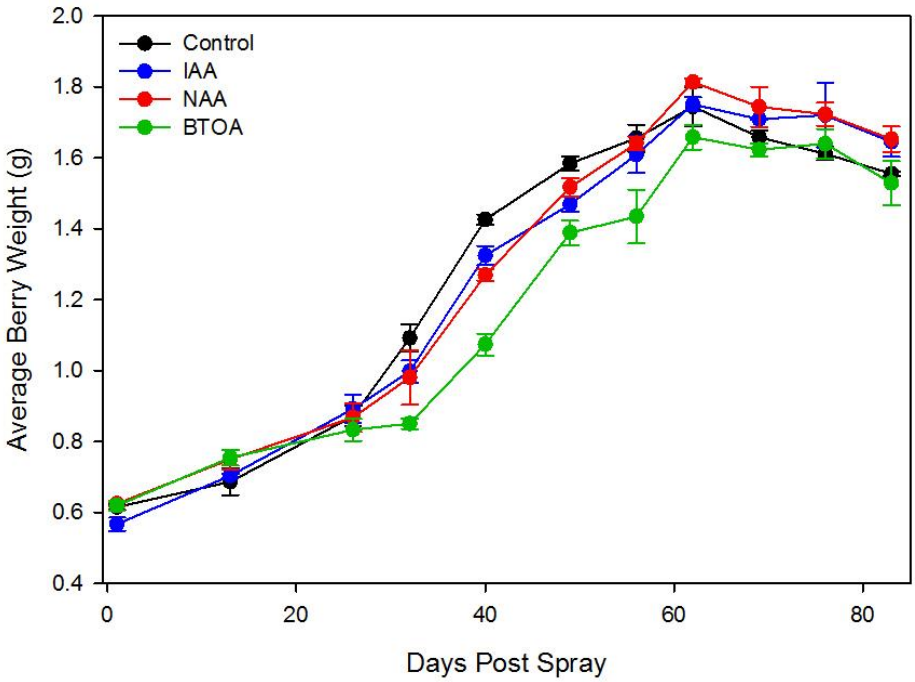



**Figure 11. Average berry weight of fruit grown in a warm climate and treated with different auxins** All data represent mean ± standard error of the mean (n = 3).

The curves for TSS accumulation (Figure 12) indicate that as for berry weight BTOA was the most effective in delaying Brix increase indicating that sugar accumulation was delayed in these fruit compared to the Control. IAA was the least effective in delaying Brix increase while NAA was more effective but not as effective as BTOA. At harvest the differences were fairly small. However, towards the end of the growing season when the air temperatures and the heating effect of incident sunlight are lower sugar accumulation is slowed and so relatively minor differences of only a few degrees Brix can mean one or two weeks of additional hang time if the fruit are to be harvested at the same final Brix level.

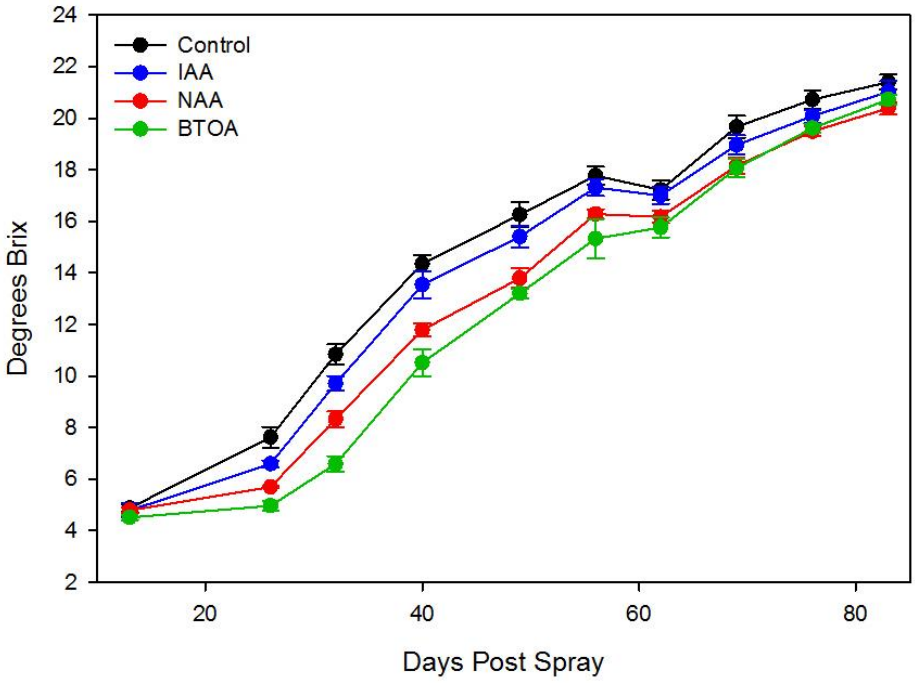



**Figure 12. Average sugar content of fruit grown in a warm climate and treated with different auxins.** All data represent mean ± standard error of the mean (n = 3).

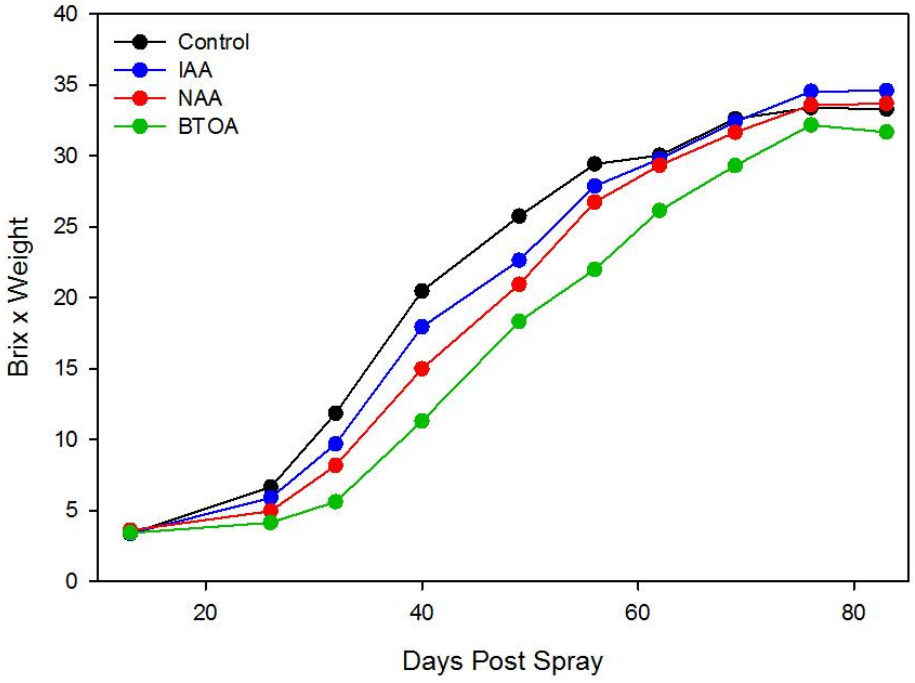



**Figure 13. Average berry weight x average Brix plotted against Days post spray.** All data represent mean ± standard error of the mean (n = 3).

Plotting the data as Brix x Weight vs Time (Figure 13) smooths the curves as it takes into account that there is a relationship between the rate of weight increase and the rate of sugar accumulation. This plot clearly shows that BTOA-treatment was the most effective in reducing weight and sugar concentration increase.

This is the first time that a significant effect of IAA on berry ripening has been observed. Previously IAA-treatments have not significantly delayed berry weight and sugar concentration increases (Figures 1, 2). As shown in other parts of this report IAA is the auxin most effectively inactivated by conjugation by the IAA amido-synthetase enzymes. The relative ineffectiveness of applied IAA in delaying ripening is therefore thought to be due to its rapid inactivation through conjugation whereas the other auxins such as NAA and BTOA, which are poor substrates for these enzymes, are more persistent and much more effective. That IAA delayed ripening on this occasion could be due to a number of reasons. The application may have been made at the exactly right time for effect as we know that the timing of auxin application is critical for effect. Work in other plants has indicated that synthetic auxins such as NAA do not bind the auxin receptors as well as the endogenous auxin IAA. The effectiveness of synthetic auxins such as NAA is more due to their longer persistence as active regulators. It would seem that NAA is not as persistent as BTOA. Relative to the timing of the initiation of ripening in the Control fruit the treatments described were later, i.e. closer to the time of veraison, than in the other experiments we have described. It may be that this was the perfect time for IAA application but that the efficacy of the synthetic auxins BTOA and NAA relies on their presence for a longer time than IAA.

## 6.9. Interactions between Ethylene and Auxin are Crucial to the Control of Grape (*Vitis vinifera* L.) Berry Ripening

For a full presentation of this work please see Böttcher C, Burbidge C, Boss PK, Davies C (2013b) Interactions between ethylene and auxin are crucial to the control of grape (*Vitis vinifera* L.) berry development, *BMC Plant Biology*, 13:(1), 222, doi 10.1186/1471-2229-13-222.

The coordinated development of plants relies on a wide range of control systems, including an intricate network of interactions between all classes of plant PGRs and other signalling compounds such as sugars (Rashotte *et al.*, 2005, Rolland *et al.*, 2002, Teale *et al.*, 2008, Weiss and Ori, 2007, Zhang *et al.*, 2009b). Our knowledge about the molecular interactions of the different classes of PGRs in the ripening process is still rudimentary (reviewed by Böttcher and Davies, 2012, reviewed by Vendrell and Palomer, 1998). An interesting target for the investigation of interplay between PGRs in fruit is the interaction between the gaseous PGR ethylene and the auxins. Ethylene plays a crucial role in the ripening of climacteric fruit which display a sharp increase in ethylene production and respiration rate at the onset of ripening (reviewed by Tucker, 1993). However the relevance of ethylene to the ripening of non-climacteric fruit, which lack the dramatic, ripening-related change in ethylene formation and respiration, is still unclear (reviewed by Böttcher and Davies, 2012). A moderate increase in ethylene concentration and respiration rate has been demonstrated for strawberrys (a non-climacteric fruit) (Iannetta *et al.*, 2006), but both events occurred after the transition into the ripening phase. The only reports of small peaks in ethylene concentration just before the initiation of ripening in non-climacteric fruit come from *Litchi chinensis* Sonn. (Wang *et al.*, 2007) and grape (*Vitis vinifera* L.) (Alleweldt and Koch, 1977, Chervin *et al.*, 2004, Düring

*et al.*, 1978). In grape, a role for ethylene is further evidenced by the developmental expression profiles of genes encoding for the ethylene biosynthesis enzymes 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO). ACS is expressed pre-veraison (Pilati *et al.*, 2007) and there is a peak in ACO transcript accumulation a short time before the initiation of ripening (Chervin *et al.*, 2004, Pilati *et al.*, 2007).

Interestingly, the treatment of pre-veraison grape berries with the ethylene-releasing compound Ethrel has been shown to delay ripening (Böttcher *et al.*, 2013c, Coombe and Hale, 1973, Hale *et al.*, 1970). The Ethrel-induced delay resembles the ripening-delaying effects caused by the pre-veraison application of auxins (Ban *et al.*, 2003, Böttcher *et al.*, 2012a, reviewed by Böttcher and Davies, 2012, Böttcher *et al.*, 2011b, Davies *et al.*, 1997, Hale, 1968). In climacteric fruit, such as tomato (*Solanum lycopersicum* Mill.) (Buta and Spaulding, 1994, Mapelli *et al.*, 1978), or banana (*Musa paradisiaca* L.) (Purgatto *et al.*, 2002) and in non-climacteric fruit like strawberry (Archbold and Dennis Jr, 1984, Symons *et al.*, 2012) and grape (Böttcher *et al.*, 2010, Cawthon and Morris, 1982, Inaba *et al.*, 1976, Zhang *et al.*, 2003) the concentrations of IAA before and during ripening were generally found to be low. The application of natural or synthetic auxins during the pre-ripening stage of fruit development has often been found to lead to a ripening delay (reviewed by Böttcher and Davies, 2012) and therefore auxins are widely viewed as ripening inhibitors (Böttcher *et al.*, 2010, Buta and Spaulding, 1994, Frenkel, 1972, Purgatto *et al.*, 2002). More detailed studies in grape berries have revealed that the concentration of IAA-Asp, an IAA-amide conjugate linked to IAA degradation (Östin *et al.*, 1998) and formed by the action of IAA-amido synthetases (GH3 proteins) (Staswick *et al.*, 2005), increased sharply at veraison and remained at high concentrations throughout the ripening phase (Böttcher *et al.*, 2010). A similar pattern of IAA-Asp accumulation has been found in tomato which suggests a more complex role for auxins in fruit ripening that requires further investigation (Böttcher *et al.*, 2010).

Morgan and Hall (1962) described the induction of ethylene biosynthesis by applied auxins upon 2,4-dichlorophenoxyacetic acid application to cotton plants cotton plants. Exogenous auxins induce the transcription of *ACS* genes in mung beans (*Vigna radiata* (L.) Wilczek) (Yoshii and Imaseki, 1982). A similar induction of *ACS* expression and in some instances also *ACO* expression has since been demonstrated for many other plant species, including *Arabidopsis* (*Arabidopsis thaliana* L.) (Abel *et al.*, 1995, Raghavan *et al.*, 2006, Tsuchisaka and Theologis, 2004), a range of climacteric and non-climacteric fruit such as apple (*Malus domestica* Borkh.) (Li and Yuan, 2008), pear (*Pyrus communis* L.) (Kondo *et al.*, 2006), peach (*Prunus persica* (L.) Batsch) (Trainotti *et al.*, 2007) and grape (Ziliotto *et al.*, 2012). The acceleration of the ripening process by auxin treatments in some climacteric fruit (Agustí *et al.*, 2003, 2004, Kondo *et al.*, 2004, Ohmiya, 2000, Yuan and Carbaugh, 2007) can therefore be interpreted as an indirect auxin effect mediated through the induction of ethylene biosynthesis (Kondo *et al.*, 2004, Li and Yuan, 2008, Trainotti *et al.*, 2007).

Conversely a dependence of ethylene action on IAA biosynthesis, perception, signalling and transport has been illustrated in studies using *Arabidopsis* mutants (Růžička *et al.*, 2007, Swarup *et al.*, 2007). However, only the recent elucidation of the main IAA biosynthesis pathway in *Arabidopsis* has revealed the full extent of the regulation of auxin biosynthesis by ethylene and the dependence of ethylene effects on auxin production. Three studies (Mashiguchi *et al.*, 2011, Stepanova *et al.*, 2011, Won *et al.*, 2011) have provided evidence that IAA is synthesised via a two-step pathway in which the family of TRYPTOPHAN

AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAA1/TAR) enzymes (Stepanova *et al.*, 2008, Tao *et al.*, 2008) converts tryptophan to indole-3-pyruvate which is then converted to IAA by the YUCCA (YUC) family of flavin-containing monooxygenases (Zhao *et al.*, 2001). The expression of *TAA1* as well as some *TAR*s in roots and apical hooks of *Arabidopsis* seedlings can be induced by ethylene treatments and the restricted expression of *TAA1/TAR* genes leading to local IAA production is thought to be the basis of tissue-specific ethylene effects (Stepanova *et al.*, 2008).

In summary, auxins and ethylene are involved in berry ripening andtheir application can alter the progression of ripening. Evidence is presented below 1) for a functional TAR/YUC pathway of auxin biosynthesis in developing grape berries which is at least in part regulated by ethylene, 2) supporting the idea that the Ethrel-induced ripening delay in grapes might be mediated by increased auxin concentrations in pre-ripening fruit, 3) for a developmentally controlled increase in the expression of ethylene biosynthesis genes that precedes elevated transcript levels of auxin biosynthesis and conjugation genes at around the initiation of ripening, indicative of a role for ethylene/auxin interactions in the control of berry ripening.

### 6.9.1. Pre-veraison Ethrel Treatment of Grape Berries Leads to Ripening Delay and Increased Auxin Concentrations

Grape berry veraison can be delayed by the pre-veraison application of the ethylene-releasing compound Ethrel (Böttcher *et al.*, 2013c, Coombe and Hale, 1973, Hale *et al.*, 1970) and auxins (Ban *et al.*, 2003, Böttcher *et al.*, 2011a, 2012a, 2011b, Davies *et al.*, 1997, Hale, 1968). The similar effects of Ethrel and auxins on the ripening behaviour of grapes combined with the increasing evidence for complex interactions between ethylene and IAA (reviewed by Muday *et al.*, 2012, and by Stepanova and Alonso, 2009) is suggestive of a link between these two plant PGRs in the control of ripening. In order to investigate the possibility of an ethylene/auxin interaction in grape berry ripening, pre-veraison Shiraz berries were treated with either Ethrel or a Control solution in two consecutive seasons. In the 2011 trial, the Ethrel treatment of berries 20 days pre-veraison delayed the onset of ripening by 13 days (veraison of Control berries was 3 February 2011). The Ethrel-treated berries had significantly lower TSS values between 31 and 54 days post spray (dps) and at 67 dps when compared with the Control berries (Figure 38A) and there was also a reduction in berry weight between 37 and 54 dps (see Böttcher *et al.*, 2013c for details).

In 2012, veraison of the Control berries (24 January 2012) occurred one week earlier than predicted, which made the two successive treatments that were applied during that season close to the veraison date (eight and one day prior to veraison). In previous studies, Ethrel-treatments within a week of veraison had no effect on sugar accumulation and berry weight (Böttcher *et al.*, 2013c). Accordingly, the ripening-delaying effect of Ethrel in the 2012 trial was less pronounced than what had been observed in 2011. Veraison of the Ethrel-treated berries was delayed by seven days only. However, from 15 days post initial spray (dpis) onwards the TSS values in the Ethrel-treated berries were significantly lower when compared with the Control berries and at 22 and 29 dpis the Ethrel-treated berries were significantly lighter (see Böttcher *et al.*, 2013b for details).

A time course of berry samples from both trials was used to determine if the Ethrel treatments had caused changes in IAA and IAA-Asp concentrations. For the 2011 experiment this was done 3–48 hours post spray (hps), which was extended for the 2012 trial to test for

differences in auxin concentrations from one hour post initial spray (hpis) through to the time of veraison of the Ethrel-treated berries.

When compared with the Control fruit at each time point, IAA and IAA-Asp concentrations in the 2011 experiment were only significantly different from the Ethrel-treated berries at the last time point analysed (48 hps), when the IAA concentration was approximately ten-fold greater and the IAA-Asp concentration approximately two-fold greater in response to Ethrel. In the 2012 samples the IAA concentration was significantly higher in the Ethrel-treated berries when the Control berries were at veraison (~ three-fold) and IAA-Asp concentrations were increased by Ethrel about two-fold 24 hpis and at veraison of the Control berries (see Böttcher *et al.*, 2013c for details).

The presented data provide evidence for an Ethrel-mediated increase in the accumulation of free and conjugated IAA in pre-veraison grape berries. The lasting elevation of auxin concentrations up to the transition into the ripening phase suggests that the Ethrel-induced ripening delay might be the result of higher than normal IAAconcentrations in pre-veraison berries.

### 6.9.2. IAA and IAA-Asp Levels and Grape Homologues of Auxin Biosynthesis Genes are Upregulated in Berries by Ethrel while Auxin Levels are Repressed by Aminoethoxyvinylglycine

Together with YUC proteins TAA1/TARs are a component of the only complete pathway of auxin biosynthesis in plants described to date (Mashiguchi *et al.*, 2011, Stepanova *et al.*, 2011, Won *et al.*, 2011). In order to investigate if the increase in auxin concentrations in Ethrel-treated grape berries might be due to a stimulation of the TAR/YUC IAA biosynthesis pathway, grapevine homologues of the *Arabidopsis* TAA1 and YUC1 proteins were identified through a BLASTP similarity search. The resulting nine TAR and ten YUC grapevine sequences were aligned to the respective protein families from *Arabidopsis* and unrooted phylogenetic trees were constructed from the alignments (see Böttcher *et al.*, 2013c for details). Any further analyses were restricted to those members of the grapevine families that were verified as being expressed in any tissues by the presence of ESTs in the NCBI database. These were VvTAR1, VvTAR2, VvTAR3 and VvTAR4 (see Böttcher *et al.*, 2013c for details), as well as VvYUC1, VvYUC2 and VvYUC3.

The expression of the putative auxin biosynthesis genes from the *TAR* and *YUC* families, as well as the previously described grapevine *GH3* genes (*GH3-1* and *GH3-2*) involved in IAA-Asp formation in berries was analysed in the same samples that had been used for the quantitation of IAA and IAA-Asp. To verify the activation of an ethylene response in the Ethrel-treated berries, the expression of the ethylene biosynthesis genes *ACS1* and *ACO1* and the expression of the ethylene receptor gene *ETR2* was also analysed. *ETR2* is commonly used as a marker for ethylene responses that has been employed in research on vegetative tissues (Binder *et al.*, 2004) as well as fruit (Ireland *et al.*, 2013).

In the 2011 experiment, a strong response to Ethrel was demonstrated by the induction of *ETR2* expression, by up to 20-fold, 3–48 h after the Ethrel spray (see Böttcher *et al.*, 2013c for details). The expression of *ACS1* (9 and 24 hps) and *ACO1* (9–48 hps) was also upregulated (two to 13-fold). *YUC3* expression was not detectable in any of the samples. The transcription of *TAR2* was increased up to 30-fold in Ethrel-treated berries between 3–24 hps. There was also an induction (two to three-fold) of *TAR4* at 24 hps and 48 hps, whereas the expression of *TAR1* and *TAR3* was not significantly different between the Control and Ethrel

samples at all time points (see Böttcher *et al.*, 2013c for details). As for *TAR1* and *TAR3* there was no significant difference in the expression of *YUC1* between the Ethrel-treated and the Control berries. Similar to *TAR4*, *GH3-1* expression was significantly upregulated by the Ethrel treatment after 24 h and 48 h (up to 4.5-fold). The transcription of *GH3-2* was also induced at 3 hps (five-fold) and at 9–48 hps (up to four-fold). The increased *GH3* expression as well as increased *TAR4* expression correlated with the higher IAA-Asp and IAA concentrations in Ethrel-treated berries 48 hps. The highly elevated expression of *TAR2* preceded the increase in free IAA and conjugate concentrations after 48 h.

In the 2012 experiment, where only a minor delay in ripening of the Ethrel-treated berries was observed, the expression analysis of *ACS1*, *ACO1* and *ETR2* also reflected a weak response to Ethrel (see Böttcher *et al.*, 2013c for details). All three genes were found to have a significant increase in transcription at only one time point. Accordingly, the changes in expression of the IAA biosynthesis and conjugation genes were also less pronounced than in the previous season.

In a recent study on the effects of ethylene on grape berry ripening, it was found that aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, had a ripening-advancing effect on berries when applied between three to one weeks prior to veraison (Böttcher *et al.*, 2013c). AVG has long been known to be a competitive inhibitor of ACS proteins (Boller *et al.*, 1979, Yamagami *et al.*, 2003), thereby blocking the conversion of S-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (Adams and Yang, 1979). AVG has also recently been described as an inhibitor of auxin biosynthesis in *Arabidopsis* with the TAA1/TAR family as its target (Soeno *et al.*, 2010). Given this data on AVG action its effect on auxin production in pre-veraison Shiraz berries was investigated in an *ex planta* experiment. Berries were exposed to AVG, Ethrel and a Control treatment for 0.5-24 h followed by the extraction and quantitation of IAA and IAA-Asp. Even at the first time point of analysis (0.5 h after the initiation of treatment) a two-fold increase in the concentration of IAA was found in the Ethrel-treated berries, whereas a three-fold reduction in the concentration of IAA was measured in AVG-treated fruit (see Böttcher *et al.*, 2013c for details). The Ethrel-induced increase in IAA accumulation was transient and only lasted for 1 h after which the IAA concentration returned to levels observed in the Control. However, AVG caused a significant reduction in IAA concentrations after 6–24 h of exposure. A similar, but delayed, trend was observed for changes in the IAA-Asp concentrations (see Böttcher *et al.*, 2013c for details). Exposure to Ethrel resulted in a significant increase in IAA-Asp accumulation after 1 h and 3 h, whereas the treatment with AVG led to a reduced IAA-Asp concentration after 6 h (two-fold). The rapid changes in auxin concentrations in response to Ethrel and AVG in the *ex planta* experiment when compared with the data obtained from berries that were sprayed in the field (see above) is most likely due to a higher uptake rate of the compounds under the *ex planta* conditions and to continuous exposure to high levels. It can therefore be inferred that changes in auxin biosynthesis and conjugation occurred within the first 30 min of exposure, which explains the unchanged expression of the *TAR*, *YUC* and *GH3* genes analysed between 0.5 and 24 h of exposure. There was also no difference in the expression of *ACS1* and *ACO1* between Control, Ethrel-treated and AVG-treated berries. The only significant change in *ETR2* expression occurred at 3 h after Ethrel exposure (two-fold increase).

It can be concluded that the TAR/YUC pathway of auxin biosynthesis is active in grape berries and can be stimulated by increased ethylene levels. As a consequence the observed ripening-delay by Ethrel may be mediated by elevated auxin formation in the berry tissue.

### 6.9.3. Changes in the Expression of Auxin and Ethylene Biosynthesis Genes During Development Suggest a Role for both PGRs in the Initiation of Grape Berry Ripening

Previous studies on grape berries as well as other non-climacteric fruit have reported low concentrations of free IAA and ethylene at the time of ripening initiation (reviewed by Böttcher and Davies, 2012). However, subtle increases in ethylene concentrations coinciding with the beginning of the ripening phase have been reported in *L. chinensis* and grapes (Alleweldt and Koch, 1977, Chervin *et al.*, 2004, Düring *et al.*, 1978, Wang *et al.*, 2007), as well as a large increase in the accumulation of IAA-Asp in ripening grapes and tomatoes (Böttcher *et al.*, 2010), suggest that both PGRs might be involved in the transition from the pre-ripening to the ripening stage. To further investigate this, the expression of *TAR1-4*, *YUC1*, *YUC3* as well as *ACS1* and *ACO1* was analysed in Shiraz berries from 3wpf through to harvest at 16 wpf. Veraison was at 8 wpf. *TAR1* was expressed at low levels in pre-veraison berries, but copy numbers increased rapidly between 10–12 wpf and remained high through to harvest after 16 weeks (see Böttcher *et al.*, 2013c for details). *TAR3* was expressed in pre-veraison berries, whereas the expression profiles of *TAR2* and *TAR4* were characterized by a significant peak in transcript levels at veraison (*TAR4*) or one week post-veraison (*TAR2*). A comparable peak in expression was also observed for *YUC1* (8–10 wpf), whereas *YUC3* was found to be expressed at very low levels in young berries (one and two wpf), but *YUC3* transcripts could not be reliably detected in berries 3–16 wpf. In grape berries, the increase in expression of the putative auxin-biosynthesis genes was preceded by high expression levels of the ethylene biosynthesis genes *ACS1* (3–7 wpf) and *ACO1* (peaking at 7 wpf) which confirmed previous results for the expression of *ACS/ACO* in developing grape berries (Böttcher *et al.*, 2013c, Chervin *et al.*, 2004, Pilati *et al.*, 2007). Similar expression patterns of all eight genes were recorded from a 3–16 wpf berry series from Cabernet Sauvignon (see Böttcher *et al.*, 2013c for details).

The data suggest that a peak in auxin biosynthesis coincides with the initiation of ripening in grape berries and that this elevated auxin production might be induced by ethylene. The post-veraison expression of *TAR1* further indicates that IAA is synthesized throughout the ripening period. An increase in IAA biosynthesis at and after veraison seems to contradict the low concentrations of this auxin in ripening berries as well as the ripening-delaying effects of high levels of auxins in pre-veraison berries. However, the sharp increase in IAA-Asp accumulation just after veraison reported by Böttcher *et al.* (2010) might indicate that all IAA produced during a short period around the start of ripening and possibly throughout ripening is sequestered via the conjugation pathway. This proposal is supported by the increased expression of *GH3-1* during this time in berry development. The long-held view that IAA-amino acid conjugates are merely means to store or degrade the free auxin (Bajguz and Piotrowska, 2009) has been challenged by a number of studies that provide evidence for an active role of IAA-Asp and other conjugates in plant development and disease response (reviewed by Korasick *et al.*, 2013). Therefore the IAA-Asp conjugate might represent a ripening signal in grapes, and possibly also in other fruit species. From the presented data, a possible role of ethylene in the ripening of grape berries could be the triggering of IAA synthesis and subsequent IAA-Asp formation at the above mentioned critical developmental stage. This hypothesis is supported by studies from Chervin *et al.* (2004, 2006), where blocking of ethylene perception close to veraison led to reduced berry size and delayed anthocyanin and sugar accumulation.

In summary, the data presented above provides evidence of a functional two-step pathway of auxin biosynthesis in grape berries and demonstrates its activation by the ethylene-releasing compound Ethrel. An observed ripening delay in Ethrel-treated fruit might therefore be due to

increased auxin concentrations in pre-ripening fruit. The expression patterns of auxin and ethylene biosynthesis genes during berry development further suggest that an increase in ethylene production prior to the initiation of ripening stimulates IAA biosynthesis at the commencement of ripening. This developmentally controlled induction of auxin biosynthesis by ethylene might be required for the rapid accumulation of the proposed ripening factor, IAA-Asp.

This data offers an explanation for previous observations (Hale *et al.*, 1970) that ethylene has a biphasic affect on berry ripening depending on the timing of application and shows that the choice of PGR and the timing of application is crucial when attempting to manipulate ripening. Again this indicates the complex set of interactions that can occur between PGR signalling systems.

## 6.10. Treatment of Shiraz with Various PGRs to Test their Effect on Ripening: 2012/2013 Season

An experiment was conducted during the 2012/2013 season to test the effect of a range of PGRs on Shiraz berry development. Three different treatments were undertaken, i.e. Control (wetting agent only), Ethrel, AVG. As described above the treatment of grape berries with Ethrel (which releases ethylene in treated berries) at certain stages pre-veraison delays the onset of ripening. It was decided to confirm this observation with a larger scale trial that would allow us to make wine at a scale suitable for both sensory and biochemical analysis. In addition, the role of ethylene was to be further tested by applying AVG to a subset of fruit. One expected effect of the application of AVG would be to reduce ethylene levels (as it inhibits the biosynthesis of ethylene) thereby advancing ripening.

The treatments were conducted as described in the Experiment Specific Method. The data for Brix is presented in Figure 47. It can be seen that the Ethrel treatment did not delay ripening as much as expected. This may have been due to a mistiming of its application, i.e. it may have been applied too close to veraison. However, it may be due to the condition of the vines as this experiment had to be abandoned part way through the growing season (49 days after the initial treatment) due to problems with the irrigation system that severely affected vine growth and berry development. The Ethrel treatment experiment described here was repeated during the 2013/2014 season (see below). Interestingly, the AVG treatment, which we think to be best applied after Ethrel and nearer to veraison, did significantly advance ripening as predicted (Figure 14). This reinforces our previous data indicating that AVG could be a valuable tool for advancing ripening and harvest as it seems effective and is a relatively inexpensive reagent used in some other horticultural industries.

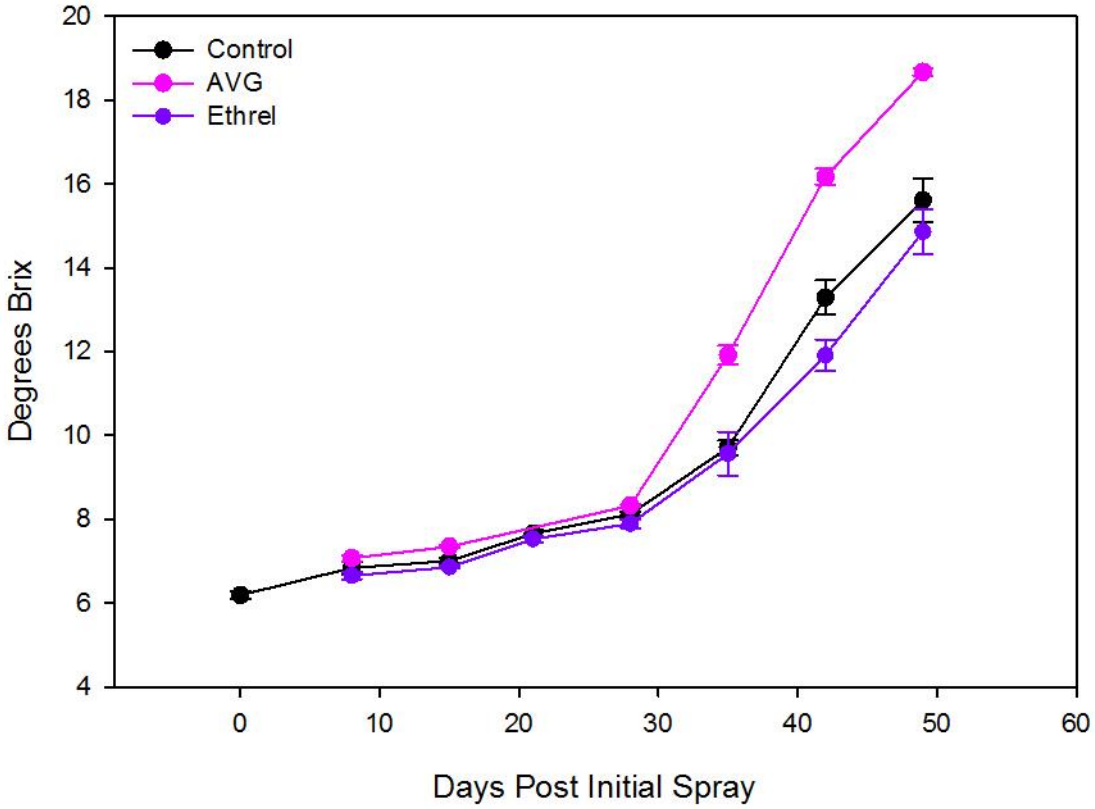



**Figure 14. Average berry weight of Shiraz berries (Blewitt Springs) treated pre-veraison with either Chemwet alone (Control), AVG or Ethrel.** Each data point represents the mean of three biological replicates, standard errors are given for each data point.

## 6.11. Shiraz Transgenic Vines Over-expressing either *VvGH3-1* or *Atetr1-1*

We have shown in the above studies that auxins appear to be pivotal PGRs in the control of the onset of grape berry ripening. The application of auxins before veraison can delay ripening and the endogenous auxin levels, which are high in early berry development, are reduced before ripening commences. Both of these observations are consistent with a role for auxins as inhibitors of ripening. Conversely, the removal of auxins from the berry earlier than normal should release the inhibition they exert over the commencement of ripening and therefore make berries ripen earlier. One convincing way of definitively establishing that auxins are inhibitors of berry ripening is to lower auxin concentrations in the berry prior to the normal timing of auxin decrease. Removing auxins to cause a decrease in auxin concentration is technically difficult. However, the reduction in auxin levels provides much better evidence for the action of a PGR than does its overproduction or application. As has been shown above for the auxin, IAA, targeted inhibitors of particular PGR metabolising enzymes can be effective tools in defining and controlling their actions. However, there can be issues with the penetration into tissues, longevity *in vivo* and specificity of inhibitors. Transgenic plants offer a targeted way to better understand the role of particular genes. For auxins one way to do this is to make use of our knowledge of the enzymes we have discovered that reduce the pool of active auxins through inactivation by conjugation to amino acids. The enzymes that do this are the family of IAA-amido synthetases also known as GH3 proteins.

By over-expressing a GH3 enzyme in grapevine we should be able to test the role of IAA in the control of ripening, the expectation is that the berries should ripen earlier. It would also demonstrate the effect of reduced auxin levels on vegetative growth. One might expect reduced vine vigour which would allow the testing of the potential of vines with greatly reduced vegetative vigour.

Shiraz grapevines were transformed with a construct containing the *VvGH3-1* gene driven by the CMV35S promoter. This promoter is designed to over-express the transgene in virtually all the plant tissues. A number of transformed lines were developed and grown in the glasshouse under PC2 conditions. The presence of the transgene was tested by PCR analysis. These vines showed a range of vegetative phenotypes (see below), but have not as yet flowered to allow the effect of reduced auxin levels on ripening to be determined. These plants continue to be grown and flowering and fruit production should occur in the near future.

Seven different transgenic lines were developed and 3-5 replicates of each transformant were produced. All of the transformed lines expressed the *VvGH3-1* transgene. A range of levels of transgene expression was detected by qRT-PCR using a primer set designed to be specific for the transgene rather than the endogenous gene (Fig.15). The small amount of expression detected in the controls may be due to a very low level of cross-hybridisation with the endogenous gene. There were a range of expression levels in the different lines. Some lines such as G1 expressed the transgene at relatively low levels, others were at much higher levels, e.g. G2.

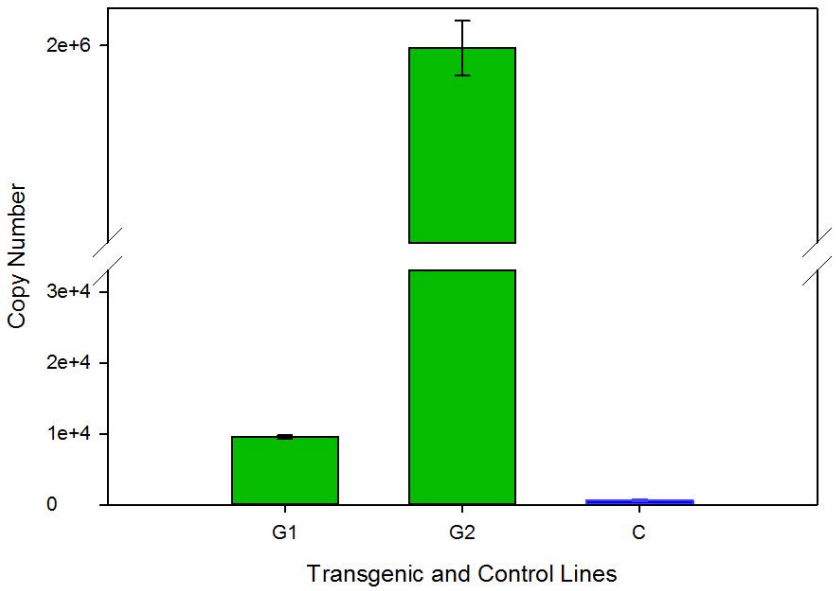



**Figure 15. Expression of the *VvGH3-1* transgene in individual lines.** Transgene expression was detected by qRT-PCR using a primer set designed to be specific for the transgene. C = control, G1, G2 = different over-expressing lines. All data represent mean ± standard error of the mean (n = 3).

The expression of a range of endogenous genes was determined but more work needs to be done to test the full implications of *GH3-1* overexpression on grapevine metabolism and development.

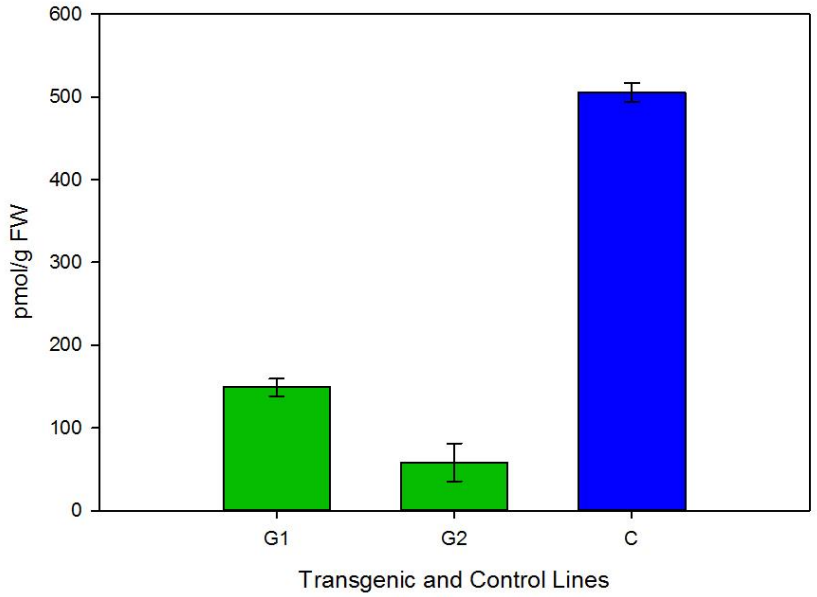



**Figure 16. Level of IAA in individual transformed GH3-1 lines.** C = control, G1, G2 = different over-expressing lines. All data represent mean ± standard error of the mean (n = 3).

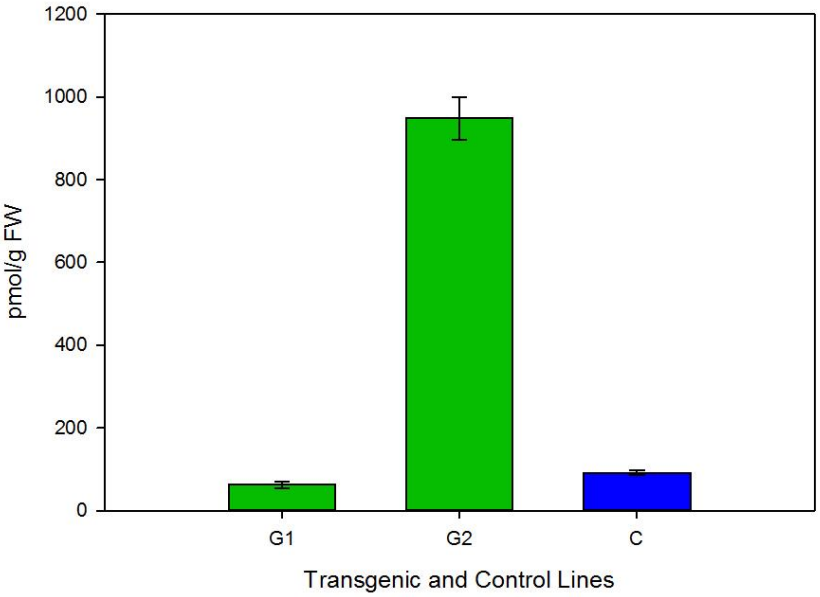



**Figure 17. Level of IAA-Asp conjugate in individual transformed GH3-1 lines.** C = control, G1, G2 = different over-expressing lines. All data represent mean ± standard error of the mean (n = 3).

The concentrations of IAA and its main conjugated form (IAA-Asp) were measured in the transgenic and Control lines using the LC-MS-based method described in the Method section (5.2.2.7). The concentration of IAA was reduced in over-expressing lines. This appears to be due to the action of GH3 activity as the levels of IAA-Asp were higher in the lines over-expressing the transgene (Figure 17). The line expressing the transgene more highly, G2 (Figure 15), had the lowest free IAA concentration (Figure 16) and the highest IAA-Asp concentration (Figure 17).

The effects on vegetative growth were quite interesting. The overall, readily visualised, effect is on organ size. This is what could be expected in plants with reduced active IAA concentration. Auxins are positive regulators of plant growth and the symptoms displayed by the transgenic plants are similar to the effects found in other low-auxin plants (Kant *et al.*, 2009). Careful analysis of various vegetative, morphological characters quantified a number of specific changes (data not shown).

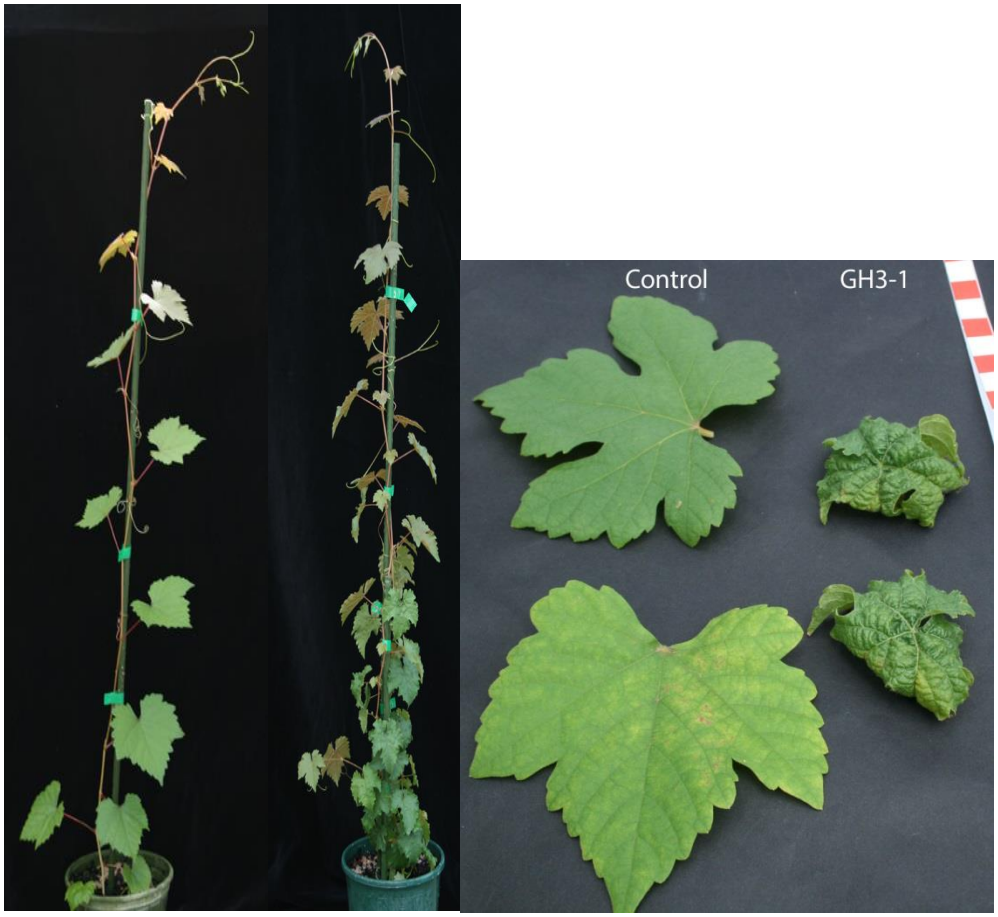



**Figure 18. A) Young transgenic *Atetr1-1* (left) and *VvGH3-1* (right) over-expressing plants.** B) Mature leaves from Control (left) and *VvGH3-1* over-expressing (right) lines.

These studies are further proof of the important role that auxins play during grapevine development. Reduced auxin concentration reduces vine growth by affecting stem and leaf structure. It also reinforces the important role that GH3 enzymes play in controlling vine growth. The most important results are yet to come due to the time it takes to get transgenic plants to flower in the glasshouse but we anticipate that we will have flowers and fruit in the near future. It will then be possible to further confirm the role played by auxins in controlling grape berry ripening.

The role of another, gaseous, PGR called ethylene, in the control of grape berry ripening is controversial. As discussed earlier in this report ethylene is pivotal in the control of the ripening of climacteric fruit such as apples and tomatoes but its role in grape berry ripening is unclear. Grapes are considered to be non-climacteric fruit and the role of ethylene in this class of fruit has not yet been accurately defined (Davies and Böttcher, 2009b).Treatment of berries with Ethrel, a compound that releases ethylene when inside plant cells, is used to improve the colour of table grapes but an extensive review of many studies (Szyjewicz *et al.*, 1984) shows that it may also accelerate sugar accumulation. In a previous section of this report we have produced evidence that Ethrel treatment early in berry development can delay ripening. This effect appears to be due to the induction of IAA accumulation by ethylene. The increased IAA levels delay the onset of veraison (see previous sections).

To investigate the role of ethylene in berry and vegetative growth in grapevine a second set of transgenics were produced in which the *Atetr1-1* gene from *Arabidopsis* was overexpressed. The *Atetr1-1* gene encodes a protein that acts as a receptor for the PGR ethylene. Receptors are the proteins that specifically bind and recognise particular PGRs and then initiate a signalling cascade that alters cellular processes. When the *Atetr1-1* version of the *ETR1* gene, which contains a mutation, is over-expressed in plants it makes them insensitive to ethylene (Bleecker *et al.*, 1988). In tomatoes this results in non-ripening fruit. As detailed above the role of ethylene during grape berry development is still the subject of debate. This experiment should give us important insights into the role of ethylene in both vegetative and reproductive growth.

Plant lines transgenic for the *Atetr1-1* construct (as determined by PCR analysis) were produced and analysed. The effects of this transgene on vegetative growth are limited. However, more work needs to be done to investigate the level of ethylene insensitivity produced and other effects on flowering and berry development.

These lines were tested for the expression of the transgene, which is quite easy in this case as the transgene is distinct in sequence to the endogenous *VvETR1* gene. All seven of the lines tested expressed the transgene and the expression levels varied widely (Figure 19). The variance in expression level could be due to differences in copy number between the lines and/or positional effects of the insertion of the transgene into the grape genome.

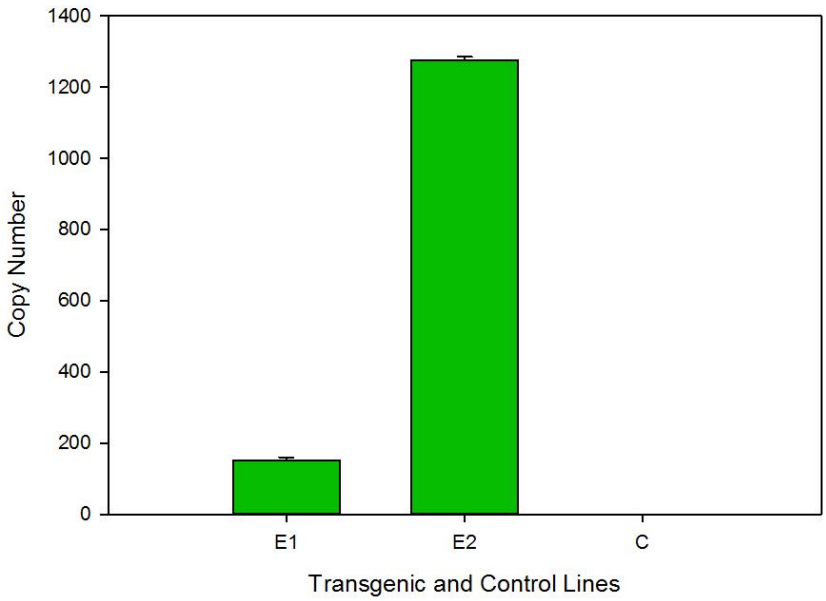



**Figure 19. Expression level of *Atetr1-1* in overexpressing lines.** Transgene expression was detected by qRT-PCR using a primer set designed to be specific for the transgene. C = control, E1, E2 = different over-expressing lines. All data represent mean ± standard error of the mean (n = 3).

As for the *VvGH3-1* over-expressing lines no flowers and fruit have yet been produced by the *Atetr1-1* lines and so at this stage we cannot comment on the effects on fruit development. The effects of both the transgenes on berry development will be fully investigated when fruit is available and will greatly help in defining the role of these two PGRs.

## 6.12. Different Wine Flavour and Aroma Compounds are Variously Influenced by Harvest Date and Fruit Sugar Content

For a full presentation of this work please see Boss PK, Böttcher C, Davies C (2014) Various influences of harvest date and fruit sugar content on different wine flavour and aroma compounds. *American Journal of Viticulture and Enology*, 65:(3), 341-353 (published after the completion of the project).

The flavour and aroma of wine can be influenced by variables throughout the production process. However, the composition of the fruit used to make the wine is still a major influence on the outcome. This is due partly to the presence of varietal flavour and aroma compounds that are transferred from the grape to the wine with little or no, chemical conversion (Dunlevy *et al.*, 2009, Ebeler and Thorngate, 2009, Robinson *et al.*, 2014). Furthermore, recent experiments have illustrated that the production of some volatile compounds during fermentation depends on the presence of precursors or potential modifiers of yeast metabolism in the grape must (Dennis *et al.*, 2012, Keyzers and Boss, 2010). Therefore, understanding the processes that influence grape composition will enable some control or prediction of wine flavour and aroma in the vineyard.

Grape composition at harvest depends on environmental variables and viticultural management before and during berry development. Geographical location of a vineyard also affects wine sensory attributes, potentially through a number of biophysical variables (Heymann and Noble, 1987, Lund *et al.*, 2009) and differences can also be observed due to vintage effects (Forde *et al.*, 2011). Management of the vineyard by techniques such as bunch-thinning and canopy management has also been shown to affect wine sensory characteristics (e.g. Diago *et al.*, 2010, Reynolds *et al.*, 2007). Another important variable is the timing of the harvest of the fruit which can change the volatile compound composition of wines (Fang and Qian, 2006).

Harvest timing is intimately linked to grape berry ripening and the accumulation of high sugar levels (Davies *et al.*, 2012). However, there is conflicting evidence about the timing of flavour compound accumulation during berry ripening and how it relates to sugar accumulation. Several studies show that a number of free and bound aroma compounds begin to accumulate in fruit at, or soon after, the onset of ripening (e.g. Battilana *et al.*, 2011, Fenoll *et al.*, 2009, Wilson *et al.*, 1984). The changes that occur at the onset of ripening, including sugar and aroma compound accumulation, may be co-ordinated or causal. For example, the same stimulus that induces sugar accumulation in the fruit may also induce pathways leading to aroma volatile production, the sudden increase in sugars during ripening may cause osmotic changes that induce other pathways including those producing aroma volatiles or these ripening processes may be controlled independently. This is indeed the case for anthocyanin production where some treatments can alter anthocyanin accumulation independent of changes in the rate of sugar accumulation (e.g. Mori *et al.*, 2005). Furthermore, sugars can act as important signalling molecules in their own right and modulate growth and development in addition to stress responses (Rolland *et al.*, 2006) and it istherefore possible that sugars regulate some aspects of fruit ripening themselves.

Anecdotal evidence suggests that in some growing regions, certain management practices or environmental conditions in some vintages alter the relationship between sugar-ripeness and flavour-ripeness. Usually it seems that the fruit has accumulated sugar before flavour has had time to develop. However, the biochemical pathways leading to the production of compounds which contribute to, or influence, wine volatile composition could also be altered by vineyard variables independent of the rate of sugar accumulation. This would mean that attempts to decrease the rate of sugar accumulation (by increasing yield for example) may not necessarily restore the sugar/flavour balance. Below experiments designed to test the relationship of the production of certain wine volatile compounds with the Brix of the berries or time of harvest as described.

Due to copyright reasons only a summary can be presented here but the full story can be found in Boss *et al.* (2014).

### 6.12.1. Chemical Analysis of the Grape and Wine Samples

The rationale behind this experiment was to test the effect of sugar and time on wine metabolites by harvesting a matrix of berry samples of the same Brix at different times and different Brix levels at the same time followed by winemaking and volatile metabolite analysis. This should enable metabolites that depend on time, sugar level or both for their levesl to be determined. Berries were sampled at four dates, 04/03/2011 (1H), 08/03/ 2011 (2H), 17/03/2011 (3H) and 23/03/2011 (4H) and were sorted into four groups based on Brix levels, i.e. 10.1-12 $^{o}$Brix (12), 12.1-14 $^{o}$Brix (14), 14.1-16 $^{o}$Brix (16) and 16.1-18 $^{o}$Brix (18), or discarded if the berry had a Brix value that fell outside this range.

Analysis of the grape samples taken at each time point shows that in each case four distinct categories of sugar levels were obtained (see Boss *et al.*, 2014 for details). While the mean glucose/fructose measurements of the fruit in these categories were not identical across the time points, each set of samples at each time point showed a trend of increasing sugar content. There were some differences observed in the mean YAN content of the samples. At the first harvest date there was an increase in YAN associated with increasing sugar content. However, this trend was not observed at any other harvest date (see Boss *et al.*, 2014 for details).

The model wines were produced with added glucose/fructose to normalise final ethanol concentrations. The wines showed a range of ethanol concentrations from 11.23% (v/v) to 12.23% (v/v) (see Boss *et al.*, 2014 for details). While it has been shown that the matrix can affect the recovery of volatile compounds using headspace SPME with ethanol having the greatest impact (Robinson *et al.*, 2009), we employed a protocol designed to minimize these effects by adding NaCl to the samples to increase extraction efficiency, diluting the wine 1:2 or 1:100 with water and adding the internal standards to the solution so that they were subject to the same matrix effects.

One hundred and thirty three volatile compounds were quantified in the experimental wines relative to the five internal standards designed to represent a range of functional groups of wine aroma compounds. Of these 133 compounds 94 were found to be significantly different ($p < 0.05$) due to either the date of harvest, the Brix of the samples or both (see Boss *et al.*, 2014 for details). Thirty nine compounds did not vary significantly when tested against Brix or harvest date. Most of these compounds were esters, which are produced by the yeast during fermentation. For these compounds it would appear that changes in fruit composition have less of an effect on their production than fermentation parameters.

Only two of the final 94 compounds chosen for further analysis were significantly different ($p < 0.05$) with respect to the fermentation replicates - hexanal and a compound tentatively identified as 2,4,5-trimethyl-1,3-dioxolane (see Boss *et al.*, 2014 for details). This suggests the variation in the small scale fermentations used in this study was small and should not affect the interpretations made with regards to the Brix and harvest date of the fruit. Seventy seven compounds were significantly different ($p < 0.05$) amongst the fermentations made on different harvest dates and 70 due to differences in the Brix of the fruit (see Boss *et al.*, 2014 for details). There were 53 compounds found to be significantly different ($p < 0.05$) due to both factors. It appears that neither factor dominates the variation observed in the concentration of these compounds in the headspace of the wines. The interaction of the harvest date and Brix variables was also tested and 32 compounds had a significant interaction effect. There are some compounds that show a strong Brix effect dependent on the harvest date (e.g. *β*-citronellyl acetate and geranyl acetate) and others that show a stronger response to harvest date at some Brix classes compared to others (e.g. (*Z*)-3-hexen-1-ol) (see Boss *et al.*, 2014 for details). These analyses suggest that there is a complex relationship between the effects of harvest date and increasing Brix on the volatile composition of the wine.

### 6.12.2. Cluster Analysis to Group Compounds with Similar Changes in Concentrations in the Experimental Wines

The compounds that were found to be significantly different in the wines in relation to the harvest date or Brix of the grape samples were analysed by *k*-means clustering. K-means cluster analysis is a powerful method to identify patterns within data sets. Data was

normalised by dividing by the maximum concentration observed for each compound, meaning the values fall between 0 and 1, to allow volatile components having diverse absolute concentrations to be readily compared. Six clusters were used to classify the data as it was found that this explained 75 % of the variation in the data and the addition of another cluster only increased this to 77 %. These clusters are shown in Boss *et al.* (2014).

### 6.12.3. Cluster 1

This cluster is made up of compounds that were significantly different in the wines but, in general, they did not show consistent changes in concentration due to harvest date or Brix content of the fruit. Having grouped these compounds together, the averaging of their profiles into the cluster mean resulted in plots that appear to vary little as subtle, but significant, changes are lost amongst the differences in relative concentrations of the compounds present in the cluster. This cluster contained 23 members (see Boss *et al.*, 2014 for details). The majority of these compounds were esters (14) or other volatiles known to be at least partially yeast-derived (6) which suggests that grape composition alters the production of these volatiles during fermentation. However, a close inspection of the changes in relative concentration of these compounds across the samples suggests that, for most of these compounds, there were no trends in the abundances due to differences in Brix or harvest date across the samples. Other vineyard variables such as pest or disease pressure, water stress or air temperature may have altered grape composition and thus caused changes in wine outcomes independent of the two main variables tested in this experiment. One of the compounds in this cluster, 1-hexanol, did show a consistent trend in relation to the Brix of the samples. In wines, the alcohol 1-hexanol originates mainly from the fruit (Dennis *et al.*, 2012, Keyzers and Boss, 2010). The relative concentration of this compound increased in association with the Brix content at each time point in agreement with the results from studies on volatile elution from fruit during these stages of berry development (Boss *et al.*, 2014, Kalua and Boss, 2009, 2010).

### 6.12.4. Cluster 2

In general, this set of compounds increased in response to an increase in Brix, but did not show a consistent response to the date of harvest. This cluster contained the most members (26) and contained a number of different classes of volatile compounds (see Boss *et al.*, 2014 for details). Nine of the compounds are aliphatic alcohols or aldehydes, or acetate esters derived from these alcohols. It is interesting that 1-heptanol, 2-heptanol, 1-nonanol and 2-nonanol all grouped into this cluster. Odd-numbered alcohols are found at lower abundance in plant tissues than the even-numbered ones due to the nature of lipid metabolism, although these compounds have been reported as being lipid oxidation products (Frankel, 1983). Given that other compounds in this cluster included the lipoxygenase pathway products hexanal and (*E*)-3-hexenol and the acetate ester produced from (*E*)-3-hexenol, this shows that wine volatiles derived from lipid oxidation are influenced by the sugar composition of the fruit. Whether this is due to differences in the level of lipid substrates, oxidation enzymes or anti-oxidant levels in the fruit is the subject of future experiments Studies on the expression of lipoxygenase genes during fruit development showed that at least one member of this multigene family increases in expression after veraison (Podolyan *et al.*, 2010) and this may increase the concentration of lipoxygenase pathway products in the fruit.

Nine monoterpene-like compounds also grouped in Cluster 2. Studies using the aromatic grape variety Gewürztraminer have shown that monoterpene glycosides increase in the fruit after veraison and this is associated with increases in the expression of terpene synthase genes

and two genes involved in the production of substrates of terpene synthases (Martin *et al.*, 2012).

### 6.12.5. Cluster 3

The curves presented in Cluster 3 suggest that the increase in abundance of some compounds is controlled by an interaction between Brix levels in the fruit and the date of harvest (see Boss *et al.*, 2014 for details). In this cluster there is a general trend of increasing concentrations with increasing Brix, but the abundances of the compounds in samples taken at the earliest time point were always lower than those taken at the latest time point. This cluster contained six members; 3-nonanone and the monoterpenes, linalool, nerol, *β*-citronellol, hotrienol and 3,7-dimethyl-1,5-octadien-3,7-diol. The monoterpenes are those found in high concentrations in Riesling wines (Rapp, 1998), and can undergo modification due to grape metabolism (Luan *et al.*, 2006, 2005), yeast activity (King and Dickinson, 2000) or the acid nature of wine (Pedersen *et al.*, 2003). The presence of the four monoterpenes in this cluster suggests that the increase of some monoterpenes in the fruit can be associated with both an increase in Brix and time.

### 6.12.6. Cluster 4

Cluster 4 represents compounds whose relative concentrations were higher in the samples harvested on the earliest date compared to the other three sampling times (see Boss *et al.*, 2014 for details). There was also a Brix effect with the abundances of the compounds decreasing in samples harvested on the latter three dates as Brix increased.
Nine of the 25 compounds in this cluster were acetate esters and many of these are acetate esters of higher alcohols. These compounds are produced during fermentation from alcohols or aldehydes present in grapes and/or yeast (Dennis *et al.*, 2012). Therefore, the abundance of compounds that may potentially become acetate ester precursors in the fruit appears to be influenced by both harvest time and Brix. The data supports this hypothesis when the changes in 1-propanol and propyl acetate are compared (see Boss *et al.*, 2014 for details).

Other members of Cluster 4 include: 2-heptanone, 1-octen-3-one and 2-undecanone (all ketones); benzaldehyde; *β*-isophorone and *β*-cyclocitral (carotenoid oxidation products); 1-ethoxy-1-methoxy ethane and 2,4,5-trimethyl-1,3-dioxolane (acetals). The presence of these ketones, aldehydes and acetals suggests that the fruit sampled on the first harvest date experienced higher levels of oxidation during fermentation which may reflect differences in antioxidant levels in the fruit across the sampling period. It may also suggest that there are oxidative enzymes present in the fruit at this time point which create more oxidation products of the substrates of these enzymes in the fruit after homogenisation and before fermentation begins.

### 6.12.7. Cluster 5

Cluster 5 consisted of compounds that decreased in concentration in response to both later harvest date and increasing Brix (see Boss *et al.*, 2014 for details). The effect of harvest date was most apparent between the first two time points. This cluster contained only three compounds and all are products of lipid oxidation. Both (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate can be traced back to the oxidation of linolenic acid via the lipoxygenase pathway, whereas (*Z*)-2-heptenal has been reported as an oxidation product from linoleic acid (Frankel, 1983). However, we have already observed the presence of lipid oxidation products in Cluster 2 and these compounds have a very different accumulation pattern across the samples. The differences could be due to changes in enzyme activities related to the production of *cis*- and

*trans*-isomers of 3-hexen-1-ol and their resulting acetate esters given that they show opposite accumulation patterns in the experimental wines (see Boss *et al.,* 2014 for details).

### 6.12.8. Cluster 6

Cluster 6 contains compounds that increased in abundance with increasing Brix on the first two harvest dates, but did not change to any great extent due to differences in Brix on the last two harvest dates (see Boss *et al.,* 2014 for details). The increase in abundance of these compounds in association with increasing Brix was greatest on the first harvest date.

There are eleven compounds in this cluster. Three of these are norisoprenoids ($\beta$-ionone, $\beta$ -damascenone and vitispirane) derived from the enzymatic cleavage of carotenoids. The abundances observed in these samples may reflect the concentrations of carotenoid precursors or dioxygenase enzyme activity required for their cleavage (Mathieu *et al.,* 2005) or that the amount of free norisoprenoids in the wine is influenced by the amount of free or glycosydically bound forms of them in the fruit.

Three of the other compounds in this cluster are acetate esters of monoterpenes, namely $\beta$-citronellyl acetate, neryl acetate and geranyl acetate. Why these monoterpenes should be esterified to a greater extent in the earlier harvested samples is not clear.

### 6.12.9. PCA Analysis of the Volatile Profiles of the Experimental Wines

To visualise the relationships between the wine samples and understand how the changes in abundance of the volatile compounds underlie these relationships, the total volatile profiles, consisting of those compounds found to be significantly different due to harvest date or Brix, were subjected to principal components analysis (PCA). The description and analysis of this data is quite complicated and is best viewed in its entirety in the published work (Boss *et al.,* 2014). In general, the PCA suggests that there is an interaction between the harvest date and Brix variables with the lower Brix, early harvest date wines appearing on the top right of the plot and with the later harvest, higher Brix wines mapping to the lower left quadrant of the plot. The PCA data supports the findings of the cluster analysis.

### 6.12.10. PLS1 Relating Harvest Date or Brix to Volatile Composition

PLS1 and PLS2 models were tested for their ability to explain the Y-variables (harvest date and Brix) using the changes in abundances of the volatile compounds (X-variables). This analysis adds further support and detail to the arguments presented above but is best read in the published work (Boss *et al.,* 2014).

The experiments described here were designed to test the hypothesis that changes in wine volatiles can be attributed to changes in Brix of the fruit, that is, wine aroma is linked to sugar accumulation. The results show that there is a complex relationship between harvest date and Brix, which is clearly illustrated by the PCA and supported by the GLM, cluster and PLS1 analyses.

The data suggests that the accumulation of varietal compounds that are often associated with positive aspects of wine flavor is mostly linked to the accumulation of sugars (i.e. Cluster 2 and a combination of some compounds in Clusters 3 and 6). Several products of lipid oxidation also follow this pattern of accumulation. Less reduced products of these oxidative processes are influenced by both harvest date and Brix (Clusters 4 and 5). The presence of a number of acetate esters in Cluster 4 is probably due to higher amounts of alcohols or aldehydes, which may become precursors of these esters, in the lower Brix and early harvest

grape samples. These ketones, alcohols and aldehydes would generally impart negative sensory attributes to wine and so their decrease during ripening and over time would be desirable. Therefore, if fruit accumulates sugar and thus ripens more quickly than normal, any negative effect on fruit and wine composition may be attributed more to the fact that compounds with potentially negative impacts on flavor and aroma are not removed to the same degree rather than the lack of accumulation of desirable varietal factors.

Over the recent past a reduction of the time required for grapes to achieve sugar-ripeness, possibly a result of climate change, has been observed in many countries. This has raised the issue of the potential disconnect between flavor-ripeness and sugar accumulation. The experimental results show that there is a complex relationship between berry sugar content, harvest date and wine volatile composition.

## 6.13. A Study of PGR Levels in Developing *Vitis vinifera* cv. Shiraz Berries Reveals a Surprising Increase in Cytokinin Levels during Ripening

For a full presentation of this work please see Böttcher C, Boss PK, Davies C (2013a) Increase in cytokinin levels during ripening in developing Vitis vinifera cv. Shiraz berries. *American Journal of Viticulture and Enology,* 64:(4), 527-531.

A number of PGRs are thought to have a role in the control of berry development and there have been numerous studies detailing changes in PGR levels in grapes (*Vitis vinifera*) (Böttcher and Davies, 2012). Recent developments in PGR analysis have meant that the outcomes of some of these studies need to be re-examined. Many plant PGRs, including gibberellins (GAs), cytokinins, brassinosteroids, abscisic acid (ABA) and auxins are at elevated levels in flowers and during early berry development then decrease to be at low levels before veraison (Böttcher and Davies, 2012). The concentrations of ABA and brassinosteroids increase again at veraison and appear to enhance or hasten ripening (Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007, Owen *et al.*, 2009, Symons *et al.*, 2006, Wheeler *et al.*, 2009). The concentrations of GAs, auxins and cytokinins have been reported to remain low during the ripening period (reviewed by Böttcher and Davies, 2012). Auxins appear to delaying ripening (Ban *et al.*, 2003, Böttcher *et al.*, 2012a, 2011b, Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007, Fujita *et al.*, 2006, Jeong *et al.*, 2004). The role of other plant growth regulators in berry development and ripening is unclear and needs to be further examined.

In addition to studying the biosynthesis of PGRs their catabolism has also been investigated during berry development. In some cases it involves the modification of a biologically active compound into an inactive or less active form through the addition of other molecules such as sugars or amino acids (Piotrowska and Bajguz, 2011), in other cases inactivation is achieved through enzymic modification of the PGR structure that often leads to degradation (Frébort *et al.*, 2011, Hedden and Thomas, 2012, Woodward and Bartel, 2005).

In this work below the quantification, using ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC ESI-MS/MS), of ABA and its metabolites and various cytokinins and GAs in the same grape berry pericarp developmental series is described.

### 6.13.1. PGR Levels during Berry Development ABA and catabolites.

ABA and all of its analysed catabolites (ABA, ABA-GE, DPA, 7'-OH-ABA, neo-PA, t-ABA) were detected at some stage of a Shiraz pericarp developmental series (Böttcher *et al.*, 2013a). The pattern of ABA accumulation was similar to that observed by previous studies with a peak shortly after veraison (8 wpf) (Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007, Owen *et al.*, 2009, Wheeler *et al.*, 2009). This peak of ABA levels has been associated with the onset of ripening and the application of ABA can promote ripening (Böttcher and Davies, 2012). The levels of DPA and ABA-GE in the pre-veraison samples confirm the pattern observed by Owen *et al.* (2009) in Merlot skin and pulp samples and indicate that ABA is being catabolized during this period through the oxidation pathway initiated by the 8'-hydroxlase enzyme (Figure 20A). ABA-GE concentrations increased rapidly during ripening to peak at 10 wpf after which they remained relatively constant throughout the rest of the ripening phase (Figure 20A). This suggests that the inactivation of ABA post-veraison occurs through glycosylation rather than through oxidation as occurs pre-veraison. There are two periods when ABA levels decrease during berry development: after flowering during the period up to veraison and after the post-veraison peak. During these periods the berries are increasing in size so that the final ABA levels are determined not only by the balance between biosynthesis and catabolism but also by dilution. A small amount of 7'-OH-ABA was present throughout the developmental series with a slight increase in the 10 and 12 wpf samples indicating that some catabolism occurred via 7'-hydroxylation.

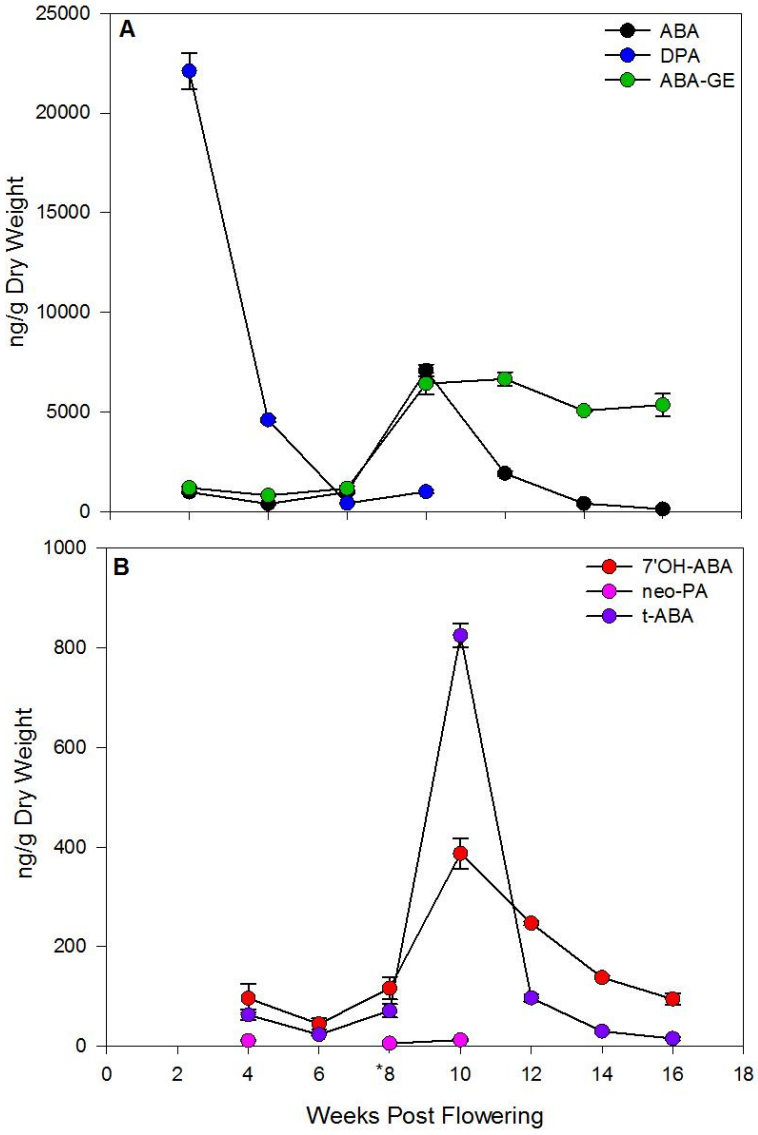



**Figure 20. Concentrations of ABA and its catabolites (ng/g dry weight) during Shiraz berry development.** (A) Levels of ABA, DPA and ABA-GE, (B) Levels of PA, 7'OH-ABA, neo-PA and t-ABA. All data represent mean ± standard error of the mean (n = 3).

**GAs.** A range of GAs were analyzed including those that are active forms, pathway intermediates and inactive catabolites (GA 1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51, 53). Only five GAs were reliably detected early in developing berries and none were reliably detected in post-veraison fruit (Figure 21). The two active GAs, GA4 and GA7, were detected at low but fairly similar concentrations in berries 4, 6 and 8 wpf only. The most populous species was GA24 which is a precursor for GA4 and GA7 in the non-hydroxylation pathway indicating that GAs 4 and 7 are likely to be actively synthesized during this period. GA34, which was present in 4, 6, and 8 wpf samples, is a catabolite of GA4 indicating that both synthesis and breakdown of GA4 is occurring at these stages.

GA19 was only detected at 4 wpf but it is an intermediate of the 13-hydroxylation pathway which leads to the active GA1 species indicating that both the non-hydroxylation and 13-hydroxylation pathways are active. Although there were some differences in the species of GAs that were detected in this study compared to other studies (Pérez *et al.*, 2009, Symons *et al.*, 2006, Zhang *et al.*, 2003) the general trends were the same, that is that active GAs were only reliably detected pre-veraison. The higher levels of active GAs in young, developing fruit are consistent with a role for GAs during the stage of rapid cell division and enlargement in grape berries.

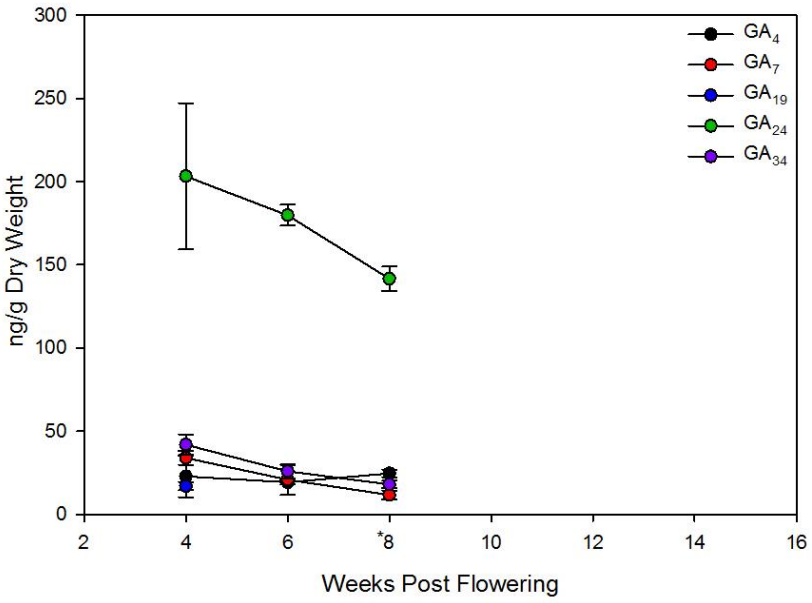



**Figure 21. Concentrations of gibberellins GA4, 7, 19, 24, 34 during Shiraz berry development.** All data represent mean ± standard error of the mean (n = 3).

**Cytokinins.** No cytokinins were reliably detected in fruit at 4 and 6 wpf. In another study high concentrations of some cytokinins were found early in berry development (Zhang *et al.*, 2003) and they are thought to be involved in setting and early growth of fruit (NeSmith, 2002, Werner and Schmülling, 2009). Low amounts of t-Z were present in berries 10, 14 and 16 wpf but much higher amounts of its glycosylated, inactive form t-ZOG were present at 14 and 16 wpf (Figure 22). Surprisingly there was an increase in the active cytokinin iP from 8-14 wpf followed by a small decline at 16 wpf. This increase in the levels of an active cytokinin during ripening has not been previously observed in grape berries. Interestingly, a rise in active forms of cytokinins (t-Z, t-ZR, iP nucleotide, t-Z nucleotide and the storage forms t-Z riboside-O-glycoside and dihydrozeatin riboside-O-glycoside) has been observed during kiwifruit development (Lewis *et al.*, 1996, Pilkington *et al.*, 2013). In kiwifruit there is no particular increase in iP levels during ripening.It can therefore be concluded that the developmental changes in cytokinin profiles are similar in both grape and kiwifruit as there is an increase in active and glycosylated cytokinin forms as ripening proceeds.

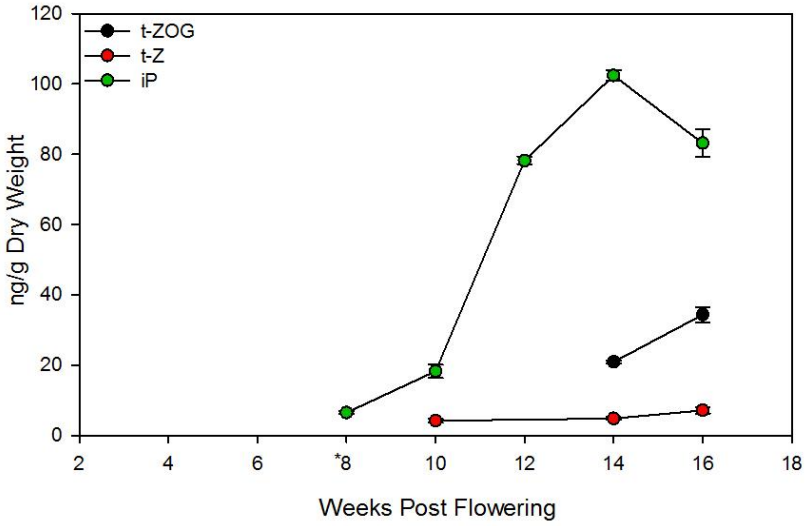



**Figure 23. Concentrations of cytokinins iP, t-Z and t-ZOG during Shiraz berry development.** All data represent mean ± standard error of the mean (n = 3).

The likely role for cytokinins early in fruit development has been mentioned above and cytokinin application can delay fruit ripening (Itai *et al.*, 1995, NeSmith, 2002, Peppi and Fidelibus, 2008) but the function of the increase in cytokinins during ripening is unknown. It has been proposed that it may be involved in reducing chlorophyll loss during maturation and indeed it may be involved in the control of tissue senescence. It is interesting that the increase in iP in grape berries parallels the observed increase in hexose levels during ripening (Figure 23, 24). As the early high levels of cytokinins have been implicated in the creation of a sink during fruit set it could be that the role of cytokinins during berry ripening is also related to developing the berry as a sink for transported photosynthate.

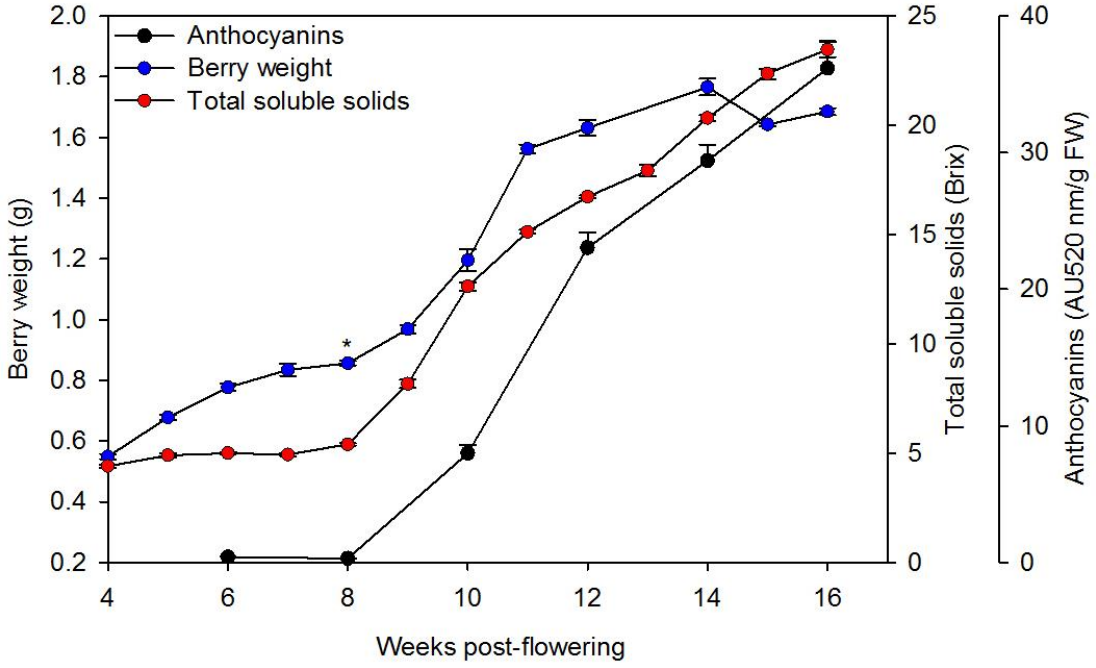



**Figure 25. Anthocyanin levels (AU520 nm), berry weight and total soluble solid levels during Shiraz grape berry development.** The asterisk represents veraison. Error bars indicate standard errors (n=3 biological replicates).

Free PGR levels appear to be reduced during the pre- and post-veraison periods in grape berries by two different methods. During the pre-veraison period enzymic catabolism appears to be a common feature, for example ABA is converted by an 8'-hydroxylase enzyme to PA which is further catabolised to DPA (Figure 20). During the post-veraison period the main mode of reduction of the levels of free forms of both ABA and cytokinins is through glycosylation rather than via enzymic catabolism (Figures 20 and 23). It has also been shown that auxin levels are controlled during the post-veraison period by conjugation to amino acids catalyzed by GH3 enzymes (Böttcher *et al.*, 2010). In other plant species there is evidence for both glycosylation and enzymic modification of castasterone (Bajguz, 2007) and this may also occur in grapes.

This work describes the quantification, using ultra-performance liquid chromatography-electrospray tandem mass spectrometry and internal deuterated standards, of the PGRs ABA, and its metabolites, and a range of cytokinins and GA species in the same grape (*Vitis vinifera* L.) berry pericarp developmental series. The most interesting finding comes from the analysis of the concentrations of certain cytokinins. It provided some previously unreported and surprising results as the concentration of iP increased rapidly at veraison and remained at elevated levels throughout ripening. The reason for this phenomenon is unknown. Cytokinins may be involved in the control of ripening and/or senescence. This warrants further investigation.

# 7. Outcome/Conclusions
## 7.1 Outcomes with Scientific and Industry impact

- Auxin (NAA) treatment of Riesling grapes delayed ripening by 15 days as measured by Brix levels. This confirms that these treatments can delay berry ripening in both white and red wine grapes.
- The delay in berry ripening differed in some ways to previous treatments using red wines grapes (e.g. Shiraz) as it delayed the Brix increase but did not significantly change the pattern of berry weight increase. Not only was the timing of sugar accumulation delayed but the rate of sugar uptake was slower. This may have important implications for obtaining good flavour without excessive sugar levels.
- The NAA treatment improved the synchronicity of berry ripening. This effect may also be a useful property of such treatments.
- Wine from NAA 'delayed-ripening' grapes had an altered volatile metabolite profile. Differences in oxidative processes resulted in higher monoterpene levels (e.g. linalool, trans-geraniol). Although there are varietal differences in response compared to the results with Shiraz some volatiles e.g. β-Damascenone increase in both varieties in response to NAA.
- Sensory analysis showed that the wines from the Control and NAA-treated fruit could be distinguished but there was no clear preference for either. This indicates that there are small differences in wine aroma and flavour but that delaying ripening does not adversely affect wine quality.
- Different auxins have different effects on ripening. Three auxins were tested extensively and differed considerably in their ability to delay ripening. The synthetic auxins NAA and BTOA are more effective than the endogenous auxin IAA, which requires very precise timing to be effective. This indicates that it might be possible to tailor auxin PGRs to fit purpose.
- A family of IAA-amido synthetase enzymes (GH3s) was characterised in grape. These enzymes were subsequently shown to be crucial in controlling IAA levels in developing berries and are therefore keys to the control of ripening. They act to reduce the free IAA levels in plant cells through inactivating it via conjugation to amino acids. Some of these conjugates themselves may have signalling activity, as has been suggested by studies in other plants.
- All members of the family of GH3 enzymes are expressed early in berry development but one is also expressed during ripening and may be critical in this process. The *GH3* genes respond to different stimuli and are differentially expressed in different grapevine organs. In addition the GH3 proteins have different amino acid substrate preferences suggesting specialised functions in plant development.
- The enzyme kinetics for three of these enzymes have been determined. These studies show that some auxins are better substrates for these enzymes than others. This explains why some forms of auxin are more effective in the field at delaying ripening than others as they last longer within the plant in an active state and are therefore more effective.
- It was shown experimentally that GH3 conjugation is directly important to IAA levels in berries and therefore a key to understanding and controlling ripening.
- A novel inhibitor was designed that inhibits GH3 enzymes in a commutative manner. This inhibitor was effective in berries as it reduced the production of an inactive auxin conjugate. This will be a useful tool in studying the role of GH3s *in planta* and could

lead to the production of cost effective inhibitors of GH3 action that could delay ripening by maintaining the inhibition by endogenous auxin.

- The 3-D structure of an IAA-conjugating GH3 enzyme has been resolved for the first time. This has helped explain the function and mechanism of these proteins and raises the possibility of designing more effective auxins that are more stable *in planta*.
- We have shown that the treatment of berries with auxins (NAA and BTOA) post-veraison does not alter berry ripening as measured by berry weight or sugar accumulation. This means that auxins cannot be used to significantly alter berry ripening after it has commenced.
- In the studies highlighted above isolated bunches were treated due to the small scale equipment available for the trials conducted. Whole vines were also treated to test the effect of auxins on whole vine development. No effects were observed on the canopy but berry ripening was still delayed as effectively as when only bunches were treated. This indicates that the large scale trials using commercial spraying equipment should still be effective without affecting vine growth or structure.
- It has been demonstrated that although there are genes that behave differently between varieties (Shiraz, Cabernet Sauvignon) when grown under similar conditions (suggesting that they may contribute to varietal differences) there are also genes that are expressed with precisely the same pattern indicating that they are involved in a basic process not involved in varietal differentiation.
- The ethylene-releasing PGR Ethrel can delay ripening as measured by weight, colour and sugar accumulation when applied sufficiently early in ripening. This offers a further opportunity to alter berry ripening using a PGR. Investigation of the cause of this effect shows that it is an indirect effect as the application of Ethrel induces an increase in endogenous auxin (IAA) levels.
- Investigations into the cause of this effect led to the characterisation of the IAA biosynthesis genes in grapevine and showed that Ethrel (through ethylene) induces IAA biosynthesis and thereby increases the levels of free, active IAA within the berry, thus delaying ripening.
- Ethylene biosynthesis genes (in two the varieties tested) are induced during berry development shortly before ripening, this is followed shortly after by an induction of auxin biosynthesis genes. This indicates that a small burst of IAA just at veraison may have biological significance.
- AVG was confirmed to advance ripening. The PGR AVG is an inhibitor of ethylene biosynthesis and auxin biosynthesis. It reduces IAA levels when applied to berries before veraison and advances berry ripening. This indicates that the naturally occurring PGR AVG which is used in other horticultural crops could be used as a cost-effective advancer of ripening under conditions where earlier ripening would be of advantage to the industry.
- Vines overexpressing the *VvGH3-1* gene (IAA-amido synthetase) have a low-auxin phenotype (as predicted) with smaller leaves and shorter internodes. Free IAA levels are reduced and IAA-Asp conjugate levels are increased. This demonstrates that the *GH3* genes can have large effects *in planta*. Once flowering occurs these plants will be used to confirm the role of auxins during berry development.
- Experimental data has been produced regarding the 'harvest date/sugar/flavour' nexus. Previously the influence of sugar has been much discussed but there are little scientifically valid data. Different wine flavour/aroma compounds are differentially affected by harvest date and sugar content in a complex relationship. The accumulation of varietal volatiles (e.g. monoterpenes), seen as positive influences on

wine flavour, are mostly linked to sugar levels. Some products of lipid oxidation are influenced by both sugar and harvest date. Some ketones, alcohols and aldehydes associated with negative aspects decrease with time. Therefore, when fruit accumulates sugar too quickly wine quality may be reduced by the lack of time for negative characters to decrease.

- The levels of a broad range of PGRs have been determined throughout berry development. This indicates that the decrease in PGR levels pre-veraison is often due to catabolism while conjugation is more frequent at and after veraison. The levels of the cytokinin iP increase in the middle and later stages of ripening and may be important for flavour development.

## 7.2. Training and development of Collaborations

This project has given us the opportunity to employ and train a new Technical Officer (Dr Crista Burbidge) in the field of grapevine research. Crista has been a great asset to the group bringing her previous experience gained in doing a PhD on organic acids in grapevine and has also greatly expanded her skill set during this project in our lab. Ms Harvey (who worked on a previous GWRDC project in this area) has continued to develop new skills (for example in sensory analysis). Her participation has been somewhat reduced due to maternity leave during the period of the grant but we have back-filled this position with other staff which has provided training for two further technical staff. The training that technical staff have received is wide ranging from detailed laboratory methods to field-based studies.

Dr Böttcher (Post-doctoral Fellow, now indefinite staff member) has continued to expand her excellent skill set and knowledge of PGRs and grape berry development and has demonstrated outstanding commitment to the project.

Successful, and ongoing, collaborations have been developed with industry in terms of the provision of experimental sites and there has been considerable interaction regarding the conduct and outcomes of the project with a range of industry members through personal discussions and more formal presentations. We are very grateful for the support provided.

Interactions with Australian and overseas scientists have continued to expand. Examples of cooperation within Australia are the interactions with Dr Grant Booker (University of Adelaide) and Drs Peat and Newman (CSME, CSIRO Parkville) regarding the determination of the 3D protein structure of the enzymes pivotal in controlling berry ripening. Opportunities to develop new mathematical methods for gene expression data analysis are continuing with Prof Glonek (University of Adelaide). The sensory analysis of some of the wines in this study was conducted in collaboration with Dr Sue Bastian (University of Adelaide). A relationship with Prof. Pezzotti's group in Verona Italy regarding the analysis of gene expression in grapes is ongoing. In a more general sense there are numerous interactions with various researchers around the world in a wide range of grape-related areas.

## 7.4. Conclusions

This project has made substantial progress in the understanding of the ripening process and its control. Improved and detailed knowledge of the control of the initiation of grape berry ripening which is the major determining factor in harvest timing has been developed. The roles of a number of PGRs involved in berry development have been better defined. These include PGRs that are delayers of veraison and promoters of veraison. Newly discovered changes in the levels of some PGRs indicate that they may be involved in the latter stages of berry development with potential consequences for the physical and metabolic state of berries at harvest. Much of the work has focused on auxins and ethylene, their interactions and their role in fruit development as they have the potential to delay ripening. The potential of another PGR, AVG to act as a promoter of ripening has been explored in some depth. All of this knowledge has been used to develop approaches to control ripening that have practical application to address industry problems including climate change related issues such as seasonal compression. In addition, important information has been developed regarding the relationship between harvest time and sugar level in regards to wine character. These outcomes have been possible through a combination of detailed scientific studies coupled with the practical testing of these ideas in commercial settings. The work has been considerably enhanced by the use of new techniques, the development of new investigative tools and through the development of complementary collaborations. We still have much to learn about berry ripening but important progress has been made which will help in the refinement of new ways to usefully manipulate grape berry ripening.

# 8. Recommendations

## 8.1 Continue Scientific Research into the Role and Action of PGRs during Grape Berry Development

There remain gaps in our knowledge regarding a number of PGRs that are likely to be involved in controlling berry ripening and are therefore targets for manipulation in the vineyard.
Areas for further investigation include:

1. An analysis of the precise roles and mechanism of action of PGRs that delay ripening (e.g. auxins and ethylene). This will require using a range of techniques and will include analysis of the synthesis, perception and action of the relevant PGRs.
2. Examine the roles of other PGRs (both established and putative), and combinations of PGRs, in berry development/ripening and flavour and aroma compound metabolism.
3. Develop new methods in grape to accurately measure a wider range of PGR classes, e.g. members of the cytokinin family of hormones that are likely to be involved in fruit set and carbon sink (sugar accumulation) determination.
4. Conduct experiments to confirm interactions in berries between the different PGR signalling pathways.

## 8.2. Further our Understanding of the Nexus between Sugar Accumulation, Harvest Time, Berry Metabolism and Wine Character

Continue to further understand the link between sugar/time/wine flavour which is important to efforts to alter ripening.

1. Investigate the effects of delaying ripening by auxin treatment on aroma/flavour volatile compounds through GC-MS and sensory analysis.
2. Conduct field experiments to further our understanding of the link between sugar accumulation, hang time and the evolution of flavour and aroma volatiles.

## 8.3. Develop and Test Methods to Manipulate Ripening in the Vineyard to Alter Harvest Timing to Improve Profitability and Sustainability

The knowledge derived from the above studies should be the basis of efforts to develop practical approaches to manipulate berry ripening in the vineyard.

1. Conduct experiments/develop methods to achieve a significant ripening delay in warmer region grapes using auxins, measure the effects on berry development.
2. In field experiments trial the ability of the ethylene-releasing compound Ethrel to significantly delay berry ripening. Initiate investigation of the effects of delaying berry ripening by Ethrel treatment on aroma/flavour volatile compounds.
3. Test the effect of other potentially useful PGRs on berry development and wine composition/sensory properties.

## Appendix 1: Communication

Articles in scientific and industry journals regarding various aspects of the control of grape berry ripening have been published (see below). Some of this work has also been presented at the AWITC. In addition, three book chapters summarising the current state of knowledge of various aspects (including the influence of PGRs) of berry ripening have been published (see below).

During this project we have had the support and interest of a number of industry individuals and companies. In particular, we have been involved in discussions with winemakers and grape growers in the Adelaide Hills and McLaren Vale regions. Both the proposed research and the results of each seasons work and its potential for the industry have been discussed. Discussions and presentations to key personnel in other regions have also occurred. The feedback we have received has been useful in guiding and refining our experimental design and research direction. We will continue to engage the industry as we progress this work.

**Talks on project work at Conferences**

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**Book chapters**

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**Journal papers**

Boss PK, Böttcher C, Davies C (2014) Various influences of harvest date and fruit sugar content on different wine flavour and aroma compounds. American Journal of Viticulture and Enology. 65, 341-353. (published after the completion of the project).

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**Industry journal article**

Researcher profile in Australian and New Zealand Grapegrower & Winemaker (2014) 603:63

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**Communication through various media outlets**

This project has generated considerable publicity and interest in a range of media.

- A media release regarding this work was completed entitled 'Time is ripe for wine grapes', this can be seen at - http://www.csiro.au/news/wine-grapes-ripening.html
- A related podcast was also produced and can be heard at - http://www.csiro.au/multimedia/Adapting-wine-grapes-to-climate-change.html
- An associated factsheet which contains further information was also prepared and can be found at - http://www.csiro.au/files/files/py33.pdf
- As a result of the above a number of radio interviews regarding this work were conducted, these included: Radio National ABC, Local ABC Radio, ABC Mildura/Swan Hill, ABC News Newcastle, Rural Life TV with ex SA premier Rob Keiran
- Discussions were also had with print journalists regarding articles: Robert Millikan of the Economist; Natasha Sherwood, the Border Mail, Victoria; Yvonne O'Hara, Allied Press, Southern Rural Life, New Zealand
- There were a number of other expressions of interest in this research by email from researchers/growers/winemakers from France, USA and rather curiously a grower from Norway.
- ABC Radio The World Today - interview with Dr Davies by Sallese Gibson, Journalist ABC News Riverland and Mallee regarding the work we are doing on controlling ripening and harvest in the current GWRDC project (March 2014).

**Talks to Winemaker and Grower Groups**

- A talk regarding this and other previous GWRDC co-funded work was given to the McLaren Vale Grape Wine and Tourism Association Meeting, Sept 22nd 2010.
- Talk presented to the Young Mudgee Grapegrowers and Winemakers group.

- Murray Valley Winegrowers Association, Mildura, 10/12/2013
- Discussions have also been held with two large agrochemical industry representatives.

## Appendix 2: Intellectual Property

The intellectual property developed in this project is outlined in the above report. Quite a deal of it has already been published in refereed or industry journals so that it is freely available. Some remaining work is still being prepared for publication and will be made available as soon as possible. Much of this work has been discussed in other, non-print forums. The IP described pertains to advances in the understanding of berry development in terms of the control of grape berry ripening and how to manipulate it and in the relationship between berry sugar levels, harvest time and wine characteristics. Some IP is contained in methods developed to measure various metabolites/PGRs and in methods for studying their action. All results and publications are checked before external discussions for any IP that may be deemed as suitable for protection. As we are trying to develop methods that will have a practical outcome we are very aware of the issues involved with intellectual property protection. One aspect of this is that ultimately we will need to involve other business (e.g. agrochemical companies) in the future development of any PGR treatments to control ripening. We have had some initial talks with different companies regarding future collaboration and progression of this work. All of these have been held under strictly confidential agreements.

## Appendix 3: References


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## Appendix 4: Staff

Dr Christopher Davies, CSIRO Plant Industry, Principal Investigator
Dr Christine Böttcher, CSIRO Plant Industry, Chief Investigator
Dr Paul Boss, Plant Industry, Co-investigator
Dr Crista Burbridge, CSIRO Plant Industry, Technical Assistant
Mrs Katie Harvey, Plant Industry, Technical Assistant (maternity leave x 2)
Various casuals and short term appointees during Mrs Harvey's absences due to maternity leave and additional requirements during peak harvest times.

## Appendix 5: Budget reconciliation

To be sent by Ms Helen Hicks as a separate document