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Wine Australia

Understanding and manipulating small signalling molecules to affect the yield/flavour ('quality') nexus

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1. Abstract

The objective was to identify small signaling molecules pivotal to berry development, to determine their mechanisms of action in controlling berry ripening. This knowledge was used to develop methods to mitigate the detrimental effects of climate change on berry composition and the timing of veraison and, consequently, harvest. Extensive vineyard and laboratory studies using techniques including sensory analysis and cutting edge analytics showed that it is possible to alter veraison and harvest timing through in-vineyard treatments without yield loss and with little, or no, impact on wine, the exception being increased pepper notes in some Shiraz plots.

2. Executive Summary

Grape berry development, and in particular the ripening phase, is a complex process our understanding of which is increasing but there is still much to learn to help sustain grape and wine quality and value in a competitive and ever-changing world. Techniques to better manage current and future challenges need to be derived from an increased knowledge of berry growth and maturation. For example, the environmental conditions under which grapes are grown are continuing to change as a result of global climate change. Greenhouse gas emissions, particularly the increase in carbon dioxide levels, are driving climate change by trapping heat within the atmosphere. The resulting increased temperatures and changing weather patterns have profound effects on plant growth and therefore on viticulture and wine production. The dynamic nature of this problem and its importance make it crucial that we generate methods to mitigate its effects.

Grapevines and grapes are very sensitive and responsive to temperature and the observed increase in temperatures is having a profound effect on the rate and timing of berry ripening and on the composition of the fruit used for winemaking, through changes in metabolism. The significant changes to berry ripening that are problematic for the grape and wine industry include: earlier onset of veraison and rapid ripening over a shorter period, rapid sugar accumulation ahead of flavour development, reduced colour/flavour development and compressed harvest seasons.

We have targeted the small signaling molecules that control grape berry development as the keys to better understanding berry development (in particular berry ripening) and as tools to manage the ripening process to the benefit of industry. The aim is to better manage the ripening process to improve grape and wine quality and harvest timing without reducing yield.

We have demonstrated previously that the small, plant growth regulator (PGR) molecule 1-naphthaleneacetic acid (NAA) has the ability to delay grape berry ripening when applied to pre-veraison berries under ‘cool’ climate conditions. This work was furthered in the current project where we showed that NAA is effective in delaying ripening and therefore harvest under warm climate conditions (McLaren Vale), making possible its use in controlling harvest timing under a broader range of conditions. To further test the potential of NAA under different conditions an experiment was conducted in the Eden Valley where veraison was significantly delayed. Post-veraison treatments with NAA were also trialed to determine whether NAA could delay ripening once it had commenced rather than delaying the onset with pre-veraison treatments. However, the progress of development, as measured by berry weight and Brix, was not altered in fruit treated post-veraison with NAA.

NAA is used in other horticultural industries as it is an effective and safe plant growth regulator and is more stable than the auxin, indole-3-acetic acid (IAA), normally found in fruit. IAA is synthesised in grapevine from the amino acid tryptophan and a sustained synthesis of IAA might be initiated if tryptophan levels were increased. The application of tryptophan did not delay ripening and it was also confirmed that the application of IAA itself was ineffective. The lack of effectiveness of IAA is almost certainly due to its rapid metabolism by specialised enzymes within the berry. Field trials demonstrated that another auxin, 4-chloroindole acetic acid, normally found in seeds and for which some evidence suggested that it may be more resistant to rapid degradation and therefore more stable within the berry, was also ineffective in delaying ripening/harvest. NAA is therefore the PGR of choice to delay berry ripening/harvest. Control over the length of the delay in veraison and harvest is also a practical consideration. A comparison of 50 mg/L and 250 mg/L applications

showed that higher concentrations increased the delay in ripening and harvest of Shiraz fruit, demonstrating that the length of the delay could be manipulated through dosage levels.

In general, the treatments with NAA that delayed ripening had little effect on both the wine volatile metabolite profiles and sensory properties. Longer delays of 2-3 weeks in ripening/harvest tended to increase the levels of some fruity esters with some minor differences in sensory properties and tasters showed no particular preference. In Shiraz, these longer delays increased the peppery character of wines with a coincident increase in the levels of rotundone, the metabolite responsible. The reasons for this specific increase are not yet known.

Other PGRs are involved in berry development and might be targets for manipulating berry quality. Cytokinins, for example, have been suggested as potential delayers of ripening. We discovered that the cytokinin isopentenyl adenine (iP), increased from veraison to high levels at harvest, the final level varying considerably between wine grape cultivars. This indicates that iP might be involved in some aspect of berry ripening. Field experiments suggested that it is not involved in the timing of ripening as its application to the low iP cultivar Pinot Noir did not affect the timing or progression of berry development. Although iP probably has some ripening-related role, perhaps related to post-veraison metabolism, e.g. sugar accumulation, we have not as yet been able to define it. To investigate the possible roles of cytokinins, the genes involved in cytokinin biosynthesis, degradation and transport in grape tissues were identified and their expression during berry development and in other tissues was studied. Cytokinins appear to be important during the early stages of berry development, during the cell division and expansion stage. Field trials over two seasons tested the effect of a range of natural and synthetic cytokinins and showed that they had no significant effects on the timing of the initiation of ripening or the progression of ripening. This clearly demonstrates that they have no role as an alternative to NAA in controlling ripening/harvest timing.

We know that jasmonate PGRs can affect the synthesis of flavour molecules in grape cells and are important in defence against herbivores. The mechanism of biosynthesis of the active form of jasmonate (the conjugate with the amino acid isoleucine) in grapes and the role of jasmonates in response to wounding (an analogue of herbivore attack) were investigated.

In summary, the use of NAA to delay the onset of ripening, and therefore harvest, seems a practical strategy to resolve some climate change-induced grape ripening issues. Where season compression causes problems for harvest and winery intake/processing, part of the vineyard could be ripening-delayed to allow harvesting at the desired stage of ripeness and allow processing and winemaking to be manageable without large increases in winery capacity.

This work was co-funded by Wine Australia and CSIRO. We would like to thank our grape and wine and agrochemical industry collaborators and collaborators from Adelaide and Verona Universities.

3. Background

Issues with sugar/flavour ripeness and harvest scheduling linked to climate change are common to most wine regions in Australia and similar trends are being observed worldwide (Mira de Orduña 2010). A study of winegrape maturity in Australia showed a significant, and widespread, trend towards early maturity in a range of both white and red cultivars (Webb *et al*. 2011). Over recent years the trend towards more rapid ripening has accelerated and is correlated with increasing air temperatures. Rapid ripening, resulting in the acceleration of sugar accumulation, can outpace the development of flavour ripeness in fruit. Our recent studies suggest that an important part of the development of 'flavour ripeness' is the gain of positive impact varietal compounds, which are often linked to increasing TSS and the loss of volatiles, and volatile precursors, that have a negative effect on wine quality and that tend to decrease with time (Boss *et al*. 2014). Longer hang times to increase flavour ripeness can lead to more dehydrated fruit which leads to reduced yield and berries with higher sugar concentration producing wines of higher alcohol content. Such increases in wine alcohol levels may have implications for human health. Higher sugar levels in berries can also increase problems with incomplete fermentation due to the resultant higher alcohol content in musts affecting yeast viability and metabolism.

Another important effect of climate change is the phenomenon of harvest season compression. The window for harvest of a particular cultivar appears to be becoming smaller as air temperatures increase (Webb *et al*. 2011) and there is more overlap between the timing of harvest ripeness in different cultivars. These effects cause difficulties in managing harvest and winery intake of fruit due to the concentration of these events over a shorter time period. A number of methods are being investigated to try to ameliorate these impacts of climate change including early/late harvesting, canopy management, e.g. late pruning, and plant growth regulator (PGR) application to delay the onset of veraison and therefore, ripening. Early harvesting means that unripe fruit, with lower yield, are fermented resulting in 'green' characters in wine that have to be 'blended away' with wine from riper fruit. The reduction of yield to try to maintain quality further impacts profitability. Late harvesting means that fruit with higher sugar levels (see above) are fermented leading to wines with 'cooked, jammy' flavours. Late pruning can delay ripening but also runs the risk of reduced harvests and when conducted later in the year, at the time when it is most effective, it can reduce productivity in the following season (Frioni *et al*. 2016).

The use of PGRs to modify the timing of berry ripening offers an attractive approach to resolving some of the issues arising from climate change. Grape berry development is a complex process, the understanding of which is important for managing the inter-relationship between yield and wine quality. Manipulating ripening is pivotal to achieving the best outcome in terms of yield, production cost efficiencies and wine 'quality' to extract maximum value in the face of difficulties arising from a changing climate. Despite its importance, berry development is still only partly understood and we need to improve our knowledge in order to successfully manipulate ripening for benefit. Various gene expression studies have shown that grape berry development is controlled through large and coordinated changes in gene expression within the berry (Cramer *et al*. 2014; Deluc *et al*. 2007; Zenoni *et al*. 2010). Around the time of ripening in particular, there are many genes whose expression levels change markedly. Changes in the concentration and/or perception of certain small, mobile molecules (PGRs and some sugars) are largely responsible for triggering these changes in gene expression. Many studies in a range of species demonstrate that PGRs control many developmental events and responses so the knowledge we gain in berries may be useful in multiple ways. Some PGRs such as auxins are at high levels early in berry development, but decrease to low levels just before veraison and act as inhibitors of the onset

of ripening. In previous work, we have shown that the auxins benzothiazole-2-oxyacetic acid (BTOA) and 1-naphthaleneacetic acid (NAA) delay ripening when applied to berries (Böttcher *et al*. 2012a; Böttcher *et al*. 2011b; Böttcher *et al*. 2010; Davies *et al*. 1997). In contrast two other PGRs, abscisic acid and castasterone, increase in concentration at veraison and can advance it (Symons *et al*. 2006; Wheeler *et al*. 2009). There are also numerous interactions between the different PGRs that affect berry development e.g. ethylene interacts with the auxin indole-3-actic acid (IAA) to affect the timing of veraison (Böttcher *et al*. 2013b). One important observation from the literature, is that the levels and perception of PGRs are tightly regulated by the plant through a range of mechanisms. One example of this is the control of the free levels of active auxins by the GH3 family of auxin conjugating amido synthetases (Böttcher *et al*. 2011a; Böttcher *et al*. 2010). There are a wide range of other PGRs and small signalling molecules that affect plants that are potential targets for further study and use in manipulating berry development.

Preliminary data suggests that it should be possible to manipulate berry growth and composition, including carbohydrate content, and wine composition (flavour and aroma metabolites) through a better understanding of the role of signalling molecules including PGRs and certain sugars. If successful, it will be possible to spread the harvest season out by delaying part of a vineyard with a PGR that delays ripening to allow sequential harvesting and winemaking. The delayed fruit will also have the benefit of ripening during a cooler part of the year that may improve fruit and wine quality and will assist not only in winery intake scheduling but should allow fruit to be harvested at the time best suited to purpose.

This project builds on our previous work which has been very successful in furthering our scientific understanding of ripening. The ability to alter ripening in a predictable way offers opportunities for increased profitability through improving grape and wine composition, and therefore their value, and by reducing winery production costs through the better management of grape intake scheduling. It is designed to provide knowledge regarding the control of the accumulation of flavour and aroma compounds and sugars in berries and how changes in particular metabolite levels alter wine composition. It will also further investigate factors controlling the timing of ripening and harvest. Our increased understanding will be used to develop tools to alter the levels of flavour and aroma compounds in relation to sugar levels in a predictable way thus improving berry and wine characteristics. This will impact on the flavour/yield relationship by allowing some flavour characteristics to be manipulated independently of yield.

4. Project Outputs and Activities

Year 1 2014-2015
A) Output: Completion of experimental work and analysis of data from 2013/2014 vintage

Activity: Complete analysis of grape and wine samples from previous season's field experiments (conducted at end of previous project). This is required because the time frame for projects allows little time at the end of the season before the end of the grant to allow the full analysis of data and wines that will not be completed until just before the end of the project. This includes analytical analysis of volatiles, sensory analysis of wine, analysis of changes in gene expression that explain the mechanism of the treatment and what processes it influences. Collate, analyse and present data.
Target date 3/11/2014

B) Output: Knowledge of auxin effects on berry development in berries with delayed ripening, samples for further detailed analyses of effects, application methods developed

Activity: Conduct field experiment to gain substantial ripening delay in Shiraz in warmer region grapes using the auxin NAA. Methods to increase delay include applying higher NAA concentration and an additional application. Measure effects of treatment on berry weight, sugar and colour accumulation, organic acid accumulation etc. Collect materials throughout experiment for later analysis. Collate, analyse and present data.
Target date 25/06/2015

C) Output: Materials for analysis of volatiles and sensory analysis of wines with ripening altered by PGR treatment

Activity: Initiate investigation of the effects of delaying ripening by NAA treatment on aroma/flavour volatile compounds (including rotundone) through GC-MS analysis. Prepare small scale wine lots, undertake sensory analysis as appropriate (to be completed in following year).
Target date 25/06/2015

D) Output: Materials for analysis of link between sugar/time/flavour metabolites

Activity: Conduct field experiments to investigate the link between sugar accumulation, hang time and the evolution of flavour and aroma volatiles. Conduct girdling experiment to alter time/sugar relationship, monitor berry development parameters, collect samples for later analysis (to be completed in following year).
Target date 25/06/2015

E) Output: Knowledge of auxin and ethylene control of ripening from transgenic plants

Activity: Investigate the precise roles and mechanism of action of auxins and ethylene by studying the effects of low auxin levels and lack of ethylene perception, respectively using transgenic plants. The plants prepared in the previous project should produce fruit in this year (if not they will in Year 2). Berry development will be monitored and samples taken and

analysed to work out the effect of the altered genotypes on the synthesis, perception and action of the relevant PGRs.
Target date 25/06/2015

F) Output: Communication of results, discussion with industry

Activity: Engage in discussions with Industry Reference Group (subject to availability but probably during Winter). Publish at least one paper in scientific journal detailing results. Attend industry and scientific conferences, as appropriate, to present work. Discuss results/proposed research with industry collaborators.
Target date 25/06/2015

Year 2 2015-2016
A) Output: Completion of experimental work and analysis of data from 2014/2015 vintage

Activity: Complete analysis of grape and wine samples from previous season's field experiments (B, C, D, E). This includes analytical analysis of volatiles, sensory analysis of wine, analysis of changes in gene expression that explain the mechanism of the treatment and what processes it influences. Collate, analyse and present data.
Target date 20/12/2015

B) Output: Knowledge of Ethrel effect in delaying ripening, samples for further investigation, application methods developed

Activity: Conduct field experiment to delay ripening significantly using the ethylene-releasing compound Ethrel. Measure effects of treatment on berry weight, sugar & colour accumulation, organic acid accumulation etc. Collect materials throughout experiment for later analysis. Collate, analyse and present data.
Target date 30/06/2016

C) Output: Knowledge of effect of Ethrel on flavour/aroma volatiles

Activity: Initiate investigation of the effects of delaying Shiraz berry ripening by Ethrel treatment on aroma/flavour volatile compounds (including rotundone) through GC-MS analysis. Prepare small scale wine lots, undertake sensory analysis as appropriate (to be completed in following year).
Target date 30/06/2016

D) Output: Knowledge of effects of other PGRs with the potential to affect ripening and flavour

Activity: Test other plant growth regulators (IAA-Asp, cytokinins, tryptophan) and combination of plant growth regulators (AVG/ABA) to alter berry development/ripening and flavour and aroma compound metabolism (testing for higher effectiveness, cheaper reagent). Collect developmental data and samples for later analysis throughout experiment (to be completed in following year).

Target date 30/06/2016

E) Output: New techniques to measure PGRs developed

Activity: Expand our ability to accurately measure a wider range of PGR classes by developing LC-MS/MS methods for the most important members of the cytokinin family of hormones that are likely to be involved in fruit set and carbon sink (sugar accumulation) determination.
Target date 30/06/2016

F) Output: Communication of results, discussion with industry

Activity: Engage in discussions with Industry Reference Group (subject to availability but probably during Winter). Publish at least one paper in scientific journal detailing results. Subject to progress publish an article in an industry journal describing study and outcomes. Attend industry and scientific conferences, as appropriate, to present work. Discuss results/proposed research with industry collaborators.
Target date 30/06/2016

Year 3 2016-2017
A) Output: Completion of experimental work and analysis of data from 2015/2016 vintage

Activity: Complete analysis of grape and wine samples from previous season's field experiments 2015/2016 (B, C, D) This includes analytical analysis of volatiles, sensory analysis of wine, analysis of changes in gene expression that explain the mechanism of the treatment and what processes it influences. Collate, analyse and present data.
Target date 30/12/2016

B) Output: Knowledge of the interacting effects that could arise from PGR application

Activity: Conduct experiments using the transgenic plants with low auxin levels and lack of ethylene perception to confirm interactions in berries between the different PGR signalling pathways. Transgenic berries will be treated with different PGRs and the effects on berry development will be monitored and samples taken and analysed to work out the extent of interaction between the various PGRs.
Target date 30/06/2017

C) Output: Information regarding the mechanism of auxins and ethylene in controlling ripening

Activity: Conduct experiments to test the importance of the ethylene-induced increase in IAA and hence IAA-Asp arising from GH3 enzyme catalysed conjugation in the control of ripening.
Target date 30/06/2017

D) Output: Further knowledge refining use of PGRs to usefully control berry development

Activity: Conduct further experiments to determine the effects of PGRs (NAA/Ethrel/cytokinins) on berry ripening/development as required and based on the outcome of the previous two seasons results. This may also include additional experiments aimed specifically at the effects related to sugar/time/yield/flavour. Collection of developmental data and berry samples, analysis of samples and collation/analysis of data.
Target date 30/06/2017

E) Output: Final project report

Activity: Collate all data from previous year's experiments, complete data analysis and interpretation of all data, prepare draft final report, develop recommendations and strategies for application of techniques developed. Communicate results to interested parties through publications, talks and discussions interested parties including the Industry Reference group. Final report with recommendations (methodologies) to be completed.
Target date Draft 30/06/2017

Variation to Output C, Year 2 2015-2016
Current Milestone

C - Output Knowledge of effect of Ethrel on flavour/aroma volatiles
Target Date 30/06/2016

C - Activity Initiate investigation of the effects of delaying Shiraz berry ripening by Ethrel treatment on aroma/flavour volatile compounds (including rotundone) through GC-MS analysis. Prepare small scale wine lots, undertake sensory analysis as appropriate (to be completed in following year).

A variation of this output was sought for the following reasons. There are interactions between ethylene (applied as Ethrel) and auxin which are important to the control of grape berry ripening and therefore harvest. Ethrel is already registered for use in wine grapes and we have shown that its application at the 'right' time can delay ripening. However, our recent experiments have shown that it is less reliable in delaying ripening in the commercial setting than the auxin NAA and the length of the delay achieved is shorter. For these reasons it was decided to concentrate more on developing the potential of NAA further. This year we have had considerable interest from Treasury Estate Wines and so we conducted a trial at a site in Eden Valley trialling the use of NAA to delay ripening in a different terroir and also using it to provide samples for further investigating the effect of NAA ripening delay on rotundone levels in berries and wine. This also has the advantage of gaining the support of one of the large companies for this work as they are searching for methods to control ripening timing.

Accepted Variation for above milestone

C - Output Knowledge of effect of NAA on berry ripening and flavour under a different terroir
Target Date 30/06/2016

C - Activity Conduct field experiment to investigate the effects of NAA in delaying Shiraz berry ripening, compare a number of different measures of berry development in Control and NAA-treated fruit. Measure the accumulation of flavour volatile compounds (including rotundone) through GC-MS analysis. The tissue will be used to track the accumulation rotundone, its putative precursors and breakdown products. Prepare small scale wine lots, undertake sensory analysis as appropriate (to be completed in following year).

Accepted variation regarding project completion

A second variation was requested and granted, 30/5/2017. This request was made because of the unusual lateness of the season meant that winemaking and sensory analysis could not be completed in the time available to complete the work.

The adjusted milestones for the new completion date (31/12/2017) are as follows:

A – Output Further knowledge refining use of PGRs to usefully control berry development
Target Date 31/12/2017

A - Activity Conduct further experiments to determine the effects of PGRs (NAA/Ethrel/cytokinins) on berry ripening/development as required and based on the outcome of the previous two seasons results. This may also include additional experiments aimed specifically at the effects related to sugar/time/yield/flavour. Collection of developmental data and berry samples, analysis of samples and collation/analysis of data.

B – Output Final Report, results communicated to relevant stakeholders.
Target Date 31/12/2017

B - Activity Collate, analyse and interpr*et al*l data from previous years' experiments.

Develop recommendations and strategies for application of techniques developed.

Communicate results through publications, talks and discussions with interested parties including the Industry Reference Group.

Prepare Final Report, including recommendations (methodologies).

5. Method

Delaying ripening/harvest in warm climate Shiraz using multiple, pre-veraison, treatments with low levels of 1-naphthaleneaceteic acid (NAA)

An experiment was conducted to test the effect of NAA in delaying ripening/harvest in a warmer climate as most of our previous studies had been based in cooler areas. Own-rooted *Vitis vinifera* L. cv. Shiraz vines in a commercial vineyard (Willunga, South Australia -35°.26, 138°.55) were used with a triplicated randomised design. Bunches (15 per replicate) were sprayed to run off three times during the pre-veraison period (Spray 1, approx. 6 WPF, 18 Dec; Spray 2, approx. 7 WPF, 26 Dec; Spray 3, approx. 8 WPF, 3rd Jan). No rain occurred for at least 24 h after treatment. Bunches were treated with either 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, Victoria, Australia, 0.5 mL/L 1N NaOH) 'Control' or with NAA 50 mg/L (Gibco BRL Life Technologies, Grand Island, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, Victoria, Australia, 0.5 mL/L 1N NaOH) 'NAA-treated'. Sodium hydroxide was included to aid solubilising the NAA. Veraison was defined as the sample date previous to the first sampling point showing a significant increase in Brix value. One-way ANOVA was performed using IBM SPSS version 20 (IBM; Armonk, NY, USA). Berries were collected at regular periods during the season, 50 berries per replicate were sampled and the average berry weight was determined. Total soluble solids (TSS) was measured by refractometer (RFM710 digital refractometer, Bellingham Stanley, Tunbridge Wells, UK) for each individual berry (50 berries per replicate, 150 in total). Sampling was completed between 1000 and 1230 h.

Testing the effects of NAA on berry development/ripening and rotundone levels in wine

NAA and Ethrel treatment of field-grown Shiraz berries

Vitis vinifera L. cv. Shiraz vines, on own roots, were grown on well-drained soil, with vertical shoot positioning trellising, at Hahndorf (Adelaide Hills, (-35º.02, 138º.84, elevation 400 m)). Limited drip irrigation was applied as required. Bunches were sprayed to run off during the pre-veraison period with NAA 50 mg/L (Gibco BRL Life Technologies, Grand Island, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, Victoria, Australia) or (2-chloroethyl) phosphonic acid as Ethrel 300 µL/L (Bayer Crop Science, East Hawthorn, Victoria, Australia) in 0.1% (v/v) Chemwet 1000 or 0.1% (v/v) Chemwet 1000 solution alone (Control). Spray dates were as follows: Control and Ethrel 16 Jan (8 days pre-veraison of Control berries) and 23 Jan (1 day pre-veraison of Control berries), NAA 23 Jan and 27 Jan (3 days post-veraison of Control berries). Veraison was defined as the sample date previous to the first sample showing a significant increase in Brix value. There was no rainfall for at least 48 h following each spray treatment. The trial was of a randomised triplicate design, the sample size per replicate was 400 bunches (1200 bunches per treatment). Samples of 60 randomly harvested berries per replicate were taken throughout development. Sampling was completed between 0930 and 1430 h. Berries were weighed and TSS were measured for each of these replicates as described below. Anthocyanins were measured at two time points, 14th Feb and at harvest (which was different for each of the treatments, the NAA-treated fruit was significantly delayed), as described below. Minimum and maximum air temperatures, light exposure and rainfall for a site located near to the vineyard (-35º.07, 138º.84) were obtained from the Australian Bureau of Meteorology (www.bom.gov.au).

Determination of anthocyanin and Total Soluble Solid levels

Frozen whole berries were ground to a powder using an IKA A11 basic analytical mill (IKA, Staufen, Germany). For the measurement of TSS (measured as degrees Brix) 100 mg of berry powder was thawed on ice, the tissue was pelleted by centrifugation at 18000 x *g* for 5 min and the supernatant was analysed with an RFM710 digital refractometer (Bellingham Stanley, Tunbridge Wells, UK). For anthocyanin determination, 300 mg of powdered sample was added to 1.5 mL of MeOH containing 1% (v/v) HCl. Anthocyanins were extracted at room temperature in the dark on a rotating mixer for one hour. The tissue was pelleted by centrifugation at 18 000 x *g* for 15 min and the supernatant retained. Depending on the developmental stage the supernatant was diluted up to 20-fold with MeOH, 1% (v/v) HCl. Total anthocyanins were measured spectrophotometrically by reading absorbance at 520 nm immediately following centrifugation. One-way ANOVA followed by Duncan's post hoc test was performed using IBM SPSS version 20 (IBM; Armonk, NY, USA).

Small scale wine making

Small scale red wine making was conducted by WIC Winemaking Services, (Urrbrae, South Australia, Australia) using the following protocol. Harvested fruit was placed at 0ºC for 12 h, the SO_2 concentrations were adjusted to 50 ppm during crushing and destemming. Yeast strain EC1118 (Lallemand, Edwardstown, South Australia, Australia) was added to 200 ppm. The ferment was conducted on skins at 18-20ºC, diammonium phosphate was added as required up to a maximum of 400 ppm. The cap was plunged 20 times twice daily. When the must reached 2ºBaume the must was pressed and then fermented to dryness and then racked. SO_2 was added to 60 ppm and the wine cold stabilised at 0ºC for 21 days. The wine was racked again and SO_2 concentrations adjusted to 80 ppm before filtering and bottling with 30 x 60 Stelvin closures (Amcor, Hawthorn, Victoria, Australia).

Sensory analysis of small scale wines

Detailed sensory profiles of all wines were generated by descriptive analysis (DA) conducted at the University of Adelaide sensory laboratory which complies with international standards for the design of test rooms (ISO 8589: 1988). The trained DA panel consisted of 12 members; eight female, four male; mean age of 36 years, ranging from 24-57 years and underwent three two-hour training sessions including evaluation of all wine samples before final assessment. The panel generated a standard list of vocabulary terms to profile the differences between the wines for appearance, aroma, palate, mouthfeel and aftertaste. Reference standards (Table 1) were developed to help clarify some of the aroma sensory attributes and ensure full agreement across assessors.

Table 1. Composition of sensory reference standards used to define aroma[a].

Attribute	*Composition*
Dark Fruit	1 tsp plum jam (Granny's Secret Recipe; Discolls)
Fresh Red Berry	1 fresh raspberry 1/6th of a fresh strawberry
Red Confectionery	1/3rd Strawberry and Cream lolly (Allen's), 5% raspberry cordial (Woolworths)
Dark Confectionery	1 g grape flavoured bubble gum (Hubba Bubba), 1 mL blackcurrant fruit juice syrup (Ribena)
Floral	1/3rd of a Parma Violet lolly (Swizzels Matlow)
Green	1/3rd gum tree leaf, 1g grape tendrils
Pepper	0.03g of ground black pepper (Saxa)
Earthy	1/4 tablespoon of soil from the Waite Campus
Dusty	Hessian fabric (4 x 1cm^2), 2.3g chalk

[a] All standards were prepared in 30 mL of Shiraz bag in box wine from Yalumba Wine Company, Angaston, Australia.

DA final assessment was carried out in triplicate in individual booths with panel members tasting up to 14 samples per day. Panellists received a sample volume of 30 mL served at 21ºC in 214 mL standardised tasting wine glasses (ISO 3591:1977). Each wine glass was covered with a watch glass to prevent headspace loss and samples were poured immediately before serving to the assessor. Samples were blind-coded with random three-digit codes and the order of sample assessment was randomised to account for first order and carryover effects. Processed water crackers and water were consumed between samples to minimise carryover effects and an inter-stimulus interval of at least a minute was chosen as a suitable time between samples with a five minute interval after flights of six wines. Panellists had access to, and were encouraged to use, all reference samples throughout final assessment. The experimental design was produced using the design generation package – Design Express (Qi Statistics, Reading, UK). Attributes were rated on 0-15 line scales, with indented extreme end word anchors for each descriptive term. Data were recorded and stored using the Fizz sensory data acquisition software (Biosystèmes, Couternon, France).

ANOVA was conducted to test the effects of Judge, Sample, Replicate, and all two-way interactions for each sensory attribute using a pseudo-mixed model with the Judge × Sample interaction as a denominator. IBM SPSS version 20 (IBM) was used for these analyses.

Non-targeted headspace volatile analysis

Solid-phase microextraction-gas chromatography-mass spectroscopy (SPME-GC-MS) was used to analyse the volatile constituents of wines produced from the Control and treated fruit. Aliquots of the wines were analysed at two different concentrations, 1 in 100 or 1 in 2 diluted with H_2O to a final volume of 10 mL. Three grams of NaCl was added to each SPME vial (20 mL) prior to sample addition.

The extraction and chromatographic conditions were identical to that described in Boss *et al*. (2014). The identity of detected volatiles was determined by comparing mass spectra with those of authentic standards and spectral libraries. A laboratory generated library (328 compounds) as well as the US National Institute of Standards and Technology-11 (NIST-11) and the Wiley Registry 9th Edition mass spectral libraries were used for identification purposes. Compounds were considered positively identified after matching of both mass spectra and linear retention indices (LRI) with that of authentic samples. LRI was calculated

from a compounds retention time relative to the retention of a series of n-alkanes (C_8-C_{26}). Other compounds were tentatively identified based upon comparison with mass spectral libraries and published LRI, or comparisons with mass spectral libraries alone.

The components of the samples were quantified using Chemstation (Agilent, Forest Hill, Victoria, Australia) relative to the relevant internal standard (d_{13}-hexanol; d_{11}-hexanoic acid; d_{16}-octanal; methyl nonanoate or d_3-linalool) using the peak area of an extracted ion.

The effect of applying Ethrel and NAA to bunches, on the concentration of volatiles in the headspace of the wines, was analysed by ANOVA using SPSS version 20 (IBM).

Rotundone quantification

The synthesis of rotundone and d5-rotundone was as described by Davies *et al*. (2015). The method used for the extraction and analysis of rotundone was derived from Siebert *et al*. (2008). Styrene-divinylbenzene SPE cartridges (SDB-L, 500 mg/6 mL; Phenomenex, Lane Cove, NSW, Australia) were conditioned with 10 mL *n*-pentane/ethyl acetate (4:1), followed by 6 mL methanol and then 6 mL of model wine (12% ethanol and 2 g/L potassium hydrogen tartrate, buffered to pH 3.2 with tartaric acid). A 100 mL aliquot of wine, containing 24 ng of d_5-rotundone in 100 µL ethanol as an internal standard, was loaded onto the SPE cartridge. The cartridge was then washed with 10 mL water followed by 2 mL *n*-pentane, and finally eluted with 10 mL *n*-pentane/ethyl acetate (9:1). This eluent was dried under a stream of N_2 and then re-dissolved in 1 mL ethanol. The extract was added to a 20 mL amber SPME vial (Chromacol; Biolab (Aust) Ltd., Clayton VIC, Australia) with 13 mL of aqueous tartrate buffer (2 g/L potassium hydrogen tartrate, buffered to pH 3.2 with tartaric acid) and analysed by SPME-GC-MS.

SPME-GC-MS analysis was performed using an Agilent Technologies 7890A gas chromatograph coupled to a 5975C mass spectrometer with a MPS2 autosampler (Gerstel, Mülheim an der Ruhr, Germany). A polydimethylsiloxane/divinylbenzene 65 µm fibre (Supelco, Bellefonte, PA) was immersed in the sample for 60 min at 40ºC with agitation, and then desorbed in the inlet at 240ºC for 1 min in pulsed-splitless mode. A pressure pulse of 25.0 psi was applied for 30 s and the flow was split with a total flow of 50 mL/min after 1 min. The fibre was cleaned for 4 min prior to extraction and also after desorption in a Gerstel fibre bake-out station at a temperature of 240ºC.

GC separation was performed on a 30 m ZB-Wax capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (Phenomenex). Ultra High Purity Helium was used as a carrier gas with a constant flow rate of 1.0 mL/min. The oven temperature was initially held at 80ºC for 1 min then increased to 220ºC at 3ºC/min, before increasing to 245ºC at 40ºC/min and holding at this final temperature for 10 min. The mass spectrometer transfer line was held at 250ºC. Selected ion monitoring (SIM) mode was used with m/z 223 and 218 as the selected ions for quantification of d_5-rotundone and rotundone, respectively, and m/z 147, 161 and 203 as the qualifying ions for rotundone and m/z 147, 161 and 208 as the qualifying ions for d_5-rotundone (dwell time 30 ms, electron impact 70eV). The analyses were performed in triplicate for each wine.

A Shiraz bag in box wine was selected for the preparation of the calibration curves as it was found to contain no rotundone using the above method. The wine was spiked in triplicate to give the rotundone concentrations of 0, 1.2, 6, 12, 30 and 60 ng/L and all samples were then analysed as outlined above. The calibration was linear throughout the range with a correlation coefficient of 0.9996 and relative standard deviations less than 5%. Using this method, the limit of detection for rotundone was 0.8 ng/L and the limit of quantification was 2.7 ng/L.

Testing the effect of different NAA concentrations on berry ripening in a warm climate

An experiment was conducted at Willunga (-35º.26, 138º.55) using *Vitis vinifera* cv. Shiraz vines grown on own roots to investigate the effect of higher levels of NAA on the length of veraison/harvest delay in warmer climates and the effect of any such delay on rotundone accumulation. A triplicated, random design of three adjacent rows, was used (see Fig. 1). The three treatments used were as follows: Control (0.1% (v/v) Chemwet 1000, 0.5 mL 1N NaOH/L), NAA 50 (as per Control but containing 50 mg/L NAA) and NAA 250 (as per NAA 50 but 250 mg/L NAA). Ten bunches were used for each of the NAA50 and NAA250 replicates, 15 bunches for each of the Control replicates. Bunches were sprayed until runoff at two times during the pre-veraison period i.e. 16/12/2014, 23/12/2014. No rain fell for at least 24 h after spraying. Samples were collected throughout the season, berry weights, anthocyanins and TSS were measured as described above. Control 1 fruit were harvested 17/2/15 at 25.5ºBrix, Control 2 fruit were harvested 27/2/2015 at 27.0ºBrix, Control 3 fruit were harvested at 10/3/2015 at 27.6ºBrix, NAA50 fruit were harvested 27/2/15 at 24.4ºBrix, NAA250 fruit were harvested on 10/3/2015 at 24.3ºBrix. Rotundone was measured as described above. Elemental analysis by inductively coupled plasma (ICP) analysis was conducted as follows: 1.5 g of ground berry tissue was digested overnight in 2 mL of 15N nitric acid then heated to 60ºC for 2 h with 1ºC/min ramping followed by 70ºC for 2 h and 115ºC for 4 h. 20 mL of water was then added, the tubes were inverted three times and the liquid filtered through Whatman grade 42 filter paper. The samples were then analysed by the Adelaide Analytical Services Unit, CSIRO Land and Water, Waite Campus, Adelaide, South Australia.

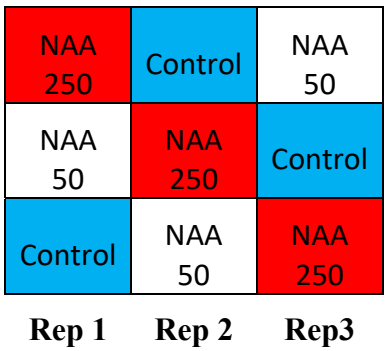


Fig. 1. Design of field experiment with Shiraz at Willunga using two concentrations of NAA with controls. Ten bunches per replicate were treated for NAA 50 mg/L (white) and 250 mg/L (red) and 15 bunches per replicate for Control (blue). Treatments are fully described above.

ICP analysis

1.5g ground berry tissue was mixed with 2ml of 70% nitric acid and left to digest overnight. The tubes were vortexed for 10 sec then placed in a heating block for 2h ramping from room temperature to 60°C at 1°C/minute. The samples were then heated at 70°C for 1 h and 115°C for 4 h. The volumes were made to 20mL with nanopure water, inverted three times to mix the contents then filtered through Whatman Grade 42 filter paper before ICP analysis by Adelaide Analytical Services (CSIRO, Urrbrae, South Australia).

Testing the effects of NAA in delaying berry ripening – different vineyard conditions

Vitis vinifera L. cv. Shiraz vines, on own roots, were grown on well-drained soil, in Eden Valley, South Australia (-34º.62, 139º.06). Limited drip irrigation was applied as required. Bunches were sprayed to run off during the pre-veraison period with NAA 100 mg/L (Gibco BRL Life Technologies, Grand Island, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, Victoria, Australia) or 0.1% (v/v) Chemwet 1000 solution alone (Control). Sprays were conducted twice, 23/12/15 and 11/01/16. There was no rainfall for at least 24 h following each spray treatment. The trial was of a randomised triplicate design (Fig. 2), three vines were used for each replicate. Samples of randomly harvested berries were taken regularly throughout development for each treatment/replicate for the measurement of berry developmental markers and biochemical analysis. Sampling was completed between 1000 and 1230 h. Berries were weighed and TSS was measured for each of these replicates using a refractometer as described above. The measurement of pH, total acid, malic acid, tartrate and YAN) was carried out by FTIR using an OenoFoss machine (Foss, Hilleroed, Denmark).

Rep 1	Rep 2	Rep 3
NAA	Control	NAA
Control	NAA	Control

Fig. 2. Design of field experiment with Shiraz at Eden Valley using 100 mg/L NAA (white) with controls (blue). Treatments are fully described in the text.

Multiple, post-veraison applications of NAA – effect on ripening and rotundone levels

This experiment was conducted using three adjacent rows of own-rooted *Vitis vinifera* L. cv Shiraz vines in a commercial vineyard (Willunga, South Australia -35°.26, 138°.55). The trial was of a randomised, triplicated design, five bunches were used per replicate. 50 mg/L NAA was applied two times post-veraison; Spray 1: 30/1/2014, at 14.8ºBrix (12 WPF), Spray 2: 6/2/2014, at 16-17ºBrix (13 WPF). Samples were taken throughout development to follow ripening progress and for rotundone measurement (see above for method).

Testing the effect of application of the precursor of indole-3-acetic acid (IAA), tryptophan, on berry development

This experiment was conducted using three adjacent rows of own-rooted *Vitis vinifera* L. cv Shiraz vines in a commercial vineyard (Willunga, South Australia -35°.26, 138°.55). The trial was of a randomised, triplicated design, each replicate consisted of 15 bunches. The Control treatment consisted of 0.1% (v/v) Chemwet 1000 and 0.5 mL 1N NaOH/L, the Trp treatment was as for the Control but included either 100 mg/L tryptophan (Spray 1) or 200 mg/L tryptophan dissolved in 0.5 mL 1N NaOH (Sprays 2 and 3). All sprays were completed pre-veraison (Spray 1, 18 Dec, Spray 2, 26 Dec; Spray 3, 3 Jan, approximately 6, 7 and 8 WPF respectively). Bunches were sprayed to runoff and no rain fell for at least 24 h after spraying.

Fifty berries were sampled at regular intervals throughout development (see Fig. 29) and berry weight and TSS were measured as described above.

Testing the effects of epigallocatechin gallate (EGCG) on berry development/ripening

To test the possibility that epigallocatechin gallate (EGCG, found at quite high purity in a very affordable green tea extract, sourced from Bulkactives Keelung City, Taiwan) could delay ripening the following experiment was conducted using three adjacent rows of own-rooted *Vitis vinifera* L. cv Shiraz vines in a commercial vineyard (Willunga, South Australia -35°.26, 138°.55). IAA (100 mg/L dissolved in 0.5 mL 1N NaOH with 0.1% (v/v) Chemwet 1000) was included to use as a control and to retest the effectiveness of the common form of auxin in plants. The EGCG treatment consisted of 1 g/L, 0.1% (v/v) Chemwet 1000, 0.5 mL/L 1N NaOH. A treatment combining both IAA and EGCG was also included. The trial was of a randomised, triplicated design (Fig. 3), each replicate consisted of 10 bunches. The Control bunches were sprayed with 0.1% Chemwet 1000 (v/v), 0.5 mL/L 1N NaOH. Bunches were sprayed to runoff, no rain fell for at least 24 h after spraying.

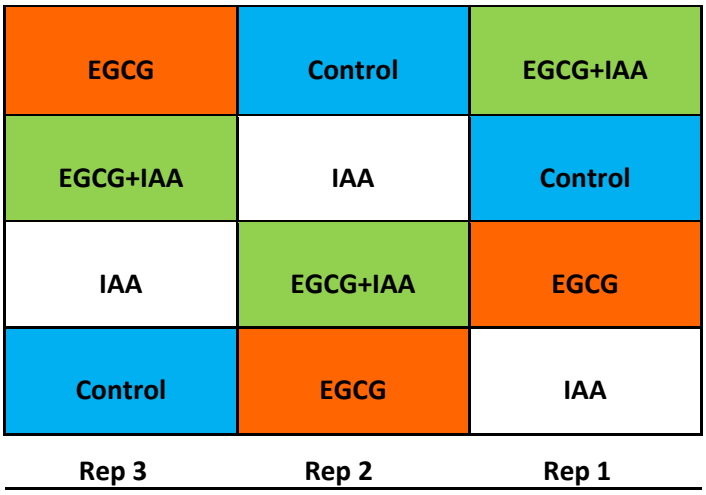


Fig. 3. Design of field experiment with Shiraz at Willunga comparing the effects of IAA (white) and EGCG alone (orange) and in combination (green), on berry ripening. Ten bunches were used for each treatment. Treatments are fully described in the text.

The metabolism and role of jasmonic acid-isoleucine (JA-Ile) formation in grape berry development

Berry developmental series and grapevine organ collection

To examine developmental changes in gene expression and jasmonate levels, *Vitis vinifera* L. cv. Shiraz berries were collected at weekly intervals as described by Böttcher *et al*. (2013b). In addition to deseeded berries from 3-16 WPF, very young berries (1-2 WPF, containing seeds) were included in this study.

Tissues used for gene expression studies in various grapevine organs were collected from Shiraz plants as follows: roots were harvested from potted canes grown in the glasshouse (CSIRO Agriculture, Adelaide, South Australia), internodes (fourth from growing tip) and tendrils (at third node from growing tip) were sampled from vines grown at the Waite Coombe Vineyard (Adelaide, South Australia, -34°.97, 138°.63) in the 1999/2000 season,

open flowers, node 1, 5 and 9 leaves and seeds from berries (60) 5, 9 and 14 WPF were collected from a commercial vineyard (Willunga, South Australia -35°.26, 138°.55) in the 2010/2011 (seeds) and the 2013/2014 season (flowers and leaves). All samples were immediately frozen in liquid nitrogen.

MeJA treatment of grapevine leaves

Leaves at nodes 4-7 on all growing shoots of mature, potted Shiraz vines grown in a shade house (CSIRO Agriculture, Adelaide, South Australia) were sprayed to run-off with 100 µM methyl jasmonate (MeJA, Sigma-Aldrich, St. Louis, MO, USA) in 40% (v/v) acetone, 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, VIC, Australia) or a Control solution (40% (v/v) acetone, 0.1% (v/v) Chemwet 1000). Three, replicate, vines were used for each treatment and three treated leaves/replicate were randomly sampled at the indicated time points. Just prior to the treatments two leaves were collected from each plant and combined to serve as the 0 h time point. All leaves were immediately frozen in liquid nitrogen and stored at -80°C until use for RNA extractions.

Wounding of grapevine leaves

Node 6 leaves on growing shoots of Shiraz vines from a commercial vineyard (Hahndorf, South Australia, -35°.02, 138°.84) were wounded by crushing the lamina across the mid-vein with forceps, achieving 15-20% of damaged leaf area. The wounded leaf and the unwounded, distal leaf (node 5) were harvested at the indicated time points (three shoots/replicate, three replicates/time point), immediately frozen in liquid nitrogen and stored at -80°C until use for RNA and jasmonate extractions.

Cloning, expression and *in vitro* activity assay of putative JA-conjugating GH3 proteins from grapevine

The coding regions of *VvGH3-7* and *VvGH3-9* were amplified by PCR from a Shiraz berry cDNA template using gene-specific primers. Heterologous expression and purification of VvGH3-7 and VvGH3-9 containing a His6-tag fused to the C-terminus were performed as described by Böttcher *et al*. (2012b; 2010).

The thin layer chromatography (TLC)-based assays for JA-amino acid conjugate formation were performed as described previously (Böttcher *et al*. 2010), except that the acyl substrates used were (±)-JA (Sigma-Aldrich, St. Louis, MO), *cis*-12-oxo-phytodienoic acid (*cis*-OPDA, OlChemIm Ltd., Olomouc, Czech Republic), *cis*-dnOPDA (OlChemIm Ltd., Olomouc, Czech Republic), salicylic acid (SA, Sigma-Aldrich, St. Louis, MO, USA), or IAA (Sigma-Aldrich, St. Louis, MO) and the reaction volume was 50 µL. TLC plates were stained with vanillin reagent (6% (w/v) vanillin (Sigma-Aldrich, St. Louis, MO, USA), 1% (v/v) sulfuric acid in ethanol) or, in the case of IAA, with Ehmann's reagent as described by Böttcher *et al*. (2010). Further confirmation of the identity of reaction products via LC-MS was done as described by Böttcher *et al*. (2010).

RNA extraction, cDNA synthesis and qRT-PCR

RNA extraction, cDNA synthesis and qRT-PCR were performed as described previously (Böttcher *et al*. 2013b) with the following modifications: qRT-PCR analyses using cDNAs from the grapevine tissue series and the leaf wounding experiment were performed in 7.5 µL reaction volumes with a final primer concentration of 0.5 µM. Each PCR was performed in triplicate with the exceptions of the tissue series (four replicates) and the leaf wounding samples (two replicates). The gene-specific primer pairs and corresponding accession numbers used for *ACT2* (actin reference gene) have been published previously (Böttcher *et al*. 2011a). The oligonucleotide primers used in this study for putative JA-conjugation and -biosynthesis genes were designed for *VviGH3-7* (GenBank: XM_002272524), *VviGH3-9* (GenBank: XM_002280702), *VviOPR3* (GenBank: NM_001281046), *VviAOS* (GenBank XM_002283744).

Extraction and quantification of JA and JA-Ile

For the quantification of JA and JA-Ile, 100 mg of grape, berry or leaf, tissue was extracted in 1 mL of 60% (v/v) isopropanol, 2.5 mM diethyldithiocarbamic acid (DDC), spiked with 250 pmol of d5-(±)-JA (CDN Isotopes, Point-Claire, Quebec, Canada) and 12.5 pmol of d2-(-)-JA-Ile (OlChemIm Ltd., Olomouc, Czech Republic) as internal standards, for 2.5 h at 4°C on a rotating mixer. After the tissue was pelleted by centrifugation at 4°C, the supernatant was removed and kept at 4°C, while the pellet was re-extracted in 1 mL of 60% (v/v) isopropanol, 2.5 mM DDC for 1 h at 4°C. Following centrifugation, the supernatant was combined with the initial extract, the organic solvent was removed *in vacuo* and the aqueous phase was acidified to pH 2 and applied to a 100 mg C18 SPE column (Waters, Wexford, Ireland). The column was washed with water pH 2 (1 mL) and then eluted with 80% (v/v) MeOH, 1% (v/v) acetic acid (2.5 mL). The dried residue was resuspended in 50 µL 60% (v/v) MeOH, 1% (v/v) acetic acid to be analysed with an Agilent liquid chromatography-mass spectrometry (LC-MS) system (1200 series HPLC coupled with a 6410 triple quad mass spectrometer). The sample (10 µL) was first separated on a Luna C18 column (75 x 4.6 mm, 5 µm, (Phenomenex, Torrance, CA)) held at 30°C using the following solvent conditions: 0-10 min, linear gradient from 60% (v/v) to 95% (v/v) MeOH, 0.05% (v/v) acetic acid, held for 5 min, linear gradient from 95% (v/v) to 60% (v/v) MeOH, 0.05% (v/v) acetic acid in 1 min, held for 6 min, 0.4 mL/min. The effluent was introduced into the ESI ion source (nebuliser pressure 35 psi) with a desolvation gas temperature of 300°C at a flow of 8 L/min, with the capillary voltage set to 4 kV. The detection was performed by multiple reaction monitoring, first in negative ion mode (0-8 min), then in positive ion mode (9-12 min). The optimisation of fragmentation was done with (±)-JA, (-)-JA-Ile (OlChemIm Ltd., Olomouc, Czech Republic) as well as the labelled standards using the Agilent MassHunter Optimizer software (version B03.01). With the collision energy ranging between 4-12 eV, the following main transitions were used for quantitation: d5-JA 214>62, JA 209>59, d2-JA-Ile 326>280, JA-Ile 324>278. The concentrations of JA and JA-Ile in the extracts were quantified in relation to their internal standards using calibrations curves that had been generated as follows: 50 µM stocks were used to prepare seven standard solutions (500 nM-20 µM for JA and 50 nM-2 µM for JA-Ile) and 50 µL of each standard solution was mixed with 250 pmol of d5-JA and 12.5 pmol of d2-JA-Ile (in triplicate). Samples were dried *in vacuo* and resuspended in 50 µL of 60% (v/v) MeOH, 1% (v/v) acetic acid resulting in internal standard concentrations of 5 pM (d5-JA) and 0.25 pM (d2-JA-Ile) in each sample. A 10 µL-aliquot of each samples was subjected to an LC-ESI-MS/MS analysis as described above and calibration curves were generated using the Agilent Quantification software (version B04.00) by plotting the known concentration of each unlabelled compound against the ratio of analyte peak area to corresponding internal standard peak area.

Statistical data analysis

Differences in gene expression due to MeJA treatment were tested at each time point using Student's t-test. Significant changes in the expression of genes or jasmonate concentrations in response to wounding over time were identified by analysis of variance (ANOVA) followed by Duncan's post hoc test. ANOVA was performed for the hormone concentrations and gene expression data collected from the berry development samples, followed by Fisher's Least Significant Difference (LSD) post hoc test to test for significant differences. Statistical testing of the various datasets was conducted using IBM SPSS Statistics ver. 20 (IBM Australia, Sydney, NSW, Australia).

Cytokinin metabolism during grape berry development/ripening

Plant material

To determine developmental changes in the expression of cytokinin-related genes and cytokinin levels, *Vitis vinifera* L. cv. Shiraz berries from a commercial vineyard were collected at weekly intervals as described by Böttcher *et al.* (2015). All tissues used for gene expression studies in various grapevine organs were collected as described by Böttcher *et al*. (2015). In addition to the Shiraz berry series, cytokinin measurements were also taken from the following samples: 1) *Vitis vinifera* L. cv. Cabernet Sauvignon and cv. Riesling, grown at a commercial vineyard (Waikerie, South Australia; -34.100°, 139.842°) and sampled every two weeks as described by Kalua and Boss (2009; 2010). Seeds were removed from frozen berries prior to grinding and cytokinin extraction. 2) *Vitis vinifera* L. cv. Pinot Noir berries, grown at a commercial vineyard (Willunga, South Australia; -35.263°, 138.553°) and sampled as in 1), but retaining the seeds. 3) Grapes of similar sugar content (19.4-20.8°Brix) collected from 13 grapevine species (11 *Vitis vinifera*, one *Vitis* hybrid and one interspecific hybrid) grown at the Waite Coombe Vineyard (Adelaide, South Australia; -34.263°, 138.553°) in the 2013/2014 season. Juice from individual berries (10 berries per replicate, three replicates) sampled from six bunches across two vines was tested for total soluble solids using a PAL-1 digital refractometer (Atago, Tokyo, Japan), followed by immediate deseeding and freezing in liquid nitrogen of berries within the above specified sugar content range. 4) Tomatoes (*Solanum lycopersicum* Mill. var. Moneymaker) grown from seed in the glasshouse (CSIRO Agriculture, Adelaide, South Australia) and harvested at five standard ripening stages as detailed by Böttcher *et al*. (2010). 5) Strawberries (*Fragaria ananassa* Duch. cv. Ablion) at four different ripening stages (small green, large green, turning, red ripe), sampled at a commercial strawberry farm (Hahndorf, South Australia; -35.038°, 138.816°). A minimum of five strawberries per stage was used for each biological replicate. For a second set of samples, achenes were removed with tweezers prior to freezing in liquid nitrogen.

Phylogenetic analysis

Grapevine sequences belonging to five families of proteins involved in the biosynthesis, activation, perception, signalling and degradation of cytokinins were identified by BLASTP searches of the non-redundant NCBI protein database (http://www.ncbi.nlm.nih.gov/) using the respective Arabidopsis sequences as queries. Phylogenetic analyses were conducted using the corresponding nucleotide sequences in MEGA6.06 (Tamura *et al*. 2013) as follows: The Arabidopsis and grapevine nucleotide sequences for each gene family were aligned using MUSCLE (Edgar 2004), all positions containing gaps and missing data were eliminated. The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al*. 1992). A bootstrap consensus tree was generated from 100 replicates (Felsenstein 1985) and branches corresponding to partitions replicated in less than 70% replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log value. The naming of grapevine genes followed the guidelines published by Grimplet *et al*. (2014).

RNA extraction, cDNA synthesis and qRT-PCR

RNA extraction, cDNA synthesis and qRT-PCR were performed as described previously (Böttcher *et al*. 2013b) with modifications as described by Böttcher *et al*. (2015). The gene-specific oligonucleotide primers and corresponding accession number used for *ACT2* (actin reference gene) have been published previously (Böttcher *et al*. 2011a). Gene expression data was analysed using the MeV software (version 4.9; http://www.tm4.org/mev/) and presented as heat maps with hierarchical clustering.

Extraction and quantification of nucleobase cytokinins

For the quantification of iP and *t*Z, 100 mg of fruit tissue was extracted in 1 mL of 70% (v/v) ethanol, 0.2 mM diethyldithiocarbamic acid, spiked with 5 pmol of d6-isopentenyl adenine (d6-iP) and d5-trans zeatin (D5-*t*Z, OlChemIm Ltd., Olomouc, Czech Republic) as internal standards, for 2 h at 4°C on a rotating mixer. After the tissue was pelleted by centrifugation at 4°C, the supernatant was removed and kept at 4°C, while the pellet was re-extracted in 1 mL of 70% (v/v) ethanol, 0.2 mM diethyldithiocarbamic acid for 1 h at 4°C. Following centrifugation, the supernatant was combined with the initial extract, the organic solvent was removed *in vacuo* and the aqueous phase was adjusted to pH 7.5 (NaOH) and applied to a 100 mg C18 SPE column (Waters, Wexford, Ireland). The column was washed with water pH 7.5 (2 mL) and then eluted with 80% (v/v) MeOH, 2% (v/v) acetic acid (2.5 mL). The dried residue was re-suspended in 50 µL 90% (v/v) 15 mM formic acid, adjusted to pH 4.0 with ammonia, 10% (v/v) methanol to be analysed with an Agilent LC-MS system (1200 series HPLC coupled with a 6410 triple quad mass spectrometer). The sample (10 µL) was first separated on a Luna C18 column (75 x 4.6 mm, 5 µm, (Phenomenex, Torrance, CA)) held at 30°C using the following solvent conditions: 0-20 min, linear gradient from 10% (v/v) MeOH, 90% 15 mM formic acid, adjusted to pH 4.0 with ammonia to 95% (v/v) MeOH, 5% (v/v) 15 mM formic acid, adjusted to pH 4.0 with ammonia, held for 5 min, linear gradient from 95% (v/v) to 10% (v/v) MeOH in 1 min, held for 6 min, 0.4 mL/min. The effluent was introduced into the ESI ion source (nebuliser pressure 35 psi) with a desolvation gas temperature of 300°C at a flow of 8 L/min, with the capillary voltage set to 4 kV. The detection was performed by multiple reaction monitoring in positive ion mode. The optimisation of fragmentation was done with iP, *t*Z (Sigma-Aldrich, St. Louis, MO, USA) as well as the labelled standards using the Agilent MassHunter Optimizer software (version B03.01). The following main transitions were used for quantitation: d6-iP 210 > 137, iP 204 > 136, d5-*t*Z 225 > 137, *t*Z 220 > 136. In addition, a qualifier ion transition was included for each compound: d6-iP 210 > 148, iP 204 > 148, d5-*t*Z 225 > 119, *t*Z 220 > 119. The sensitivity of the analysis was enhanced by monitoring d5-*t*Z and *t*Z in a different retention window (0-15 min) to d6-iP and iP (15-22 min). The concentrations of iP and *t*Z in the extracts were quantified in relation to their internal standards using calibration curves that had been generated as follows: 50 µM stocks were used to prepare eight standard solutions (1 nM-500 nM) and 50 µL of each standard solution was mixed with 5 pmol of d6-iP and d5-*t*Z (in triplicate). Samples were dried *in vacuo* and resuspended in 50 µL of 90% (v/v) 15 mM formic acid, adjusted to pH 4.0 with ammonia, 10% (v/v) methanol resulting in internal standard concentrations of 100 nM each. A 10 µl-aliquot of each samples was subjected to an LC-ESI-MS/MS analysis as described above and calibration curves were generated using the Agilent Quantification software (version B04.00) by plotting the known concentration of each unlabelled compound against the ratio of analyte peak area to corresponding internal standard peak area. The limits of detection (signal-to-noise ratio > 3) gained from the calibration curves were 0.2 fmol/µL for *t*Z and 0.08 fmol/µL for iP, the limits of quantification (signal-to-noise ratio > 10) were 0.67 fmol/µL for *t*Z and 0.25 fmol/µL for iP.

Statistical data analysis

Significant differences in TSS contents and cytokinin concentrations were identified by analysis of variance (ANOVA) followed by Duncan's post hoc test. ANOVA was performed for the gene expression data from the Shiraz berry development samples, followed by Fisher's Least Significant Difference (LSD) post hoc test to test for significant differences. Statistical testing of the various datasets was conducted using IBM SPSS Statistics ver. 20 (IBM Australia, Sydney, NSW, Australia).

Post-veraison cytokinin application to the low cytokinin variety Pinot Noir

A triplicated, random design experiment, using three adjacent rows (Fig. 4) was developed to test the possible effects of cytokinin application to a 'low' berry cytokinin cultivar, Pinot Noir, on wine volatile formation. The naturally occurring cytokinin iP was sprayed onto bunches to run off at a concentration of 100 mg/L (also containing 0.1% Chemwet, 0.5 mL NaOH/L). The berries were sprayed on 12/1/2015 and were at 18.9°Brix when treated. No rain fell for at least 24 h after spraying. Samples were taken at 0, 1, 24 h after treatment and prior to commercial harvest (29/1/2015 at approximately 23°Brix). Brix, pH, yeast available nitrogen (YAN), primary amino nitrogen (PAN) and glucose/fructose were measured by FTIR as described above. Small scale wines were made and headspace volatile analysis conducted on the harvest samples as described above.

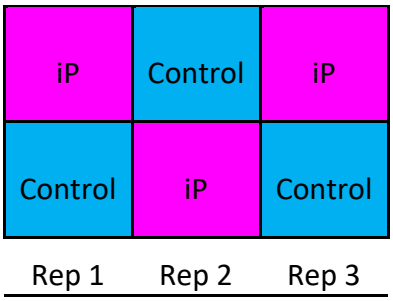


Fig. 4. Design of field experiment with Pinot Noir at Willunga comparing the effects of iP (pink) and Control (blue) on berry ripening. Treatments are fully described in the text.

Pre-veraison application of auxin and cytokinin PGRs, alone and in combination, to Shiraz grape berries

Shiraz vines at Langhorne Creek (-35.367, 139.009) were used for this experiment to test the effect of auxin and cytokinin PGRs on the progression of ripening and berry composition. A triplicated random design was used for the six treatments (Fig. 5). Fifteen bunches over two vines were used for each replicate. The treatments were: Control: 1 mL/L 1N NaOH in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, Victoria, Australia); NAA: NAA 100 mg/L (Gibco BRL Life Technologies, Grand Island, USA), dissolved in 1 mL 1N NaOH in 0.1% (v/v) Chemwet 1000; IAA: 100 mg/L IAA (Sigma-Aldrich) prepared as for 'NAA'; 4-Cl-IAA: 100 mg/L 4-chloroindole-3-acetic acid (4-Cl-NAA, Toronto Research Chemicals, Ontario, Canada) prepared as for 'NAA'; BA: 100 mg/L 6-Benzylaminopurine (BA, Sigma-Aldrich) prepared as for 'NAA'; NAA+BA: 100 mg/L each of NAA and BA prepared as for 'NAA'. Bunches were sprayed to run off at two time points (14/12/20015 and 21/12/2015, approximately 6 and 7 WPF respectively) during the pre-veraison period. No rain fell for at least 24 h following spraying.

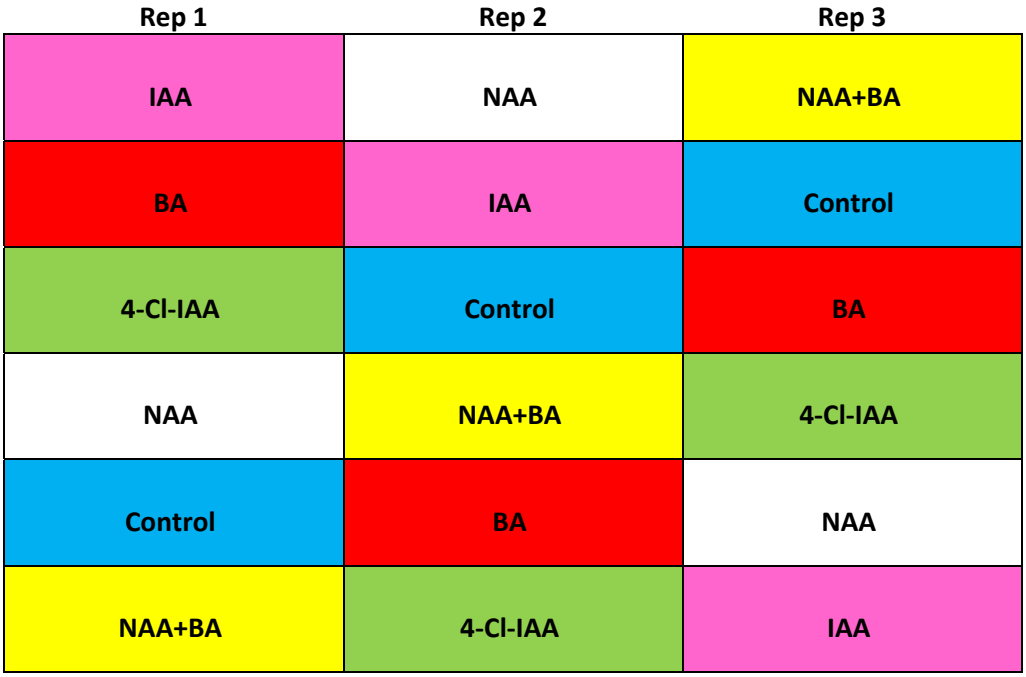


Fig. 5. Design of field experiment with Shiraz at Langhorne Creek comparing the effects of different auxins, cytokinins and a combination of an auxin and a cytokinin on berry ripening. Control (blue), IAA (pink), NAA (white), BA (red), 4-Cl-IAA (green), NAA+BA (yellow). 15 bunches were used for each replicate. Treatments are fully described in the text.

Four berries per bunch were sampled at regular intervals throughout the season. The following measurements were done for all samples: berry weight, pH as measured by pH electrode, anthocyanins by spectroscopy (see above), Brix by refractometry (see above). From and after 14/1/2016 the samples were also assayed by FTIR (OenoFoss analyser) for other parameters including YAN and organic acid content.

Testing the effects of natural and synthetic cytokinins on the timing and progression of ripening

Vitis vinifera L. cv. Cabernet Sauvignon vines, grown on own roots, at Seppeltsfield, Barossa Valley (-34.496, 138.892) were used for this study. Limited drip irrigation was applied as required to these vines whose rows ran roughly North/South. A triplicated, randomised design was used (Fig. 6) Five treatments were applied: Control = 0.1% (v/v) Chemwet 1000, 2 mL EtOH/L. iP and BA = 20 mg taken up in 2 mL ethanol added to 1 L of 0.1% (v/v) Chemwet1000. Prestige, 1/500 dilution in water, no wetting agent or ethanol added, final concentration of active ingredient 20 mg/L. Thidiazuron 500, 1/25000 dilution in 0.1% (v/v) Chemwet1000 containing 2 mL ethanol/L final concentration of active ingredient 20 mg/L. Bunches were sprayed twice during the pre-veraison period, i.e. 5/01/2017 (42 DPF) and 17/01/2017 (54 DPF). 16 bunches were used for each replicate. Samples were taken regularly throughout ripening, 4 berries/bunch, 64 in total, which were then analysed for berry weight and Brix and for other parameters by FTIR (OenoFoss analyser).

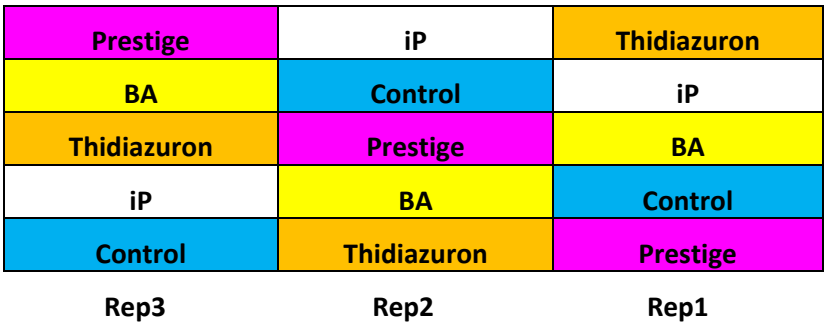


Fig. 6. Design of field experiment with Cabernet Sauvignon in Barossa Valley comparing the effects of different cytokinins (20 mg/L), both synthetic (commercial preparations) and naturally occurring, on berry ripening. Control (blue), Prestige (pink), iP (white) Thidiazuron (orange), BA (yellow). Treatments are fully described in the text.

6. Results

Delaying ripening/harvest in warm climate Shiraz using multiple, pre-veraison, treatments with low levels of 1-naphthaleneaceteic acid (NAA)

Previous studies (Wine Australia report CSP 0905, (Böttcher and Davies 2012; Davies and Böttcher 2009) have shown that PGRs can be used to manipulate the timing of veraison and therefore, harvest. One useful outcome of an ability to manipulate ripening would be to reduce the problems with winery intake scheduling issues that result from compressed harvest seasons. The auxin NAA in particular has proven to be effective in delaying ripening (Böttcher *et al*. 2012a; Böttcher *et al*. 2011b; Böttcher *et al*. 2010). Much of the work so far has been done under cooler climate conditions where berry development takes longer than in warmer regions which makes these experiments easier to conduct at a technical level. As delaying ripening may be more commercially useful in warmer regions, this experiment was conducted at Willunga to confirm previous work and to test if repeated pre-veraison treatments with low levels of NAA might provide a long enough ripening delay to help manage fruit intake. To this end bunches were treated three times prior to veraison using a low dose of auxin, 50 mg/L NAA.

Figure 7 shows that the repeated NAA treatment delayed the rapid phase of berry weight increase by approximately 14 days. Weight of the Control berries increased rapidly after 21 days post initial spray (DPIS) while for NAA fruit it was only after 35 days that a similar rate of increase was observed. Berry weight is dependent on seed number (total seed weight in fact), which is quite variable and other factors including soil conditions, water supply and canopy status can also affect berry size. As only 50 berries were sampled per replicate this has meant that the variability within each replicate was quite high. For this reason, there was only one time point (49 DPIS) when there was a significant difference detected, (at the $p<0.05$ level), by ANOVA between the Control and NAA-treated berries. Although the NAA berries appeared to weigh more at harvest this difference was not statistically significant.

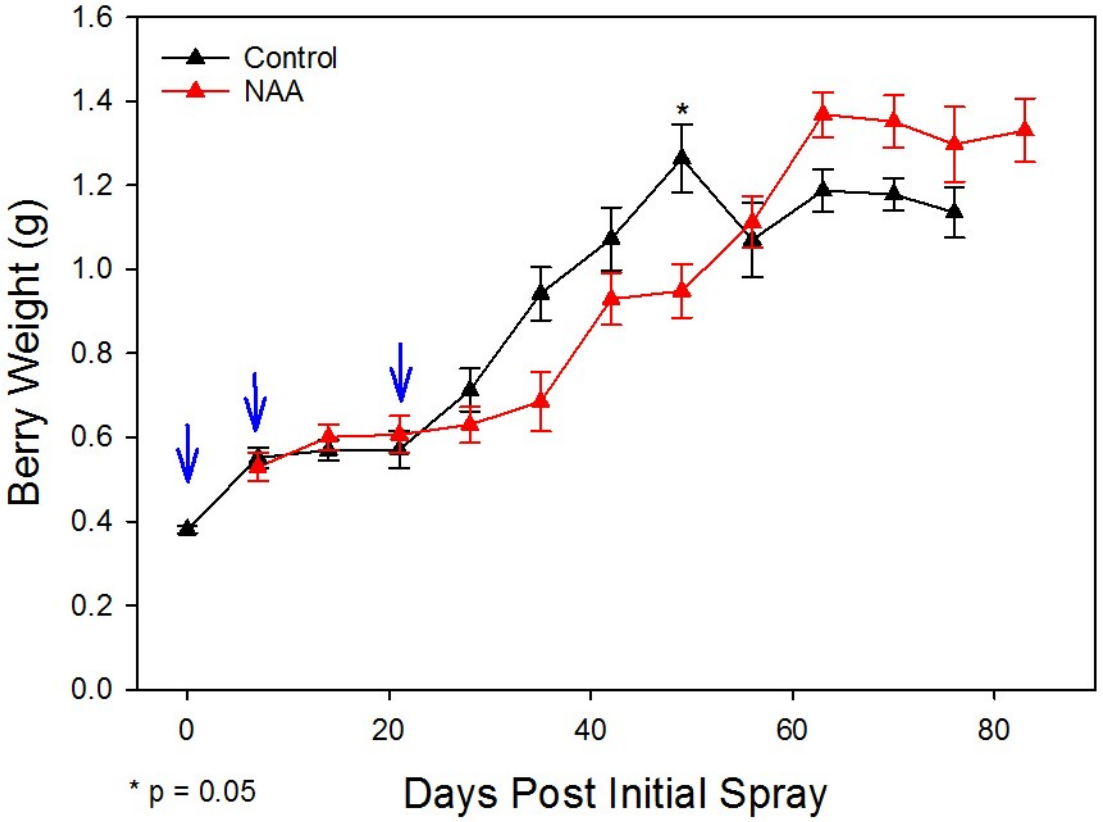


Fig. 7. Average weights (g) of Control (black line and triangle) and NAA-treated (red line and triangle) berries. The blue arrows indicate the timing of the NAA treatments (18 Dec, 26 Dec, 3 Jan). The asterisk indicates the single time point with a significant difference between Control and NAA-treated fruit as determined by ANOVA (p<0.05)

Measuring total soluble solids (Brix) is frequently used to gauge the stage of berry ripening and virtually all of the increases in Brix during ripening are due to increases in the hexoses glucose and fructose. Generally, Brix measurements of individual berries are much less variable than, measurements of berry weight, probably because sugar accumulation is largely unaffected by seed number. Due to less variability the Brix data showed a very clear and statistically significant difference between the TSS accumulation profile of the Control and NAA fruit from 28 DPIS (Fig. 8). TSS accumulation was delayed by NAA treatment. After 21 DPIS the Control berries had higher TSS levels at all but one time point, only at the final harvest point did the NAA-treated fruit 'catch up'. The rate (slope of the line) of TSS accumulation was much slower in the NAA fruit between 21 and 56 DPIS.

Taken together the delay in berry weight increase and sugar accumulation in NAA-treated fruit show that the repeated treatments with low concentrations of active ingredient were effective in delaying berry veraison. This delay in the initiation in ripening was translated into a delay of approximately 14 days at harvest. Being able to delay fruit maturity by two weeks should be helpful in managing grape harvest/winery intake in compressed harvest seasons that are now regularly encountered due to the effects of climate change.

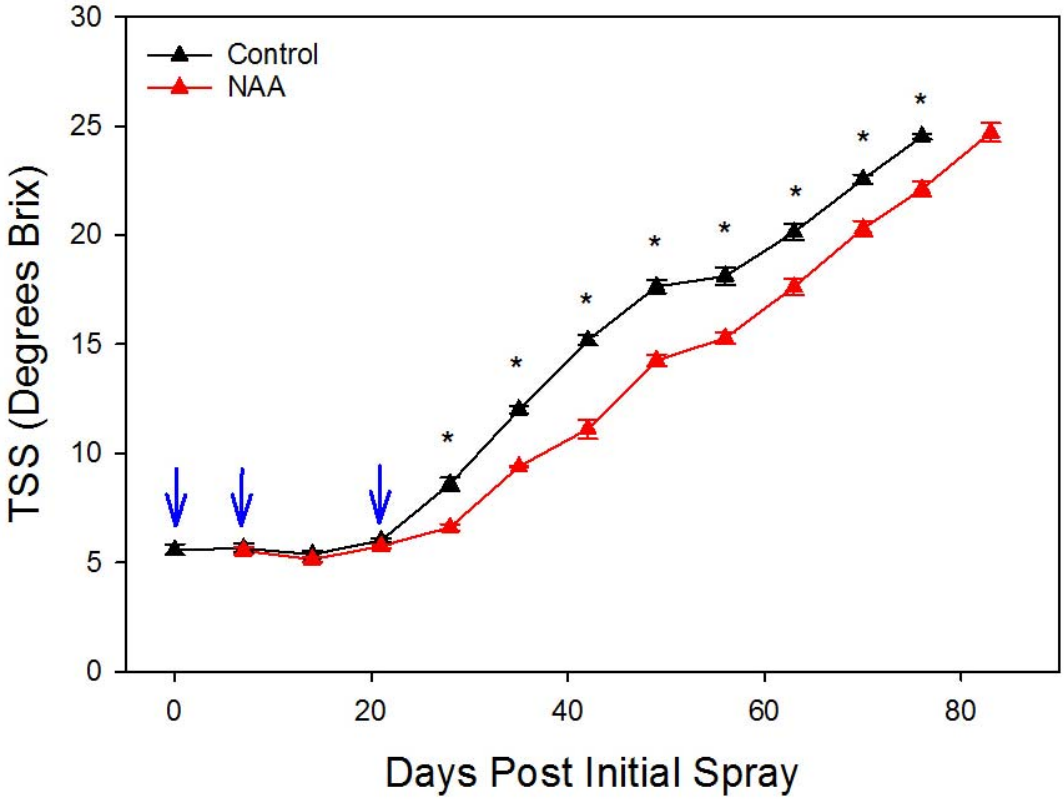
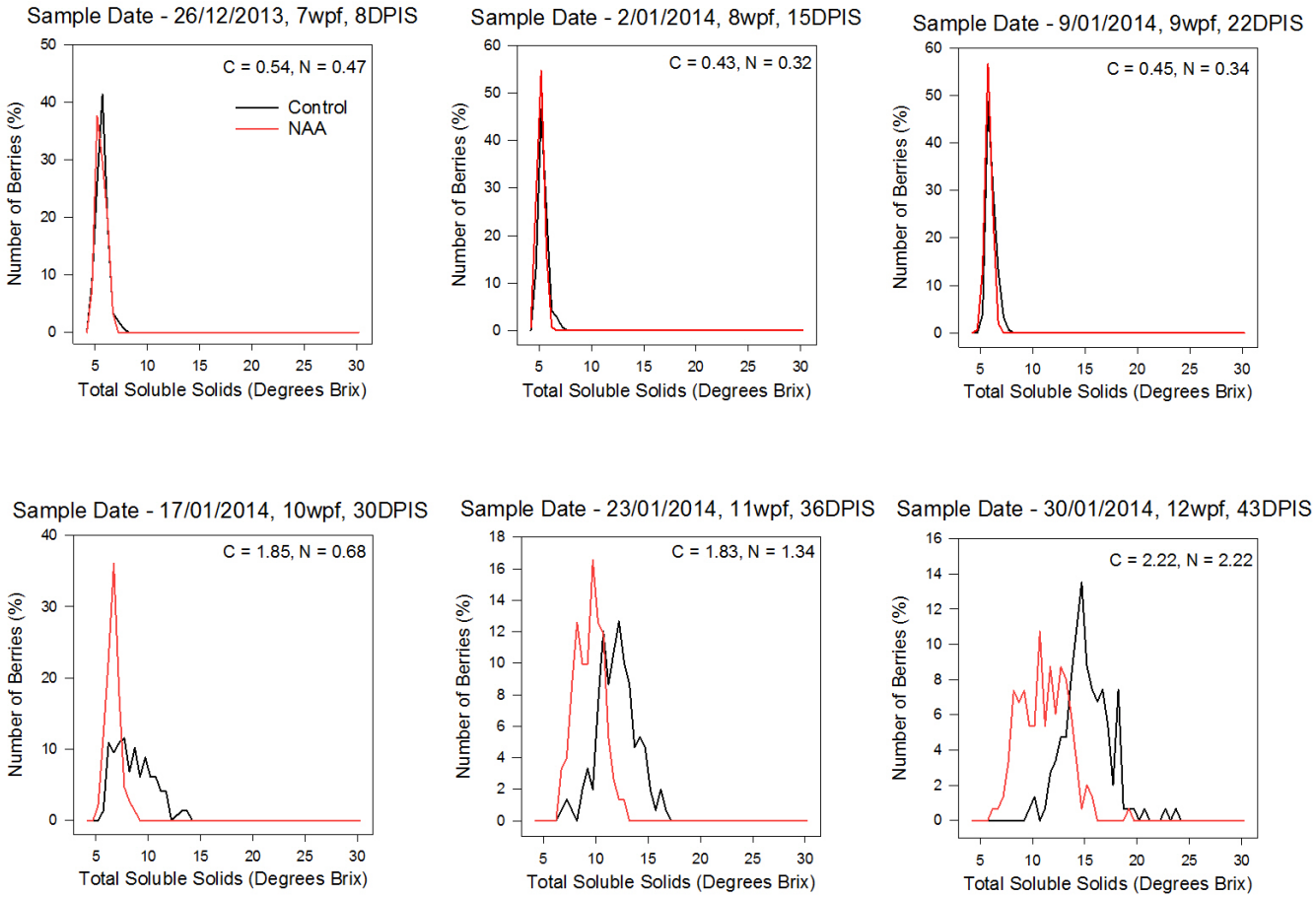


Fig. 8. Average TSS levels (degrees Brix) of Control (black line and triangle) and NAA-treated (red line and triangle) berries. The blue arrows indicate the timing of the NAA treatments. The asterisks indicate time-points when there is a significant difference between Control and NAA-treated fruit as determined by ANOVA ($p<0.05$).

Previous work has indicated that the NAA ripening-delaying treatments discussed above may also have an effect on the synchronicity of berry ripening (Böttcher *et al*. 2012a; Böttcher *et al*. 2010). Fig. 9 shows the individual berry Brix data plotted for both Control and NAA-treated berry populations. This data allows us to determine the 'shape' of the population as defined by Brix values. This in turn gives a measure of the degree of synchronicity of ripening. As can be seen from the graphs the Control berries began ripening first, the black 'curve' moves to the right of the graph. As ripening begins the peak spreads, demonstrating an unevenness in the initiation and/or progression of ripening. The NAA-treated fruit then began to ripen and followed a similar course to the Control berries. The standard deviation gives a measure of the synchronicity of the population. For nine out of 11 time points (Fig. 9) the standard deviation for the NAA fruit was lower than that for the Control fruit indicating that the NAA treatments had reduced variation within the population during most of ripening.

Fig. 9. Individual berry Brix data plotted for Control (black line) and NAA-treated (red line) berries. The Brix value for approximately 150 individual berries for each treatment at each time point was assigned into Brix classes of 0.5°Brix increments for plotting. The standard deviation for the populations at each time point is given at the top of the panels. DPIS = days post initial spray.



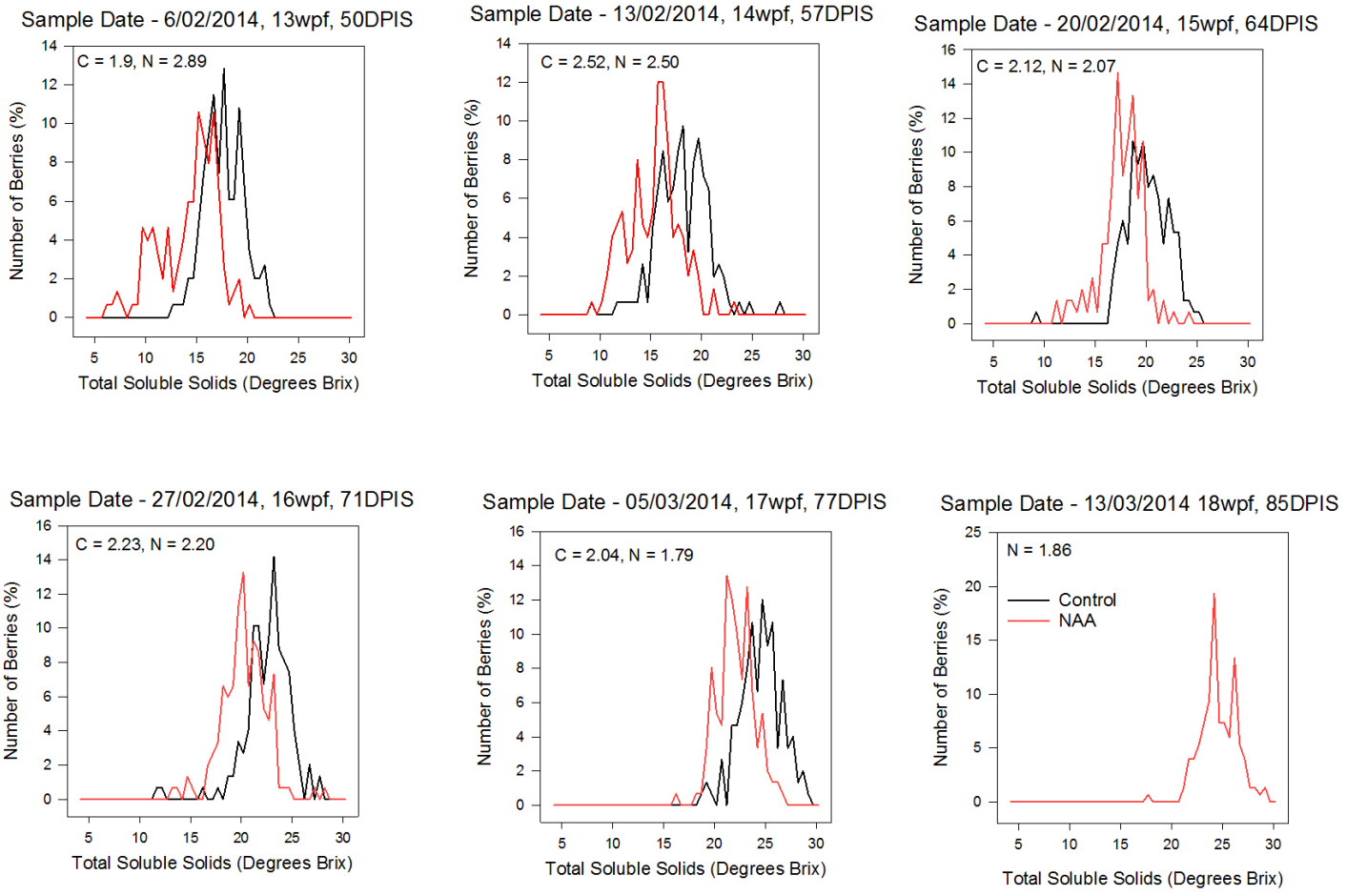

Sample Date - 6/02/2014, 13wpf, 50DPIS
C = 1.9, N = 2.89
Sample Date - 13/02/2014, 14wpf, 57DPIS
C = 2.52, N = 2.50
Sample Date - 20/02/2014, 15wpf, 64DPIS
C = 2.12, N = 2.07
Sample Date - 27/02/2014, 16wpf, 71DPIS
C = 2.23, N = 2.20
Sample Date - 05/03/2014, 17wpf, 77DPIS
C = 2.04, N = 1.79
Sample Date - 13/03/2014 18wpf, 85DPIS
N = 1.86
Control
NAA
Number of Berries (%)
Total Soluble Solids (Degrees Brix)

Fig. 9 Contd.

These results clearly show that the repeated treatments of NAA at low levels (50 mg/L) were effective in delaying the onset of ripening (veraison) and hence commercial harvest under warm climate conditions. Delaying ripening under warmer climate conditions is more challenging than under cooler conditions where ripening usually takes somewhat longer anyway. The commercial application for delaying ripening is likely to be in warmer regions, where the effects of climate change on seasonal compression are more severe. The use of multiple sprays with low amounts of PGR ensures that the timing of at least one treatment is likely to be at the most effective time of development to delay ripening/harvest. However, more knowledge regarding the window of opportunity where treatments are most effective means that methods using a single spray of higher NAA concentration should be possible.

As has been seen in most previous examples where we have delayed ripening, and therefore harvest, with NAA, the delaying treatment appears to make berry ripening more synchronous (Böttcher *et al*. 2012a; Böttcher *et al*. 2010). This can be seen from the 'sharper', taller peaks in most of the NAA-treated berry Brix profiles shown in Fig. 9 and is evidenced by the generally lower standard deviations at most time points. These differences are modest but suggest that there is a possibility that there may be ways to better synchronise ripening in asynchronous populations if desired.

Testing the effects of NAA and Ethrel on berry development/ripening and rotundone levels in wine

As described in our previous work, PGRs can be used as tools to either speed up or delay ripening and therefore harvest (Böttcher and Davies 2012; Davies and Böttcher 2009). In addition to changing the timing of ripening events there is also the possibility that the application of PGRs may affect berry composition and therefore wine flavour and aroma, either directly or as a consequence of the altered timing of ripening and harvest. Previous studies on the delay of ripening, in both red and white grapes, has have resulted in small, or no significant, changes in wine aroma/flavour perception and metabolite levels.

In an experiment with Shiraz, wines made from NAA-treated berries had small differences in the concentration of flavour/aroma volatiles as measured by SPME-GC-MS analysis and could not be distinguished from the Control wines by sensory analysis (Böttcher *et al*. 2011b). A similar experiment with Riesling resulted in only modest differences in measured flavour/aroma volatiles, but, distinct to the Shiraz example, a sensory panel could distinguish the wines made from NAA-treated berries from those made from Control fruit (Böttcher *et al*. 2012a).

In this experiment Shiraz berry ripening was delayed using two different PGRs, Ethrel (which releases ethylene) and NAA. Both of these treatments altered the sensorial properties of wine made from treated fruit when compared with Control fruit. The profiles of a range of volatile flavour/aroma metabolites were determined. Differences in the concentrations of rotundone emerged as a critical factor in the perception of differences between the wines.

Both Ethrel and NAA treatments delayed Shiraz berry ripening and harvest

The pre-veraison treatment of Shiraz berries with either Ethrel or NAA delayed the phase of rapid berry weight increase as can be seen by comparison with the Control (Fig. 10). Once the Control berries had begun to expand they did so rapidly but the Ethrel and NAA-treated fruit increased weight more slowly until later in development when the rate of increase was higher than at any stage of Control berry development. Despite these trends there were no significant differences between the treatments and this is probably due to the inherent 'noise'

in the measurement of berry weights (as discussed above). At harvest maturity the average weight of berries from all three treatments was very similar (Fig. 10).

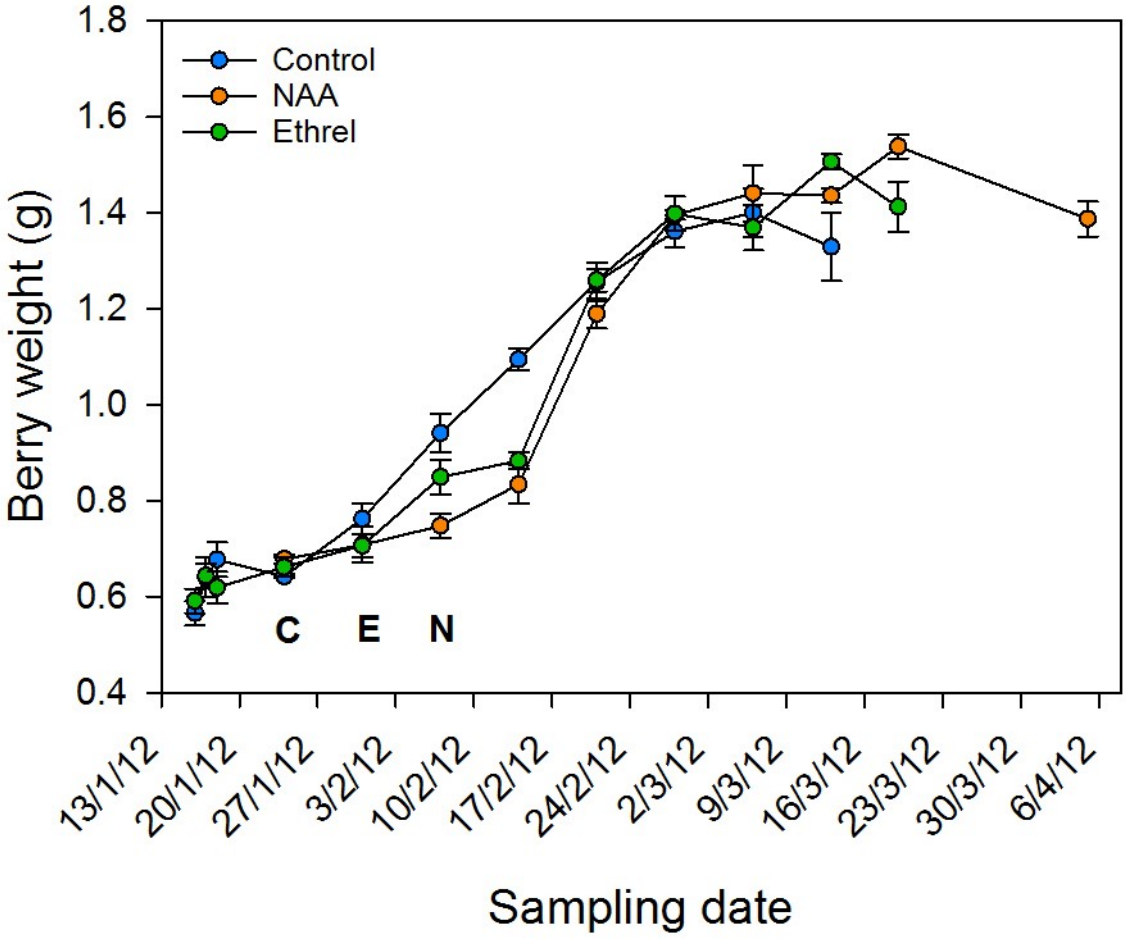


Fig. 10. Effect of Ethrel and NAA treatments on average berry weight (g). All data represent means ±STERR (n=3). There were no significant differences between treatments as determined by one-way ANOVA. C, E and N indicate the time of veraison for Control (blue symbol), Ethrel-treated (green symbol) and NAA-treated (orange symbol) fruit respectively.

As with the increase in berry weight the phase of rapid increase in sugar accumulation (Brix) was also delayed by both Ethrel and NAA treatments (Fig. 11). TSS levels are a good marker for the commencement of ripening. After these differences in the onset of ripening, the rate of TSS increase was similar for all fruit, as can be seen from the similar slopes of the curves. At most of the time points after the first sampling, the TSS levels of all three treatments were significantly different (p<0.05). The delay resulting from Ethrel treatment was less pronounced than that produced by NAA treatment. In this experiment, Ethrel was applied earlier during berry development than NAA in order to have the desired, delaying effect because earlier application had previously been shown to be effective (Böttcher *et al*. 2013b; Böttcher *et al*. 2013c; Coombe and Hale 1973; Hale *et al*. 1970). A previous study showed that ethylene can induce an increase in the biosynthesis and accumulation of the endogenous auxin, IAA, through the induction of IAA biosynthesis gene expression (Böttcher *et al*. 2013b). The increase in IAA concentration is the most likely reason for the observed delay in ripening. NAA is more effective than IAA in delaying ripening probably because NAA is a

poor substrate for IAA-amino acid synthetases, the enzymes that inactivate IAA through conjugation (Böttcher *et al*. 2011a) Therefore, NAA has a longer half-life within the berry. However, although less effective than NAA, it seems that an Ethrel-induced increase in IAA concentrations was sufficient to delay the onset of ripening (Fig. 11). This may be because applied IAA does not easily enter through the berry skin and so IAA produced within the berry may be at a higher concentration and be produced over a longer period thus making it effective.

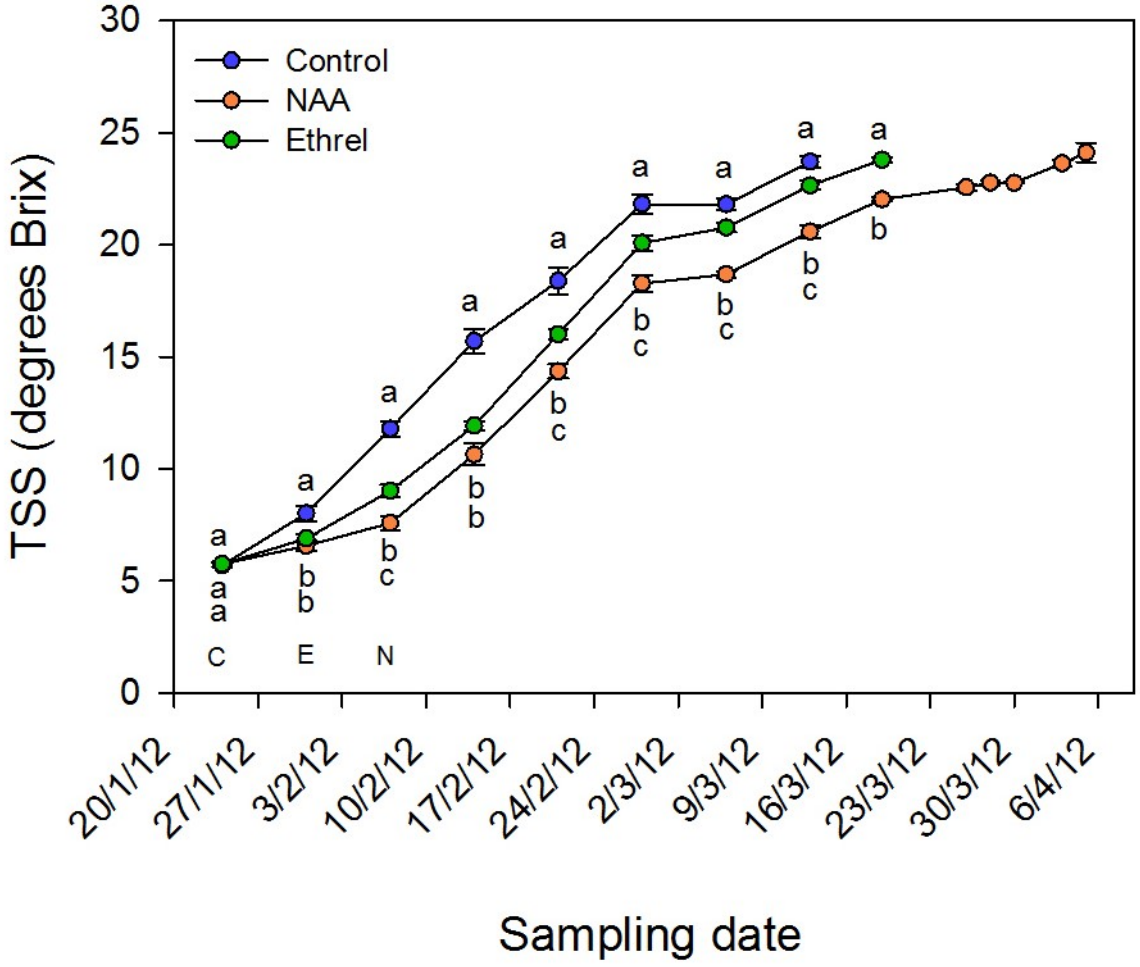


Fig. 11. Effect of Ethrel and NAA treatments on sugar accumulation. All data represent means ±STERR (n=3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test. C, E and N indicate the time of veraison for Control (blue symbol), Ethrel-treated (green symbol) and NAA-treated (orange symbol) fruit, respectively.

The harvest of Ethrel- and NAA-treated fruit was delayed by six and 23 days, respectively (as measured by differences in TSS). Despite the changes in ripening resulting from the treatments the final mean berry weights for all three at harvest were similar (Control 1.33 g/berry, Ethrel 1.41 g/berry, NAA 1.39 g/berry, ANOVA $p=0.583$). There was also no significant difference in titratable acidity at harvest (Table 2). There was a statistically significant but small difference in Baume and Brix between Control and NAA-treated fruit at harvest.

Table 2. Measurements of Baume, Brix, pH and T.A. of fruit at intake.

TREATMENT	BAUME	BRIX	PH	T.A. (G/L H^2T)
CONTROL	13.7 [a]	24.1 [a]	3.4 [a]	6.4 [a]
NAA	13.4 [b]	23.4 [b]	3.4 [a]	6.6 [a]
ETHREL	13.6 [ab]	24 [a]	3.4 [a]	6.4 [a]

Values represent means (n = 3) and different letters denote significant differences between treatments at p<0.05 using one-way ANOVA followed by Duncan's post hoc test.

Anthocyanin accumulation was also delayed by Ethrel and NAA treatments. Three weeks after veraison of the Control fruit (14/2/2012), Control berries contained a significantly higher amount of anthocyanins, than Ethrel- and NAA-treated fruit, which reflected the differences in TSS. In contrast, the anthocyanin concentrations in berries of similar TSS levels (approximately 24°Brix) at harvest were not significantly different (Fig. 12).

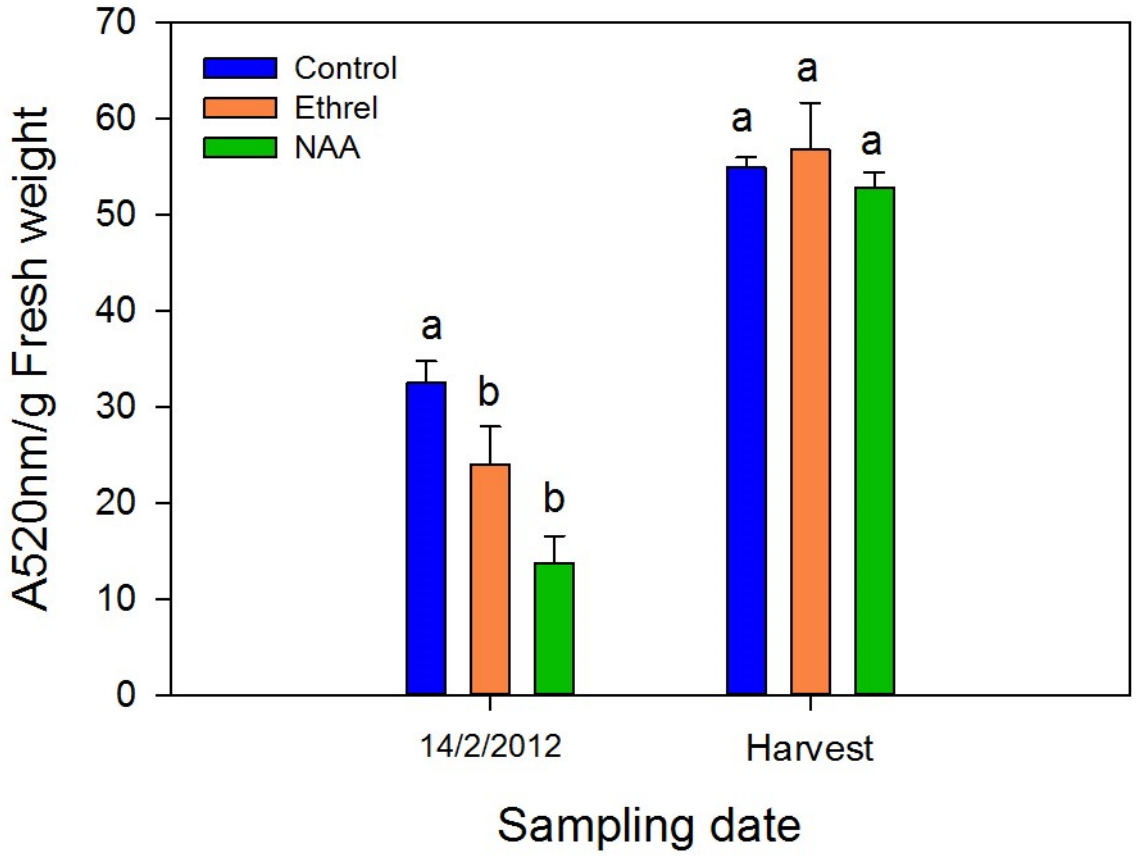


Fig. 12. Effect of Ethrel and NAA treatments on anthocyanin accumulation (A520 nm). All data represent means ±STERR (n=3) and different letters denote significant differences between treatments at p<0.05 using one-way ANOVA followed by Duncan's post hoc test.

These data indicates that changes in both primary and secondary metabolism that occur during ripening were delayed by the pre-veraison application of Ethrel, and to a larger extent, NAA.

Wines were made from Control, Ethrel- and NAA-treated fruit for sensory and biochemical analyses. At the completion of winemaking various basic measurements of wine properties were taken. Small, but statistically significant, differences were observed in % alcohol, acetic acid, pH and titratable acidity (Table 3), but these are unlikely to have impacted on the sensory properties of the wine.

Table 3. Measurements of percent alcohol, acetic acid, pH and T.A. of wines at bottling[a]

TREATMENT	ACETIC ACID (G/L)	PH	T.A. (G/L H^2T)	% ALCOHOL (V/V)
CONTROL	0.36^{a}	3.72^{b}	6.8^{a}	14.6^{a}
NAA	0.27^{b}	3.85^{a}	6.6^{ab}	14.2^{c}
ETHREL	0.30^{b}	3.80^{a}	6.3^{b}	14.4^{b}

Values represent means (n = 3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test.

Descriptive sensory analysis of the wines

Twenty four wine sensory attributes (two attributes related to appearance, eight related to odour, 11 to palate characteristics and three related to wine mouthfeel properties) were scored for the wines by a trained panel. Significant differences were identified between the wines for the two appearance attributes, four aroma attributes, three palate attributes and one mouthfeel attribute (Table 4, Fig. 13).

Table 4. Sensory attributes found to be significantly different between the wines through descriptive analysis.

Attribute	*Control*	*Ethrel-treated*	*NAA-treated*	*p-value*
Transparency	8.50 b	5.61 c	9.70 a	<0.001
Pepper Flavour	4.41 b	4.58 b	7.59 a	<0.001
Colour	7.59 b	6.03 c	9.06 a	0.001
Dark Fruit Aroma	8.05 b	6.91 c	9.40 a	0.003
Pepper Aroma	2.99 b	2.63 b	6.01 a	0.003
Earthy/Dusty Aroma	1.84 b	2.63 ab	3.46 a	0.007
Tannin quantity	8.89 a	8.00 ab	7.39 b	0.015
Red Berry Flavour	4.66 b	5.71 a	3.99 b	0.025
Red Confection Aroma	6.53 a	6.54 a	4.95 b	0.033
Earthy/Dusty Flavour	2.18 b	2.97 ab	3.65 a	0.044

Values represent means (n=3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test.

Wines produced from grapes treated with NAA were found to have more 'colour' and 'transparency' than those from the Control and Ethrel-treated fruit (Table 4, Fig. 13). Other sensory attributes that scored higher in the wines from the NAA-treated fruit included 'dark fruit', and 'pepper' aromas, and 'pepper' flavour on the palate (Table 4, Fig. 13). Scores for 'red confection' odour and 'red berry' flavour were significantly lower in the wine produced

from NAA-treated grapes than the Control and Ethrel-treated samples (Table 4). 'Earthy/dusty' aroma and flavour were scored higher in the wines produced from the NAA treatment compared to the Control wines, but were not significantly different from the wines from the Ethrel treatment (Table 4). In contrast, 'tannin quantity' was found to be significantly lower in the wines from the NAA-treated fruit compared to Control wines, but was not different from the wines from Ethrel-treated bunches (Table 4). The Ethrel treatment also had a significant effect on wine attributes with higher scores than the Control and NAA wines for 'red berry' flavour, but lower scores for wine 'colour', 'transparency' and 'dark fruit' aroma. Some of the differences in character between wines from Control and NAA-treated fruit are graphically represented in Fig. 13. Those attributes that were not significantly different due to the treatment of the fruit are listed in Table 5.

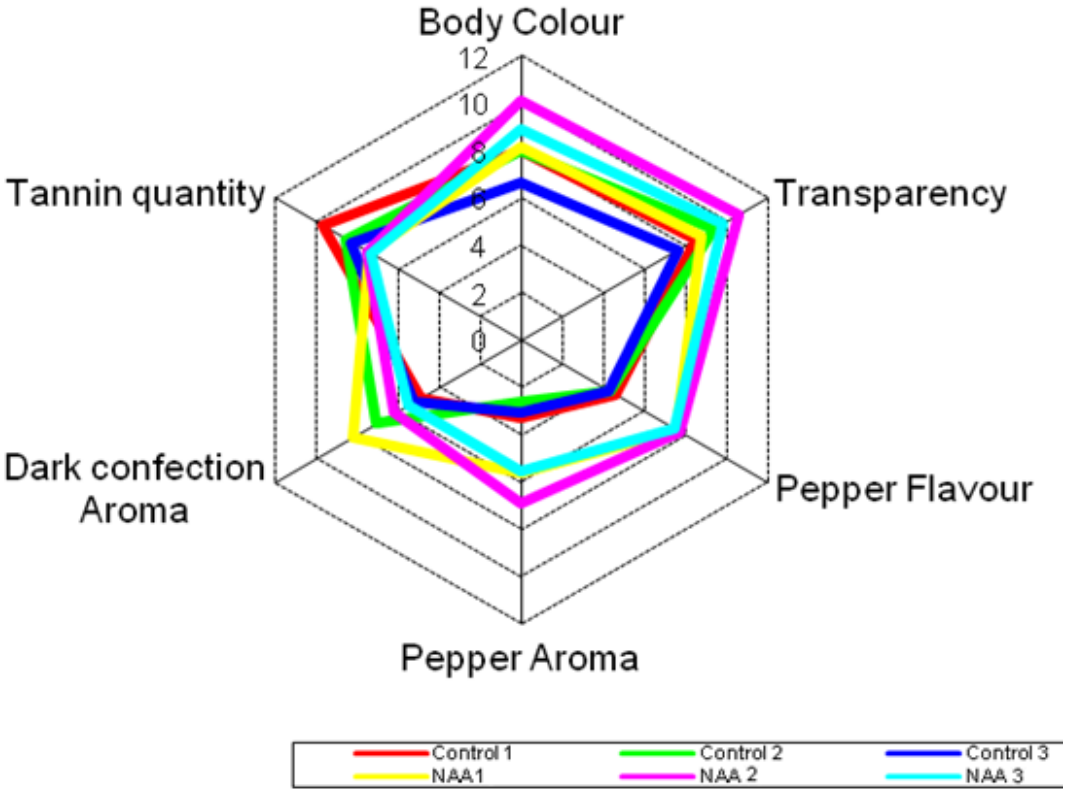


Fig. 13. Diagram showing the differences between scores, on a replicate basis, for six of the sensory characters scored as significantly different between Control and NAA wines. Pepper aroma and flavour clearly stand out most clearly as different.

Table 5. Wine sensory attributes found to be not significantly different through descriptive analysis.

Attribute	*Control*	*Ethrel-treated*	*NAA-treated*	*p-value*
Red Berry Aroma	5.07	5.90	4.14	0.096
Dark Confection Aroma	5.63	5.40	6.59	0.274
Floral Aroma	7.34	6.30	6.08	0.099
Green Aroma	1.30	1.82	1.48	0.582
Acidity	7.44	6.94	6.19	0.265
Body (mouthfeel)	7.79	7.13	6.65	0.233
Dark Fruit Flavour	7.99	7.82	8.66	0.456
Red Confection Flavour	5.48	5.64	5.07	0.709
Dark Confection Flavour	5.43	5.06	6.27	0.370
Green Flavour	2.60	2.57	2.76	0.822
Flavour Length	8.44	8.37	7.81	0.230
Tannin Quality	7.61	6.87	7.04	0.353
Bitterness	4.72	4.93	4.09	0.310
Flavour Intensity	8.20	7.49	7.88	0.539

Values represent means (n=3) and *p*-values represents the effect of the treatments on the attribute.

Analysis of wine volatile compounds

Significant differences were observed in volatile profiles of the wines as determined using SPME-GC-MS analysis. Of the 164 compounds quantified, eight were found in greater amounts in the headspace of wines made from NAA-treated grapes compared with the Control wines and 17 were more abundant in Control wines than those made from the NAA-treated fruit (Table 6). No compounds were in significantly greater concentration in the wine from Ethrel-treated fruit compared with the other two treatments, but, two compounds (ethyl salicylate and hotrienol) were at a lower concentration in these wines compared with wines from both the Control and NAA-treated fruit (Table 6). However, 18 compounds were in lower abundance in the wines produced from Ethrel-treated grapes compared to the Control wines (Table 6). The concentration of 2-heptanol, 1-hexanol and (Z)-3-hexen-1-ol was intermediate in the wines from Ethrel-treated fruit and lowest in wine from NAA-treated berries (Table 6). The differences in abundance were modest, being mainly less than two-fold.

Four compounds, 2-heptanol, ethyl (Z)-3-hexenoate, ethyl (E)-2-hexenoate and (E)-3-hexen-1-ol, were previously found at higher concentrations in Control wine compared with wine from NAA-treated, ripening-delayed grapes, in either, or both, Riesling or Shiraz (Böttcher *et al*. 2012a; Böttcher *et al*. 2011b). This shows that NAA treatments affect the headspace concentrations of some volatile compounds in a similar manner in different years and between different varieties. In contrast, hexyl acetate and (Z)-3-hexenyl acetate were higher in Control wines in the current experiment (Table 6) but had previously found to be at higher

concentrations in wines made from NAA-treated Riesling grapes (Böttcher *et al*. 2012a). Hexyl acetate was also previously shown to be at higher concentrations in wine from NAA-treated Shiraz berries compared with wine from Control-treatment berries (Böttcher *et al*. 2011b). The above results indicate that NAA-treatment which delayed ripening significantly altered the abundances of relatively few of the volatile compounds measured. Changes in the accumulation of some compounds in response to NAA treatment are consistent between experiments but the concentrations of other compounds vary independently and must be under more complex control.

Some compounds derived from the lipoxygenase pathway were in lower abundance in the wines produced from NAA-treated grapes compared with the Control wines. These were 1-hexanol, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol as well as the esters hexyl acetate, ethyl (Z)-3-hexenoate, ethyl (E)-2-hexenoate, (Z)-3-hexenyl acetate which are derived from C6 alcohols and aldehydes produced by the lipoxygenase pathway (Dennis *et al*. 2012). Other lipid oxidation products at higher concentration in Control wines than in wines from NAA-treated berries were 2-heptanol and 1-penten-3-ol. In general, the compounds that were significantly more abundant in the headspace of the Control wines compared with those from the NAA treatment were two-fold, or less, different. Exceptions to this are isoamyl isovalerate and (Z)-3-hexenyl acetate, which were a little more than two-fold higher in the Control wines than the NAA treatment wines.

Some aromatic compounds were more abundant in the wines from NAA-treated fruit compared with the levels in wine from Control fruit. These were ethenyl benzene, ethyl salicylate, phenylethyl butyrate and 1,1,6-trimethyl-1,2-dihydro-napthalene, the latter being derived from the degradation of carotenoids, the others arise from benzenoid metabolism. None of these compounds were more than two-fold different in abundance between the wines. Some compounds derived from the lipoxygenase pathway (1-hexanol, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol and the esters hexyl acetate, ethyl (Z)-3-hexenoate, ethyl (E)-2-hexenoate, (Z)-3-hexenyl acetate) that were lower in the wines from NAA-treated fruit compared with that from Control fruit (Table 6) are generally described as ‘green’ for the alcohols and ‘green/fruity/apple’ for the esters. However, green characters were not found to be significantly different amongst the wines. It is possible that these compounds are influencing the perception of ‘dark fruit’ versus ‘red confection’ in the wines, that is, higher amounts in the Control wines make the fruit characters more ‘fresh’ and ‘red’ compared to the lower amounts in the wines from the NAA treatment where the fruit characters are described more as ‘cooked fruit’ or ’dark’.

Table 6. Volatile compounds significantly different in wines produced from Control, Ethrel-treated and NAA-treated berries.

Compound	Compound ID[a]	Control[b]	Ethrel	NAA
	More abundant in headspace of Control wines cf NAA-treated and/or Ethrel-treated wines			
(*Z*)-3-Hexenyl acetate	A	0.035 a	0.015 b	0.016 b
Isoamyl isovalerate	B	0.015 a	0.013 ab	0.007 b
Hexyl acetate	A	1.862 a	1.037 b	0.936 b
2-Heptyl acetate	B	0.016 a	0.006 b	0.009 ab
(*Z*)-3-Hexen-1-ol	A	0.268 a	0.197 b	0.154 c
Ethyl (*E*)-2-hexenoate	A	0.080 a	0.053 b	0.052 b
Theaspirane A	B	0.028 a	0.019 b	0.019 b
Ethyl (*Z*)-3-hexenoate	A	0.102 a	0.061 b	0.071 b
(*E*)-3-Hexen-1-ol	A	0.021 a	0.015 b	0.015 b
Diethyl malate	A	0.035 a	0.021 b	0.025 b
2-Heptanol	A	0.029 a	0.025 b	0.021 c
1-Hexanol	A	2.104 a	1.809 b	1.591 c
2-Ethyl furoate	A	0.049 a	0.044 a	0.037 b
1-Penten-3-ol	A	0.021 a	0.016 b	0.017 b
4-Methyl-1-pentanol	A	0.119 a	0.104 b	0.096 b
Theaspirane B	B	0.026 a	0.019 b	0.021 b
Citronellyl acetate	A	0.006 a	0.005 b	0.005 b
α -Terpineol	A	0.465 a	0.406 b	0.386 b
2-Phenylethanol[c]	A	0.305 a	0.253 b	0.264 ab
Hotrienol	B	0.006 a	0.005 b	0.006 a
	More abundant in headspace of NAA-treated wines cf Control wines			
α-Calacorene	B	0.039 b	0.062 b	0.184 a
Cadalene	B	0.014 b	0.014 b	0.033 a
3-Ethoxy-1-propanol	A	0.032 b	0.039 ab	0.051 a
Ethenyl benzene	A	3.445 b	3.906 b	4.912 a
Methionol	A	0.106 b	0.123 ab	0.135 a
Ethyl salicylate	B	0.004 b	0.002 c	0.005 a
Phenylethyl butyrate	A	0.013 b	0.013 b	0.016 a
1,1,6-Trimethyl-1,2-dihydro-napthalene	B	0.058 b	0.061 b	0.069 a

[a]Abbreviations: A, identity confirmed by matching mass spectra and LRI with that of authentic standards; B, tentative assignment based upon comparison with mass spectral libraries and published LRIs. [b]Values represent means (n = 3) of the areas under the peaks of a selected ion relative to the relevant internal standard. Different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test. [c]Compound quantified in the 1:100 dilution.

The concentration of some sesquiterpenes was elevated by NAA treatment

Among the compounds in higher concentrations in the headspace of the wine from NAA-treated fruit were two sesquiterpenes tentatively identified as α-calacorene and cadalene.

These volatile compounds were found in concentrations 4.7- and 2.4-fold higher in the wines produced from the NAA-treated berries compared with the Control wines (Table 6). Shiraz is a grape cultivar that can produce wines with a distinctive 'peppery' character depending on climate, viticultural practice and winemaking practice (Iland and Gago 2002). The 'peppery' aroma and taste is attributed to the grape bicyclic sesquiterpene rotundone (Wood et al. 2008). The detection threshold of rotundone in red wine has been reported to be 16 ng/L and approximately 80% of participants of a sensory panel were able to detect this character. Rotundone has been detected at varying concentrations in a range of red and white varieties e.g. Cabernet Sauvignon, Durif, Mourvedre, Schioppettino, Vespolina and Grüner Veltliner (Mattivi et al. 2011; Wood et al. 2008). The concentration of rotundone in berries is extremely low at veraison but increases rapidly during ripening to be highest at harvest (Caputi et al. 2011). Rotundone concentrations vary considerably between season and within vineyards but no definitive reasons for this variation have been demonstrated (Caputi et al. 2011; Mattivi et al. 2011; Scarlett et al. 2014; Wood et al. 2008). As α-calacorene and cadalene have mass spectra typical of sesquiterpenes and because terpene synthase enzymes can make multiple products (Lücker *et al*. 2004) the concentration of rotundone was analysed in the wines using SPME-GC-MS. Rotundone was not detected in the Control wines, was present in low concentrations (2 ± 0.4 ng/L) in the wines made from Ethrel-treated fruit, but was 14.5-fold higher in concentration (29 ± 1.6 ng/L) in NAA treatment wines (Fig. 14). The concentration in the wines produced from NAA-treated fruit is above the odour threshold for rotundone which has been determined to be 16 ng/L in red wine, for those sensitive to the compound (Wood *et al*. 2008).

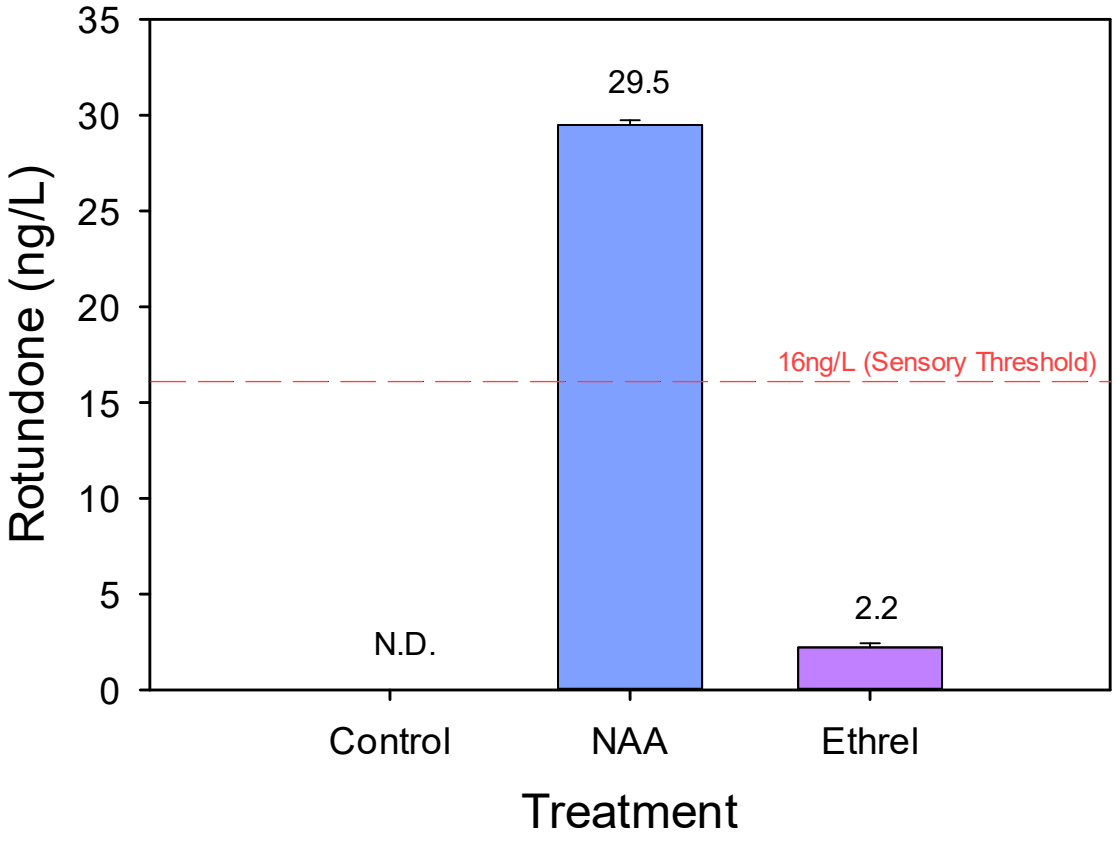


Fig. 14. Rotundone levels in the wines from Control, NAA-treated and Ethrel-treated berries. N.D. = not detected. The sensory threshold for rotundone in wine is indicated at 16 ng/L

The increase in ‘pepper’ aroma and flavour of the wine made from NAA-treated fruit was particularly noteworthy as it was clearly distinguishable during sensory testing (Table 4, Fig. 13). The molecule responsible for ‘peppery’ character in grapes has been identified as rotundone (Wood *et al*. 2008) and appears to be associated with the ‘peppery’ aroma and flavour detected in the sensory analysis of the wines in this study. Although rotundone was at a higher concentration in wines made from Ethrel-treated grapes compared with the Control, the concentration was not above the odour threshold in red wine of 16 ng/L (Wood *et al*. 2008) and there was no significant difference in ‘pepper’ characters detected between the wines from Control and Ethrel-treated grapes. The large increase in rotundone in wine from NAA-treated grapes was detected sensorially (Table 4, Fig. 13). Rotundone concentrations have been shown to vary considerably within a vineyard (Scarlett *et al*. 2014). In the experiment described here the sample replication in the field was maintained throughout winemaking, sensory analysis and metabolite analysis and so the effects of any field variation can be tested by statistical analysis using ANOVA. While the treatment effect on rotundone concentration was significant (p=0.002) neither the effect of row (p=0.321) nor the interaction of treatment and row (p=0.164) were statistically significant at the 0.05 level.

We have no evidence to indicate the mechanism involved in the increase in rotundone, i.e. is it via increased biosynthesis, decreased catabolism, or a combination of both? However, there are a number of possible explanations for the increased concentration of rotundone in wines from Ethrel-, and in particular, NAA-treated grapes. As described above, it is feasible that auxins are the common factor in both NAA and Ethrel treatments due to the interaction of auxins and ethylene (Böttcher *et al*. 2013b; Böttcher *et al*. 2013c). This may mean that there is a direct effect of auxins on rotundone metabolism that results in a sustained increase in rotundone concentration and that the effect of NAA is greater than that of IAA due to the greater persistence of NAA in berries.

An alternative explanation is that the increased concentration of rotundone, especially in wines made from NAA-treated grapes, was due to the delay in veraison. Certainly, the longer the delay in veraison, and therefore harvest, the more rotundone was accumulated. The increase in rotundone could be due to a lengthening of the period before veraison caused by the treatments. As rotundone appears to only accumulate after veraison (Caputi *et al*. 2011) an increase in rotundone that was caused by a delay in ripening would have to be due to an increase in rotundone precursor molecules during this period. The precursor for rotundone has been identified as α-guaiene which can be converted to rotundone through oxidation (Huang *et al*. 2014). Another possibility is that the delay in veraison causes ripening to occur under different climatic conditions and that might be more favourable for rotundone accumulation. For example, it has been suggested that lower temperatures may result in increased ‘pepper’ character (rotundone) accumulation (Caputi *et al*. 2011; Iland and Gago 2002; Scarlett *et al*. 2014). There was no obvious change in the pattern of minimum and maximum temperatures or rainfall during the period when rotundone accumulated (Figs. 15 and 16). Therefore, any effect of air temperature on rotundone accumulation is either quite subtle or there are other factors that have a significant impact. However, there did appear to be a decrease in sunlight (global exposure, Fig. 16). Reduced light levels could either encourage the production of rotundone or may slow its degradation. Reduced sunlight may also impact on berry temperature, an effect that is not apparent from the air temperature measurements. Fruit that is directly exposed to sunlight is warmed by the incident light effect and so we might expect that the delayed ripening fruit was lower in temperature due to reduced light associated heating. One other parameter that differed between the Control and NAA-treated fruit in particular was the length of time from veraison to harvest which was longer by approximately eight days for NAA-treated fruit compared to Control fruit. As rotundone can be formed via the oxidation of α-guaiene (Huang *et al*. 2014) it could be that

the longer hang-time of the NAA-treated fruit after harvest resulted in higher rotundone concentrations. Along with the increase in rotundone concentration the concentrations of two other sesquiterpenes (α-calacorene and cadalene, Table 6) were higher in wines from NAA-treated fruit indicating that there may be a more general effect of the treatment on sesquiterpene metabolism.

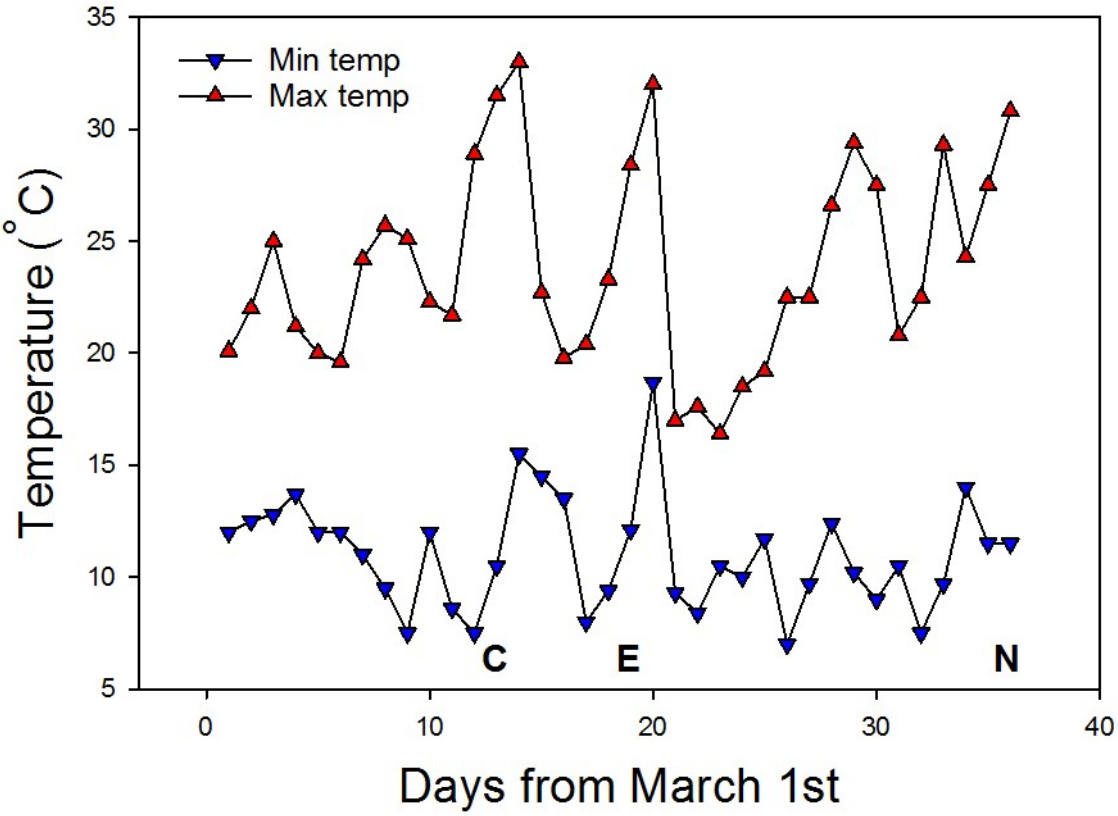


Fig. 15. Vineyard minimum (red triangles) and maximum air temperatures (blue triangles) from March 1, 2012. Letters along the horizontal axis refer to the harvest times of Control (C), Ethrel (E) and NAA (N) treated grapes. Data from BOM Adelaide.

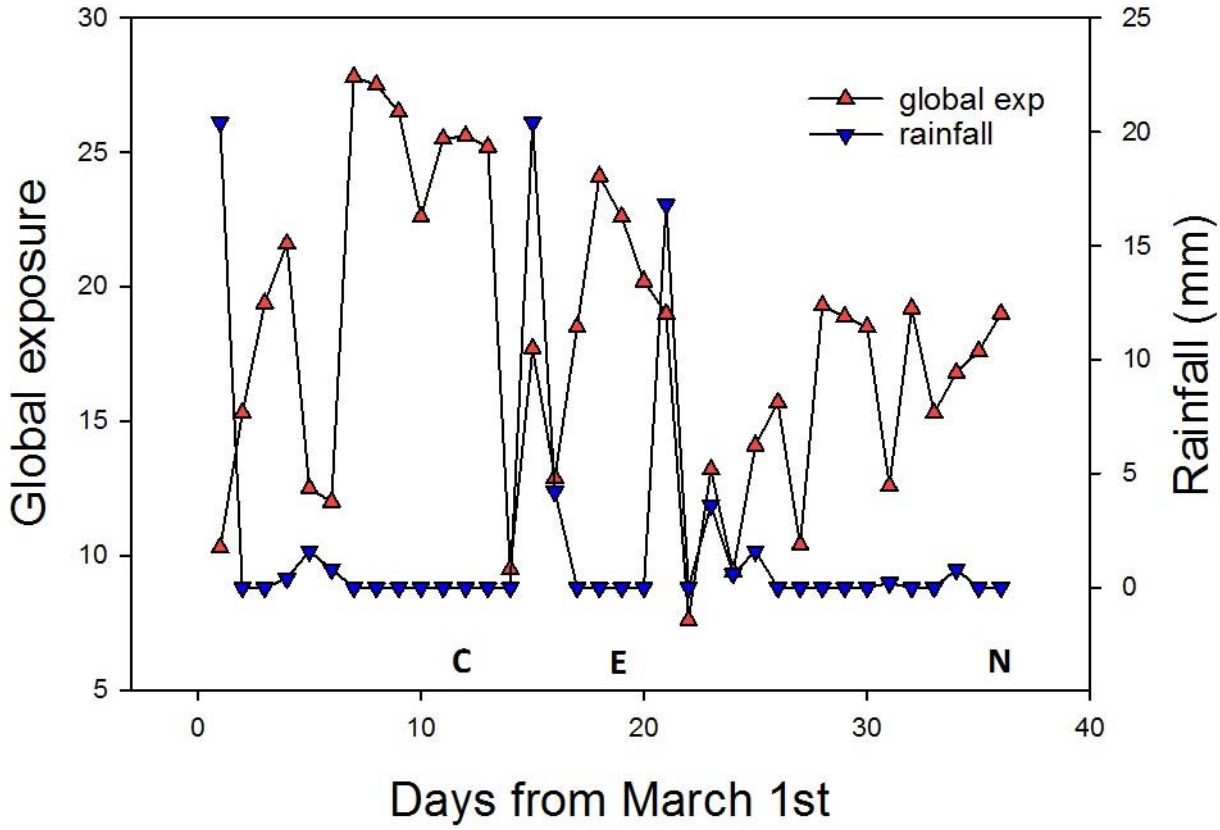


Fig. 16. Vineyard rainfall (blue triangles) and global exposure (red triangles) from March 1, 2012. Letters along the horizontal axis refer to the harvest times of Control (C), Ethrel (E) and NAA (N) treated grapes. Data from BOM Adelaide.

To summarise, both Ethrel and NAA treatments can delay the onset of ripening, which in turn results in a delay in harvest. NAA treatment delayed ripening to a greater extent than Ethrel, probably due to its longer half-life in the berry than that of the IAA induced by ethylene arising from Ethrel. Both Ethrel and NAA treatments significantly altered wine volatile metabolite levels, but greater differences were induced by NAA treatment. This is in line with the longer delay engendered by NAA treatment. The sensory character of the wines produced from these fruit were also different. This could be the result of changes in a range of metabolites such as esters but it appears that the difference in 'peppery' character, which is quite distinctive, may be due to higher concentrations of rotundone in these wines. The reason for the greatly increased rotundone concentration in the NAA wines is unknown but could arise from a direct effect of NAA on rotundone accumulation e.g. specific changes to the activity of the enzymes involved in its synthesis, or be an indirect effect arising from the altered timing of ripening initiation and harvest phase affecting sesquiterpene metabolism.

While previous results have shown relatively small changes in wine flavour/aroma due to NAA-induced ripening delay, it seems that varieties where rotundone plays a central role in wine character might be a special case. Rotundone is highly active sensorially and has a distinctive flavour/aroma such that changes in its level are readily detected by many tasters. The increase in rotundone due to harvest delay could be seen as a positive factor in Shiraz styles where this character is highly valued. This is in addition to the benefit of NAA treatments assisting with winery intake scheduling.

Testing the effect of different NAA concentrations on berry ripening

We have shown that NAA can be successfully used to delay ripening/harvest and may be useful in a commercial setting to ameliorate some of the negative consequences of climate change. The timing of application is critical to success as there is a window of opportunity when such treatments are effective. If applications are too early there can be little or no effect. This could be due to two reasons, the first is that if too early the level of NAA may be reduced to low levels by endogenous metabolic processes before it is required to be present to delay ripening. If applied too late then no significant delay will be achieved. Obviously getting the timing right is an important issue, however, the concentration of NAA applied is another parameter that needs to be tested to see if the application of higher levels may be able to reduce the risk of mistiming the application and to extend the ripening delay.

In this experiment, two different concentrations of NAA were applied. The lower concentration, 50 mg/L, has been used previously and if the application is timed accurately it can induce a significant delay in veraison and harvest dates (see above). A concentration five time higher (250 mg/L NAA) was also applied to test if higher concentrations can induce a longer delay in ripening. As with previous data the measurements of berry weight were more variable than those of Brix. However, there was a trend for a delay in berry weight increase in the '50 mg/L' fruit which was even more pronounced in the '250 mg/L' fruit (Fig. 17). Towards the end of development there was no significant difference in berry weight.

Ripening, as measured by changes in TSS (measured as Brix), was clearly delayed by auxin treatment, with the delay resulting from treatment with the lower NAA concentration (50 mg/L) substantially less than that caused by the higher NAA concentration (250 mg/L) (Fig. 18).

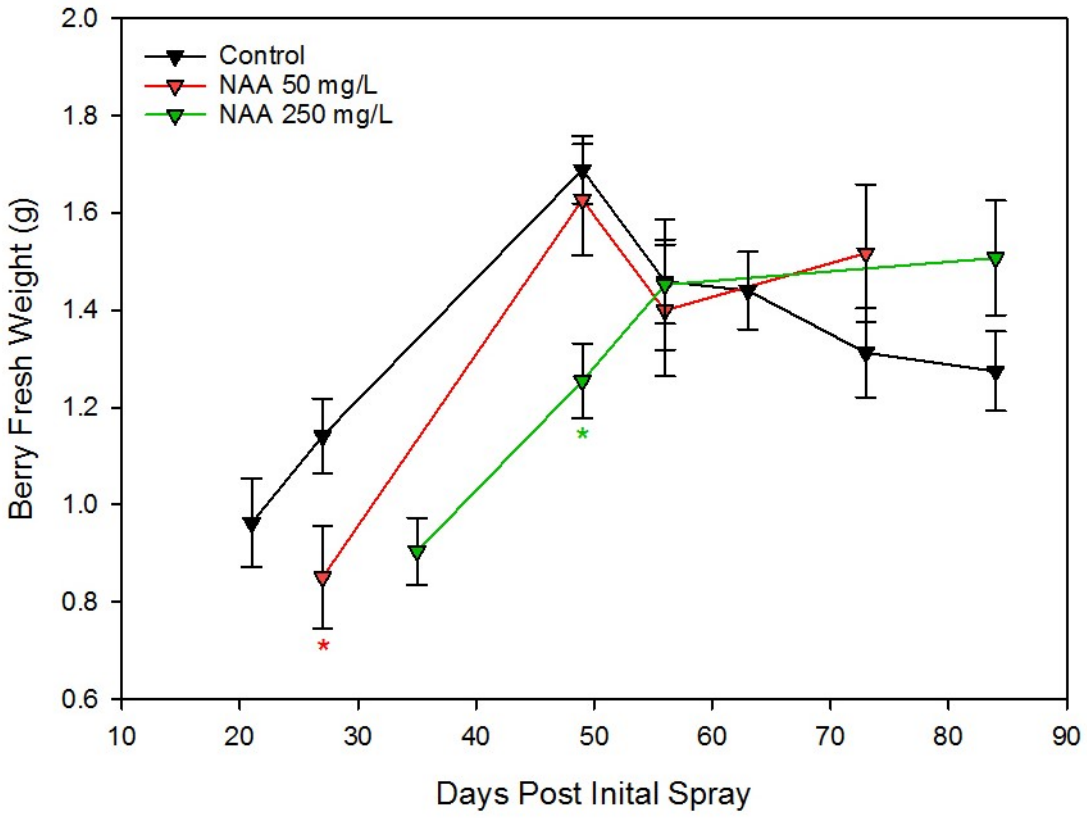


Fig. 17. The effect of the pre-veraison application of different NAA concentrations on the delay of ripening of Shiraz berries as measured by berry weight. Black = Control, Red = NAA 50 mg/L, Green = NAA 250 mg/L.*=significantly different at $p<0.05$ by t-test.

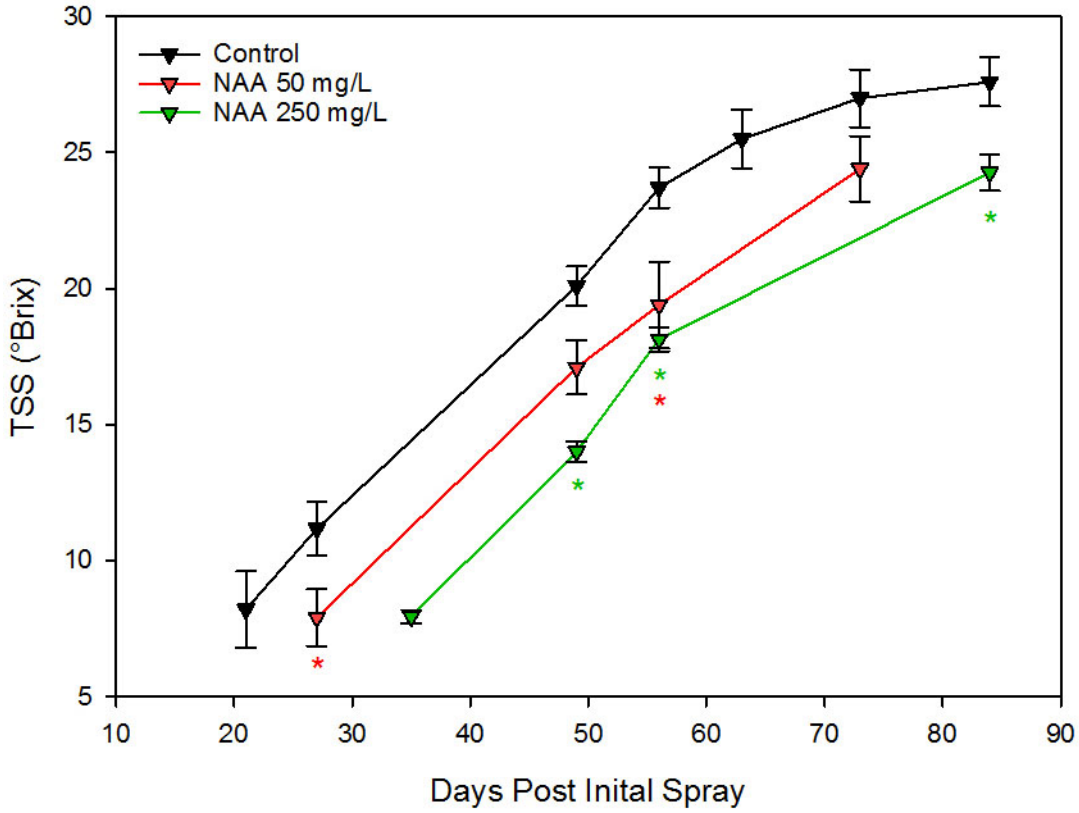


Fig. 18. The effect of the pre-veraison application of different NAA concentrations on the delay of ripening as measured by ºBrix. Black = Control, Red = NAA 50 mg/L, Green = NAA 250 mg/L. *=significantly different at $p<0.05$ by t-test.

The increase in anthocyanins after veraison is another measure of ripening that is commonly used and may reflect changes in secondary metabolism rather than the primary metabolism as is measured by changes in TSS. Figure 19 shows that the NAA50 treatment delayed anthocyanin accumulation and the NAA250 treatment was even more effective in delaying colour development. The delaying treatment did not seem to reduce the anthocyanin level as the much delayed NAA250 fruit at 84 DPIS (24.3ºBrix) had very similar A520nm levels as the Control at 63 days post initial spray even though the Control fruit had a slightly higher TSS (25.5ºBrix).

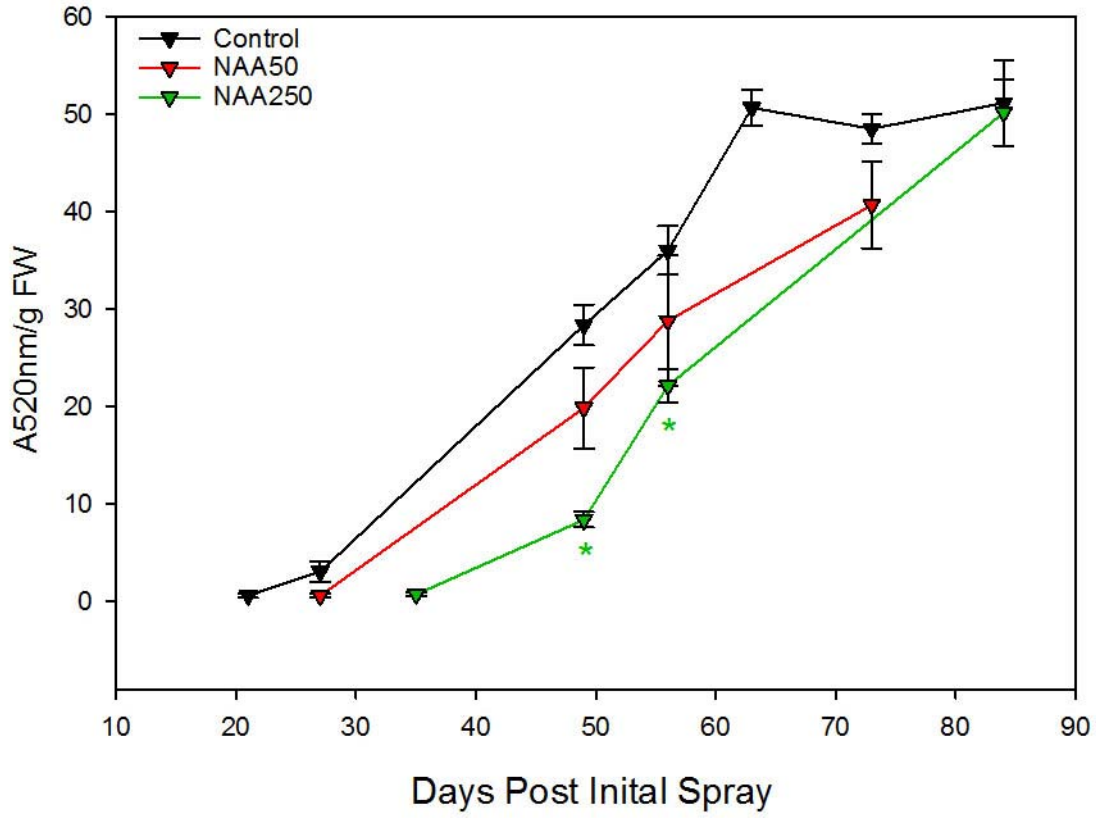


Fig. 19. The effect of the pre-veraison application of different NAA concentrations on the delay of anthocyanin increase during ripening as measured at A520nm. Black = Control, Red = NAA 50 mg/L, Green = NAA 250 mg/L. *=significantly different at $p<0.05$ by t-test.

We have observed that the delaying of Shiraz berry ripening by NAA application can result in an increase in rotundone levels (see above). In this experiment, Control 1, at 25.5ºBrix (Fig. 20), contained a fairly low level of rotundone, i.e. below the reported sensory detection threshold in red wine of 16 ng/L (Wood *et al*. 2008). Ten days later (27/2/2015) the Control 2 Brix level had increased to 27.0 but the rotundone levels had not changed significantly. At the same time (27/2/2015) the NAA50 treatment fruit were at 24.5ºBrix, i.e. at a similar Brix to the control (Control 1) from 10 days earlier with a similar rotundone concentration. By the time the NAA250 treatment fruit had reached a similar Brix of 24.3º (11 days later, 10/03/2015) the rotundone had dramatically increased to be well above the sensory threshold level. At these levels one would expect that wine made from these fruit may well have a peppery character due to the additional rotundone.

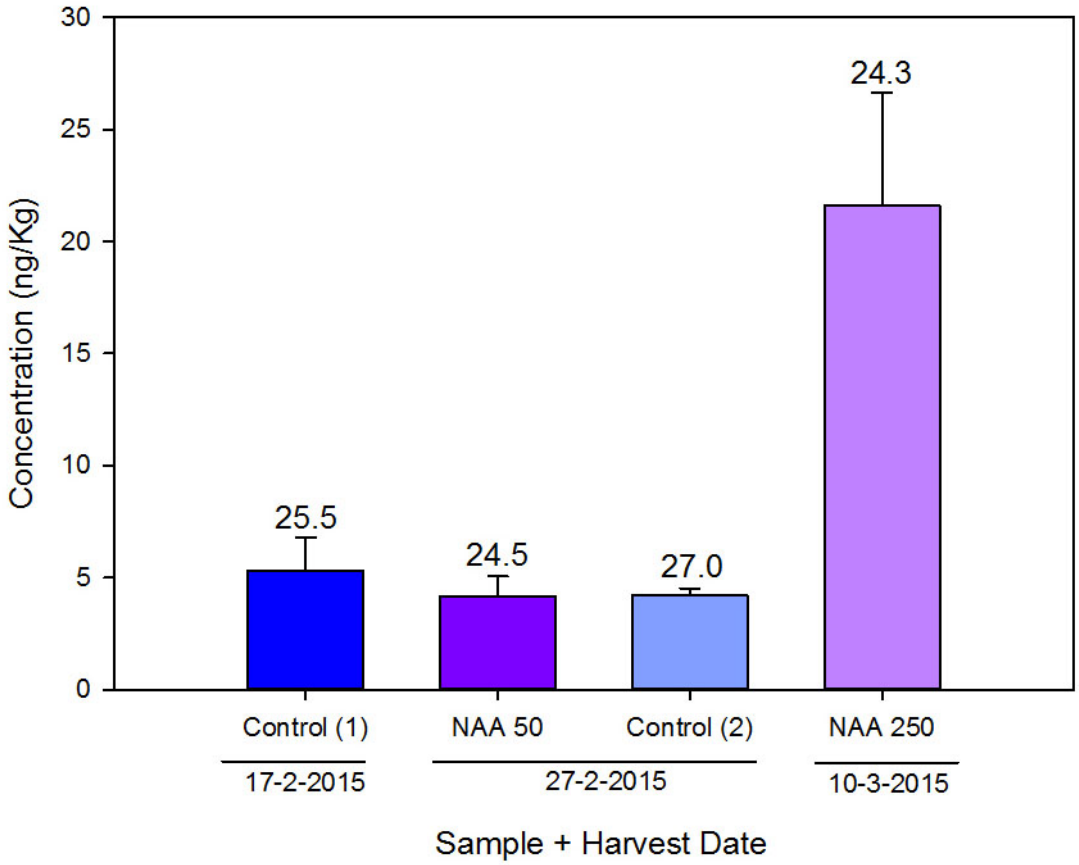


Fig. 20. Rotundone concentration in Control and NAA-treated fruit.

In summary, the higher concentration of NAA (250 mg/L) applied to berries by spraying considerably increased the delay in both veraison and achieving harvest ripeness over the delay resulting from 50 mg/L NAA. This demonstrates that it may be possible to control the length of the delay by varying the concentration of the applied NAA. Although the 50 mg/L NAA treatment was successful in delaying ripening somewhat, it was only the longer delay engendered by the 250 mg/L treatment that significantly increased rotundone levels. It is not possible to tell from this experiment whether this increase in rotundone concentration was due to a direct effect of NAA on rotundone production or the extended, delayed, maturation process.

Testing of the effects of NAA in delaying berry ripening – different vineyard conditions

For a viticultural intervention to be commercially useful it needs to be tested under a variety of different conditions. An experiment was designed to test the effects of NAA in delaying ripening in a commercial vineyard in the Eden Valley. Sufficient fruit, both Control and NAA-treated, was sprayed to allow small-scale winemaking for volatile and sensory analysis (difference testing and descriptive analysis as required). The fruit were sprayed twice during the pre-veraison period (23/12/15 and 11/01/16). Unfortunately, this experiment was not taken to full completion due to the vineyard operator mistakenly harvesting the fruit before the completion of the trial. However, as can been seen below, useful data regarding the effect of NAA in delaying ripening was still obtained.

As expected from previous work (see above) the NAA treatment delayed berry development. The increase in berry weight was delayed in NAA-treated fruit (Fig. 21) but the berry weights at 58 DPIS were not significantly different.

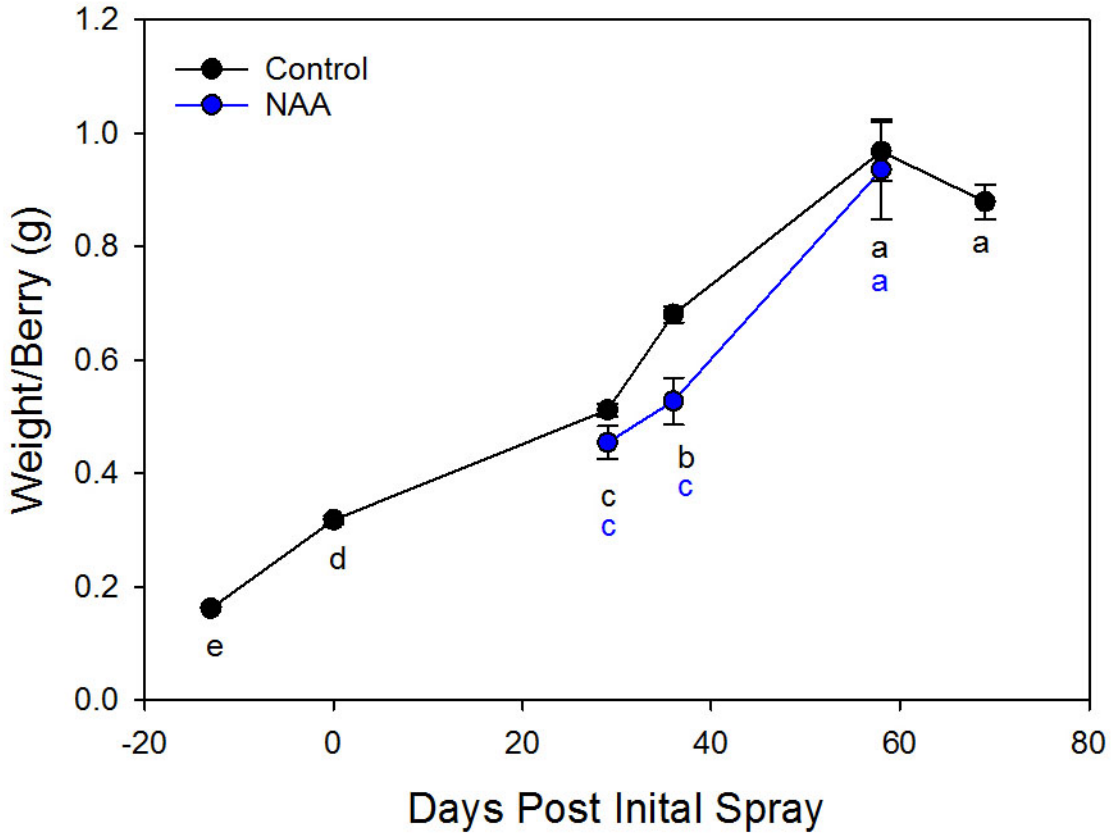


Fig. 21. Berry weights of Control and NAA-treated fruit. All data represent means ±STERR (n=3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test.

The Brix values for the NAA-treated fruit were significantly lower than those for the Control berries at all time points tested (Fig. 22). Again, this shows that the NAA treatment was effective in delaying the initiation of berry ripening.

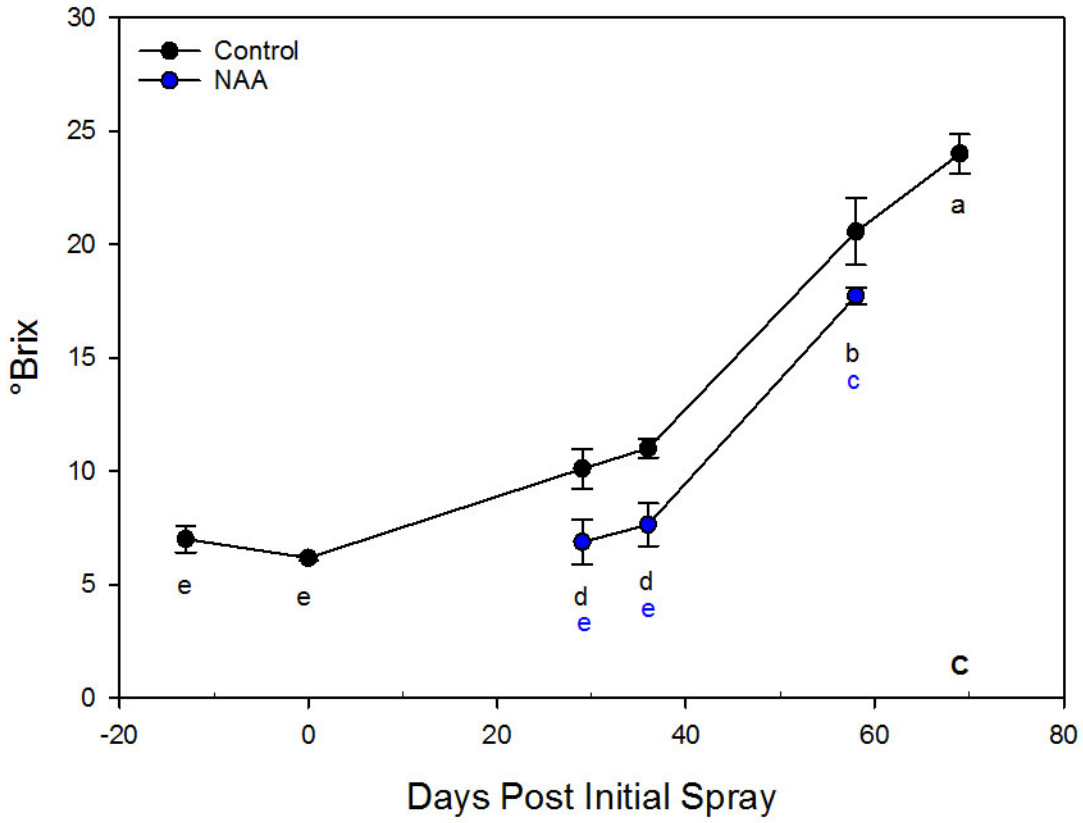


Fig. 22. Brix of Control and NAA-treated fruit. All data represent means ±STERR (n=3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test.

Delayed ripening was also indicated in the NAA-treated fruit by the significantly higher level of total acid at 58 DPIS, even though the pH was not significantly different (Table 7).

Table 7. TSS (ºBrix), pH and total acids (g/L) in Control and NAA-treated berries at 58 DPIS as measured by FTIR.

	Control	NAA
°Brix	22.5	18.2*
pH	3.3	3.2
Total Acid	6.1	8.4*

*significantly different ($p<0.05$) from Control (Student's t-test)

The retardation of colouring (anthocyanin accumulation) resulting from NAA treatment is shown in the photographs of Control and NAA-treated berries taken on consecutive weeks. Fig. 23 shows that at on 21/01/2016 there was no colour in the NAA fruit but the Control fruit had begun to 'colour up' with some berries exhibiting the characteristic dark purple skin colouration. In Fig. 24, taken a week later, the Control fruit was highly coloured with very few of the NAA-treated berries showing any colour.

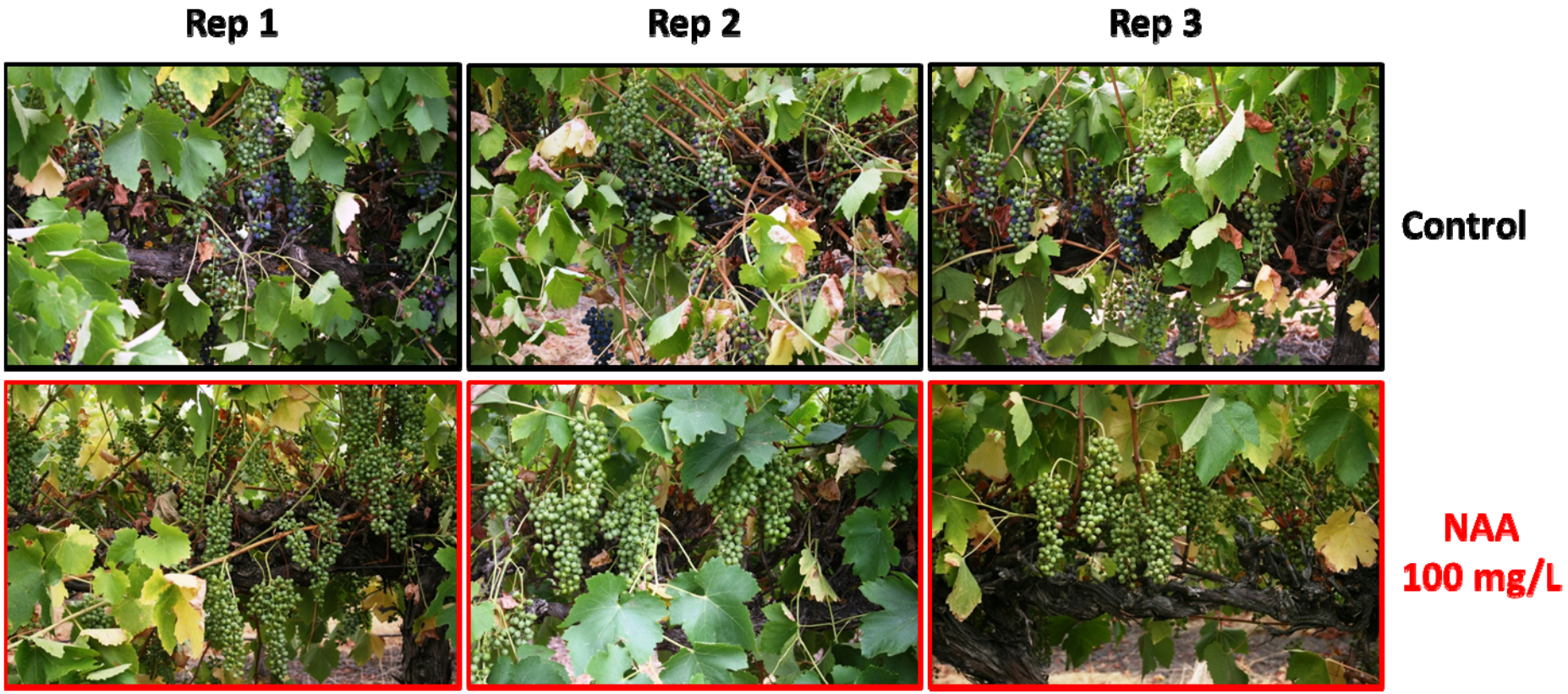


Fig. 23. Shiraz trial, Eden Valley, 2015/16. Photographs of Control and NAA-treated berries taken 21/01/2016, showing delay caused by NAA application, all three replicates are shown.

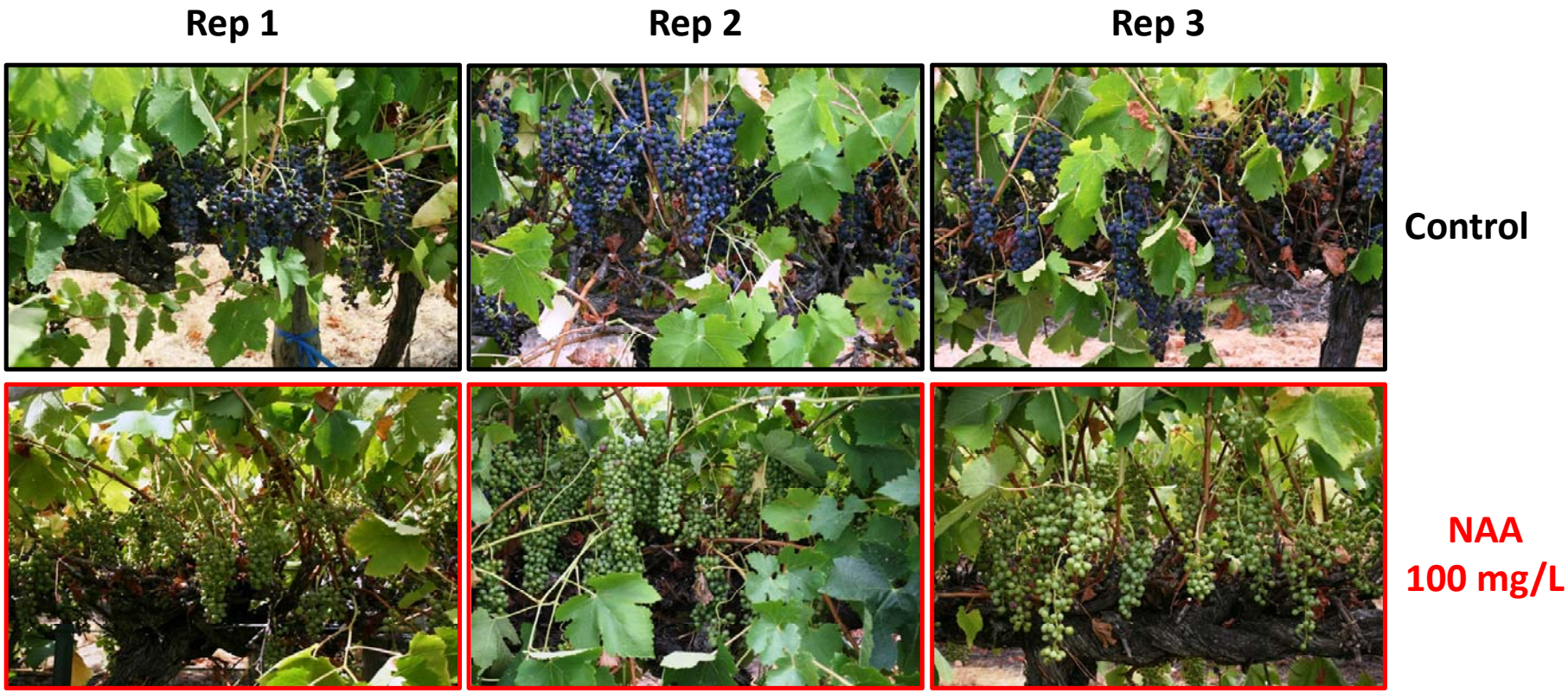


Fig. 24. Shiraz trial, Eden Valley, 2015/16. Photographs of Control and NAA-treated berries taken 28/01/2016, showing delay caused by NAA application, all three replicates are shown.

The delay in anthocyanin accumulation is also clearly shown by the changes in A520nm throughout ripening (Fig. 25).

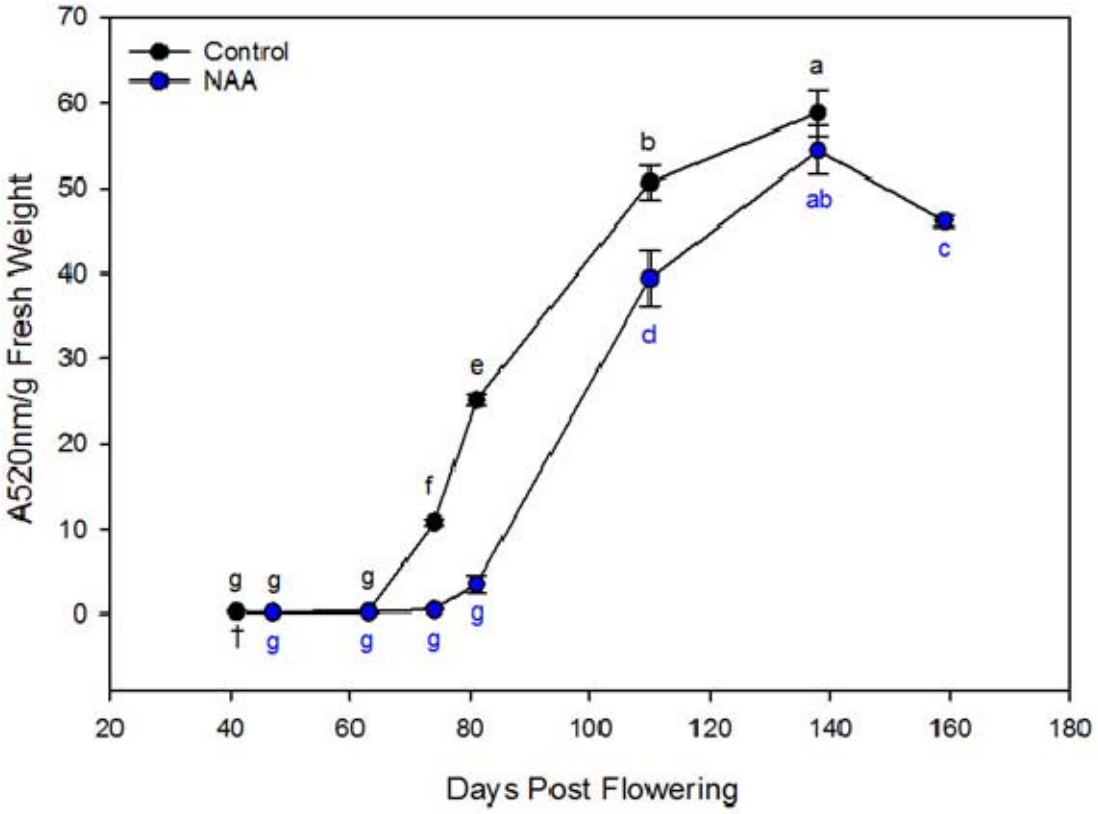


Fig. 25. Anthocyanins as measured by absorbance at 520nm in Control and NAA-treated fruit. All data represent means ±STERR (n=3) and different letters denote significant differences between treatments at $p<0.05$ using one-way ANOVA followed by Duncan's post hoc test.

Even though foreshortened, this experiment showed that NAA delayed Shiraz ripening at a different site than used previously, demonstrating that the process should be robust. As seen previously at other sites and in other seasons the entire ripening process is most likely retarded as the increases in berry weight, sugar levels and anthocyanin levels were all similarly delayed. Unfortunately, the effect of the delaying treatment on wine could not be determined for this study as the NAA-treated fruit was not available.

Shiraz Willunga – two post-veraison treatments

The delaying of veraison, and therefore harvest, by auxin treatment has previously been shown to increase the levels of rotundone, the compound in Shiraz responsible for the peppery character in some wines (see above). The reason for this increase is unknown and could be due to a range of effects including, a direct effect of NAA on the synthesis of rotundone, or its precursors, a result of the longer pre-veraison period or some other indirect effect of the delay such as altered air temperature during ripening later in the season. If it is a direct effect then one could suggest that the post-veraison treatment of berries with NAA might increase rotundone levels without affecting the timing of ripening. To test this, NAA

was applied to Shiraz fruit at Willunga at two times post-veraison, i.e. 30/1/2014 when the fruit was at 14.8ºBrix (12 WPF) and on 6/2/2014 when the fruit was at 16-17ºBrix (13 WPF). Sampling was carried out throughout development for any, unexpected, change in the progress of development and to test for any change in rotundone levels at harvest.

The two post-veraison NAA treatments did not alter ripening as measured by TSS increase as there was no significant difference between the Control and NAA-treated fruit at any sampling point (Fig. 26). This indicates that the delay in harvest observed as a result of pre-veraison NAA treatments is due to the delay NAA causes in the timing of veraison rather than a slowing down effect on the ripening phase itself.

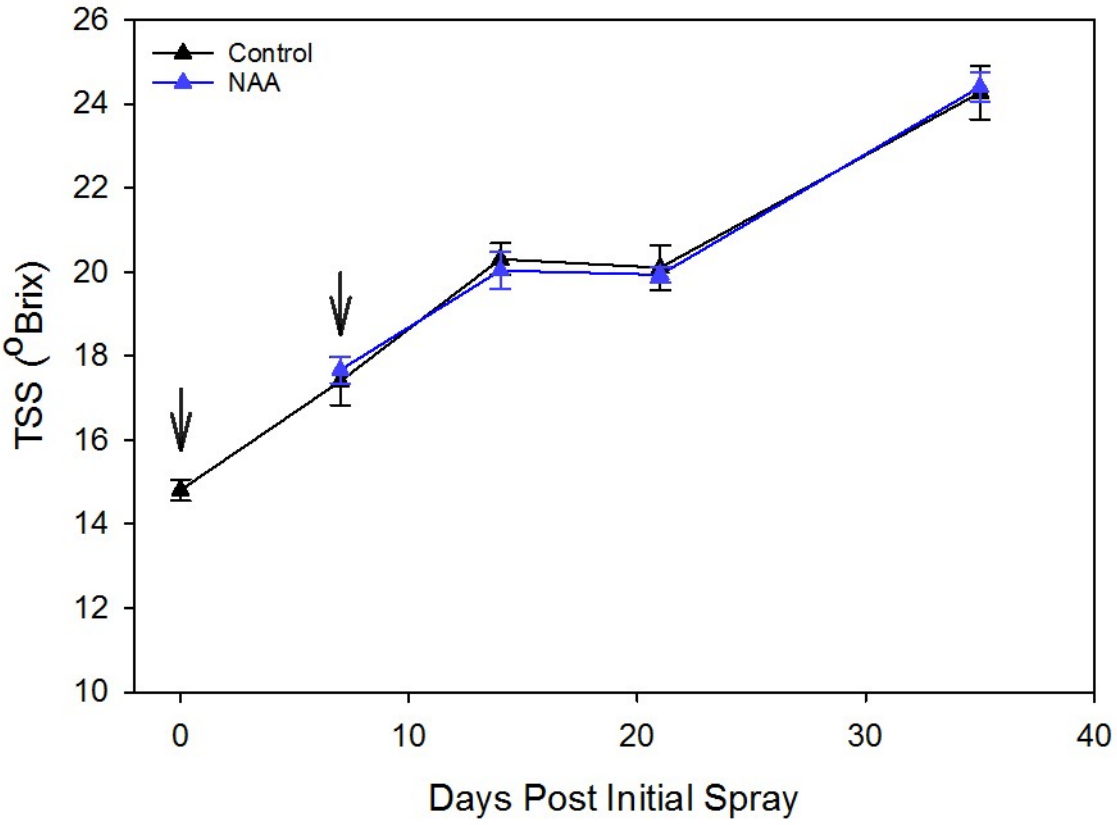


Fig. 26. TSS (ºBrix) of Control and NAA-treated berries following post-veraison treatments. All data represent means ±STERR (n=3). The arrows indicate the two treatment time points.

The concentration of rotundone (the peppery compound in Shiraz and some other cultivars) was measured in Control and NAA-treated fruit when they were both at 24ºBrix (Fig. 27).

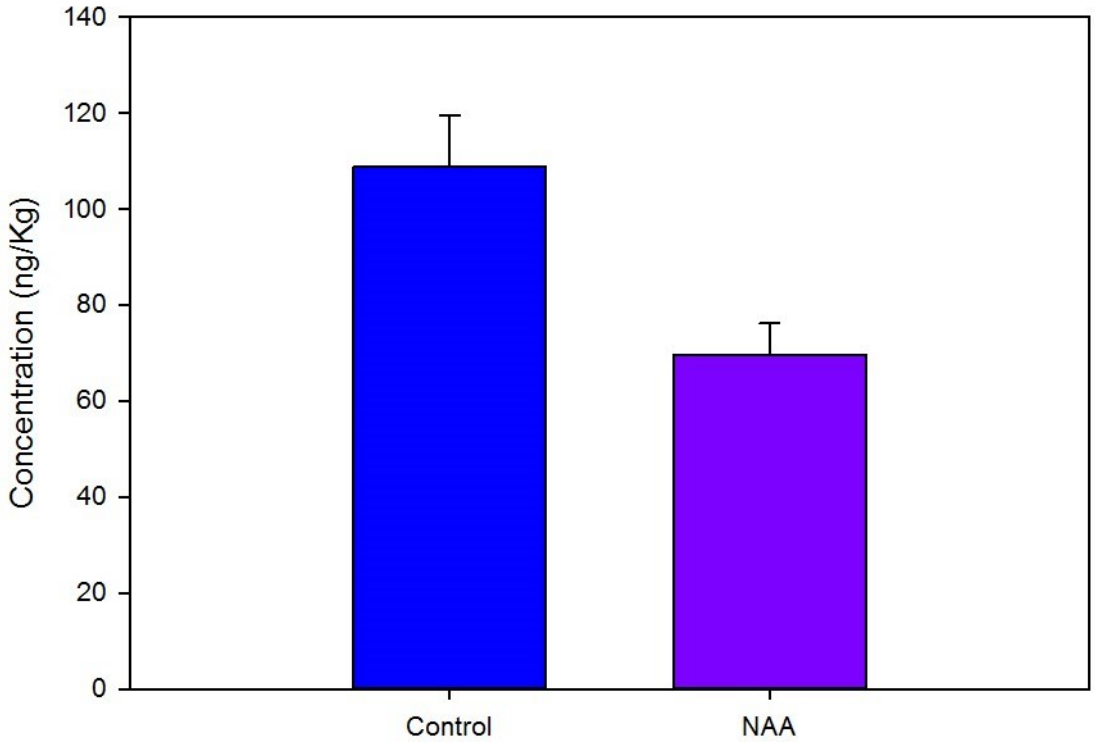


Fig. 27. Rotundone concentration in Control and NAA-treated fruit harvested 5/3/2014 at 24.3 and 24.4ºBrix respectively. All data represent means ±STERR (n=3). The values for Control and NAA are statistically different (P = 0.0363).

The reduced concentration of rotundone in the NAA-treated, delayed, fruit was quite unexpected. From previous results we might have predicted that the rotundone levels might have been similar to, or even higher than, the Control fruit compared with the NAA-treated fruit. Grapes from the two treatments were at near identical Brix levels suggesting that they were at equivalent developmental stages. This experiment could be repeated but if the results are proven it indicates a more complex relationship between NAA application and rotundone accumulation than previously suspected.

This experiment also demonstrates that the timing of the NAA treatment has to be prior to veraison to be effective. Once commenced, auxins cannot delay or retard the progress of ripening. This further indicates that auxins are involved in delaying the initiation event, i.e. preventing the triggering of the ripening event and are not effective once the ripening developmental process is underway.

Testing the effect of application of the precursor for IAA, tryptophan, on berry development

We have shown that the onset of ripening appears to be controlled by auxins levels. Endogenous auxin (IAA) concentration is high in flowers and young berries and decreases before the onset of ripening (Böttcher *et al*. 2010). IAA is inactivated by a group of enzymes that conjugate it to amino acids (Böttcher *et al*. 2011a; Böttcher *et al*. 2010). IAA application to berries is ineffective in delaying ripening, probably due to it being inactivated by the mechanism mentioned above. However, application of the closely related, synthetic, auxin NAA, is effective, probably due to it not being a particularly good substrate for the

deactivating enzymes (Böttcher *et al*. 2011a). It is conceivable that if we could boost the levels of the endogenous IAA auxin we may be able to use it to delay ripening despite its lability. One way of doing this might be to increase the levels of IAA precursors. IAA is formed via a simple two step pathway from the amino acid tryptophan (Fig. 28). By increasing tryptophan levels through spray application, it might be possible (if precursor substrate levels were the limiting factor) to increase endogenous IAA concentration in the berry.

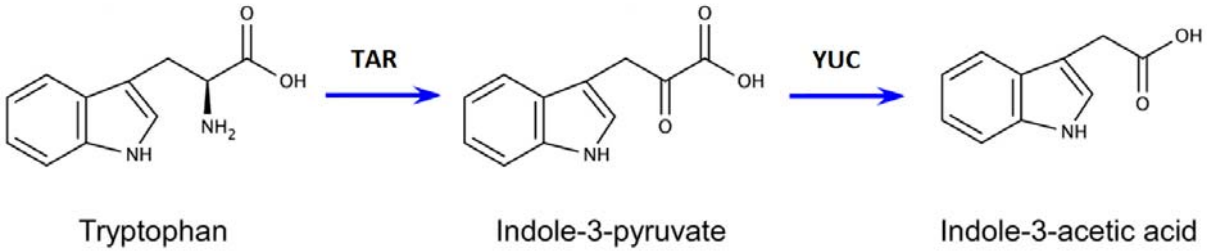


Fig. 28. Biosynthetic pathway (with substrates, products and enzymes) for IAA production from the amino acid tryptophan in plants. TAR = tryptophan aminotransferase related, YUC = flavin containing monooxygenase

Repeated treatments were carried out using relatively high levels of pure tryptophan (see Method). Figure 29 shows the effect of these treatments on average berry weight (A) and TSS respectively (B). It seems that applying exogenous tryptophan, the precursor for the naturally occurring auxin IAA, to berries to delay ripening will not achieve its aim. There are a number of possible reasons for this. First, the waxy skin of the berry may not allow enough tryptophan to enter the flesh to make a difference and what does enter the skin may not diffuse well throughout the berry. Second, the level of the precursor may not be the limiting factor in IAA synthesis, it may well be that in order to get more IAA made, the amount of biosynthetic enzymes present may need to also be increased. Third, the berry homeostatic response may rapidly dampen any increase in IAA. Important players in this response are the previously reported GH3 enzymes that conjugate IAA to amino acids to inactivate it. We have shown that the two most important members of this family inactivate IAA very efficiently and so may rapidly decrease any tryptophan-induced increase in IAA levels. However, the delay of ripening through Ethrel treatment, with the accompanying increase in IAA concentration (Böttcher *et al*. 2013b), indicates that under some circumstances IAA may be capable of delaying ripening.

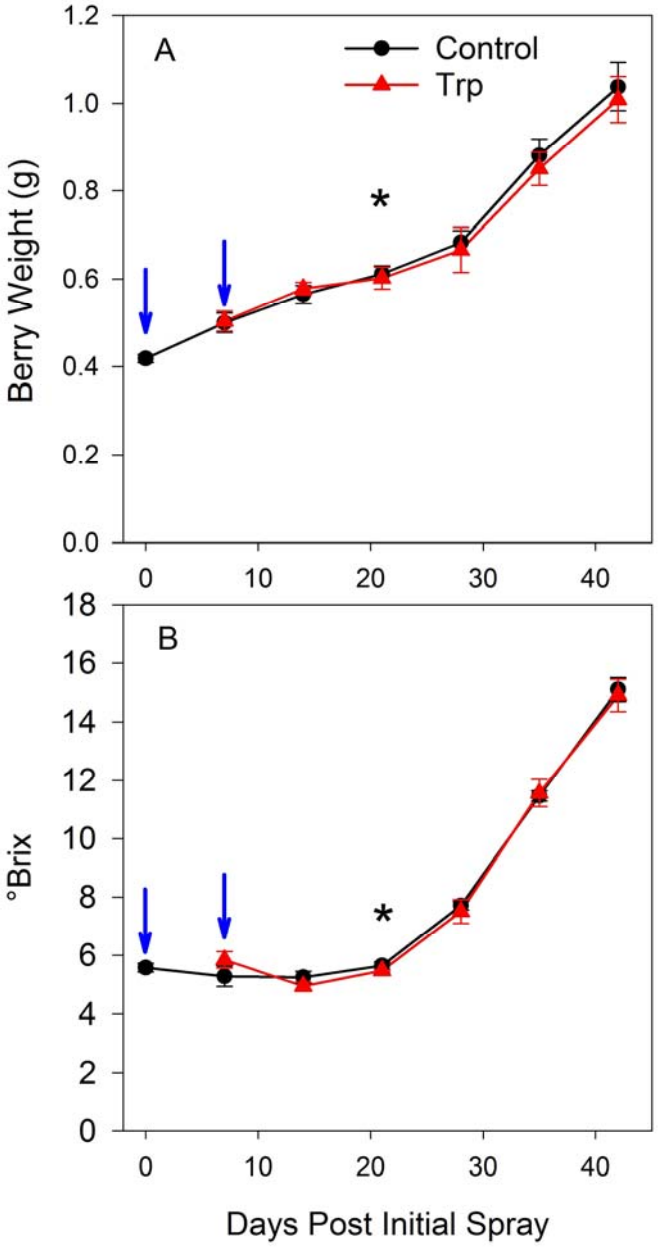


Fig. 29. (A) Berry weight of Control (black) and tryptophan-treated (red) berries, (B) TSS (ºBrix) of Control (black) and tryptophan-treated (red) berries. Blue arrows indicate treatment dates, asterisk indicates date of veraison. All data represent means ±STERR (n=3).

Testing the effects of epigallocatechin gallate (EGCG) on berry development/ripening

In an attempt to test whether any cheap, readily available compounds other than NAA might be able to delay ripening, an experiment was conducted to re-test the possible use of IAA sprays (to make absolutely sure that they were ineffective). In addition, another naturally occurring compound, epigallocatechin gallate (EGCG), was tested for its ability to delay berry ripening. EGCG is a tannin that has been shown to bind specifically into the active site of the pectin methylesterase enzyme and inhibit its activity (Lewis *et al*. 2008). This enzyme is important to plant cell wall structure as it de-esterifies methylated pectin molecules which makes the pectin more susceptible to breakdown by polygalacturonases, a process involved in

cell expansion and fruit softening. It should be noted that the extensibility of plant cell walls also requires the action of additional enzymes. Berry softening is the first easily detected change that occurs at the commencement of ripening and usually precedes sugar and colour accumulation by a few days. Changes to the cell wall, in part mediated by pectin modifying enzymes, have to occur to allow fruit softening and cell expansion which in turn results in berry expansion due to the accumulation of hexoses and the accompanying influx of water. It is most likely that ripening would not happen, or be delayed, if the cell expansion resulting from cell wall changes did not occur. If it is possible to get EGCG into the berry it may an alternative way to delay ripening.

Neither, the IAA, EGCG or IAA+EGCG treatments had any statistically significant effect on berry weight (Fig. 30A). TSS values were only significantly different at the final time point when all treatments were a little higher than the Control (Fig. 30B). It has previously been shown that although the endogenous IAA is likely to inhibit ripening, applied IAA does not seem to have this effect (Böttcher *et al*. 2011a; Böttcher *et al*. 2013b). This may be because IAA does not pass through the skin in sufficient quantities to have an effect, coupled with its inactivation by berry homeostatic responses. EGCG also did not delay berry expansion or sugar accumulation. This might be due to the molecules larger size not allowing easy penetration into the berry at a concentration sufficient to be effective. It is also possible that the active site in grape PMEs has a different structure to the enzymes tested from other plants (citrus, tomato, *Cuscuta pentagona*, *Castilleja indivisa*) that were previously shown to be inhibited by EGCG (Lewis *et al*. 2008).

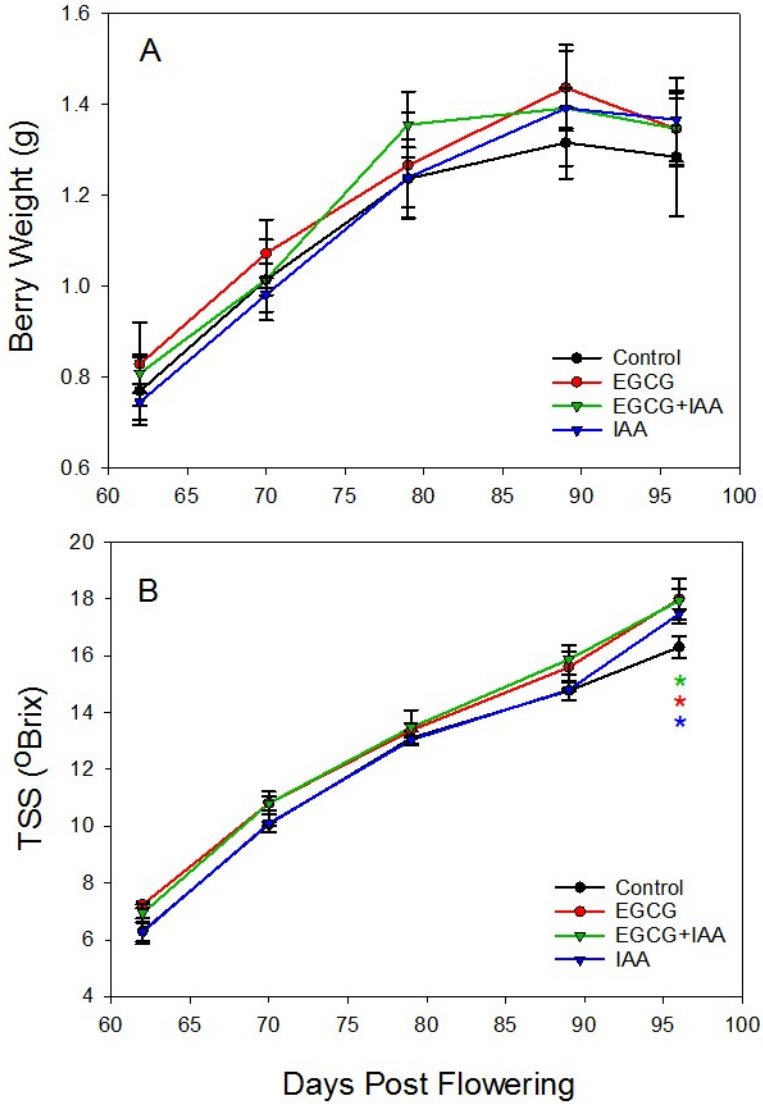


Fig. 30. Effect of EGCG and IAA on average berry weight (A) and TSS (B). All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

The analysis of total acids, malic acid, tartaric acid, anthocyanins, YAN and pH did not show any significant differences between the treatments and Control at any particular time point (data not shown). The above data indicate that EGCG is unlikely to be useful as a tool to delay berry ripening.

Two JA-conjugating GH3 enzymes from grapevine have overlapping substrate specificities

Jasmonates are a class of plant hormones derived from the metabolism of unsaturated membrane fatty acids that regulate a wide range of processes such as responses to abiotic and biotic stresses, reproductive development and growth. It has also been shown that jasmonate can stimulate the biosynthesis of secondary metabolites including sesquiterpenes, proanthocyanidins and stilbenes in grape cell cultures (D'Onofrio *et al*. 2009).

This diverse group of molecules includes jasmonic acid (JA), its methyl ester (MeJA) and amide conjugates with amino acids, such as JA-isoleucine (JA-Ile). Conjugation to the amino acid to form JA-Ile is required to confer biological activity (Fonseca *et al*. 2009; Staswick and Tiryaki 2004; Thines *et al*. 2007). The formation of JA-amino acid conjugates is catalyzed by acyl-amido synthetases of the Gretchen Hagen3 (GH3) family, which comprise a group of plant-specific proteins also known to conjugate amino acids to other plant hormones, such as auxins (see above and Staswick *et al*. 2005; 2002). JA-conjugating GH3 enzymes from a range of different species have been shown to have roles in wounding, pathogen attack and herbivore attack (e.g. (Fukumoto *et al*. 2013; Kang *et al*. 2006; Shimizu *et al*. 2013; Suza *et al*. 2010; Svyatyna *et al*. 2014; Wakuta *et al*. 2011; Wang *et al*. 2008a).

Jasmonates are also essential for the formation of functional reproductive organs (Ishiguro *et al*. 2001; Li *et al*. 2001; Li *et al*. 2004; Stintzi and Browse 2000). Our understanding of the role of JA-conjugation in fruit is scant, despite a number of indications that jasmonates might be involved in various stages of fruit development. Transient increases in endogenous JA and MeJA concentrations during the onset of ripening in tomato and apple (*Malus domestica* Borkh.) have been reported (Fan *et al*. 1998), suggesting a role for JA in the initiation of ripening initiation in these fruit. In grape (*Vitis vinifera* L.) skins (Kondo and Fukuda 2001) or strawberries (*Fragaria ananassa* Duch.) (Gansser *et al*. 1997), high levels of MeJA, and/or JA were found early in fruit development, which decreased to be low before the initiation of ripening and remained low during ripening. In addition, JA or MeJA application to fruit on the plant, detached fruit or to cultured cells, can induce the production of secondary metabolites known to accumulate in ripening fruit, such as anthocyanins, carotenoids, sesquiterpenes, tannins and stilbenes (D'Onofrio *et al*. 2009; Kondo *et al*. 2000; Pérez *et al*. 1993; Wang *et al*. 2008b). Furthermore, the application of MeJA to grapes at veraison increased levels of terpenes and norisoprenoids in wines made from the treated fruit (Gómez-Plaza *et al*. 2012).

The grape VviGH3-7 and VviGH3-9 proteins conjugate isoleucine to JA to produce the active jasmonate hormone

Previously, two predicted grapevine sequences, VviGH3-7 and VviGH3-9, were identified as dicot Group I GH3 proteins (Böttcher *et al*. 2011a). Characterised members of this group

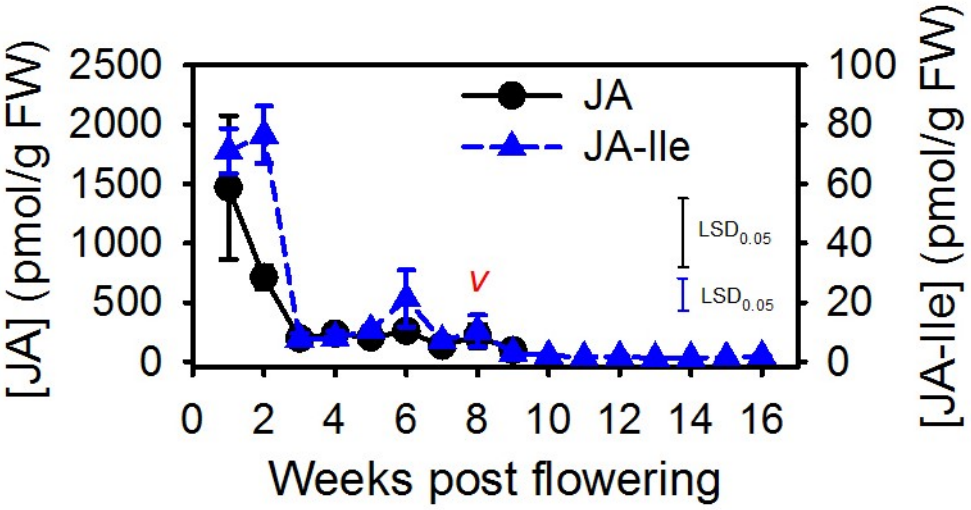
have been described as JA-conjugating enzymes that catalyse the formation of a range of JA-amino acid conjugates, including JA-Ile (Staswick and Tiryaki 2004; Suza *et al*. 2010; Svyatyna *et al*. 2014; Wakuta *et al*. 2011). Purified, recombinant VviGH3-9 protein was found to conjugate a total of 12 amino acids to JA (data not shown). The most readily conjugated amino acids (detected by TLC) were Ile, Leu, Met, Val, Phe, Ser, Thr, Cys while His, Ala, Gln and Trp were poorer substrates that were only detected by LC-MS analysis (data not shown). Other acyl substrates tested were SA, IAA, and the JA-precursors OPDA and dnOPDA, but, as described for AtJAR1 (Staswick *et al*. 2002), none of them were substrates for VviGH3-9 (data not shown). VviGH3-7-catalysed conjugation of JA to Ile, Leu, Met, Val Ser, Thr, Cys and His (data not shown) whereas SA, IAA, OPDA and dnOPDA were not accepted as acyl substrates (data not shown). The substrate specificity of VviGH3-7 therefore resembled that of VviGH3-9.

The regulated formation of JA-Ile by the JA-amido synthetase type of GH3 proteins is crucial for the development and survival of plants (Wasternack and Hause 2013). All monocot and dicot species studied to date express at least two JA-conjugating GH3s (Staswick and Tiryaki 2004; Suza *et al*. 2010; Wakuta *et al*. 2011). Both VviGH3-7 and VviGH3-9 conjugated Ile to JA (data not shown). Further evidence for this activity comes from the presence of highly conserved target residues for JA binding in both, VviGH3-9 and VviGH3-7, sequences (Peat *et al*. 2012).

JA biosynthesis and conjugation during grape berry development and in different grapevine organs

The concentration of JA and JA-Ile was measured in Shiraz berries from one week post flowering (WPF) through to harvest at 16 WPF (Fig. 31A) to determine berry developmental stages characterised by high jasmonate activity. The highest concentration of both jasmonates was detected in young berries (1-2 WPF), followed by a rapid decrease to low or non-detectable (JA, 10-16 WPF) levels of JA and JA-Ile throughout the rest of development. In all samples where JA was detected, its concentrations were found to be 10 to 20-fold higher than JA-Ile concentrations. The jasmonate profile in developing berries was mirrored by *VviGH3-7* expression levels, whereas the post-veraison increase in *VviGH3-9* transcripts, which were generally more abundant than *VviGH3-7* transcripts (~10-fold), was not reflected by changes in JA-Ile concentrations (Fig. 31B). JA production in berries was further assessed by measuring changes in expression of genes central to JA biosynthesis. Allene oxide synthase (AOS) catalyses a step near the start of the JA biosynthesis pathway (Schaller 2001); the grapevine gene used here, *VviAOS,* is thought to be an orthologue of the AOS gene from *Arabidopsis* however, as this is a large gene family in grape, this cannot be determined without functional analysis. *VviOPR3* is the grapevine orthologue of the *Arabidopsis OPDA-reductase 3* (*OPR3*) gene (74% amino acid sequence identity), which has been shown to catalyse the last specific step in the JA biosynthesis pathway (Schaller *et al*. 2000). The pattern of *VviAOS* expression during berry development (Fig. 31B), distinguished by a sharp increase at veraison, did not align with JA accumulation which suggested that this gene might not be involved in JA biosynthesis in berries. The high transcript abundance of *VviOPR3* in pre-veraison berries, 1-8 WPF (Fig. 31B), was suggestive of sustained JA production during this period. The expression of *VviGH3-7*, *VviGH3-9, VviAOS* and *VviOPR3* was also analysed in a range of other grapevine tissues. In general transcript levels were found to be similar to those detected in pre-veraison berries (Fig. 32). *VviGH3-7* was most highly expressed in node 5 and 9 leaves, was expressed at moderate levels in flowers, young leaves (node 1), tendrils, internodes and roots and either had low transcript numbers (seeds 9 WPF), or was below detection, in seeds. *VviGH3-9* was expressed in all organs, with the highest transcript accumulation in node 9 leaves, seeds (9 WPF) and internodes, whereas *VviOPR3* was mainly expressed in flowers, tendrils, internodes and roots.

A


[JA] (pmol/g FW)
[JA-Ile] (pmol/g FW)
2500
2000
1500
1000
500
0
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Weeks post flowering
JA
JA-Ile
LSD0.05
LSD0.05
v

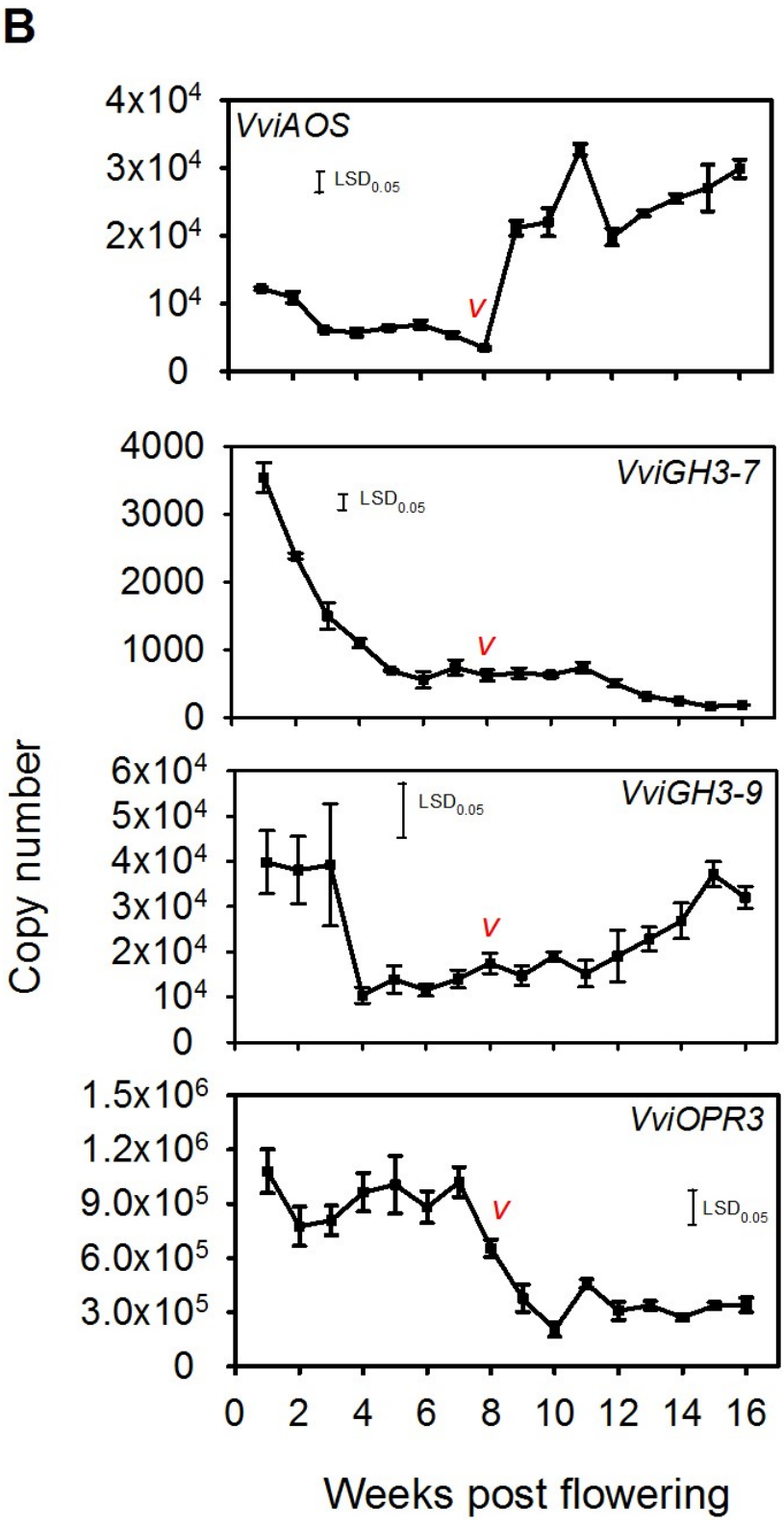


Fig. 31. JA biosynthesis and conjugation to isoleucine in developing grape berries
Field-grown Shiraz berries from 1-16 WPF were used to **(A)** determine JA and JA-Ile concentrations and **(B)** study changes in the expression of JA biosynthesis (*VviAOS*, *VviOPR3*) and conjugation *(VviGH3-7*, *VviGH3-9*) genes. JA was not detected in 10-16 WPF berries. The red "v" indicates veraison as determined by the last time point before a significant increase in total soluble solids levels was recorded. All data represent means ± SE (n = 3 biological replicates) and LSD values were determined at the $p<0.05$ significance level. FW, fresh weight.

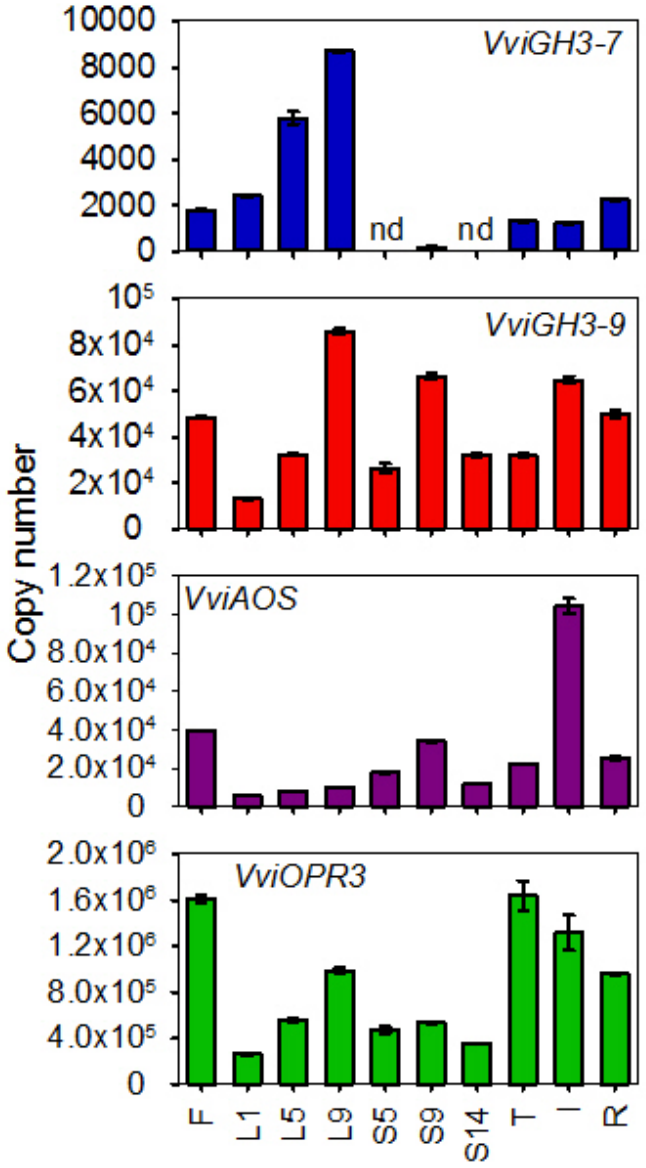


Fig. 32. Expression of genes involved in JA biosynthesis and conjugation in different grapevine organs
Transcript accumulation of *VviGH3-7*, *VviGH3-9*, *VviAOS* and *VviOPR3* in different organs of either field grown (flower, seeds, leaves, tendril, internode) or glasshouse grown (root) Shiraz plants. Bars represent means ± SE (n = 3 technical replicates). F, flower; I, internode; L, leaf (node indicated by number); R, root; S, seed (WPF indicated by number); T, tendril; nd, not detected.

The developmental changes of JA/JA-Ile concentrations and *VviAOS/VviOPR3/VviGH3-7/VviGH3-9* expression levels in grape berries reported above (Fig. 31) suggest a role for jasmonates early in fruit development, i.e. in fruit set and cell division in grapes. The stimulating effect of jasmonates on the production of secondary metabolites (reviewed by Pauwels *et al*. 2009), has been previously reported, this includes the induction of sesquiterpene and phenylpropanoid pathways in cell cultures derived from pre-veraison Cabernet Sauvignon berries (D'Onofrio *et al*. 2009). However, a role for JA or JA-Ile in the post-veraison accumulation of secondary metabolites in grapes is not supported by the presented data. This is in accordance with a previous study that reported a rapid decrease in

JA concentrations in 'Pione' grapes between 3-5 WPF after which there was no increase during the rest of berry development (Kondo and Fukuda 2001). The transcript levels of genes encoding jasmonic acid conjugating enzymes are not always correlated with the detected jasmonate concentrations (Suza and Staswick 2008), as observed for the late post-veraison increase in *VviGH3-9* expression that was not reflected by changes in the concentration of JA-Ile (Fig. 31). Therefore, tissue-specific differences in the expression of grapevine Group I GH3 genes (Fig. 32) might not reflect corresponding differences in jasmonate levels.

Grapevine *GH3-7* and *GH3-9* differ in transcriptional response to wounding and MeJA treatment

To gain more information about possible differential functions of VviGH3-7 and VviGH3-9, changes in expression of the corresponding genes were analysed in response to two treatments known to stimulate a JA response. When plants are exposed to volatile MeJA, a series of JA-dependent defence responses, including the transcriptional induction of the complete JA biosynthesis pathway, is triggered (Baldwin 1998; Li *et al*. 2002; McConn *et al*. 1997; Wasternack *et al*. 2006). Induction of the JA biosynthesis pathway in MeJA-treated grapevine leaves was evidenced by increases in the expression of *VviAOS* and *VviOPR3*, which were at a maximum at 4 h and returned to Control levels 6 h after treatment (Fig. 33). A similar response was observed for *VviGH3-9* with a three-fold induction at the 4 h time point. However, there was no change in the transcript levels of *VviGH3-7* which were not different between Control leaves and MeJA-treated leaves at any of the time points tested (Fig. 33). Similar to MeJA treatments, mechanical wounding is known to stimulate JA production and signaling, not only at the wound site, but also in unwounded, distal parts of the plant (Schilmiller and Howe 2005). Differential expression of *VviGH3-7* and *VviGH3-9* was observed in wounded (node 6) and unwounded, distal Shiraz leaves (node 5), (Fig. 34A). As described above for MeJA treatment, the expression of *VviGH3-9* was similar to that of *VviOPR3*, a marker for early gene induction in response to mechanical wounding (Koo *et al*. 2011; Li *et al*. 2005; Strassner *et al*. 2002), with a strong induction (17- and 24-fold, respectively) in wounded leaves after 2 h and a less pronounced, delayed increase in transcript levels, peaking at 6 h after wounding, in the unwounded, distal leaves (7.5- and two-fold, respectively). *VviAOS* expression also increased in the wounded leaves. Significant changes in jasmonate concentrations were only detected for JA-Ile in wounded leaves (Fig. 34B). Changes in JA-Ile were similar to those observed for the expression levels of *VviOPR3* and *VviGH3-9*, as JA-Ile concentration peaked in leaves 2 h after wounding (four-fold induction), but was still significantly elevated at the 6 h time point. However, *VviGH3-7* transcript levels were unchanged in wounded leaves at all time points tested, but were significantly increased in the distal leaves 2 h (2.5-fold) and 6 h (3.5-fold) after the wounding of the basal leaves (Fig. 34A).

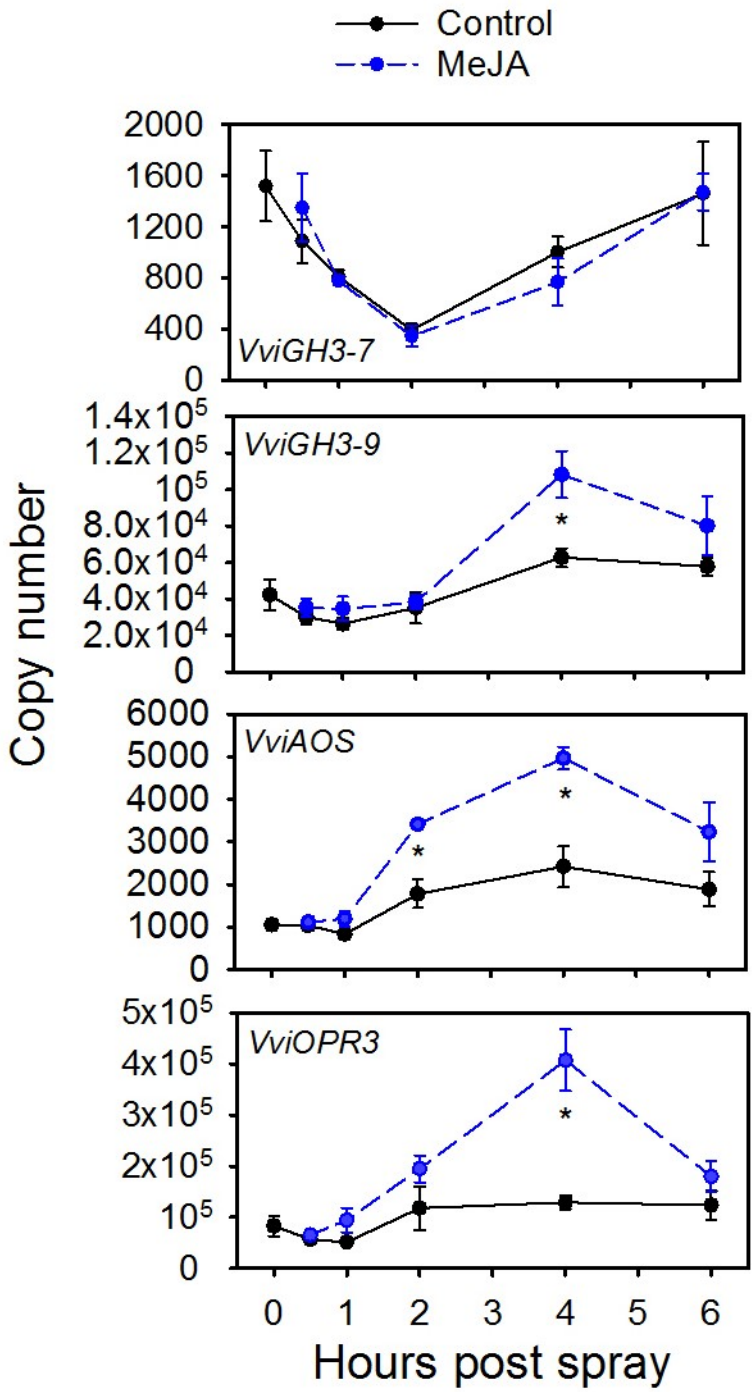


Fig. 33. Changes in the expression of genes involved in JA biosynthesis and conjugation in response to MeJA treatment of Shiraz grapevine leaves

The expression of *VviGH3-7*, *VviGH3-9*, *VviAOS* and *VviOPR3* in leaves was analysed by qRT-PCR at five time points after treatment with a Control- or 100 µM MeJA-solution. All data represent means ± SE (n = 3 biological replicates). Asterisks indicate significant differences of the mean values of MeJA-treated samples from the mean values of Control samples as determined with Student's t-test (*=p<0.05).

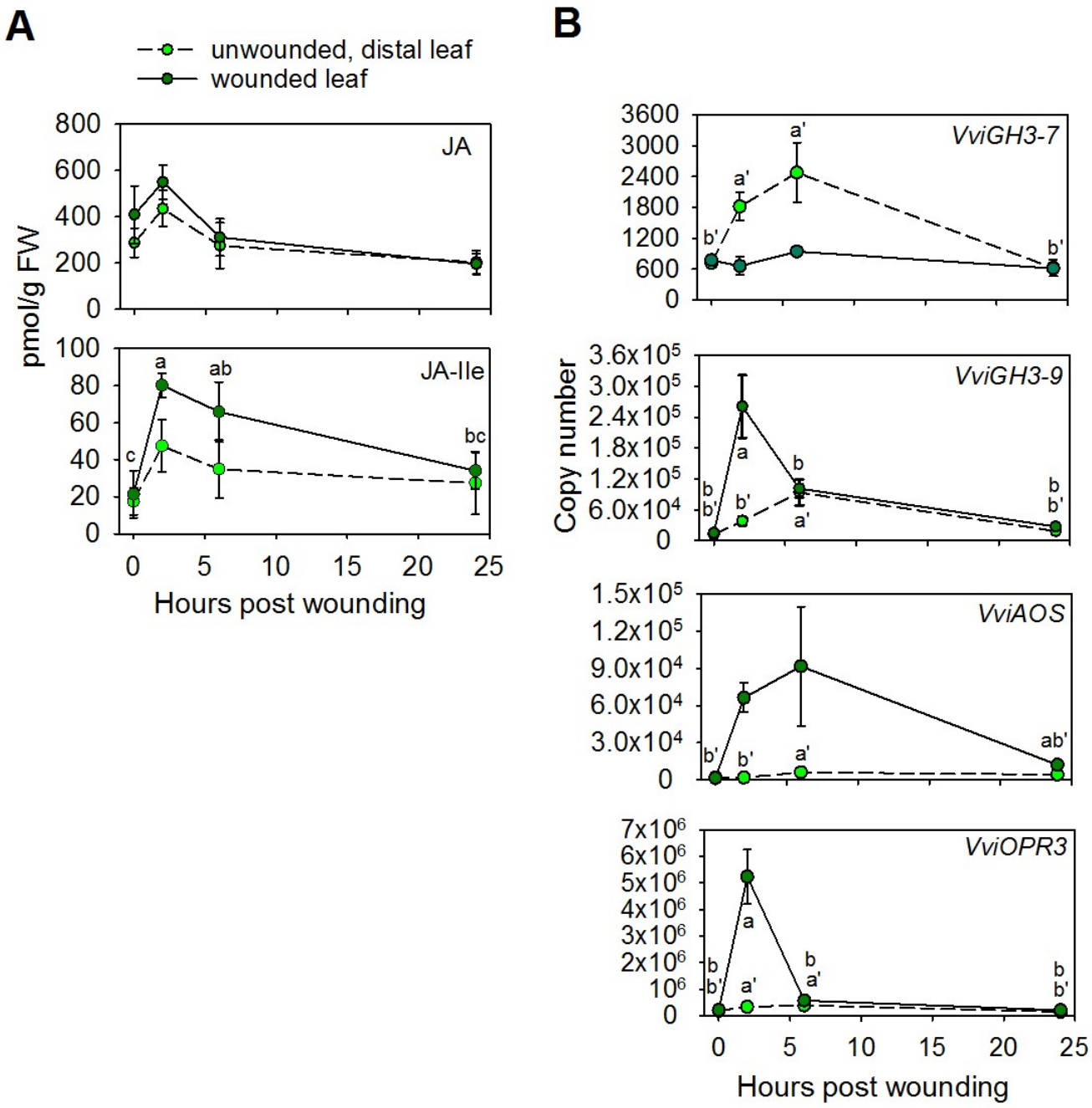


Fig. 34. Wound-induced changes in JA biosynthesis and conjugation in Shiraz grapevine leaves

Wounded node 6 leaves on growing shoots of field-grown vines and unwounded node 5 leaves were sampled at the indicated time points and analysed for changes in jasmonate concentrations and related gene expression. **(A)** The transcript levels of *VviGH3-7*, *VviGH3-9*, *VviAOS* and *VviOPR3*, **(B)** JA and JA-Ile concentrations. All data represent means ± SE (n = 3 biological replicates). One-way ANOVA followed by Duncan's post hoc test was employed to evaluate changes in jasmonate concentration or transcript accumulation over time in either wounded or unwounded, distal leaves. Data points denoted by a different letter (a-c, wounded leaf; a'-b', unwounded, distal leaf) differ significantly (p<0.05).

The differences in transcription observed in grape berries and other grapevine organs between *VviGH3-7* and *VviGH3-9* demonstrated variations in the regulation of these genes. This was confirmed by the analysis of gene expression in response to MeJA, a treatment known to

quickly elicit a series of JA-dependent defence responses (Baldwin 1998; Li *et al*. 2002; McConn *et al*. 1997). Since Arabidopsis jar1 mutants (the equivalent to a grapevine *VviGH3-9* mutant) confer the same level of resistance to both JA and MeJA and because MeJA is not a substrate for AtJAR1 it is assumed that demethylation and subsequent JA-Ile formation is required to trigger this response (Staswick *et al*. 1998). As previously reported for the tomato gene *SlJAR1* (Suza *et al*. 2010), *VviGH3-9* was MeJA-inducible, whereas *VvGH3-7* expression was not affected by the treatment (Fig. 33). In Arabidopsis both Group I GH3 genes, *AtJAR1* and *AtGH3-10*, have been described as unresponsive to MeJA (Staswick and Tiryaki 2004; Yuan *et al*. 2013), but in another study *AtGH3-10* expression was reported to be MeJA-inducible (Zheng *et al*. 2006). Therefore, from the available data it seems unlikely that Group I subgroup I and II genes can be distinguished by their response to MeJA, but further investigations are required. *VviGH3-7* and *VviGH3-9* also differed in their response to wounding in local and unwounded, distal leaves. The transient increase in *VviGH3-9* and *VviOPR3* transcripts in wounded grapevine leaves, with a peak 2 h after wounding, was similar to the wound-induction in leaves of the orthologue genes in other species such as, tomato (Li *et al*. 2005; Strassner *et al*. 2002; Suza *et al*. 2010), Arabidopsis (Heitz *et al*. 2012; Koo *et al*. 2011; Suza and Staswick 2008), rice (Wakuta *et al*. 2011) and tobacco (Paschold *et al*. 2008; Wang *et al*. 2008a). In contrast, *VviGH3-7* transcript levels in wounded leaves remained unchanged over the 24 h period of the experiment (Fig. 34A). The concentration of JA-Ile peaked at the same time as the expression of *VviOPR3* and *VviGH3-9* peaked (Fig. 34B), reaching 80 pmol/gFW, 2.5-25-fold lower than what has been reported in similar experiments from other species such as Arabidopsis (Heitz *et al*. 2012; Koo *et al*. 2011; Koo *et al*. 2009; Suza and Staswick 2008), tomato (Suza *et al*. 2010), tobacco (Kang *et al*. 2006) and rice (Wakuta *et al*. 2011). However, JA-Ile concentrations similar to those reported here grapevine have recently been published from rice (Svyatyna *et al*. 2014). For all plant species mentioned above a transient burst in JA concentrations has also been reported, whereas no significant changes were detected in grapevine leaves (Fig. 34B). This is most likely attributable to the high basal level of JA (410 pmol/g FW) measured in grapevine leaves, which is much higher than the levels below 20 pmol/g FW typically observed in Arabidopsis (Suza and Staswick 2008), tomato (Strassner *et al*. 2002; Suza *et al*. 2010), tobacco (Kang *et al*. 2006) and rice (Svyatyna *et al*. 2014; Wakuta *et al*. 2011). Since the grapevine plants used in this study were grown in a commercial vineyard, exposure to abiotic stresses, such as wind and mechanical wounding due to vineyard management practices, as well as pathogen and herbivore attack are possible causes for the elevated JA levels discussed above. Such high levels would not be expected in a controlled environment. Plant defence responses initiated at the wound site spread rapidly to unwounded, distal parts of the plant (Schilmiller and Howe 2005) and the crucial role played by jasmonates in transducing wound signals has been demonstrated using tomato mutants defective in JA biosynthesis and perception (Li *et al*. 2005; Li *et al*. 2002). The nature of the transduced signal is still much debated and some evidence points to species-specific differences in the mechanism of the systemic wound response (reviewed by Howe 2004). While JA biosynthesis was not required for a wound response in unwounded, distal tomato leaves (Li *et al*. 2002) and the expression of JA biosynthesis and conjugation genes, including *SlOPR3* and *SlJAR1*, were unchanged (Strassner *et al*. 2002; Suza *et al*. 2010). A study in Arabidopsis reported a strong and rapid induction of *AtOPR3* and a slightly delayed induction of *AtJAR1* in distal, unwounded leaves (Koo *et al*. 2009). The same pattern of induction in unwounded, distal leaves was observed for grapevine *VviOPR3* and *VviGH3-9*, with peaks of expression 2 h and 6 h, respectively, after wounding of the basal leaves, but maximal transcript levels were still 15-fold (*VviOPR3*) and three-fold (*VviGH3-9*) lower than in the wounded leaves (Fig. 34A). No significant changes in JA and JA-Ile concentrations were detected in the distal leaves (Figure 34B), which, as described for the wounded leaves, was likely due to unusually high basal

levels of both compounds. Intriguingly, the expression of *VviGH3-7* increased up to 3.5-fold (6 h) in the unwounded, distal leaves, indicating a possible induction by a transmitted wound signal distinct from JA or JA-Ile (Fig. 34A).

In summary, recombinant VviGH3-7 and VviGH3-9 proteins have been shown to conjugate JA to a range of amino acids including isoleucine (data not shown). JA-Ile is the active form of jasmonate (Fonseca *et al*. 2009; Staswick and Tiryaki 2004; Thines *et al*. 2007) and so in this case conjugation has the opposite effect of the conjugation of IAA to Asp which is thought to inactivate it. Gene expression studies of *VviGH3-7*, *VviGH3-9* and the JA biosynthesis genes *VviAOS*, *VviOPR3* in berries and other grapevine tissues and wounded and MeJA-treated leaves, together with JA and JA-Ile measurements suggested a primary role for JA signaling early in berry development and in stress responses of vegetative tissues. Despite JA application stimulating the biosynthesis of secondary metabolites such as sesquiterpenes, proanthocyanidins and stilbenes in grape cell cultures (D'Onofrio *et al*. 2009) there is no evidence for a direct role for jasmonates in the control or ripening. A detailed investigation into the transcriptional regulation of *VviGH3-7* and *VviGH3-9* and the further exploration of possible additional acyl substrates are required to elucidate the functional commonalities and differences of these grapevine enzymes.

Cytokinin metabolism during grape berry development/ripening

Naturally occurring cytokinins have diverse functions in plant growth and development and are molecules of great biological and agricultural importance. The four most abundant cytokinins found in plants, trans-zeatin (tZ), N6-(Δ2-isopentenyl)-adenine (iP), cis-zeatin (cZ), and dihydrozeatin, differ in the structure of their isoprenoid side chains but little is known about the physiological relevance of these differences (Sakakibara 2006). In addition to their role in regulating cell division and differentiation (Amasino 2005), cytokinins are involved in a range of processes including leaf senescence (Gan and Amasino 1995; Kim *et al*. 2006a), control of shoot-to-root balance (Werner *et al*. 2003; Werner *et al*. 2001), nutritional signalling (Samuelson and Larsson 1993; Takei *et al*. 2001b) and stress tolerance (Argueso *et al*. 2009). Quantity and composition of cellular cytokinins are regulated through a balance of biosynthesis, transport, inter-conversion of distinct forms, transient inactivation by conjugation, and irreversible inactivation by side chain cleavage (Kudo *et al*. 2010). The targeted disturbance of this balance in rice (Ashikari *et al*. 2005) and Arabidopsis (Bartrina *et al*. 2011), has indicated the importance of cytokinins in reproductive development and hence crop productivity. High cytokinin activities or concentrations have been reported in immature seeds and fruit from a large number of species, including pea (Quesnelle and Emery 2007), tomato (Desai and Chism 1978), strawberry (Lis *et al*. 1978), kiwifruit (Lewis *et al*. 1996), raspberry (Miret *et al*. 2014) and grape (Alleweldt *et al*. 1975; Chacko *et al*. 1976; Inaba *et al*. 1976). Cytokinin activities/concentrations usually peak shortly after fertilisation coinciding with periods of high rates of cell division during fruit set and early growth (Gillaspy *et al*. 1993; Srivastava and Handa 2005). Applications of synthetic cytokinins such as 6-benzylaminopurine, N-(2-Chloro-4-pyridinyl)-N'-phenylurea (CPPU) and thidiazuron (TDZ) have been widely used in fruit such as grape (Zabadal and Bukovac 2006), kiwifruit (Kim *et al*. 2006b), blueberry (NeSmith 2002), apple (Stern *et al*. 2003) and pear (Flaishman *et al*. 2001) to improve fruit set and/or increase fruit size. In contrast, the role of cytokinins during later stages of fruit development is poorly understood, partly due to the reported decrease in cytokinin activities/concentrations following the initial growth phase (Davies and Böttcher 2014). Treatment of fruit with synthetic cytokinins has produced inconsistent effects on the progression of ripening depending on the fruit species and the cytokinin used. For example, CPPU-treated grapes showed a delayed accumulation of sugars and anthocyanins and delayed softening compared with control berries (Peppi and Fidelibus 2008). A similar

CPPU-induced ripening delay has been described in blueberry (NeSmith 2002). However, the opposite effect was observed in kiwifruit, where CPPU treatment increased sugar accumulation, decreased acidity and reduced flesh firmness (Famiani *et al*. 1999). TDZ had the same ripening-advancing effect on kiwifruit as CPPU (Famiani *et al*. 1999), whereas TDZ-treatment delayed the ripening of persimmon fruit (Itai *et al*. 1995). In contrast, treatment with 6-benzylaminopurine had no effect on the ripening progression of persimmon (Itai *et al*. 1995). While application studies have not given any clear indications of possible functions for endogenous cytokinins during ripening, the asynchronous ripening of siliques and reduced viable seed production in cytokinin-deficient Arabidopsis mutants suggest the involvement of these hormones in fruit maturation (Werner *et al*. 2003). In addition, two recent studies on kiwifruit (Pilkington *et al*. 2013) and grape berries (Böttcher *et al*. 2013a) have reported a significant increase in active cytokinin concentrations in the flesh of ripening fruit. In the case of kiwifruit, the main contributor to this increase was tZ, whereas iP was the main cytokinin species accumulating in ripening grapes.
The aim of this study was to further investigate the possible role of the ripening-related increase in iP concentrations in grapes.

Berries of different grape cultivars exhibit a similar increase in cytokinin accumulation during fruit development but iP concentrations at full ripeness vary

The recent discovery of a large increase in iP concentrations in ripening Shiraz berries has provided the first evidence for a possible involvement of cytokinins in grape berry ripening (Böttcher *et al*. 2013a). To evaluate if the ripening-associated accumulation of iP is common in grapes, berries from three grapevine cultivars, sampled from two weeks post flowering (WPF) to commercial harvest (15-17 WPF), were analysed for their iP content (Fig. 35). The concentrations of the only other active cytokinin present in detectable amounts in grape berries, tZ (Böttcher *et al*. 2013a) were generally found to be low (below 1 pmol/g fresh weight (FW)) and were only significantly elevated at one time point in Cabernet Sauvignon (Fig. 35A, 4 WPF), Riesling (Fig. 35B, 2 WPF) and Pinot Noir (Fig. 35C, 6 WPF). The biggest increase in tZ concentration was recorded for Pinot Noir berries (~20-fold), which, unlike Cabernet Sauvignon and Riesling berries, had not been deseeded prior to cytokinin extraction. In berries from all three cultivars tested, iP concentrations had increased significantly by four weeks after veraison and continued to increase for the rest of development (Fig. 35). However, absolute iP concentrations at harvest varied greatly, being highest in Cabernet Sauvignon (73.9 pmol/g FW), followed by Pinot Noir (31.5 pmol/g FW) and lowest in Riesling (14.6 pmol/1 FW).

To further investigate cultivar-specific differences in berry iP concentrations, grapes from 13 cultivars grown in the same vineyard were sampled at a similar TSS content (19.4-20.8°Brix) and subjected to iP quantification (Table 8). iP concentrations differed up to 14-fold, ranging from 4.46 pmol/g FW in Viognier to 62.90 pmol/g FW in Shiraz. iP abundance was not associated with berry skin colour. Whilst the iP concentration in Cabernet Sauvignon berries (Table 8) was comparable to berries in the same TSS range sampled in a different year and from a different vineyard (Fig. 35A), it was lower in berries from Riesling, Pinot Noir (Table 8, Fig. 35B, C) and Shiraz (Table 8, Fig. 36A).

Table 8. iP concentration in berries (19.4-20.8°Brix) of 13 grape cultivars.
iP values represent means (n = 3) ± SE and different letters indicate significant differences between the cultivars as determined by one-way ANOVA (p<0.05) followed by Duncan's post hoc test.

Species-Cultivar	Skin Colour	iP (pmol g FW^{-1})
V. vinifera cv. Viognier	White	4.46±0.77 ^{f}
V. vinifera cv. Verdelho	White	5.33±0.87 ^{f}
V. vinifera cv. Pinot Noir	Red	5.69±0.60 ^{f}
V. vinifera cv. Riesling	White	6.11±0.77 ^{f}
Vitis hybrid cv. Rubired	Red	7.97±0.37 ^{ef}
V. vinifera cv. Muscat Gordo Blanco	White	8.79±3.83 ^{ef}
V. vinifera cv. Barbera	Red	12.82±0.44 ^{def}
V. vinifera cv. Sauvignon Blanc	White	15.59±4.17 ^{cde}
Interspecific hybrid cv. Chambourcin	Red	20.27±4.17 ^{cd}
V. vinifera cv. Pedro Ximénez	White	21.16±1.19 ^{cd}
V. vinifera cv. Durif	Red	21.85±5.90 ^{c}
V. vinifera cv. Cabernet Sauvignon	Red	40.77±1.72 ^{b}
V. vinifera cv. Shiraz	Red	62.90±0.43 ^{a}

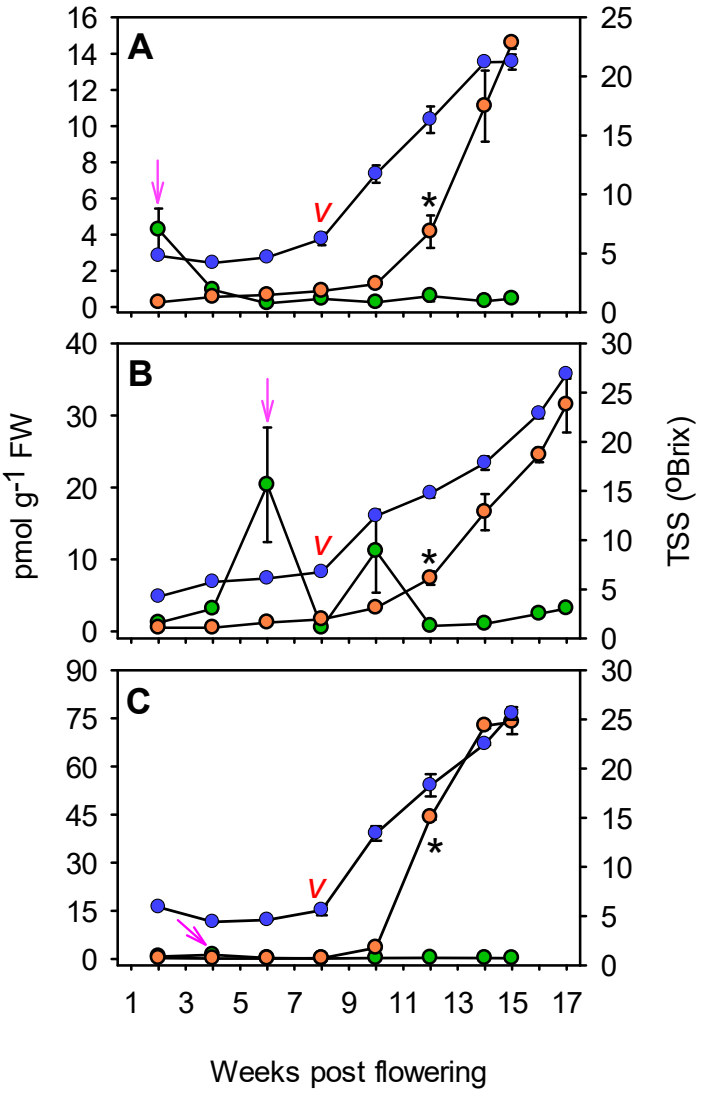


Fig. 35. Concentrations of iP and *tZ* in developing berries from three grapevine cultivars. iP and *tZ* were quantified by LC-MS/MS in developing berries of field-grown **(A)** Cabernet Sauvignon, **(B)** Riesling and **(C)** Pinot Noir. All data represent means (n = 3) ± SE. The blue symbols indicate TSS, the orange symbols indicate iP concentration and the green symbols represent tZ concentration. The red 'V' indicates veraison, as determined by the last time point before a significant increase (p<0.05) in TSS levels was recorded. Asterisks mark the start of a significant increase in iP concentrations. In each cultivar, the concentration of *tZ* was significantly higher (p<0.05) at one time point compared to the others, and this is denoted by an arrow. FW, fresh weight.

Grapevine has multigene families encoding genes with roles in cytokinin biosynthesis, activation, perception, signalling and catabolism

To investigate if the post-veraison increase in grape berry iP concentrations is due to local changes in cytokinin biosynthesis, activation and/or catabolism, grapevine genes belonging to the families of isopentenyltransferases (IPTs), LONELY GUY (LOG) cytokinin nucleoside 5'-monophosphate phosphoribohydrolases and cytokinin oxidases/dehydrogenases (CKXs) were identified by sequence similarity to the respective Arabidopsis genes (Fig. 36). Cytokinin histidine kinase (CHK) receptors and type-A and type-B response regulators (RRs, Fig. 37) were included in the analysis as a functional perception/signal transduction system is required for the detection of, and response to, changed iP concentrations.

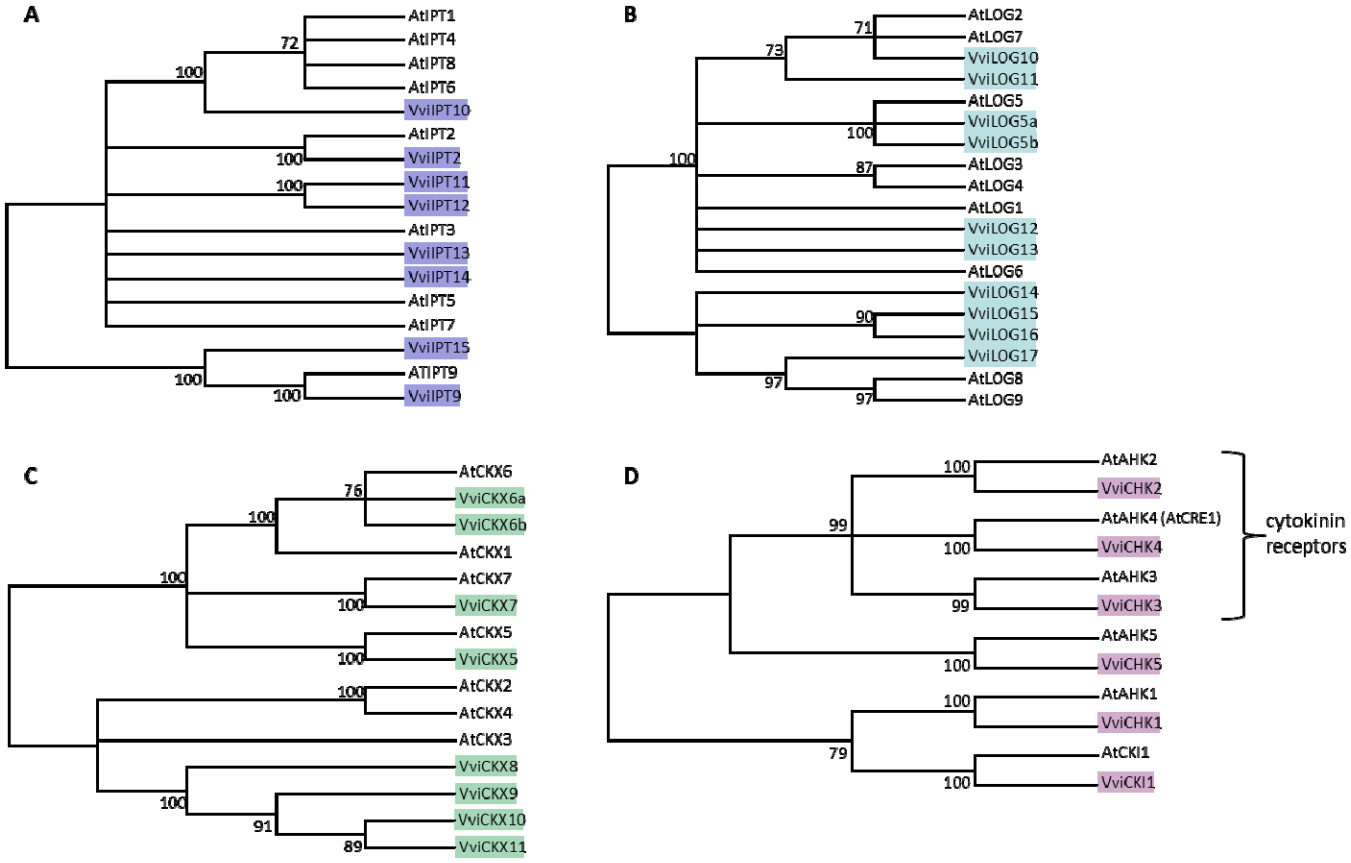


Fig. 36. Phylogenetic relationship of IPT, LOG, CKX and CHK coding sequences from grapevine and Arabidopsis. Unrooted trees of **(A)** IPT, **(B)** LOG, **(C)** CKX and **(D)** CHK sequences. The naming of grapevine sequences followed the guidelines published by Grimplet *et al*. (2014). Predicted grapevine proteins are highlighted with a coloured background.

Adenylate IPTs catalyse the first step in the main cytokinin biosynthesis pathway, i.e. the formation of iP-riboside 5'-phosphates (Kakimoto 2001; Takei *et al*. 2001a). The isoprenoid side chain can subsequently be hydroxylated by the cytochrome P450 enzymes CYP735A1/CYP735A2 to produce *tZ*-ribotides (Takei *et al*. 2004). However, the gene putatively responsible for this conversion was not expressed in berries (data not shown) and cytokinin species conversion seems unlikely. tRNA-IPTs catalyse the addition of an isopentenyl group to adenine bases in tRNAs, which can release *cZ* and iP upon hydrolysis (Murai 1994). The grapevine genome was found to encode eight IPTs, six of which clustered with the Arabidopsis adenylate IPTs and two orthologues (VviIPT2, VviIPT9) of the respective Arabidopsis tRNA-IPTs (Fig. 36A). Inactive cytokinin ribotides produced by the action of adenylate IPTs can be converted to active nucleobases by LOG phosphoribohydrolases (Kurakawa *et al*. 2007). Ten grapevine *LOG* genes were identified (Fig. 36B). Inactivation of cytokinins occurs by CKX-catalysed oxidative cleavage of the isoprenoid side chain (Galuszka *et al*. 2007; Hare and van Staden 1994). Out of the eight grapevine CKXs, four were close orthologues of Arabidopsis CKXs (Fig. 36C). One-to-one

orthologues were identified for all five grapevine CHK sequences (Fig. 36D), three of which (VviCHK2-VviCHK4) represented the *bona fide* cytokinin receptors (Hwang and Sheen 2001). The downstream targets of the His-Asp phosphorelay of the cytokinin signalling pathway are RRs, which are classified as negative (type-A) or positive (type-B) regulators of cytokinin signalling (D'Agostino *et al*. 2000; D'Agostino and Kieber 1999; Imamura *et al*. 1999). In contrast to Arabidopsis, more type-A (11) than type-B (8) RRs (Fig. 37) were identified in the grapevine genome.

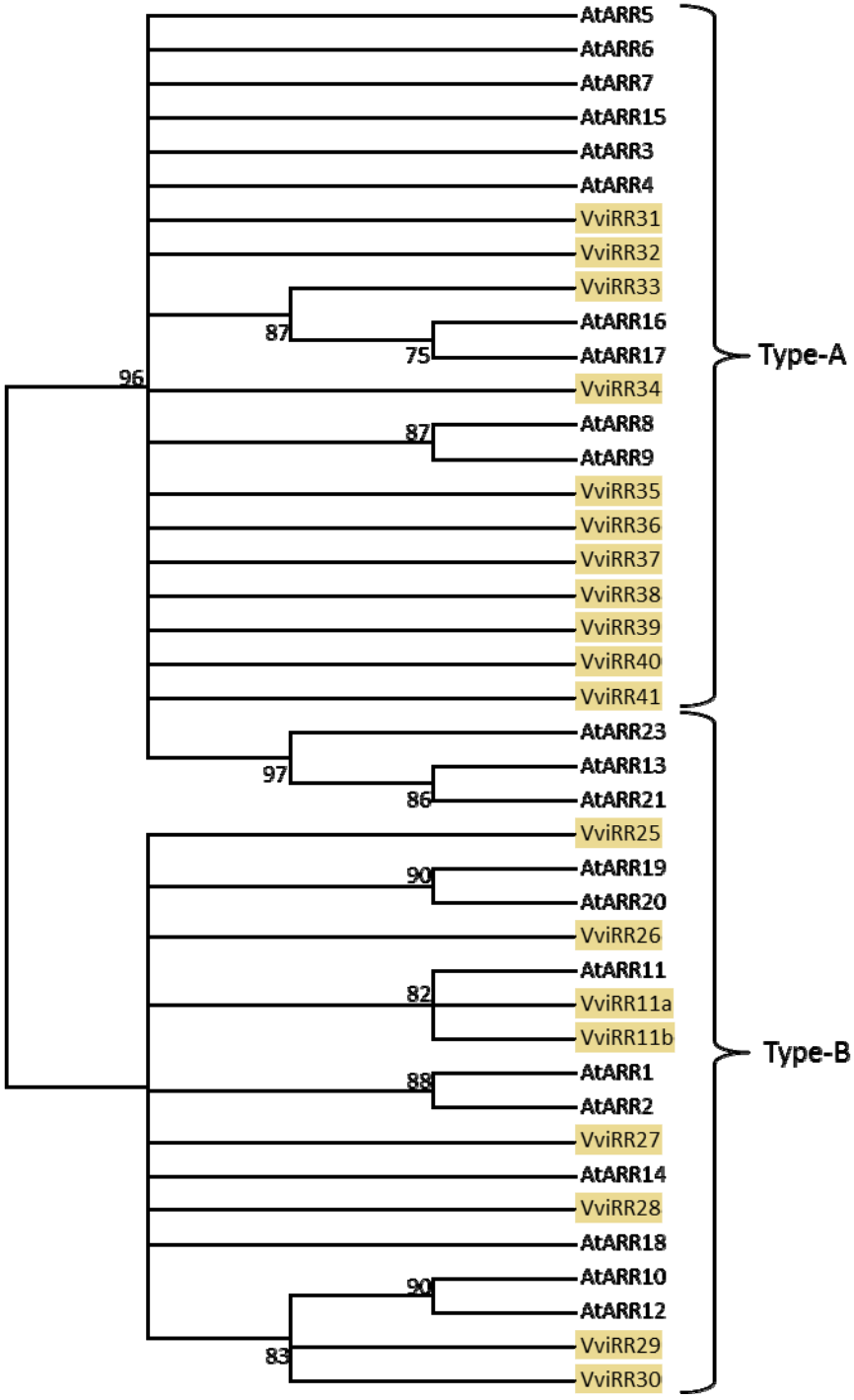


Figure 37. Phylogenetic relationship of RR coding sequences from grapevine and Arabidopsis. The naming of grapevine sequences followed the guidelines published by Grimplet *et al*. (2014). Predicted grapevine proteins are highlighted with a coloured background.

A subset of cytokinin-related genes is expressed coincidently with the accumulation of iP during berry development

In an attempt to discover causal relationships between the post-veraison accumulation of iP and the transcript abundance of genes involved in the control of cellular cytokinin accumulation, cytokinin nucleobases and the expression of 48 cytokinin-related genes were quantified in developing Shiraz berries (Fig. 38). Copy numbers and statistical data analyses for those genes expressed at more than two time points (29) are provided in Figure 39. VviCHK1, VviCHK5 and VviCKI were not included in this study due to their uncertain contribution to cytokinin perception/signal transduction (Choi and Hwang 2007; Yamada *et al*. 2001).

The changes in cytokinin concentration in Shiraz berries during development (Fig. 38A) followed a similar pattern to those observed in Cabernet Sauvignon, Riesling and Pinot Noir (Fig. 35). These results confirm and expand our previous data obtained for a subset of the Shiraz samples using different methods of extraction and quantification (Böttcher *et al*. 2013a). tZ concentrations remained low throughout development whereas a significant increase in iP concentrations was recorded from 11 WPF onwards reaching a maximum of 98.7 pmol/g FW at 15 WPF (Fig. 38A).

In total, 38 cytokinin-related genes, were expressed in berry tissue and hierarchical clustering formed the gene expression profiles into six distinct clusters (Fig. 38B). Cluster 1 contained four genes, a *LOG*, a *CHK* and two *RR*s, with the highest expression between 1-4 WPF and moderate to low transcript levels for the remainder of development. Cluster 2 contained nine genes, two *IPT*s, one *LOG*, one *CHK* and five *RR*s, which had peaks of expression between 1-4 WPF and 11-16 WPF with the highest transcript abundance in the post-veraison peak. Cluster 3 consisted of *IPT12* and *RR11a*, which displayed a peak in transcript levels between 5-8 WPF, and also at 16 WPF for *RR11a*. The expression the genes in Cluster 4 (one *CKX*, two *RR*s) was mainly at the 4 WPF time point. Cluster 5 was the most populous cluster, containing 15 genes representing all five families of the cytokinin-related genes analysed. These gene were predominantly expressed in young berries (1-4 WPF). Cluster 6 contained two *LOG* genes only, which were expressed between 9-16 WPF. *LOG13* and CKX6a were not included in the clusters and their transcripts were only detected at one time point (2 WPF and 10 WPF, respectively), whereas *RR37* was poorly expressed in young berries (1-2 WPF) but highly expressed from 14-16 WPF.

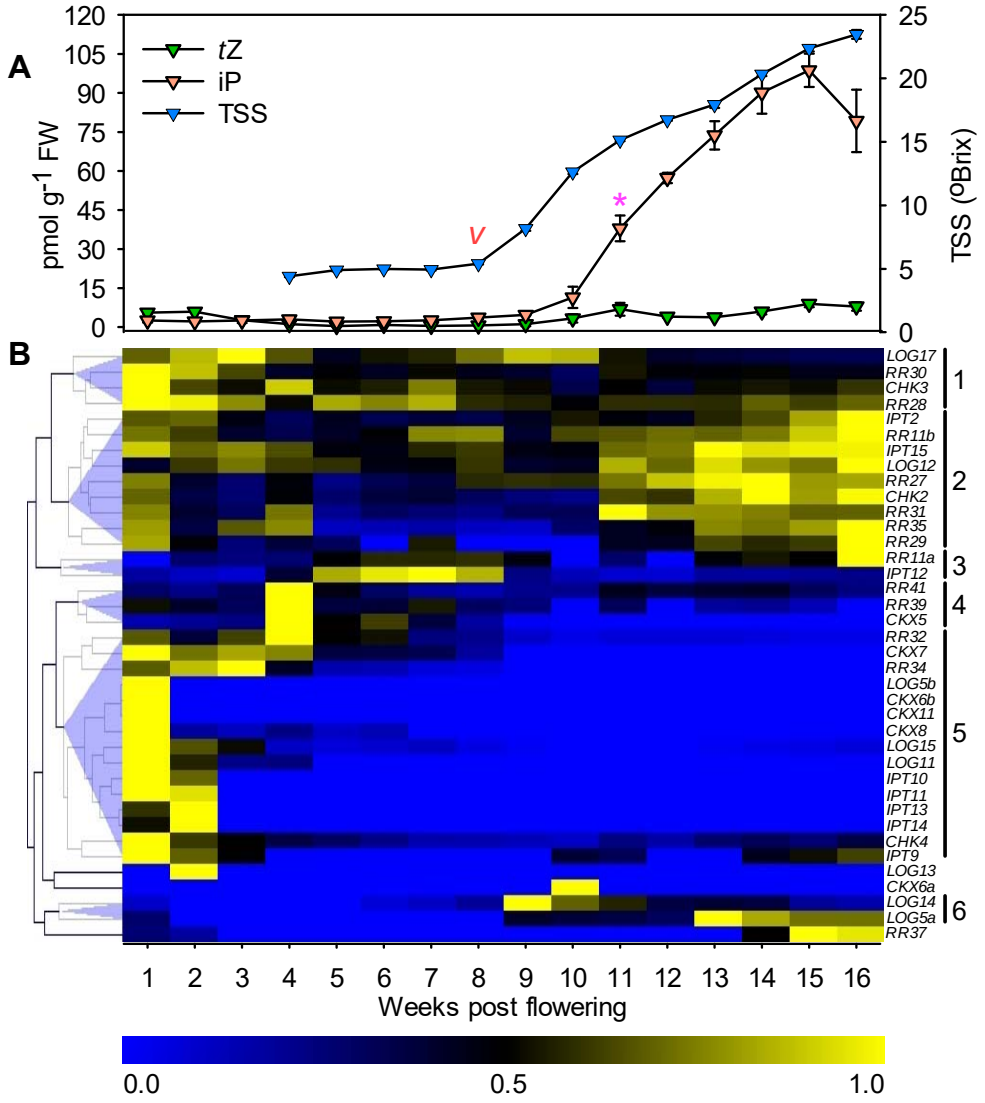


Fig. 38. Changes in iP and tZ concentrations and the expression of cytokinin-related genes in developing Shiraz grape berries.

(A) Changes in TSS, iP and tZ concentrations in field-grown Shiraz berries. All data represent means (n = 3) ± SE. The red 'V' marks veraison as determined by the last time point before a significant increase (p<0.05) in TSS levels was recorded. The pink asterisk indicates commencement of a significant increase in iP concentrations (p< 0.05). FW, fresh weight. **(B)** Heat map showing changes in transcript levels of cytokinin-related genes in berries. To adjust for differences in absolute copy numbers between the genes, the mean (n = 3) expression values for each transcript were scaled by dividing by the maximum copy number obtained from the berry developmental series so that all values fall between 0 and 1. Hierarchical clustering was used to group genes with similar expression profiles.

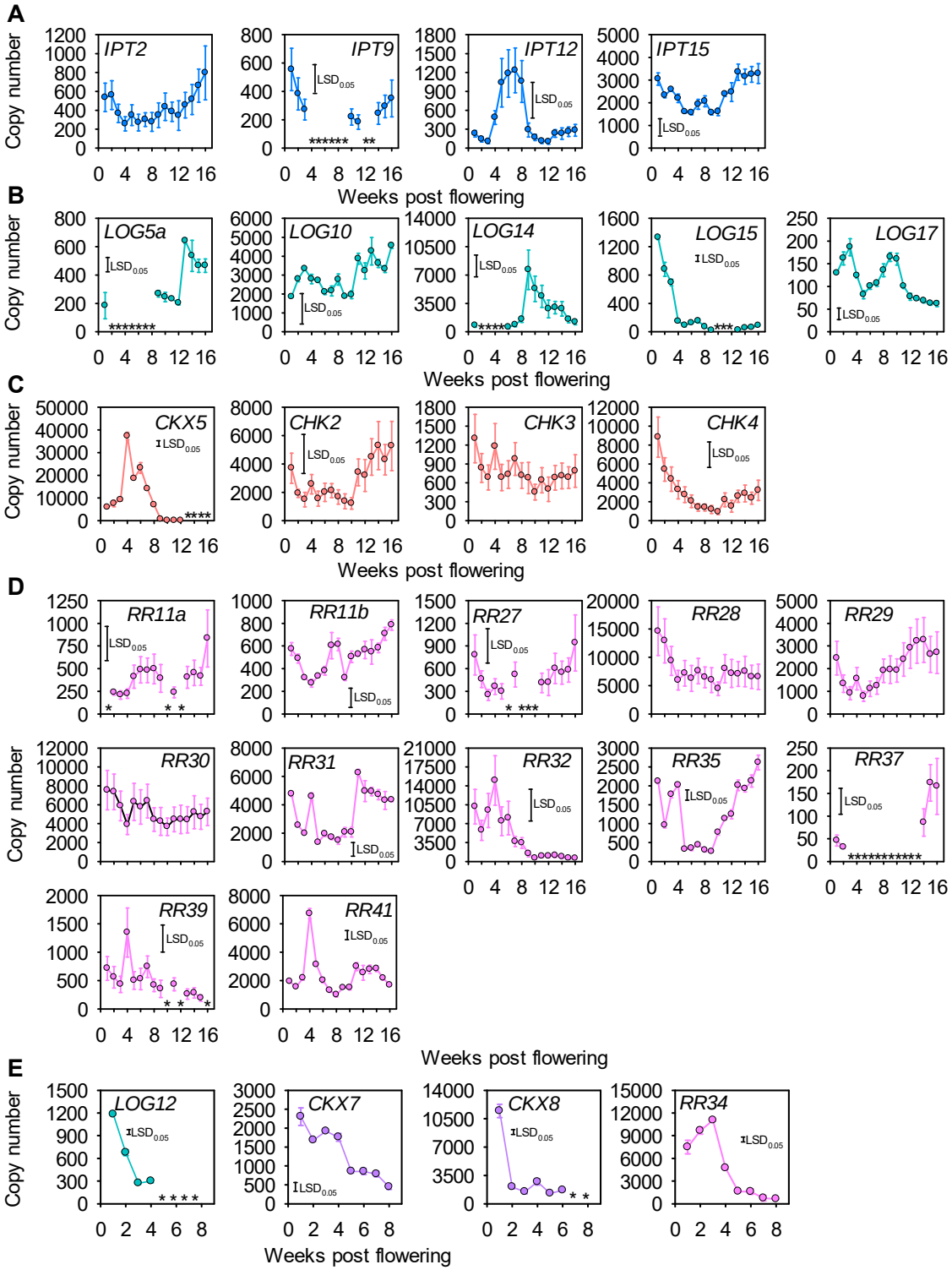


Fig. 39. Transcript accumulation of 39 CK-related genes expressed at two or more time points in a Shiraz developmental series as measured by RT-PCR, **(A)** *IPT*, **(B)** *LOG*, **(C)** *CKX*, **(D)** *RR* and **(E)** pre-veraison specific genes. LSD was determined at the $p<0.05$ significance level (n=3).

The reason for the large variation in maximal iP concentration between different grapevine cultivars observed in this study (Figs. 35 and 38A, Table 8) is unknown but genetic as well as environmental influences are likely factors. The well-described stimulatory effect of

cytokinins on anthocyanin accumulation in plants (Deikman and Hammer 1995; Guo *et al*. 2005; Nakamura *et al*. 1980) suggested a possible link between the post-veraison accumulation of iP and anthocyanins in red cultivars. However, iP data obtained from red and white skinned cultivars at a similar berry sugar level, showed that, although the three cultivars with the highest iP concentrations were red skinned, there was no consistent correlation between skin colour and iP content. For example, the iP concentration of Rubired berries, which produce anthocyanins in the flesh and skin, could not be distinguished from white cultivars with low iP concentrations, e.g. Riesling or Viognier (Table 8).

Cytokinin-related genes are expressed with diverse profiles in a range of grapevine tissues

To better understand the possible roles of cytokinin-related genes in grapevine development the expression the transcript accumulation of the cytokinin-related genes discussed above was also analysed in a range of other grapevine tissues (Fig. 40). Transcripts of 46 genes, including eight genes that were not expressed in berries, were detected in one or more organs/tissues. Their gene expression profiles clustering into seven groups (Fig. 40). Cluster 1 (*RR34* and *LOG12*), was characterised by expression in node five (L5) and nine (L9) leaves and in seeds 5 WPF (S5; *RR34*). Cluster 2 genes, *RR35* and *CKX6b*, were expressed in flowers and roots. Cluster 3 consisted of five genes, one *LOG* and four *RR*s, with transcripts detected in all tissues and highest expression in flowers, L9, S5, S9 or roots. The cluster containing the largest number of genes (Cluster 4, 21 genes) was mainly expressed in tendrils and roots. *CKX5* and *CXK6a* were also highly expressed in S5 (seeds 5 WPF). Cluster 5 contained eight genes, representing all five families of cytokinin-related genes analysed, with highest expression in L9 or roots. The common feature of *RR26*, *CKX11* and *LOG13* was specific expression in S14 while genes in Cluster 7 were mainly detected in flowers and seeds. Three genes were not included in clusters: *LOG5b* was mainly expressed in stem internodes, *LOG5a* showed expression in all tissues except seeds and RR40 was only found in roots. Copy numbers of all expressed genes are provided in Figure 41.

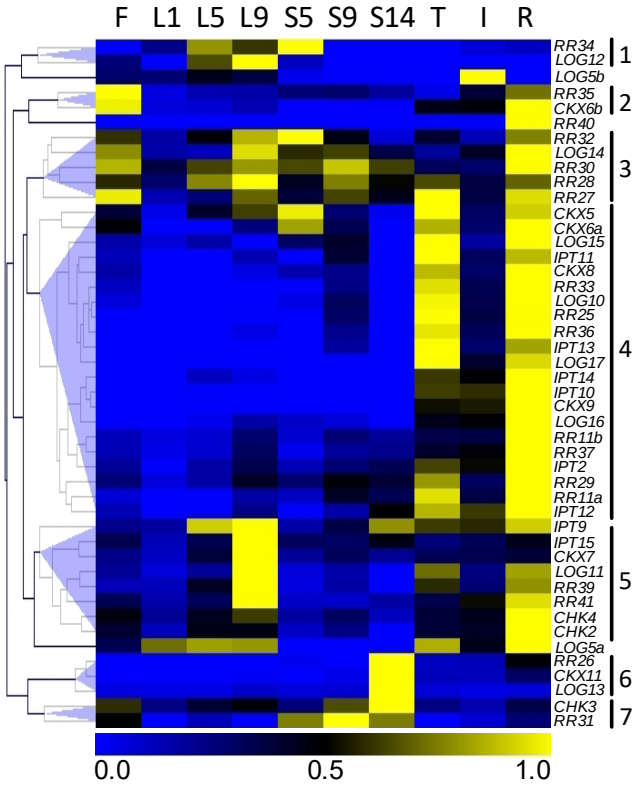
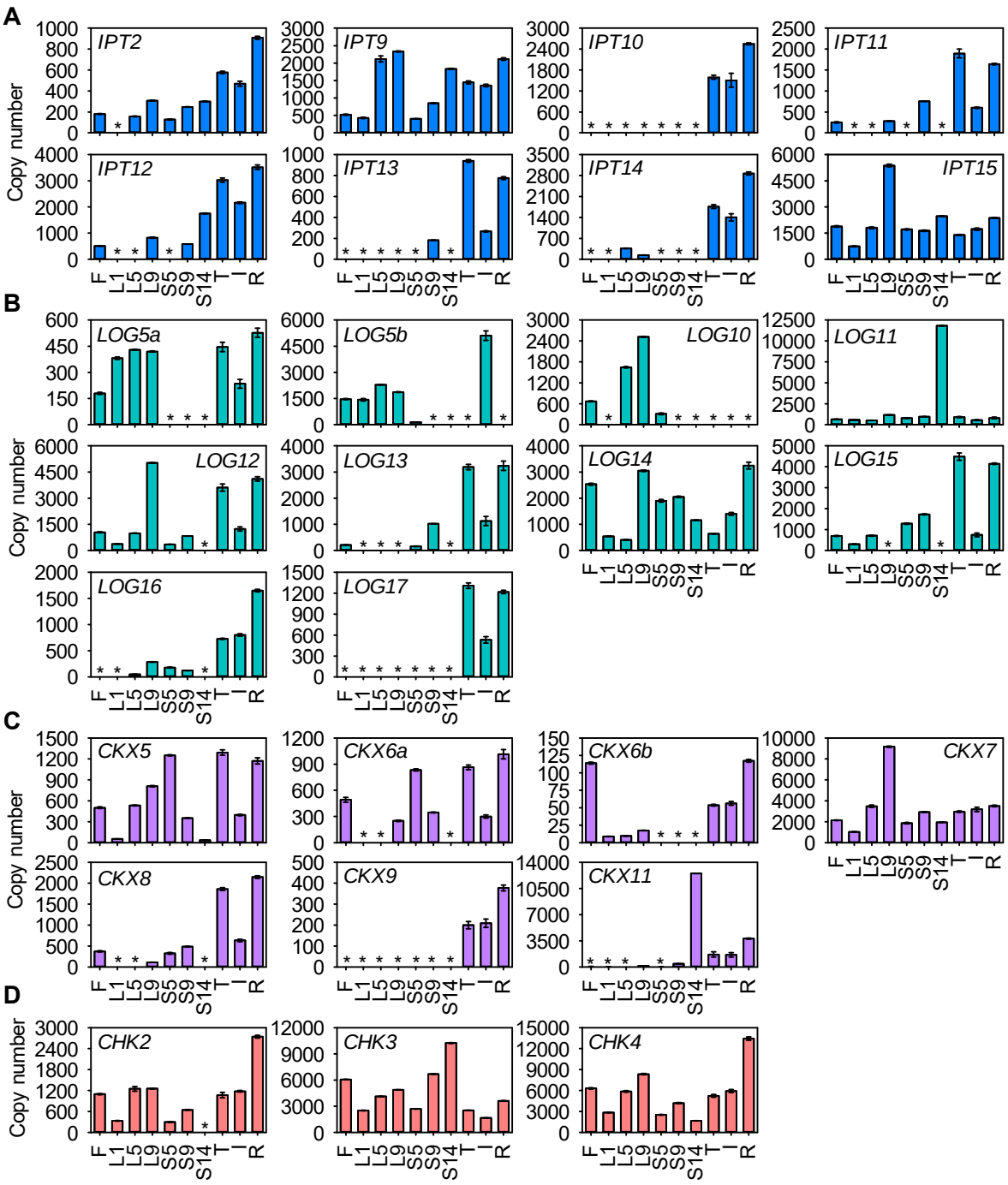


Fig. 40. Expression profiles of 46 cytokinin-related genes in different Shiraz grapevine organs/tissues.

Heat map showing transcript levels of cytokinin-related genes expressed in different organs/tissues of either field grown (flower, seeds, leaves, tendril, internode) or glasshouse grown (root) Shiraz plants. In order to adjust for differences in absolute copy numbers between the genes, the mean (n = 3 technical replicates) expression values for each transcript were scaled by dividing by the maximum copy number obtained from the tissue series, making all values between 0 and 1. Hierarchical clustering was used to group genes with similar expression profiles. Copy numbers for all expressed genes are given in Figure 41. F, flower; I, internode; L, leaf (node indicated by number, increasing from the shoot apex); R, root; S, seed (WPF indicated by number); T, tendril.


A
B
C
D
Copy number
IPT2
IPT9
IPT10
IPT11
IPT12
IPT13
IPT14
IPT15
LOG5a
LOG5b
LOG10
LOG11
LOG12
LOG13
LOG14
LOG15
LOG16
LOG17
CKX5
CKX6a
CKX6b
CKX7
CKX8
CKX9
CKX11
CHK2
CHK3
CHK4
F
L1
L5
L9
S5
S9
S14
T
I
R

E

Fig. 41. Transcript accumulation of CK-related genes in a different Shiraz tissues as measured by RT-PCR, **(A)** *IPT*, **(B)** *LOG*, **(C)** *CKX*, **(D)** *CHK* and **(E)** *RR* genes. All data represent means ±STERR (n=3).

iP concentrations also increase during tomato and strawberry ripening

The accumulation of iP during the ripening phase of fruit has not been reported from any fruit species other than grape (Böttcher *et al*. 2013a). In order to investigate if the ripening-associated iP increase also occurs in other fruit, nucleobase cytokinins were measured in several developmental stages of tomato (climacteric) and strawberry (non-climacteric) fruit (Fig. 42). In tomato, *t*Z concentrations were below the limit of quantification and iP concentrations were low, below 1 pmol/g FW, in all stages tested (Fig. 42A). However, in red firm fruit, iP concentration was significantly increased. In strawberry, *t*Z could only be detected in receptacles of pre-ripening fruit (Fig. 42B). In small green fruit removal of the achenes prior to cytokinin extraction significantly decreased the concentration of *t*Z. Similar to tomato, iP concentrations in strawberry receptacles were low, but significantly increased in turning fruit and were even higher in fully mature, red ripe strawberries (Fig. 42B). At the last developmental stage, achene-containing receptacles contained significantly higher iP concentrations than receptacles without achenes.

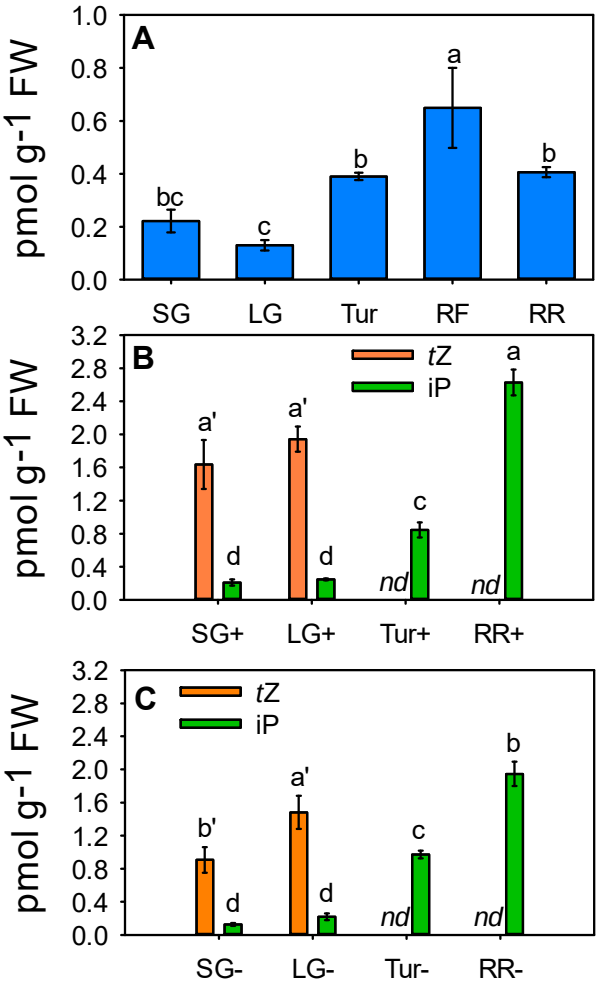


Fig. 42 Concentrations of iP and tZ in developing tomatoes and strawberries.
iP and *t*Z in **(A)** small green (SG), large green (LG), turning (Tur), red firm (RF) and red ripe (RR) tomatoes and in **(B, C)** small green (SG), large green (LG), turning (Tur) and red ripe (RR) strawberry receptacles with (+, B) and without (-, C) achenes. *t*Z concentrations were below the limit of quantification in tomato. FW, fresh weight; nd, not detected. Bars represent means ± SE (n = 3) and are denoted by a different letter (a-d, iP; a'-b', *t*Z) if the means for each time point differed significantly ($p<0.05$) using one-way ANOVA followed by Duncan's post hoc test.

Most of the published studies on cytokinins in fruit (Alleweldt *et al*. 1975; Chacko *et al*. 1976; Desai and Chism 1978; Inaba *et al*. 1976; Kim *et al*. 2006b; Letham and Williams 1969; Ohkawa 1973; Prakash and Maheshwari 1970; Vitulo *et al*. 2014) have used bioassays, based on physical parameters changes in cell proliferation or pigment accumulation, to determine the concentration of active cytokinins. Across all fruit species, high cytokinin activity was reported in young fruit during cell division, but low or undetectable activities were found in ripening fruit. This seems to contradict the ripening-associated increase in iP concentrations reported here for four grapevine cultivars (Figs. 35 and 38A) and tomato and strawberry (Fig. 42), but the above mentioned bioassays were mostly using *t*Z, and never iP, as the reference cytokinin and therefore may have not detected changes in iP concentration. Detectable *t*Z concentrations were found to be restricted to pre-ripening strawberries (Fig. 4) and in pre-veraison grapes, seeds were the main *t*Z source as evidenced by a high *t*Z concentration in seed-containing Pinot Noir berry tissue at 6 WPF (Fig. 35C). The

accumulation of *t*Z during early grape seed development has previously been reported (Pandey and Singh 1989; Zhang *et al*. 2003). Although both, *t*Z and iP, are classified as cytokinins they need to be considered as different and independent molecules in regard to their localization and transport within the plant, signalling outputs and biological effects. Experiments n Arabidopsis with mutants impaired in the *trans*-hydroxylation step that converts iP to *t*Z have revealed that tZ exclusively regulates cell proliferation in the shoot apical meristem (Kiba *et al*. 2013). In addition, the application of Z-type cytokinins to maize (*Zea mays* L.) leaves led to the induction of *ZmRR1*, but no changes in *ZmRR1* expression were observed in response to iP-type cytokinins (Takei *et al*. 2001b). Furthermore, CHK receptors (Choi *et al*. 2012; Romanov *et al*. 2006; Shi and Rashotte 2012; Stolz *et al*. 2011) and members of the CKX degradation pathway (Bilyeu *et al*. 2001; Galuszka *et al*. 2007) differ in their preference for iP and *t*Z. Differential roles for *t*Z and iP in long distance signalling pathways in plants has long been discussed. Xylem sap has been found to mainly contain *t*Z in the form of its ribosides and ribotides (Beveridge *et al*. 1997; Hirose *et al*. 2008; Takei *et al*. 2001b), whereas iP ribosides and ribotides are transported via the phloem (Corbesier *et al*. 2003; Hirose *et al*. 2008).

From the evidence listed above it is feasible that changes in fruit iP concentrations have previously escaped detection due to its lack of activity in the chosen bioassays. Where iP has been quantified throughout the development of fleshy fruit, grapes (Böttcher *et al*. 2013a, this study) were shown to accumulate up to 100-fold more iP during the ripening phase than tomato (this study), strawberry (this study) and kiwifruit (Lewis *et al*. 1996; Pilkington *et al*. 2013) while no increase in iP concentration was detected during the later stages of raspberry ripening (Miret *et al*. 2014). The iP concentrations in tomato, strawberry and kiwifruit fall are in a similar range to those in Arabidopsis seedlings (Kasahara *et al*. 2004; Zhang *et al*. 2013), maize roots, leaves and kernels (Veach *et al*. 2003), young 'Microtom' tomato ovaries (Matsuo *et al*. 2012), rice inflorescence meristem (Ashikari *et al*. 2005) and various soybean tissues (Le *et al*. 2012), whereas the iP quantities detected in grape berries are unprecedentedly high. This indicates a specific relevance for iP accumulation in grapes and might, for example, be related to the expansion-driven post-veraison growth and the high rate of sugar accumulation in these berries (Davies *et al*. 2012). A study utilizing Arabidopsis microarray analysis revealed the induction of 30 cell-wall-related genes by cytokinins (Brenner *et al*. 2012), confirming previously reported cytokinin-induced changes of cell wall characteristics, such as increased extensibility (Thomas *et al*. 1981), or decreased wall thickness (Jung *et al*. 2008). It is therefore possible that berry cell the post-veraison expansion is at least in part controlled by the observed increases in iP concentration. The induction of cell wall invertase genes and the large number of cytokinin-regulated genes involved in trehalose-6-phosphate metabolism (Brenner *et al*. 2012) give further credence to a possible role for iP in the maintenance of sink strength in ripening berries. Cytokinins are positive regulators of sink strength in vegetative organs, attracting carbohydrates and amino acids to sites of high cytokinin concentration (Kuiper 1993; Mothes and Engelbrecht 1963; Mothes *et al*. 1961; Werner *et al*. 2008). Studies on *Chenopodium rubrum* L. cell suspension cultures (Ehness and Roitsch 1997) and leaf senescence in tobacco (*Nicotiana tabacum* L.) (Gan and Amasino 1995; Lara *et al*. 2004) indicate that sink strength is most likely mediated by cytokinin-inducible cell wall invertases and hexose transporters, which are linked to the apoplastic phloem unloading pathway and maintain a sucrose gradient between source and sink organs (Ho 1984). A shift from symplastic to apoplastic phloem unloading, coinciding with the start of the ripening phase coupled with increased expression of invertases and hexose transporters, has been described for grape berries (Davies *et al*. 1999; Zhang *et al*. 2006). The expression of a cell wall invertase gene in Cabernet Sauvignon with a profile resembling the post-veraison pattern of iP accumulation further supports a possible role of iP

in the maintenance of post-veraison berries as strong sink organs (Deluc *et al*. 2007; Hayes *et al*. 2007).

Cytokinin nucleobases, ribosides and ribotides, including low levels of iP-type cytokinins, have been detected in the bleeding sap of Shiraz vines at budbreak (Field *et al*. 2009) so it is possible that the post-veraison iP accumulation reported in this study (Figs. 35 and 38A) resulted from iP import from the phloem. However, the spatial expression patterns of cytokinin-related genes in kiwifruit (Pilkington *et al*. 2013) and tomato (Matsuo *et al*. 2012) indicated that local cytokinin biosynthesis and degradation occur in fruit and play an important role in fruit development. This was confirmed in grapes, where genes regulating cytokinin biosynthesis, activation, degradation, perception and signalling were expressed in all stages of berry development (Figs. 38B and 39). Transcripts of all eight grapevine *IPT*s were detected in berries, some only during pre-veraison stages, others were expressed pre- and post-veraison, including during the period of iP accumulation (Fig. 38). The expression of specific *IPT* genes at certain developmental stages seems to be highly regulated since *IPT12*, which peaked between 5-8 WPF, is also under control by post-transcriptional silencing (Carra *et al*. 2009). Judging from the expression of *IPT* genes in various grapevine organs (Figs. 40 and 41) and in agreement with reports from Arabidopsis (Miyawaki *et al*. 2004), tomato (Matsuo *et al*. 2012) and soybean (Le *et al*. 2012), local cytokinin biosynthesis seemed to occur throughout the plant, especially in roots, tendrils, and mature leaves. The LOG-dependent pathway of producing active cytokinin nucleobases from ribotide precursors, has been established as the main cytokinin-activating mechanism in rice (Kurakawa *et al*. 2007) and Arabidopsis (Kuroha *et al*. 2009). All ten LOG genes were found to be expressed with distinct patterns in at least one of the grapevine tissues tested, with predominant transcript accumulation in the same organs as IPTs (Figs. 40 and 41).The LOD-dependent pathway also appeared to be active early (1-3 WPF) and late (9-16 WPF) in berry development, since *LOG12* and *LOG17* were expressed in pre- and post-veraison fruit, four additional *LOG*s were expressed during pre-veraison stages and expression of *LOG5a* and *LOG14* was post-veraison-specific (Fig. 38B) with the transcript accumulation of *LOG5a* closely matching the pattern of iP increase (Fig. 38).

The irreversible degradation of cytokinins by CKX enzymes, a vital part of the regulation of local cytokinin concentrations (Werner *et al*. 2006), is restricted to early developmental stages (1-4 WPF, Fig. 38A) in grape berries. The progressive decrease, with increasing development, of *CKX5* transcripts has previously been reported in microarray studies investigating transcriptional changes in developing grape berries (Deluc *et al*. 2007; Pilati *et al*. 2007). The lack of cytokinin degradation in post-veraison grapes might contribute to the large increase in iP concentrations, as iP is more susceptible to CKX-catalysed degradation than other cytokinins (Bilyeu *et al*. 2001; Galuszka *et al*. 2007).

All three grapevine cytokinin receptor genes were expressed in every organ (Figs. 40 and 41) and berry developmental stage analysed (Figure 38B), *CHK3* and *CHK4* showed higher transcript accumulation in pre-veraison berries while *CHK2* was characterised by a significant increase in expression during the late, high-iP, post-veraison phase. The Arabidopsis orthologue of VviCHK2 has been reported to preferentially bind iP, whereas the other two receptors prefer *t*Z (Shi and Rashotte 2012). The post-veraison increase in expression of *CHK2* might therefore amplify any iP-specific responses during the ripening phase. Supporting this hypothesis is the expression of a set of post-veraison-specific *RR*s, including four B-type *RR*s and three A-type *RR*s which could translate the iP signal into a ripening-specific, transcriptional response (Fig. 38B). Pre-veraison berries were characterised by the expression of a separate set of *RR* genes (two B-type *RR*s, four A-type *RR*s), whereas no *RR* genes were expressed in both pre- and post-veraison berry stages (Fig. 38B). In other grapevine organs, roots showed the overall highest expression of *RR*s, but *RR* transcripts

were found in all tested tissues, with nine *RR*s expressed ubiquitously and nine *RR*s restricted to specific organs (Figs. 40 and 41).

Conclusions

The present study provides evidence for the occurrence of a ripening-associated increase in iP concentrations in a number of different grapevine cultivars, strawberry and tomato. This suggests a role for this cytokinin in the regulation of fruit ripening processes. The uniquely high concentrations of iP found in post-veraison grape berries suggest a specific relevance for iP accumulation in these fruit, possibly related to the high concentrations of sugar stored in grapes. Developmental changes in the expression of genes related to cytokinin biosynthesis, activation, perception, signalling and catabolism indicate that the regulation of berry cytokinin concentrations and the response to specific cytokinin species can be controlled locally and provide a possible explanation for the post-veraison accumulation of iP. Distinct expression patterns within each gene family in berries and a range of other grapevine tissues display spatial and temporal specification suggesting a complex system of regulation.

Effect of cytokinin application on the low cytokinin variety Pinot Noir

Our previous work has shown that the concentration of the cytokinin iP increases in grape berries during ripening (Böttcher *et al*. 2013a). The reasons for this increase and the effects it has on berry metabolism and development are unclear. However, as the increase is so dramatic in many cultivars and as this occurs at veraison it could play important roles in the ripening grape.

In this experiment we attempted to test the effect of cytokinin application (iP) on Pinot Noir metabolism. Pinot Noir is a 'low' iP cultivar (see above) and one way of investigating the role of iP during berry ripening, when iP levels in many cultivars increase to substantial levels, is to increase the levels and look for a response. Unfortunately, the vines in this experiment suffered burning damage due to a sulfur application applied by the grower on a hot day. Due to this, the experiment was harvested earlier than preferred. During the abbreviated time course, the cytokinin treatment did not affect TSS levels compared to the control (data not shown). Small scale ferments were carried out to investigate any effects on volatile composition. Only three volatile compounds were significantly different in ferments made from Control and iP-treated fruit, all were less than two fold different. 1-nonanol was approximately 1.6 fold higher in iP-treatment wines, two unknowns were higher in Control wines (data not shown). Due to issues with the experiment it cannot be stated unequivocally that iP had little effect on volatile/volatile precursor production as measures of variation differed considerably among the replicates. This experiment may need to be repeated, or other experiments designed to test the role of iP during ripening.

Testing the effectiveness of auxins and cytokinins, alone and in combination, in delaying the veraison and harvest of Shiraz grape berries

We have previously shown that auxins applied at certain stages during the pre-veraison period can delay ripening, and therefore, harvest (Böttcher *et al*. 2012a; Böttcher *et al*. 2011b; Böttcher *et al*. 2010; Davies *et al*. 1997). In previous experiments the auxin normally found in grapes, IAA, has not been effective in delaying ripening but the closely related, synthetic auxin, NAA, is very effective. One of the reasons for this is that NAA is a much poorer substrate for the GH3, auxin inactivating, enzymes that conjugate auxins to amino acids (Böttcher *et al*. 2011a) and so NAA appears to be more persistent in the berry following spray application. 4-chloroindole-3-acetic acid (4-Cl-IAA) is a naturally occurring auxin that is common in seeds (Simon and Petrášek 2011). Interestingly, 4-Cl-IAA is a poor substrate

for the Arabidopsis GH3 enzymes that, like those in grapevine, conjugate auxins within the plant and so inactivating them depending on the amino acid that is conjugated (Staswick *et al*. 2005). With this in mind, it was thought that it might be useful to test the ripening delaying ability of 4-Cl-IAA in grapevines. It could be that 4-Cl-IAA might be more effective than NAA and so it could be commercially useful and offer a protectable product.

Cytokinins are a group of plant growth regulators with a role in regulating cell division and differentiation (Amasino 2005) and are involved in a wide range of processes (Argueso *et al*. 2009; Cooper and Long 1994; Gan and Amasino 1995; Kim *et al*. 2006a; Samuelson and Larsson 1993; Sasaki *et al*. 2014; Takei *et al*. 2001b; Werner *et al*. 2003; Werner *et al*. 2001). Some evidence indicates that cytokinin can be used to delay grape berry ripening. For example, grapes treated with CPPU, a synthetic cytokinin, were delayed accumulation of sugars and anthocyanins and softened later when compared with control berries (Peppi and Fidelibus 2008). One aim of this experiment was to test if cytokinins might be a useful, vineyard tool, either alone or in combination with an auxin, to delay berry ripening. In this experiment we used the synthetic cytokinin 6-Benzylaminopurine (BA). It could be that auxins and cytokinins have a synergistic effect which could increase the ripening-delay effect when used together and lead to a protectable formulation that might aid commercialisation. This experiment was designed to test both of the above questions.

Two pre-veraison treatments of auxins and cytokinins were applied, 14/12/15 and 21/12/15. The positive control for this experiment was the NAA application that was shown to delay ripening when applied alone, or in combination with, the cytokinin BA. Photographs of berries taken at two time points 14/01/2016 (Fig. 43) and 25/1/2016 (Fig. 44) clearly show that anthocyanin accumulation was delayed. Measurements of the fruit presented in the photographs confirmed this delay, for example, NAA and NAA+BA-treated fruit had lower TSS values at both time points (Tables 9 and 10). Anthocyanin measurements confirmed this delay (Fig. 45).

NAA-treated and NAA+BA-treated fruit were delayed in berry weight increase. NAA berries were significantly smaller than Control berries at 31 and 42 DPIS, NAA+BA berries were significantly smaller at 31 DPIS (Fig. 46). NAA and NAA+BA-treated samples were significantly lower in TSS levels compared to the Control at all time points and the fruit treated with 4-Cl-IAA was also lower at two time points. This delay in ripening was further evidenced by other measurements taken of these berries using FTIR spectroscopy. The ripening-associated decrease in total acid and malic acid was also delayed in both NAA and NAA+BA-treated fruit (Figs. 45 and 47). The YAN data seemed highly variable and fluctuated considerably but the delayed increase in its levels in the first three samples also indicated a delay (Fig. 47). The pH data, as measured by electrode, also seemed to be variable but showed a general trend towards increasing with time (Fig .48). For the berry developmental parameters berry weight, TSS, total acid, malic acid, YAN and anthocyanins, there was no evidence that the cytokinin BA had any effect on berry development/ripening. These data suggest that BA, IAA and 4-Cl-IAA had little or no effect on berry ripening, while NAA was effective in delaying ripening as predicted.

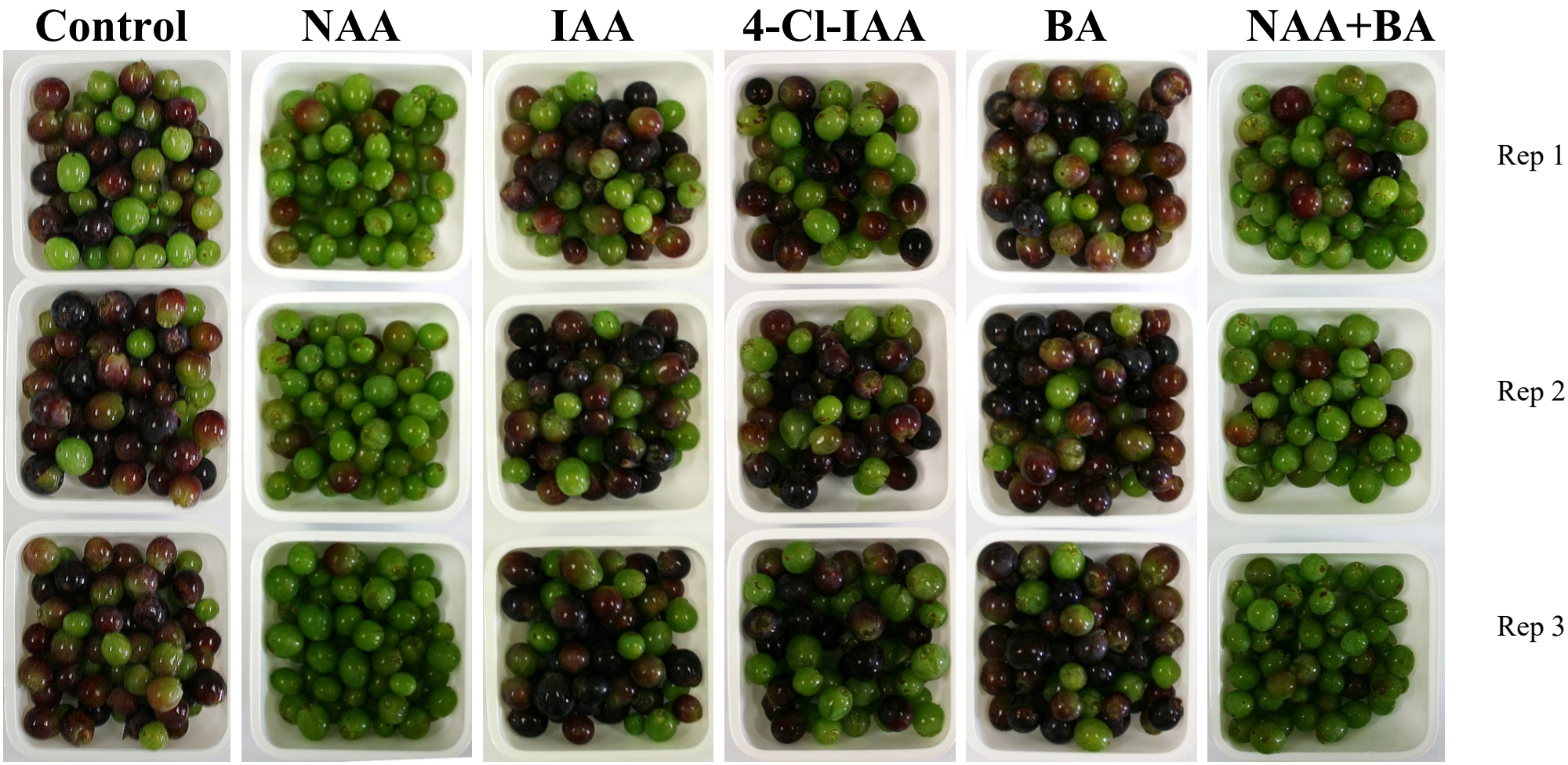


Fig. 43. Photographs of randomly selected berries taken 14/01/2016. Three replicates for each treatment are shown. NAA – 1-naphthalene acetic acid; IAA – indole-3-acetic acid, BA – 6-benzylaminopurine, 4-Cl-IAA – 4-chloroindole-3-acetic acid

Table 9. TSS (Brix, determined by FTIR) and berry weights for Control, NAA, IAA, 4-Cl-IAA, BA and NAA+BA berries, on 14/01/2016 as for Fig. 43, sprayed 14/12/15 and 21/12/15.

	Control	NAA	IAA	4-Cl-IAA	BA	NAA+BA
TSS (°Brix)	11.8	7.3*	11.6	10.6	12.7	8.2*
Weight (g)	0.95	0.75	0.92	0.94	1.06	0.78

* significantly different from Control at $p<0.05$ Student's t-test)

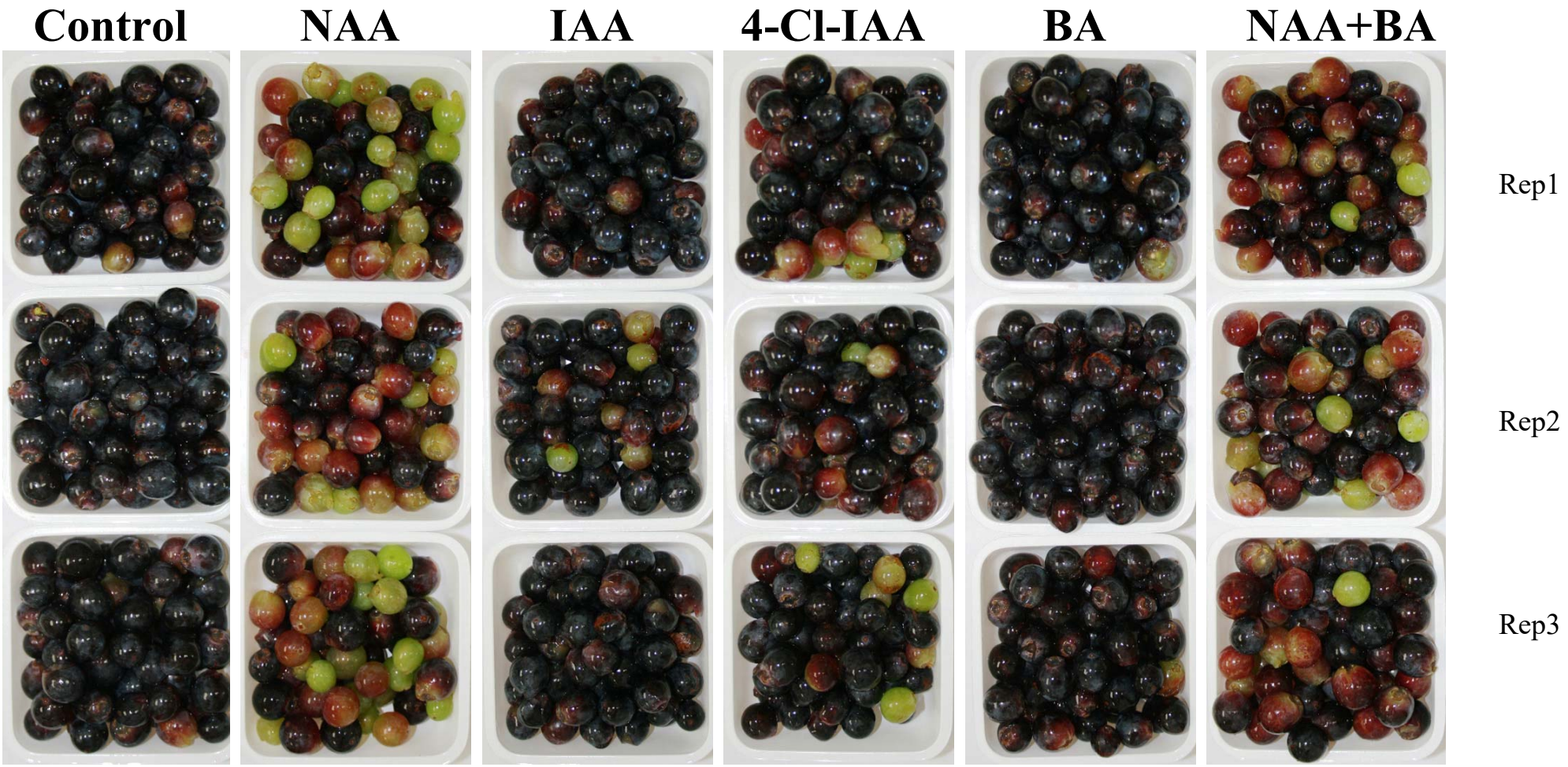


Fig. 44. Photographs of randomly selected berries taken 25/01/2016. Three replicates for each treatment are shown. NAA – 1-naphthalene acetic acid; IAA – indole-3-acetic acid, BA – 6-benzylaminopurine, 4-Cl-IAA – 4-chloroindole-3-acetic acid

Table 10. TSS (Brix), pH, total acid (TA), as determined by FTIR, and berry weights for Control, NAA, IAA, 4-Cl-IAA, BA and NAA+BA berries, on 25/01/2016, as for Figs. 44 and 45, sprayed 14/12/15 and 21/12/15.

	Control	NAA	IAA	4-Cl-IAA	BA	NAA+BA
TSS (°Brix)	17.0	13.0*	17.1	16.2	17.5*	13.6*
pH	3.2	3.1*	3.2	3.2	3.3	3.2
TA (g/L)	10.3	15.7*	10.6	11.1	9.3	12.7
Weight (g)	1.32	1.13	1.26	1.38	1.35	1.22

*significantly different from Control at $p<0.05$ Student's t-test)

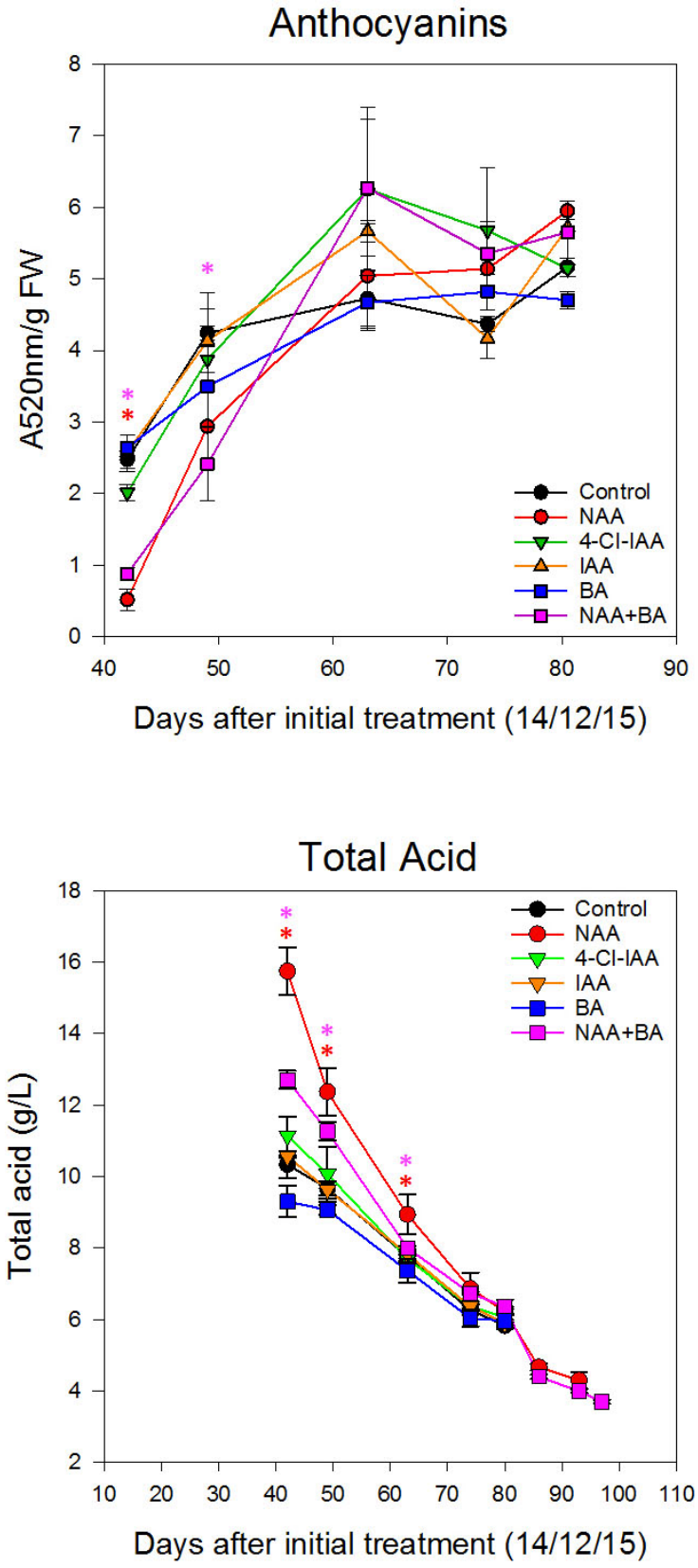


Fig. 45. Anthocyanin (A520nm) and total acid (by FTIR spectroscopy) content in auxin and BA-treated fruit. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

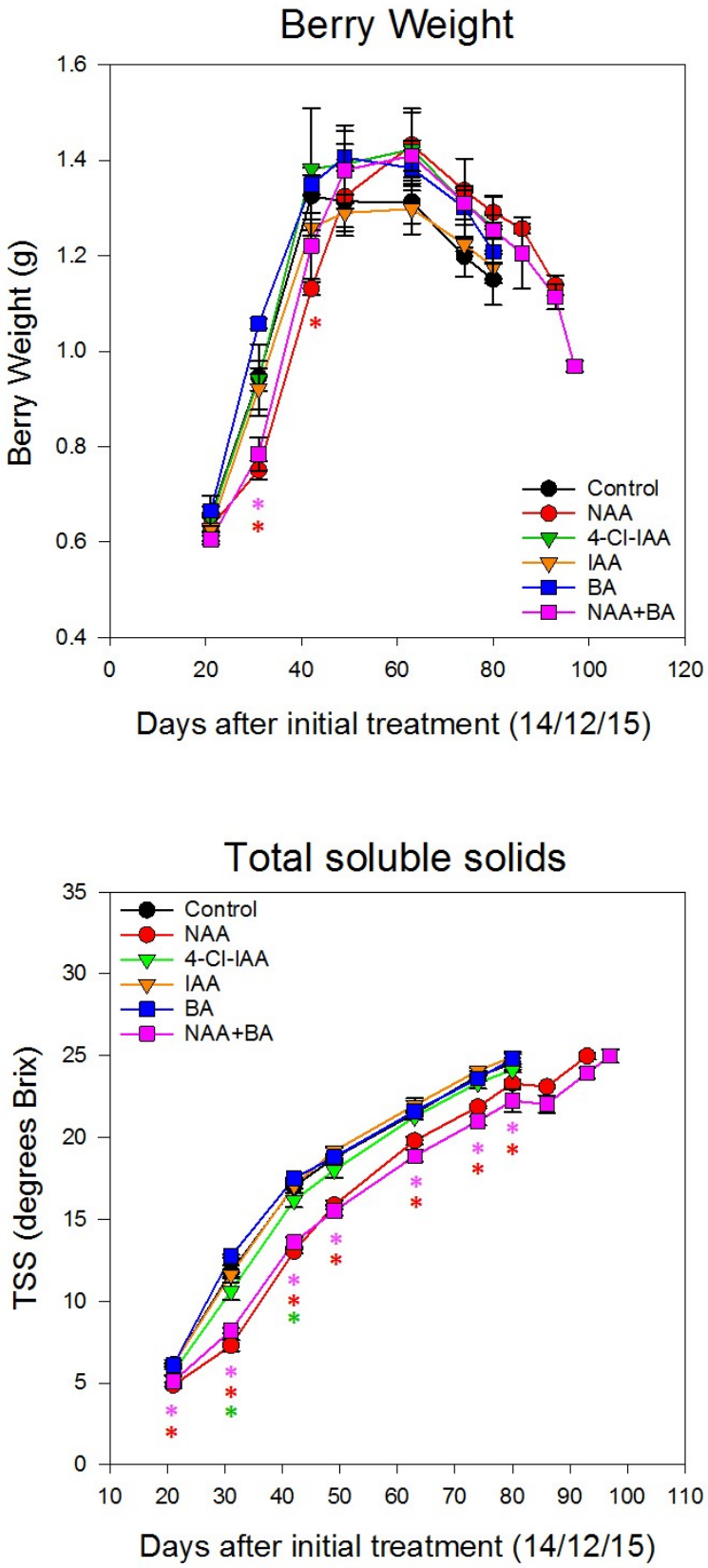


Fig. 46. Berry weight and TSS (by refractometer) of Control, auxin and BA-treated fruit. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

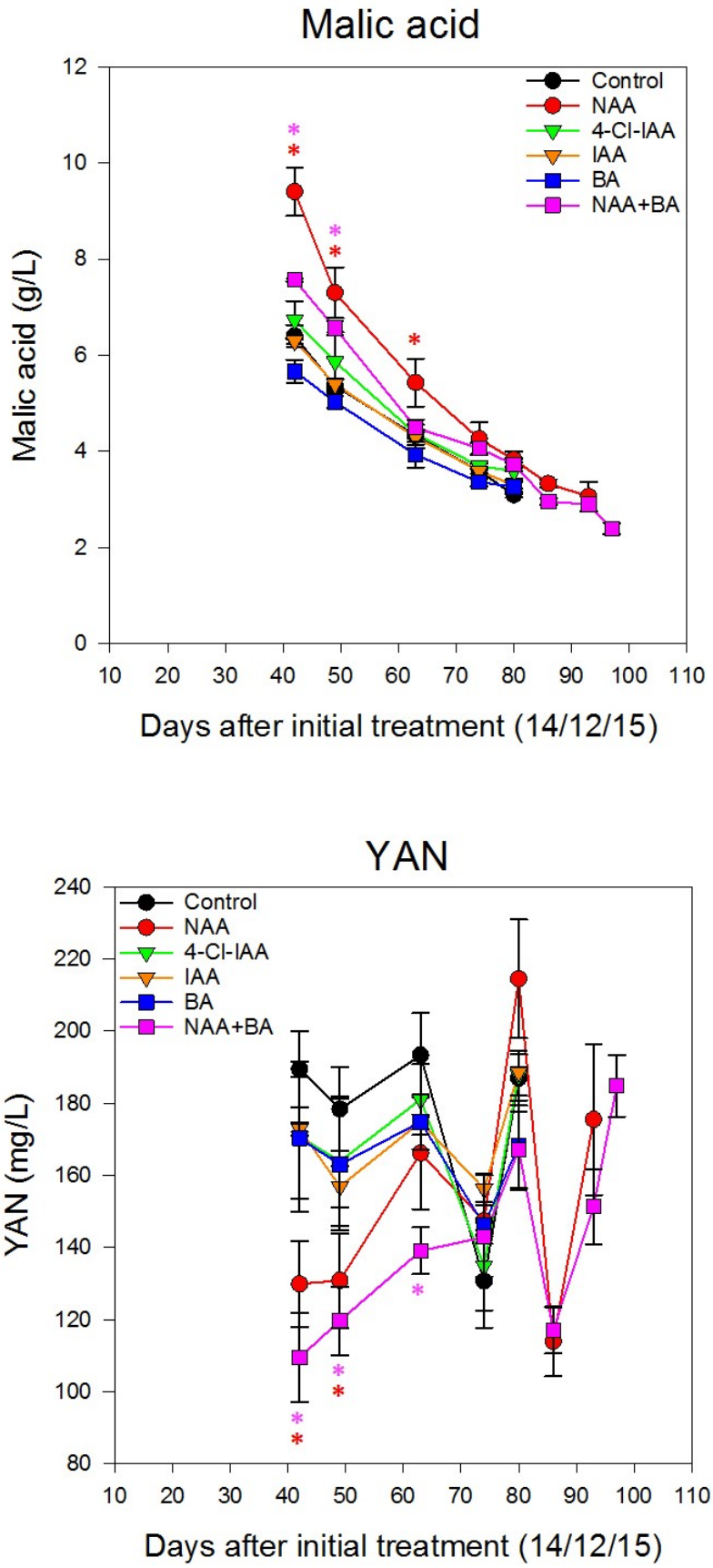


Fig. 47. Malic acid and YAN (by FTIR spectroscopy) content in auxin and BA-treated fruit. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

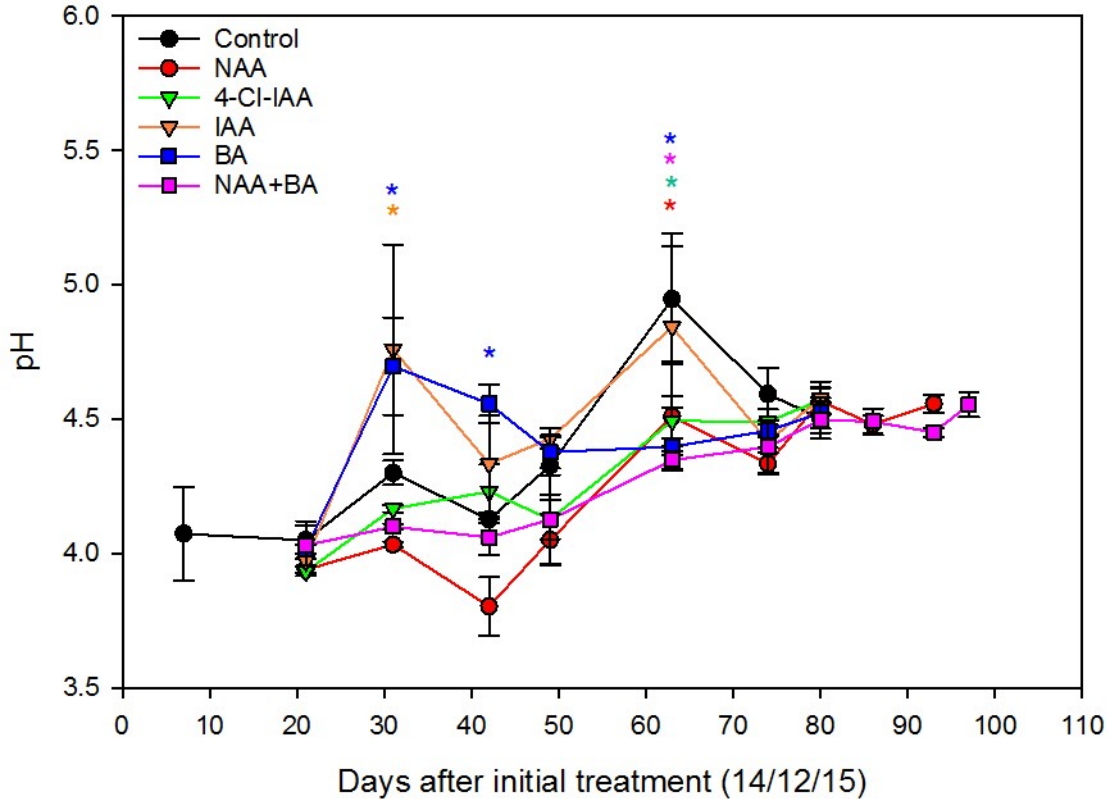


Fig. 48. pH (by electrode) of auxin and BA-treated fruit. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

In summary, these results confirm earlier results that the auxin NAA is effective in delaying ripening, while IAA is not. As discussed previously, this is most likely due to IAA being a better substrate for the inactivating, conjugating GH3 enzymes (Böttcher *et al*. 2011a), which probably results in applied IAA having a short half-life in the berry. NAA, by contrast, is longer lasting as it is a poor substrate for these enzymes and so might be expected to be more effective. The attempt to delay berry ripening with 4-Cl-IAA was also unsuccessful. The logic for trying this, naturally occurring, auxin was that some evidence suggested that it may not be a particularly good substrate for GH3 enzymes and therefore, may be longer acting and more effective, similar to NAA (Staswick *et al*. 2005). Apparently, in grape, either the levels of 4-Cl-IAA are reduced by catabolism quite effectively or this auxin is not effective in delaying ripening because it does not enter the berry efficiently or does not elicit the delaying response even when present. Either way it seems to be of no use in a commercial setting. The cytokinin BA was also shown to be ineffective in delaying berry ripening under the experimental conditions used. We have now tested two different cytokinins and neither have shown the potential for delaying ripening, and therefore, harvest. There is a wide range of cytokinins and cytokinin-like molecules available and so to complete the story it may be worth trying others, such as CPPU, which is the cytokinin previously reported to have some effect on grape berry ripening.

The development of transgenic plants to discover the mechanism of auxin control of vegetative and reproductive development in grapevine

As can be seen from the above studies auxins have many roles in plants and their roles in vegetative growth and fruit development in grape berries is of particular interest. Despite

their importance in the control of the timing of grape berry ripening we still have little knowledge of the mechanisms involved. To resolve this, we have used knowledge and materials from our previous studies to produce transgenic wines with low auxin levels. The grape GH3-1 enzyme is indole-3-acetic acid-amido synthetase that reduces the active concentrations of IAA through conjugation to, primarily, the amino acid aspartic acid (Böttcher *et al.,* 2010). By over-expressing the corresponding gene in grapevine, we have created vines with low auxin levels. The plants have smaller internodal length with smaller, wrinkled leaves. For various reasons, flowering of these vines has been limited and the study of fruit is ongoing. However, some initial studies into the effects of low auxin on gene expression in leaves have been conducted. Forty genes were overexpressed, including the transgenic *GH3-1* gene, but the dominant theme is the over-expression of many receptor-like proteins that suggests a reprogramming of signalling genes has occurred. Some of the under-expressed genes are involved directly in controlling the auxin response, including one that has been shown to be involved in the control of growth. The under expression of many heat shock proteins is very interesting but currently unexplained.

Testing the effects of a larger range of natural and synthetic cytokinins on the timing and progression of ripening

In the previous experiment, testing the possibility that cytokinins may be able to delay ripening, and therefore, harvest BA was the synthetic cytokinin used. It did not have any significant effect on berry development when used on its own and also did not significantly enhance the delaying effect of NAA when used in combination (Figs. 45-48). To confirm that cytokinins are not effective in delaying ripening in wine grapes when applied during the pre-veraison period, an experiment was conducted using one naturally occurring and three synthetic cytokinins. The naturally occurring cytokinin was iP, the synthetic cytokinins were BA, CPPU, present in the commercial formulation Prestige® 10 EC (Sumitomo) at 10 g/L, and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea present in the commercial formulation Thidiazuron 500 GenFarm (Landmark Operations) at 500 g/L. The commercial formulations were diluted considerably to obtain a solution with the desired concentration of active ingredient (see Method). All cytokinins were used at a final concentration of active ingredient of 20 mg/L.

Despite two pre-veraison treatments (5/01/2017, 17/01/2017) none of the cytokinins applied greatly altered berry development. The berry weight was significantly greater than the Control at two time points in Prestige-treated fruit and at one time point for Thidiazuron-treated fruit (Fig. 49) so it is possible that they had a small effect on size. There was also little effect of cytokinins on TSS levels as Prestige-treated fruit had a higher Brix than the Control at the second sampling and BA-treated fruit had lower Brix at the third time point (Fig. 50).

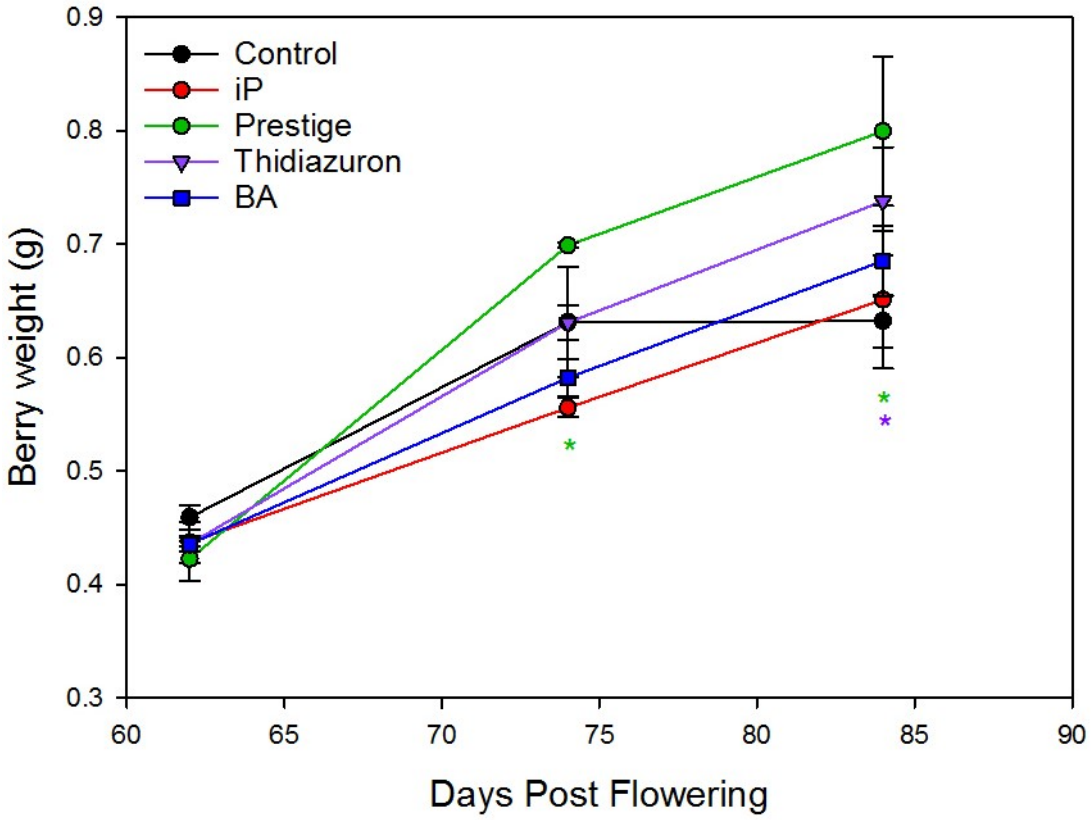


Fig. 49. The effect of four cytokinins on berry weight, Control, black; iP, red; Prestige green; Thidiazuron, purple; BA, blue. All cytokinins were applied at an active ingredient concentration of 20 mg/L. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

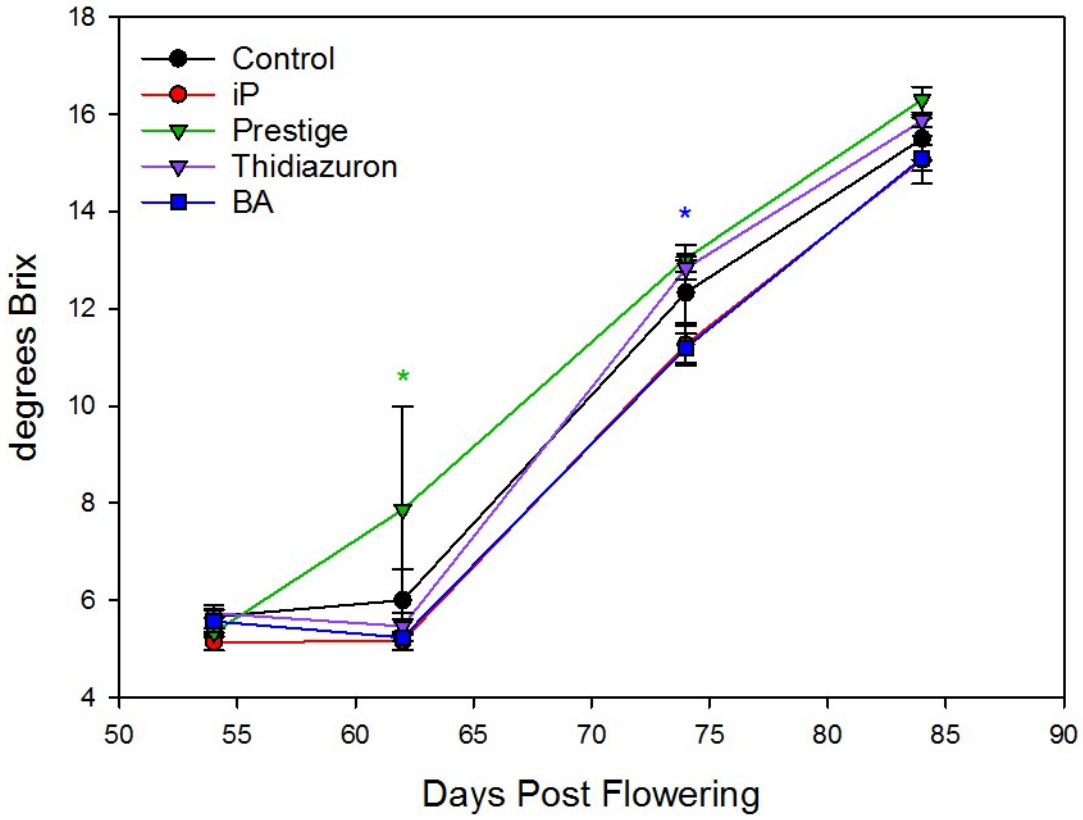


Fig. 50. The effect of four cytokinins on berry TSS measured as Brix, Control, black; iP, red; Prestige green; Thidiazuron, purple; BA, blue. All cytokinins were applied at an active ingredient concentration of 20 mg/L. All data represent means ±STERR (n=3). Asterisks denote treatments significantly different from Control at $p<0.05$ as determined by one-way ANOVA followed by Duncan's post hoc test.

Prestige and Thidiazuron had an effect on YAN and malic acid levels and on total acid as these parameters were lower than in the Control, iP or BA-treated samples. pH was also slightly higher than the Control in the iP, Prestige and Thidiazuron-treated fruit (Fig. 51). There were no significant effects on anthocyanin levels (Fig. 52).

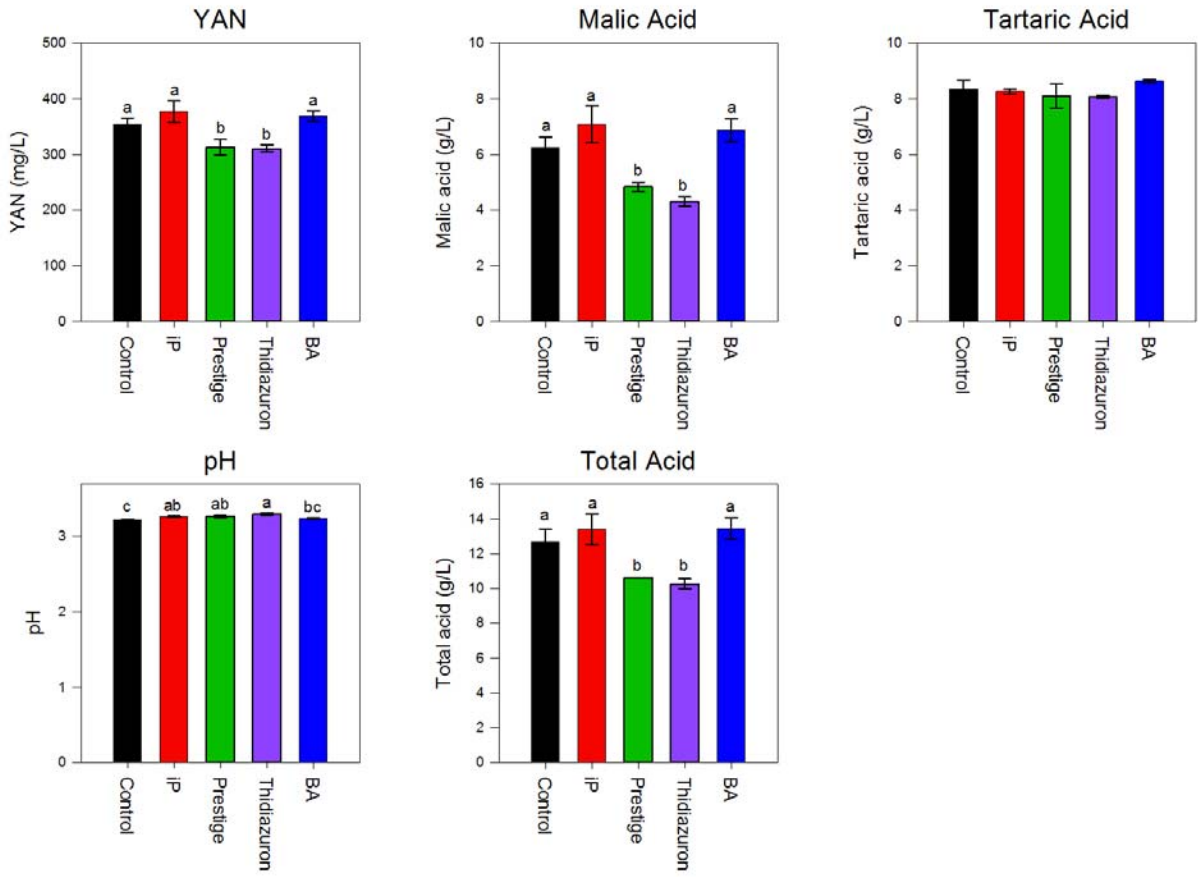


Fig. 51. The effect of treatment with four cytokinins on berry YAN, malic acid, tartaric acid, pH and Total acid as measured by FTIR (OenoFoss). Control, black; iP, red; Prestige green; Thidiazuron, purple; BA, blue. All data represent means ±STERR (n=3). Different letters indicate if the means for each treatment differed significantly (p<0.05) using one-way ANOVA followed by Duncan's post hoc test.

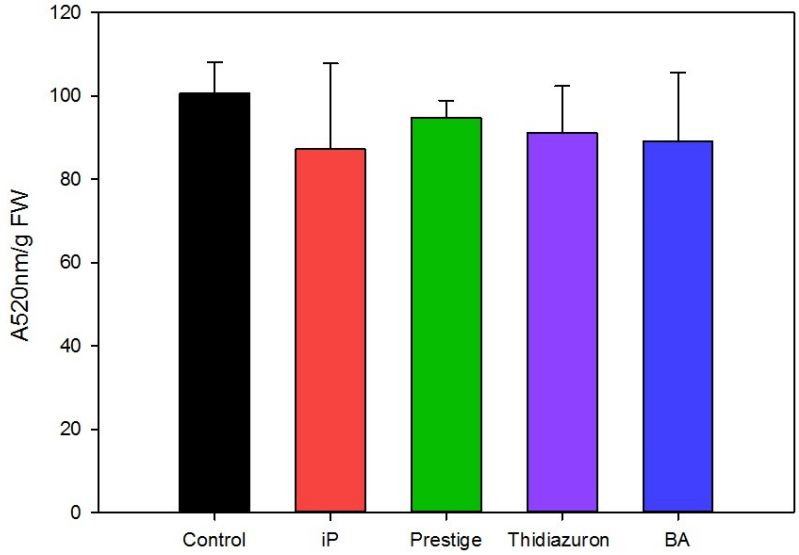


Fig. 52. Treatment with four cytokinins had no significant effect on berry anthocyanin content at harvest. Control, black; iP, red; Prestige green; Thidiazuron, purple; BA, blue. All data represent means ±STERR (n=3). FW: fresh weight.

This experiment has not produced any evidence to support the contention that the pre-veraison treatment of berries with cytokinins can delay veraison and therefore, ripening. If anything, the reduced acid and YAN levels in Prestige and Thidiazuron-treated fruit suggest that ripening may be slightly speeded up (Fig. 51). The effectiveness of CPPU in delaying ripening described in a previous report (Peppi and Fidelibus 2008) may be due to differences in a range of factors including grape cultivar, application rate, timing of application etc. The most important difference may be that Peppi and Fidelibus (2008) applied CPPU only one week after fruit set, during the period of rapid cell division and that the application increased berry size of the table grapes they were using. An increase in berry size might be expected to delay ripening as larger berries require more sugars to get to a certain Brix value than smaller berries. CPPU treatment also reduced anthocyanin accumulation (Peppi and Fidelibus 2008) which would not be satisfactory to the wine industry. The application of cytokinins using the conditions described in the Method section did not increase berry weight which may be the reason why a delay in ripening was not observed.

In conclusion, our experiments indicate that cytokinins are unlikely to be useful in delaying ripening in a controlled fashion without greatly affecting the final berry composition. Auxins still appear to be the agent of choice to delay veraison and therefore, delay ripening in an increasingly warm environment.

7. Outcomes and conclusions

The broad objectives of this project were to better understand the process(s) of berry ripening with the aim of developing methods to control ripening to the benefit of industry. This project was successful in achieving most of the originally planned outputs and performance targets. There were two agreed variations, one that related to a slight change in direction due to information gathered during the progress of the project. Given the success of auxins in delaying ripening the alternative possible method using ethylene received less emphasis as this method acted through a less direct pathway and the risk of detrimental effects might be slightly elevated. The second variation involved a six month delay in the delivery of the final report. This was due to the lateness of the final season and some issues with malolactic fermentation of the experimental wines that together greatly delayed analysis of the wine.

A range of PGRs was tested for their ability to delay ripening and therefore harvest. The auxin NAA was selected as the first choice agent as it reliably delayed ripening with few or minor changes in wine flavour/aroma metabolite levels and sensory character. Other agents were found wanting in that they were less effective, or not effective, or had other detrimental properties. These choices were all based on sound and extensive experimental analysis in commercial vineyards. Although in most cases flavour was not affected by the delaying treatments there was one exception. In Shiraz fruit that had been delayed for two or more weeks, there was an increase in wine pepper character due to an increase in the sesquiterpene molecule responsible, rotundone.

The ability of NAA to delay ripening, and therefore harvest, has significant potential to resolve some of the climate change-induced problems that have arisen due to increased temperatures and CO_2 levels. The earlier harvests combined with seasonal compression (i.e. the phenomenon that varieties that once had spread out harvest times now come due for harvest over a much shorter window) mean that there is much more pressure on harvesting, winery intake and processing than before. The practice of picking fruit too early or too late because of the problem reduces fruit quality and wine price. Being able to slightly retard, through auxin application, a portion of winery intake will be a useful management tool. This should have benefits for the growers in being able to deliver consistently high quality/value fruit, should improve fruit intake and processing scheduling and result in wine of improved quality.

The role of two other types of PGRs, cytokinins and jasmonates, potentially involved in ripening was evaluated. Cytokinins are involved in the early stages of berry development but have no value as delayers in ripening, contrary to some previous suggestions. However, it was found that one cytokinin may play a role during berry ripening. Jasmonates can affect secondary metabolism but seem mainly involved early in berry development and in the response to wounding/herbivore attack.

We have used the most up to date analytical methods available and have conducted the overwhelming proportion of experiments in the field under conditions that relate as near as possible to common commercial conditions. There are always more experiments that can be done given time and materials but the choices made seem appropriate and have yielded sturdy and valuable results.

8. Recommendations

The work described in this report has the potential to provide a tool to the grape and wine industry to control the timing of grape harvest by delaying the onset of ripening. The data are sufficient to show that this can be done effectively and should be reliable but numerous variables remain to be tested. More testing needs to be done over successive years, at different sites/under different weather conditions and with different cultivars as deemed appropriate. There are some quite practical considerations that require thorough examination such as testing different concentrations of the auxin to demonstrate that the length of the delay in ripening/harvest can be reliably manipulated by this means. Compatibility with other agrochemicals could be tested to allow co-spraying to be conducted if required, this could reduce tractor pass numbers making it cost neutral and preventing the increase in soil compaction. The effectiveness of different spray adjuvants and spray systems could also be tested. For all the above studies it would be valuable to study the effects on harvest timing, berry metabolism, berry and wine composition and sensory properties. It will also be important to investigate if there are any residues of the auxin treatment to be found in grapes and wine and if these might change with time.

The timing of the application of the auxin treatment has been shown to be critical to the effectiveness in delaying ripening/harvest. Experiments need to be conducted to determine the most effective time during development and then to develop a simple and reliable test that can be used in the field to determine the best time to apply the reagent. This, in simple terms, will be a predictor of veraison to allow time to make the application within the optimal time window.

In the longer term there could be further opportunities to develop methods to control other aspects of berry and vine development as our understanding of the way that PGRs are involved in grapevine development improves. For example, there may be novel ways to control vine vigour and bunch and vine architecture. We have made some progress on better understanding the relationship between sugar accumulation, secondary metabolism and grape/wine composition but as this is such a complex issue, more needs to be done.

Appendix 1: Communication

The outcomes from this research have been communicated through a number of methods. A number of discussions both informal and structured have been held with our industry collaborators in both smaller and larger companies. For example, a presentation with discussion has been held each season with the Technical Viticulture group from Treasury Wine Estates, planning experiments in their vineyards and discussing the outcomes (August 2015, August 2016, June 2017). In addition, a formal presentation was given at the annual Viticulture Meeting at TWE 10 Nov 2016.

As part of the AWRI 2017 Roadshow Seminars, talks on the above work were presented by Dr Davies at Rutherglen (24 Oct), Avoca (25 Oct) and Bendigo (26 Oct). Title: 'Understanding & manipulating grape berry development/ripening'

Publications in industry journals

Davies C (2014) Exploring the potential to regulate grape ripening. Australian & New Zealand Grapegrower & Winemaker. Winetitles Media, 61-63.

Davies C, Böttcher C and Boss P. (2015) Grape ripening: Delayed grape ripening - more spice. Wine & Viticulture Journal **30**, 41-42.

Conference presentations

Böttcher C, Boss PK and Davies C (2014) Auxins or ethylene- who controls berry ripening? ComBio. Canberra, Australia.

Davies C, Böttcher C and Boss PK (2014a) The control and manipulation of berry development by plant growth regulators and the consequences for wine composition. Macrowine. Stellenbosch, South Africa.

Davies C, Böttcher C, Boss PK, Peat T and Newman J (2014b) Understanding and manipulating grape berry development in a changing world. 7th International Table Grape Conference. Mildura, Australia.

Davies C, Böttcher C, Burbidge C and Boss PK (2014c) Understanding and manipulating grape berry development in a changing world. Queenstown Molecular Biology Meeting. Queenstown, New Zealand.

Boss PK, Maffei SM, Nicholson EL, Böttcher C and Davies C (2016) Developmental changes in grape composition and the implications for wine volatile profiles. X International Symposium on Grapevine Physiology and Biotechnology. Verona, Italy.

Böttcher C, Boss PK and Davies C (2016) Hormonal control of grape berry ripening. Australia-Indonesia Science Symposium. Canberra, Australia.

Davies C, Böttcher C and Boss PK (2016) Grape berry ripening - Who's in control? Australia - Italy Grape & Wheat Symposium. Adelaide, Australia.

Publications in international scientific journals

Grimplet J, Adam-Blondon A-F, Bert P-F, Bitz O, Cantu D, Davies C, Delrot S, Pezzotti M, Rombauts S and Cramer G. (2014) The grapevine gene nomenclature system. BMC Genomics **15**, 1077.

Böttcher C, Burbidge CA, Boss PK and Davies C. (2015a) Changes in transcription of cytokinin metabolism and signalling genes in grape (*Vitis vinifera* L.) berries are associated with the ripening-related increase in isopentenyladenine. BMC Plant Biology **15**, 223.

Böttcher C, Burbidge CA, di Rienzo V, Boss PK and Davies C. (2015b) Jasmonic acid-isoleucine formation in grapevine (*Vitis vinifera* L.) by two enzymes with distinct transcription profiles. Journal of Integrative Plant Biology **57**, 618-627.

Davies C, Nicholson EL, Böttcher C, Burbidge CA, Bastian SEP, Harvey KE, Huang A-C, Taylor DK and Boss PK. (2015) Shiraz wines made from grape berries (*Vitis vinifera*) delayed in ripening by plant growth regulator treatment have elevated rotundone concentrations and "pepper" flavor and aroma. Journal of Agricultural and Food Chemistry **63**, 2137-2144.

Robinson S, Glonek G, Koch I, Thomas M and Davies C. (2015) Alignment of time course gene expression data and the classification of developmentally driven genes with hidden Markov models. BMC Bioinformatics **16**, 196.

Adam-Blondon A-F, Alaux M, Pommier C, Cantu D, Cheng Z-M, Cramer GR, Davies C, Delrot S, L Deluc L, G Di Gaspero G, Grimplet J, Fennell A, Londo JP, Kersey P, Mattivi F, Naithani S, Neveu P, Nikolski M, Pezzotti M, Reisch BI, Töpfer R, Vivier MA, Ware D and Quesneville H. (2016) Towards an open grapevine information system. Horticulture Research **3**, 16056.

Boss PK, Kalua CM, Nicholson EL, Maffei SM, Böttcher C and Davies C. (2017) Fermentation of grapes throughout development identifies stages critical to the development of wine volatile composition. Australian Journal of Grape and Wine Research, DOI: 10.1111/ajgw.12319. This article will be presented in the project report to WA prepared by Dr. Boss.

Davies C, Böttcher C, Boss PK, Peat T and Newman J. (2017) Understanding the control of grape berry ripening and developing opportunities for its manipulation. ISHS Acta Horticulturae 1157: IX International Symposium on Grapevine Physiology and Biotechnology, 1-10.

The following two articles were published in Open Access journals and so are available online:

Robinson S, Glonek G, Koch I, Thomas M and Davies C. (2015) Alignment of time course gene expression data and the classification of developmentally driven genes with hidden Markov models. BMC Bioinformatics 16, 196.

This work was completed through collaboration with Adelaide University to develop a mathematical/statistical-based method for the alignment of gene expression data from different length developmental series to allow comparison of such data sets. The method was developed using grape berry transcriptomic data. This expands the usefulness of this data in helping define possible functions for genes and processes.

Grimplet J, Adam-Blondon A-F, Bert P-F, Bitz O, Cantu D, Davies C, Delrot S, Pezzotti M, Rombauts S and Cramer G. (2014) The grapevine gene nomenclature system. BMC Genomics 15, 1077.

This article reports on work done through the International Grapevine Genome Project (IGGP) to improve the use of grapevine genetic and transcriptomic data through the development of a unified, universal gene nomenclature system. Dr Davies is a member of the IGGP Steering Committee and of the Nomenclature Committee. The paper by Adam-Blondon *et al.* (Horticulture Research (2016) 3, 16056; doi:10.1038/hortres.2016.56, C. Davies as co-author, see above) is the next stage in this work which has put a case, together with guidelines/design for the development of a system based on FAIR principles off data management and data stewardship (Findable, Accessible, Interoperable, and Reusable) to improve the use and value of the large number of genomic, genetic, transcriptomic, metabolomic and phenotypic data sets. This paper could not be reproduced as is not in an open access journal. We are involved in writing grant proposals to seek funding to further this initiative.

Appendix 2: Intellectual Property

The intellectual property (IP) and valuable information from this project falls into two categories. The first is the knowledge of how plant growth regulators (PGRs) control berry development/ripening and other processes such as vegetative growth and the response to wounding. This forms the knowledge base and basic framework for the second category of information that is related to application of the basic knowledge to issues important to the grape and wine industry. Much of the knowledge regarding the roles of endogenous PGRs and the effects of exogenous PGR application has been published in scientific and industry journals. This and further information has been developed to demonstrate the reliability and effectiveness of proposed methods and to outline any limitations. Information that we think might be useful for the commercialisation of the process and getting it out to industry concerns the more detailed practical aspects of formulation and application. This IP will be used to assist in this process.

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Appendix 5: Supplemental Material

See attached.

Appendix 6: Budget Reconciliation

Submitted online via Wine Australia's Clarity Investment Management System.

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RESEARCH ARTICLE Open Access

Alignment of time course gene expression data and the classification of developmentally driven genes with hidden Markov models

Sean Robinson[1*], Garique Glonek[1], Inge Koch[1], Mark Thomas[2] and Christopher Davies[2]

Abstract

Background: We consider data from a time course microarray experiment that was conducted on grapevines over the development cycle of the grape berries at two different vineyards in South Australia. Although the underlying biological process of berry development is the same at both vineyards, there are differences in the timing of the development due to local conditions. We aim to align the data from the two vineyards to enable an integrated analysis of the gene expression and use the alignment of the expression profiles to classify likely developmental function.

Results: We present a novel alignment method based on hidden Markov models (HMMs) and use the method to align the motivating grapevine data. We show that our alignment method is robust against subsets of profiles that are not suitable for alignment, investigate alignment diagnostics under the model and demonstrate the classification of developmentally driven genes.

Conclusions: The classification of developmentally driven genes both validates that the alignment we obtain is meaningful and also gives new evidence that can be used to identify the role of genes with unknown function. Using our alignment methodology, we find at least 1279 grapevine probe sets with no current annotated function that are likely to be controlled in a developmental manner.

Keywords: Alignment, Classification, Hidden Markov models, Time course microarray experiment

Background

Alignment of time course gene expression data is an important problem since, 'biological processes have the property that multiple instances of a single process may unfold at different and possibly non-uniform rates in different organisms, strains, individuals, or conditions' [1]. Such different rates may affect the timing of gene expression, which will be manifest in the observed expression profiles.

We consider a time course microarray experiment conducted on grapevines (*Vitis vinifera* L., Cabernet Sauvignon) at the 'Willunga' and 'Clare' vineyards in South Australia. The experiment was run over the duration of the development cycle of the grape berries, from the closed-flower to ripe-red stage of the berries themselves. For each gene, we have a pair of expression profiles, one from each of the Willunga and Clare vineyards. Pairs of expression profiles for four example genes can be seen in Fig. 1. For each pair of profiles, we aim to obtain a single profile that captures the relevant gene expression information over the development cycle of the grape berries from both vineyards. The common representations can then be used for an overall analysis of the gene expression.

The rate of development of the grape berries was different at the Willunga and Clare vineyards. Differences between the vineyards such as soil conditions, viticultural management and climate are likely causes of the different rates of berry development [2]. During the experiment, the length of the development cycle was 19 weeks at Willunga and 17 weeks at Clare. Since the experiment called for weekly measurements, the expression profiles from Willunga have length 19 while the expression profiles from Clare have length 17 (Fig. 1). Hence we require an alignment between the different length profiles.

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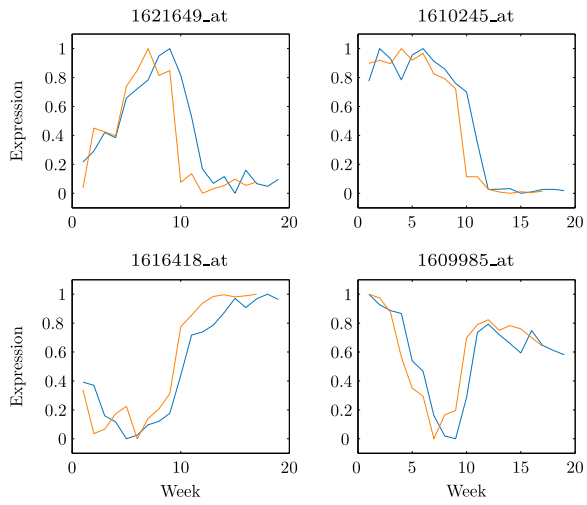

Fig. 1 Expression profiles for four example genes from the Willunga (blue) and Clare (orange) vineyards

The basic underlying pattern of berry growth and ripening was the same at both the Willunga and Clare vineyards, which suggests a common underlying framework of gene expression control. Hence in spite of the different conditions, if a pair of expression profiles exhibit the same basic shape at both vineyards and are suitable for alignment, this is strong evidence that the corresponding gene is likely to be developmentally controlled. On the other hand, pairs of profiles with different shapes are not suitable for alignment and the corresponding gene is unlikely to be driven by the development process but by other factors.

A recent survey of grapevine genes [3] indicated that the annotation of 44 % of genes is 'poorly informative' (including 29 % having no Blast hit and 9 % with function unknown). Actual functional data is available for only a small subset of those genes with an assigned function and most often function is defined on the basis of sequence similarity with genes from other species. Additionally, the assignment of a biochemical function does not define whether a gene has a mainly developmental role or is merely responding to external cues.

Hence considering whether a pair of profiles is well aligned will give important additional evidence that can be used to identify genes as either likely to be developmentally driven or not.

The time sparsity and variability of the grapevine data is typical of longer term time course gene expression experiments. Interpolation of the expression values between observed time points is not readily justified as significant non-linear variations in expression could conceivably occur between adjacent time points. Rather than the expression levels week by week, the biological relevance is in the general expression behaviour over the entire development cycle, which is where both the available data and current biological understanding lie.

Non-model based alignment methods such as discrete time warping (DTW) have been used for alignment of time course gene expression data [1]. However, for the grapevine data, DTW invariably produces pathological results. For example, >3 time points mapped to a single time point from Willunga to Clare immediately followed by the same from Clare to Willunga has no reasonable interpretation when each time step is a week and especially when the alignment differs for different pairs of profiles. Simply considering the lag between profiles would also not be a suitable model for the timing differences between vineyards and would violate the experimental set-up.

In order to work with the typical sparsity of the grapevine data, as well as to provide a principled way to obtain a common alignment across both vineyards, we turn to hidden Markov model (HMM) based alignment methods.

Left-right HMMs

Lin *et al.* [4] aligned gene expression profiles using an HMM by constraining the Markov chain component to be a 'left-right' model. In a left-right HMM a state can never be revisited once it has been left and transitions away from a state may only occur to a single other state. Hence an alignment is achieved between the expression profiles by considering the different times the state transitions occur in the corresponding Viterbi paths.

A left-right HMM can be altered to allow for less restrictive transitions between states while keeping the same alignment idea, for example allowing the 'leapfrogging' of states. Schliep *et al.* [5] considered such an alignment, however their main focus was a model-based 'soft' clustering method for expression profiles using mixtures of HMMs.

We aim to capture the basic pattern of each pair of profiles, which may be different from any other pair (Fig. 1). Hence approaches that constrain the Markov state transitions to the extent that all realised state sequences must share the same basic shape are not suitable in this case.

Pair HMMs

Pair HMMs are the standard model for the alignment of genomic sequence data [6]. However, Pair HMMs require discrete emission random variables to model the genomic sequences of interest. In addition, the conditional information of a previous emission observation is not the actual observed value but whether the observation was a pair or single nucleotide symbol. Since we aim to interpret the underlying Markov structure as capturing distinct quantitative levels of the expression profiles, we

require more than the binary pair/single nucleotide symbol dynamics of the Markov chain component of a Pair HMM.

Extensions of Pair HMMs

Two ways in which Pair HMMs could be extended to model time course gene expression data are to:

- Retain the binary dynamics of the Markov chain component of the model and consider continuous emission random variables; or
- Incorporate additional information into the model so that the Markov structure encodes more than just binary dynamics.

Note that these possible extensions do not explicitly take alignment into account, although the motivation in considering such extensions is that the established alignment method of Pair HMMs could be carried over.

Binary Markov dynamics with continuous emissions

Yuan and Kendziorski [7], and Yoneya and Mamitsuka [8] both proposed extensions of a Pair HMM that retain the binary dynamics of the Markov chain component of the model. Both modelled time course gene expression data and hence considered continuous emission random variables. Yuan and Kendziorski [7] did not aim to obtain an alignment between expression profiles, and it is not clear how their model could be adapted for this purpose. Although the model of Yoneya and Mamitsuka [8] could be used as the basis of an alignment, their model requires strict assumptions about the shape of the expression profiles, assuming average expression levels except for at least one spike feature. Most genes in the grapevine data do not display expression profiles with such patterns (Fig. 1) so this approach is not suitable.

Additional information incorporated into the model

Listgarten *et al.* [9] proposed a 'Continuous Profile Model' (CPM), which they consider to be a 'continuous analogue' to a Profile HMM. Also widely used for the alignment of genomic sequence data, Profile HMMs are closely related to Pair HMMs [6]. Under a CPM, each time series is modelled as an emission sequence and the corresponding realisation of the state sequence is a mapping to an additional input sequence or 'latent trace'. The latent trace has a higher number of time points than the observed time series (approximately double), which allows the mapping to 'slow down' and 'speed up' relative to 'latent time' and hence constitute an alignment.

The CPM was developed for mass spectrometry and speech waveform time series that were sampled frequently enough in time that interpolating smoothly between time points was a reasonable approach. The assumption of smoothness in time necessary for the 'continuous' CPM alignment is not reasonable for the grapevine data. Therefore, it would not be appropriate to apply the CPM alignment method to the grapevine data.

Our approach

We will model the expression profiles as multiple emission sequences of an HMM so that each pair corresponds to a common underlying state sequence. The emission sequences are aligned under the model in that aligned emission random variables are conditioned by the same state random variable. We will assume that the underlying Markov state sequence represents a common expression profile at both vineyards and that the Markov states represent distinct quantitative levels of gene expression.

Like the CPM, our alignment HMM is conceptually similar to a Pair HMM. However, in contrast to Pair HMMs, the alignment in our model is not determined by the underlying Markov chain but through 'gap position' parameters, which we incorporate into the model as additional information. Rather than the latent trace and continuous time warping of Listgarten *et al.* [9], this coarse approach to alignment is necessitated by the sparsity of our data.

We use our alignment HMM to achieve an alignment of the grapevine data and quantify how well each pair of profiles is aligned. We show that our method of training the model is computationally efficient and also robust against subsets of profiles that do not align. We then consider diagnostics under the model and demonstrate that genes can be classified as either likely to be developmentally driven or not by how well they align.

Methods

Grapevine data

In addition to being from spatially distinct vineyards, the time course microarray experiment was run in the 2004 grape growing season at Willunga and in the 2005 grape growing season at Clare. Gene expression levels were measured weekly at both vineyards using Affymetrix grapevine GeneChips (Santa Clara, CA, USA, Part #520054). We discard the expression profiles not differentially expressed in time at the 0.001 % significance level using LIMMA [10], as well as those without at least a 2-fold change in expression level. We also discard all profiles corresponding to the *Vitis vinifera* Array (non vinifera / non 3 prime) Mask. We average the replicate expression observations at each time point and then linearly scale each profile individually so that all observed expression levels lie in the interval [0,1] (Additional file 1: Figure S1). We refer to the resultant 8644 pairs of profiles as the 'grapevine data'.

Alignment model

We present our alignment methodology based on an HMM for the scaled time course gene expression grapevine data. The conditional independence graph of the alignment model is given in Fig. 2. Each pair of expression profiles is modelled as the two sequences of emission random variables $W_{1:19}$ and $C_{1:17}$ (indexed by time) for the Willunga and Clare vineyards respectively. The alignment is obtained based on the assumption that both emission sequences arise from a single state sequence $S_{1:19}$. The time points for the Willunga sequence $W_{1:19}$ correspond directly to those of the common state sequence $S_{1:19}$, while the time points for the Clare sequence $C_{1:17}$ are obtained via 'gap positions' $1 < g_1 < g_2 \leq 19$. In our approach the gap positions are treated as parameters of the model to be estimated from the data.

For a single pair of expression profiles, there is usually insufficient information to identify optimal gap positions. However, since the grapevine data have been scaled so that all observed expression levels lie in the interval [0,1], the Markov state space and conditional emission distributions can be considered common for all genes. This allows us to estimate a single set of gap positions by pooling the data from all pairs of profiles.

The state random variables $S_{1:19}$ that form the Markov chain component of the alignment HMM are discrete valued and take values in a common state space $\Omega_S = \{1, 2, \ldots, N\}$. For convenience we use $p(x)$ to symbolise both a probability density function and a probability mass function, in addition to using the event '$X = x$' as an argument.

Let $a = (a_1, a_2, \ldots, a_N)^\mathsf{T}$ be the $N \times 1$ vector of initial state probabilities and $A = \{a_{ij}\}$ be the $N \times N$ state transition matrix of the Markov chain state sequence where

$$a_i = p(S_1 = i)$$

for $i = 1, 2, \ldots, N$ and

$$a_{ij} = p(S_t = j | S_{t-1} = i)$$

for $i, j = 1, 2, \ldots, N$.

Let $B = \left\{\mu_1, \sigma_1^2, \mu_2, \sigma_2^2, \ldots, \mu_N, \sigma_N^2\right\}$ be the set of all parameters of the Gaussian emission distributions so that

$$p\left(x | S_t = j\right) = b\left(x | \mu_j, \sigma_j^2\right)$$

where

$$b\left(x | \mu_j, \sigma_j^2\right) = \frac{1}{\sqrt{2\pi\sigma_j^2}} \exp\left\{ -\frac{1}{2\sigma_j^2}(x - \mu_j)^2 \right\}$$

for $j = 1, 2, \ldots, N$.

In the general case for the k^{th} gene, we consider an underlying state sequence,

$$S_1^{(k)}, S_2^{(k)}, \ldots, S_T^{(k)}$$

and model the L expression profiles for each gene as the emission sequences

$$X_{l,1}^{(k)}, X_{l,2}^{(k)}, \ldots, X_{l,T_l}^{(k)}$$

where $T_l \leq T$ for $l = 1, 2, \ldots, L$. The alignment of the l^{th} expression sequence to the underlying common state sequence is defined by values

$$1 \leq \tau_{l,1} < \tau_{l,2} < \cdots < \tau_{l,T_l} \leq T$$

that indicate the state positions corresponding to each observed expression value.

Taking the set of HMM parameters to be

$$\lambda \equiv \{a, A, B\}$$

and the set of alignments to be

$$\boldsymbol{\tau} = \{\tau_{l,t} \mid l = 1, 2, \ldots, L \text{ and } t = 1, 2, \ldots, T_l\}$$

the general alignment HMM log-likelihood can be written as

$$\ell(\lambda, \boldsymbol{\tau} | \boldsymbol{x}) = \sum_{k=1}^{K} \log \left[\sum_{(s_1, s_2, \ldots, s_T)} p(s_1, s_2, \ldots, s_T) \times \prod_{l=1}^{L} \prod_{t=1}^{T_l} p\left(x_{l,t}^{(k)} | S_{\tau_{l,t}} = s_{\tau_{l,t}}\right) \right]$$

where

$$p(s_1, s_2, \ldots, s_T) = a_{s_1} \prod_{t=2}^{T} a_{s_{t-1} s_t}$$

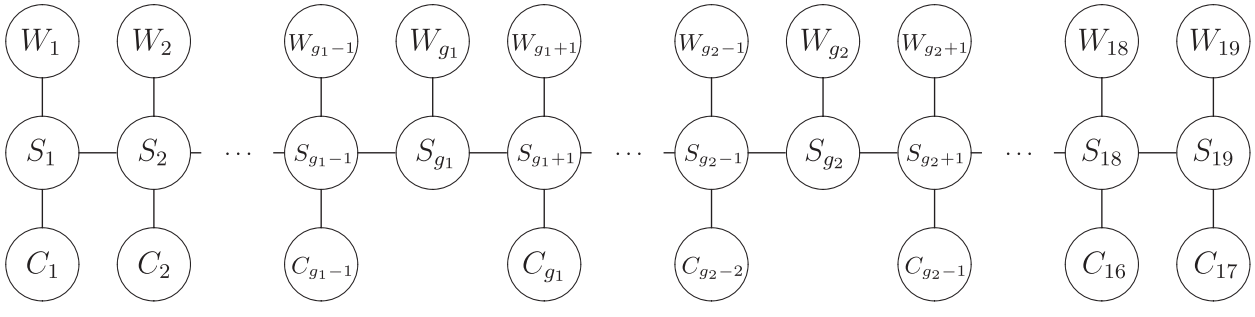

Fig. 2 Conditional independence graph of the alignment HMM for the grapevine data

and

$$p\left(x_{l,t}^{(k)}|S_{\tau_{l,t}}=s_{\tau_{l,t}}\right)=b\left(x_{l,t}^{(k)}|\mu_{s_{\tau_{l,t}}},\sigma^2_{s_{\tau_{l,t}}}\right).$$

The alignment is determined by maximising ℓ with respect to the HMM parameters λ and the alignment points $\boldsymbol{\tau}$. The model underlying this likelihood allows each gene its own unique state sequence but imposes a common alignment over all genes.

For the grapevine data, $K = 8644$, $L = 2$ and $T = 19$. Taking $\boldsymbol{w}$ and $\boldsymbol{c}$ to represent the expression data from Willunga and Clare respectively ($x_{1,t}^{(k)} = w_t^{(k)}$ and $x_{2,t}^{(k)} = c_t^{(k)}$ for $k = 1,2,\ldots,8644$ and $t = 1,2,\ldots,T_l$), we have $T_1 = 19$, $T_2 = 17$ and

$$\tau_{1,t} = t \text{ for } t = 1,2,\ldots,19.$$

The alignment is then determined by choosing

$$1 = \tau_{2,1} < \tau_{2,2} < \cdots < \tau_{2,17} \leq 19$$

which can be specified by equivalently choosing two gap positions $g_1 < g_2$ in the sequence $2,3\ldots,19$. That is,

$$\tau_{2,t} = \begin{cases} t & \text{for } t = 1,2,\ldots,g_1-1 \\ t+1 & \text{for } t = g_1,g_1+1,\ldots,g_2-2 \\ t+2 & \text{for } t = g_2-1,g_2,\ldots,17, \end{cases}$$

as represented by the conditional independence graph in Fig. 2. Note that due to the experimental set-up, we constrain the first expression values from Willunga and Clare to align ($\tau_{1,1} = \tau_{2,1} = 1$). The log-likelihood of the alignment model for the grapevine data is then

$$\begin{aligned}\ell(\lambda,g_1,g_2|\boldsymbol{w},\boldsymbol{c}) = \sum_{k=1}^{8644}\log\Bigg[&\sum_{(s_1,s_2,\ldots,s_T)} p\left(s_1,s_2,\ldots,s_T\right) \\ &\times\prod_{t=1}^{19} p\left(w_t^{(k)}|S_{\tau_{1,t}}=s_{\tau_{1,t}}\right) \\ &\times\prod_{t=1}^{17} p\left(c_t^{(k)}|S_{\tau_{2,t}}=s_{\tau_{2,t}}\right)\Bigg].\end{aligned} \quad (1)$$

There are well established methods for efficient calculation of the likelihood, finding the Viterbi paths and estimating the model parameters for a standard HMM [11]. These methods are readily adapted to our alignment model defined by (1) if the gaps g_1 and g_2 are given. Note that our alignment HMM is a special case of a hidden semi-Markov model [12].

Alignment model fitting method

We fit the alignment HMM to the grapevine data by maximising the log-likelihood $\ell(\lambda,g_1,g_2)$ with respect to the HMM parameters λ and the gap positions g_1 and g_2. A profile likelihood approach could be implemented by applying the Baum-Welch algorithm [11] to obtain an estimate $\hat{\lambda}^*(g_1,g_2)$ for the HMM parameters for each pair (g_1,g_2) and then maximising the profile likelihood $\ell(\hat{\lambda}^*(g_1,g_2),g_1,g_2)$ with respect to g_1 and g_2.

We propose a two-step approach with a much lower computational requirement and greater robustness to non-aligned expression profiles. In the first step, an estimate $\hat{\lambda}$ for the HMM parameter is obtained, independent of the pairing and of the gap positions. In the second step, the log-likelihood $\ell(\hat{\lambda},g_1,g_2)$ is evaluated for each pair (g_1,g_2) and the maximum likelihood estimates are selected from the enumeration. The estimate $\hat{\lambda}$ is obtained from modelling each individual expression profile at both Willunga and Clare by a standard HMM [11] in which the same parameters λ apply to both vineyards. Such a model is implied by (1) when dropping the constraint that each pair of emission sequences correspond to a common state sequence.

The computational advantage of this approach is that it requires only a single maximisation of the HMM likelihood rather than one for each pair of gap positions. More importantly, it is also robust against the influence of expression profiles not suitable for alignment. The notion of a common alignment is plausible for developmental genes but not for those driven by environmental factors such as temperature. Since the non-developmental genes are not known in advance, they cannot be removed and their presence may produce significant bias in the estimate $\hat{\lambda}^*(\hat{g}_1,\hat{g}_2)$. A minor issue is that the standard HMM model from which $\hat{\lambda}$ is obtained is inconsistent with the alignment HMM (1) because of the gaps in the Clare sequence. However, it is reasonable to assume that any bias arising from this inconsistency is minor compared to that arising from non-aligned expression profiles in the full likelihood estimate $\hat{\lambda}^*(\hat{g}_1,\hat{g}_2)$.

To summarise, we produce an alignment for the grapevine data in the following steps:

1. The gene expression profiles are filtered so that only those with significant differential expression and at least 2-fold change in expression over the time course are retained.
2. Each expression profile is linearly rescaled to lie in the interval [0,1].
3. A standard HMM is fitted to the data to obtain the estimated HMM parameters $\hat{\lambda}$.
4. The gap positions are estimated by maximising the alignment HMM log-likelihood $\ell(\hat{\lambda},g_1,g_2)$ with respect to g_1 and g_2.
5. A single representation of the aligned expression profiles can be obtained either by averaging the aligned expression profiles or by finding the Viterbi path.

We implemented our methodology in MATLAB by adapting the code provided in the HMM Toolbox [13].

Results and discussion

A standard HMM with $N = 5$ states was fitted to the grapevine data. The variances of the emission distributions were constrained so that $\sigma_j^2 \geq 0.001$ for $j = 1, 2, \ldots, 5$. This constraint was applied to avoid difficulties arising from the fact that the distribution of scaled expression values has point masses at the endpoints 0 and 1 (Additional file 1: Figure S1). The gap positions that maximise the log-likelihood $\ell(\hat{\lambda}, g_1, g_2)$ were found to be $\hat{g}_1 = 2$ and $\hat{g}_2 = 11$ (Fig. 3). The single peak in Fig. 3 indicates that the gap positions are well determined for the grapevine data.

Figure 4 shows the aligned expression profile for gene 1621649_at, together with the Viterbi path and average profile representations. For this gene, the alignment HMM has produced a suitable alignment. The method performs similarly for the other genes shown in Fig. 1. On the other hand, Fig. 5 shows poorly aligned expression profiles for genes 1622520_at and 1616700_at. For gene 1622520_at, the expression profiles at Willunga and Clare have very different shapes and cannot be aligned. The expression profiles for gene 1616700_at have similar shapes at Willunga and Clare but are not well aligned by the estimated gaps $\hat{g}_1 = 2$ and $\hat{g}_2 = 11$.

For the purpose of comparison, the parameters λ were also estimated from the alignment HMM (1) with fixed gaps $\hat{g}_1 = 2$ and $\hat{g}_2 = 11$. The estimated emission distributions for $\hat{\lambda}$ and $\hat{\lambda}^*(\hat{g}_1, \hat{g}_2)$ are shown in Fig. 6. In both cases the estimated means are spaced evenly across the range $[0, 1]$. However, for $\hat{\lambda}^*(\hat{g}_1, \hat{g}_2)$, the estimated variances are noticeably larger. An explanation for this difference is the presence of genes with expression profiles that are not suitable for alignment. In particular, the presence of misaligned profiles will lead to very different expression values being aligned at the same time point and equally contributing to the parameter estimates for the same state, hence inflating the estimated variance.

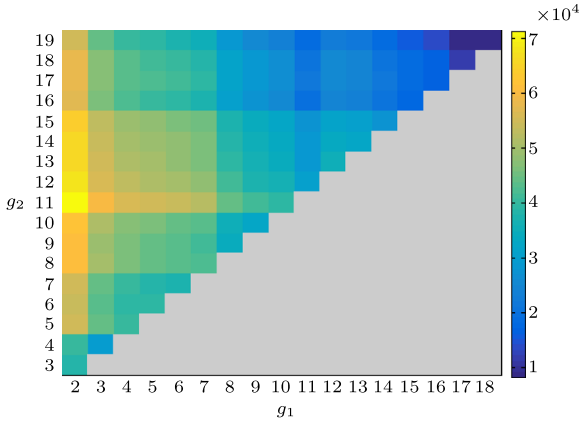

Fig. 3 Heat-map of the alignment HMM log-likelihood for the grapevine data (1) evaluated using $\hat{\lambda}$ and each possible combination of the gap positions $1 < g_1 < g_2 \leq 19$

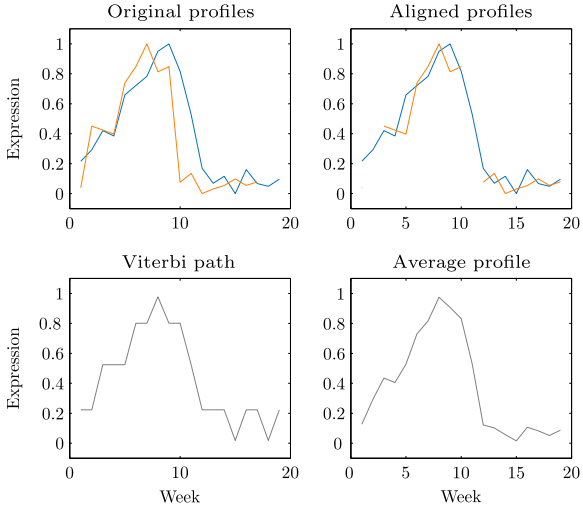

Fig. 4 Expression profiles from the Willunga (blue) and Clare (orange) vineyards, aligned expression profiles, Viterbi path and average profile representation for gene 1621649_at. The Viterbi path is plotted at the estimated means of the emission distributions

We consider the robustness of the estimates of the gap positions. In a simulation experiment, even with up to 80 % of the data not suitable for alignment, the true gaps can clearly still be found through the log-likelihood (Additional file 2: Figure S2). For subsets of simulated profiles with different true gap positions, the maximum peak in the log-likelihood heat-map becomes less concentrated and spreads out (Additional file 2: Figure S2). For the grapevine data, the log-likelihood is sharply peaked (Fig. 3) and the estimated gaps additionally conform with other physiological features measured on the berries during the experiment. For example, both total soluble solids (sugar content) and berry weight were also measured weekly at Willunga and Clare and the same gap positions appear to work well for this additional data (Additional file 3: Figure S3).

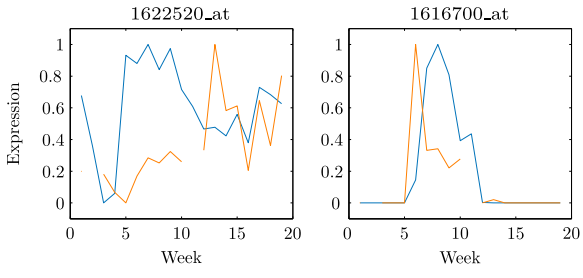

Fig. 5 Poorly aligned expression profiles for two example genes from the Willunga (blue) and Clare (orange) vineyards

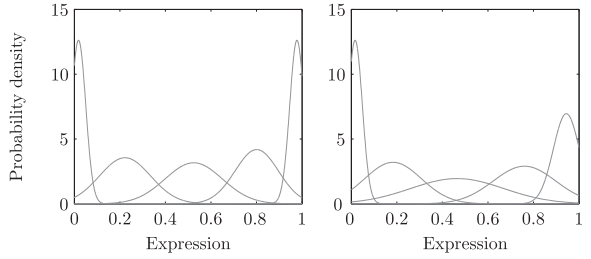

Fig. 6 Estimated emission densities corresponding to $\hat{\lambda}$ (left) and $\hat{\lambda}^*(\hat{g}_1, \hat{g}_2)$ (right)

We also consider fitting the alignment model with different choices of the number of states N. The estimated emission densities and heat-maps for $N = 3$ and $N = 7$ are given in Additional file 4: Figure S4. We can see that the same maximum likelihood gaps are found in both cases. It appears that $N = 3$ states is not enough over the range of the data while $N = 7$ is too many as two of the emission densities coincide.

It is the difference between the estimates $\hat{\lambda}$ and $\hat{\lambda}^*(\hat{g}_1, \hat{g}_2)$ seen in Fig. 6 that suggests the presence of poorly aligned profiles in the grapevine data. To identify the well and poorly aligned expression profiles we consider the Hamming distance between the Viterbi path for each pair of aligned profiles and the Viterbi paths obtained for the individual profiles. Let $\hat{S}^{(k)}_{1:19}$ be the alignment HMM Viterbi path for the k^{th} pair of profiles, and let $\hat{S}w^{(k)}_{1:19}$ and $\hat{S}c^{(k)}_{1:17}$ be the standard HMM Viterbi paths for the k^{th} Willunga and Clare profiles respectively. The Hamming distance between the Viterbi paths for the k^{th} pair of expression profiles is

$$H(k) = \sum_{t=1}^{19} I\left\{\hat{S}^{(k)}_{\tau_{1,t}} \neq \hat{S}w^{(k)}_t\right\} + \sum_{t=1}^{17} I\left\{\hat{S}^{(k)}_{\tau_{2,t}} \neq \hat{S}c^{(k)}_t\right\}.$$

The Hamming distance $H(k)$ has a negative linear relationship to log-likelihood (Additional file 5: Figure S5). Table 1 shows the Hamming distances and the log-likelihoods for the example expression profiles shown in Figs. 1 and 5. Well aligned expression profiles typically have high log-likelihood and low Hamming distance while conversely, the poorly aligned expression profiles typically have low log-likelihood and high Hamming distance. Not all profiles are obviously well or poorly aligned. Note that the aligned profiles for gene 1622520_at have relatively high log-likelihood because they are well aligned for all the time points when the gene exhibits low expression (Fig. 5). While the Hamming distance is purely a measure of the quality of alignment as determined by the Viterbi paths, the log-likelihood incorporates other aspects of model fit such as the distance from the expression values to the state means. For this reason we recommend the Hamming distance to identify poorly aligned expression profiles.

Table 1 Log-likelihood and Hamming distance for the example pairs of profiles given in Figs. 1 and 5

Affy ID	Figure	Log-likelihood	$H(k)$
1621649_at	Fig. 1	17.4670	7
1610245_at	Fig. 1	41.7735	6
1616418_at	Fig. 1	25.6842	7
1609985_at	Fig. 1	18.3318	10
1622520_at	Fig. 5	-43.0573	18
1616700_at	Fig. 5	24.1855	10

As previously outlined, how well a pair of expression profiles align across vineyards is evidence for whether the corresponding gene is likely to be developmentally driven. To illustrate the potential for identification of biological function from alignment, a set of 198 genes were considered as test data (Additional file 6). Although this test data were also used to train the alignment model, we never made use of the labels in the model fitting. Our classifier arises out of the diagnostics of the alignment HMM as we assume there is a correspondence between 'well aligned' and 'developmental'.

The left side of Fig. 7 shows the distribution of Hamming distances for all pairs of expression profiles in the grapevine data. The right side of Fig. 7 shows the receiver operating characteristic (ROC) curve for classifying genes as 'developmental' or 'non-developmental' (temperature responsive) based on whether the Hamming distance is below or above a given threshold. The area under the curve is 0.91, indicating a good level of discrimination for this data. When the threshold is taken as $H(k) = 10$, the true positive rate is 85.3 % and the false positive rate is 21.9 %. This suggests that applying the same threshold is a potentially useful filter for the classification of developmentally controlled genes amongst a set of genes of unknown function.

Grimplet *et al.* [3] surveyed the current gene function annotation for grapevines. Assigning a developmental role

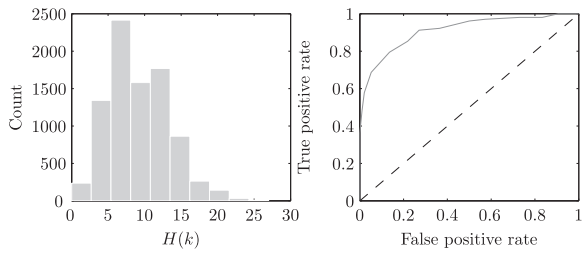

Fig. 7 Histogram of $H(k)$ for the grapevine data (left) and ROC curve for classifying 'developmental' or 'non-developmental' (temperature responsive) genes based on whether they are above or below a given $H(k)$ threshold (right)

to genes based on the putative function of the proteins they encode, as determined by sequence similarity to other genes of known function and without reference to their expression patterns, is an uncertain practice. For example, so-called 'heat shock' proteins with similar protein sequences may be either developmentally controlled or may be induced by changes in temperature, or both. Additionally, differences in the promoter sequences of genes encoding similar proteins may determine whether a gene is involved in a developmentally controlled process or not.

By comparing the expression of genes under different growth conditions, as has been done in this paper, we are able to gain evidence regarding the reproducibility of gene expression patterns indicative of a role in development as opposed to a response to external signals. This information can be used as additional evidence in the further investigation of gene function. For example, using the annotation of Grimplet *et al.* [3], of the 8644 genes represented in the grapevine data, 1968 have no description of possible function ('no function', 'no hit', 'unknown' or 'unknown function') and of these we find 1279 probe sets with $H(k) \leq 10$. That is, 1279 genes with no current annotated function are well aligned between the Willunga and Clare vineyards and therefore we now have additional information that these genes are likely to be controlled in a developmental manner Additional file 7.

The proposed alignment method could be extended and refined in a number of ways. In particular, potential improvements may be obtained through more detailed modelling of the emission distributions in the HMM. In the present paper, we have applied Gaussian emission distributions to the expression profiles averaged over replicates within vineyards. This approach could be refined by considering the replicates as multivariate observations instead of averaging and also by considering alternatives to the Gaussian emission assumption. Autoregressive emissions as well as higher-order Markov components of HMMs have been investigated and found to improve performance in the identification of over-expressed genes [14]. The incorporation of this structure into our framework may more realistically model the expression profiles with potential improvements in performance. The implementation and evaluation of these improvements are the subject of future research.

Conclusion

We have presented a novel alignment method based on an HMM and demonstrated the alignment on the grapevine data. This is a model suitable for sparse time course data where interpolation is not appropriate. The estimated model parameters have simple interpretations and the estimated gap positions are well determined for the grapevine data. We have demonstrated that the estimates of the HMM parameters as well as the gap positions are robust against subsets of profiles that are not suitable for alignment. For pairs of expression profiles that are well aligned, the Viterbi paths or average profile representations can be used as the input to downstream analysis of the data. This allows for an integrated analysis of multiple site time course gene expression data such as the Willunga and Clare grapevine data.

We have demonstrated the use of the Hamming distance and the log-likelihood as a measure of quality for the alignment of a pair of expression profiles. Pairs of profiles that are well aligned will have high log-likelihood and a small Hamming distance while the poorly aligned pairs will have low log-likelihood and a large Hamming distance. We have also shown, for a set of genes with known function, that classification of genes according to the Hamming distance has reasonable predictive power for the classification of developmentally driven genes. This both validates that the alignment we obtain is meaningful and also suggests the potential for helping to identify the role of genes with unknown function.

Availability of supporting data

The MATLAB code and grapevine data to obtain all of the output described in this paper are provided as Additional files. The raw gene expression data is stored at NCBI in the GEO database as GSE7677 (Willunga) and GSE8445 (Clare) Additional file 8.

Additional files

Additional file 1: Figure S1. Histogram of the scaled expression levels for the grapevine data overlaid with a mixture density of the estimated emission densities where the mixture coefficients are the stationary Markov transitions of the estimated alignment HMM parameters $\hat{\lambda}$.

Additional file 2: Figure S2. Heat-maps corresponding to a number of simulation experiments. Top row: 1000 pairs of profiles were simulated using the estimated HMM parameters $\hat{\lambda}$ and with true gap positions ($g_1 = 5, g_2 = 13$). Pairs of profiles not suitable for alignment were obtained by permuting the pairing information of a subset of profiles. From left to right: Heat-maps calculated using the simulated data and parameters $\hat{\lambda}$ with an increasingly large subset of profiles not suitable for alignment. Middle row: Same simulation set-up with true gaps of either (5, 13) or (8, 16). From left to right: Heat-maps calculated using the simulated data and parameters $\hat{\lambda}$ with an increasingly mixed proportion of pairs of profiles with different true gaps. Bottom row: Same simulation set-up with true gaps of either (4, 9) or (12, 17).

Additional file 3: Figure S3. Total soluble solids (left) and average berry weight (right) measured over the development cycle at the Willunga (blue) and Clare (orange) vineyards with the same alignment as found for the grapevine expression data. Note that these measurements did not commence at the beginning of the experiment.

Additional file 4: Figure S4. Estimated emission densities and heat-maps when fitting the alignment model with $N = 3$ (top) and $N = 7$ (bottom) states to the grapevine data.

Additional file 5: Figure S5. Log-likelihood under the alignment HMM by Hamming distance for each pair of expression profiles in the grapevine data.

Additional file 6: The set of 198 labelled genes (test data). From a separate experiment, 96 of these genes had been identified as 'temperature responsive' genes through the response of gene expression to changes in temperature. The remaining 102 genes were selected from the Grapevine Affymetrix array probe list on the basis of annotated function where selected genes were thought to be involved in a developmental process in grapevine (and often in other plant species) and, where possible, on the basis of gene expression patterns throughout development.

Additional file 7: Final grapevine output. Log-likelihood under the alignment HMM, Hamming distance and current annotation [3] for all 8644 genes in the grapevine data.

Additional file 8: MATLAB code and grapevine data. The MATLAB code and grapevine data to obtain all of the output described in this paper.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the time course microarray experiments: MT CD. Performed the experiments: MT CD. Developed the alignment methodology: SR GG IK. Implemented the methodology: SR. Analysed the data: SR GG IK. Contributed to the analysis and drafting of the paper: IK MT. Wrote the paper: SR GG CD. All of the authors have read and approved the final manuscript.

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The grapevine gene nomenclature system

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Abstract

Background: Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops in the world and serves as a valuable model for fruit development in woody species. A major breakthrough in grapevine genomics was achieved in 2007 with the sequencing of the *Vitis vinifera* cv. PN40024 genome. Subsequently, data on structural and functional characterization of grape genes accumulated exponentially. To better exploit the results obtained by the international community, we think that a coordinated nomenclature for gene naming in species with sequenced genomes is essential. It will pave the way for the accumulation of functional data that will enable effective scientific discussion and discovery. The exploitation of data that were generated independently of the genome release is hampered by their heterogeneous nature and by often incompatible and decentralized storage. Classically, large amounts of data describing gene functions are only available in printed articles and therefore remain hardly accessible for automatic text mining. On the other hand, high throughput "Omics" data are typically stored in public repositories, but should be arranged in compendia to better contribute to the annotation and functional characterization of the genes.

Results: With the objective of providing a high quality and highly accessible annotation of grapevine genes, the International Grapevine Genome Project (IGGP) commissioned an international Super-Nomenclature Committee for Grape Gene Annotation (sNCGGa) to coordinate the effort of experts to annotate the grapevine genes. The goal of the committee is to provide a standard nomenclature for locus identifiers and to define conventions for a gene naming system in this paper.

Conclusions: Learning from similar initiatives in other plant species such as *Arabidopsis*, rice and tomato, a versatile nomenclature system has been developed in anticipation of future genomic developments and annotation issues. The sNCGGa's first outreach to the grape community has been focused on implementing recommended guidelines for the expert annotators by: (i) providing a common annotation platform that enables community-based gene curation, (ii) developing a gene nomenclature scheme reflecting the biological features of gene products that is consistent with that used in other organisms in order to facilitate comparative analyses.

Background

As for many other major model plant species, the release of the grapevine genome in 2007 [1] led to a rapid accumulation of "Omics"-scale data and a burst of high-throughput studies. In 2010, the *V. vinifera* cv. PN40024 genome sequence was updated from 8X to 12X coverage [2] and is, to date, the reference genome for *V. vinifera*. The gene models and their putative functions have been automatically predicted from the genome sequence and have been used in many functional studies. The results from these published studies were deposited in general-purpose gene databases such as NCBI, but also in other independent repositories. These data are a highly informative resource to help curate the automatic prediction. Another resource, consisting of manually curated gene families associated with heterogeneous levels of functional evidence is also growing rapidly [3-6] but lacks a central storage system allowing coordination of gene nomenclature. Previous important efforts have been made in the past to curate the automated functional annotation [7]. These data are publicly available, but are not well integrated into major genomic databases such as NCBI and EBI.

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To streamline the new nomenclature initiative from the sNCGGa, a set of directives, addressing the most important issues, has to be provided to allow a better integration of the various, diverse resources into an improved global annotation of the grapevine genome, both at the structural and functional levels. These directives are aimed at facilitating exchanges between international genomic repositories to assist the analysis of gene experimental functional data and comparisons with other species.

In addition to the sequencing of the nearly homozygous PN40024 genome, other genomic resources for *V. vinifera,* and related species, continue to be generated, including the sequencing of the genomes of other varieties [8-10], EST sequencing, integrated genetic maps, and the whole genome re-sequencing for polymorphism discovery of other *Vitis* varieties and species [11]. The EST and genome resources have permitted the design of a wide variety of microarrays for large-scale mRNA expression profiling studies (for example: [12]), but microarrays are being replaced by RNA-seq (for example: [9]). A majority of the expression data are maintained in the PLEXdb database [13]. However, heterogeneity in the design of the microarray platforms, both in terms of the version of the annotation and in technical design, requires considerable bioinformatic effort to identify the probes or probesets corresponding to a unique gene. Besides, the assembly of the genome of other varieties [9,10] and the elucidation of their transcriptomes [14], produce varietal specific sets of genes that will have to be traced. These under-exploited resources can be better used to improve the annotation of the reference genome.

The availability of the annotated genome sequence also facilitates the identification of proteins resulting from mass spectrometry analyses and increases the effectiveness of high throughput proteomics studies in grapevine [15]. Proteomic analyses have been used to characterize differential expression of proteins underlying diverse aspects of grapevine physiology in the berry or vegetative tissues [15,16]. Furthermore, information acquired from these studies on the potential functional role of the genes coding for these proteins would benefit gene annotation curation. Conversely, the continuous improved annotation will impact favourably on expression and proteomics analyses, provided this annotation remains easily accessible.

To achieve our goals, a network of annotation experts with a clearly defined strategy and *modus operandi* is needed. From the several plant genomes sequenced in recent years, only *Arabidopsis* has really benefited from a comprehensive monitoring and a real refinement of data generated automatically. This was mainly because of the existing large scientific community, supplied with significant financial support from granting agencies, allowing the development of resources such as TAIR [17]. Rice [18] and tomato [19] are at an intermediate level; their data curation structures have been established. The herein proposed directives have been inspired by the sets of rules for gene nomenclature that are available for *Arabidopsis* [20], rice [21], *Medicago* [22] and tomato [23].

The grapevine genomics community at large is mostly structured around the International Grape Genome Program (IGGP; www.vitaceae.org) whose mission is to facilitate the networking of grapevine researchers in order to develop common and publicly available resources. These resources facilitate the elucidation of the genetic and molecular basis of biological processes in *Vitis* and should lead to a more efficient exploitation of the *Vitis* biological resources for the development of new cultivars and clones that have improved quality and reduced economic and environmental costs. It may also allow for more efficient vineyard management.

It is therefore the IGGP's objective to provide a common platform for continuous improvement of the annotation of grapevine genes. This objective will be coordinated by the Supernomenclature Committee for Grapevine Gene Annotation (sNCGGa), and was supported by the Grape Research Coordination Network (funded by the United States National Science Foundation in the USA). The first milestone presented here is the development of a standardized protocol for gene naming, with names that have to be unique, consistent with other plant models and sustainable. This report proposes guidelines for the nomenclature of the genes from the latest version of the gene structural annotation, promoted by the COST (European Cooperation in Science and Technology) ACTION FA1106 (funded by the European Union), and performed on the assembly (V2) of the scaffold from the 12X version of the reference genome performed in a collaboration between the Institut National de la Recherche Agronomique (INRA) and the Istituto di Genomica Applicata (IGA). The automatic annotation of the genes was performed with the Eugene software [24] at the Vlaams Instituut voor Biotechnologie (VIB) and released through the ORCAE website that will be used for community annotation [25]. The important points addressed in each section of the manuscript to help gene annotators to address specific issues that they may encounter are highlighted in Figure 1.

Results & discussion

Nomenclature and definition of the gene naming system and convention

There are three main categories of nomenclature that need to be addressed for each gene (Table 1). In the first place, the Locus Identifier (Locus ID), will represent the unique identifier of the gene in the genome. This identifier is not intended to be related to a physical position on the chromosome. The second and the third places correspond

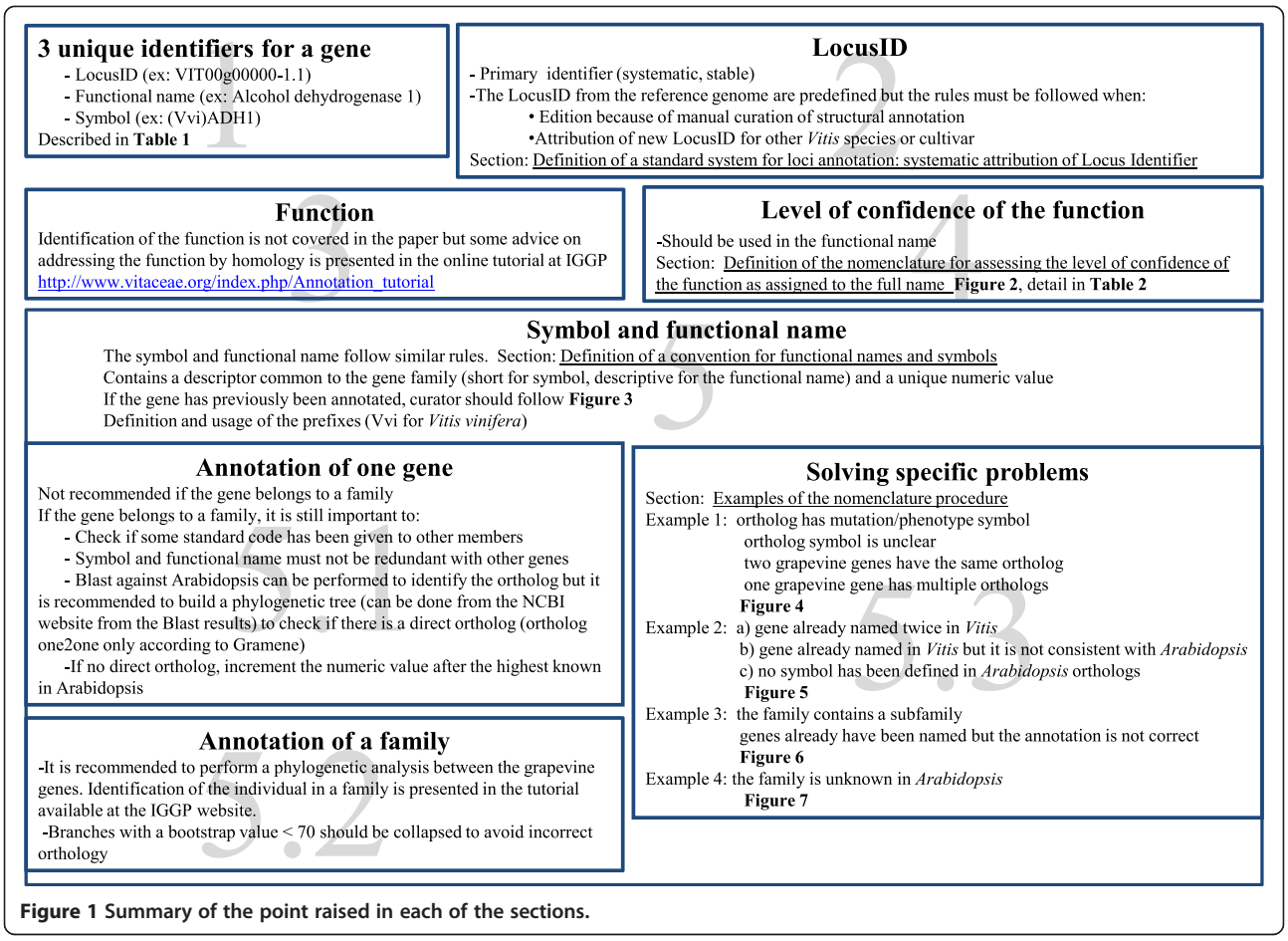

Figure 1 Summary of the point raised in each of the sections.

to the Full Name and the Symbol, respectively, and refer to the description of the functional role of the protein encoded by the gene. The Symbol is a short abbreviation of the full name. To deal with pre-existing naming schemes we propose to add synonyms. These correspond to other types of names that have been encountered in the literature; they can be symbols or full names.

Definition of a standard system for loci annotation: systematic attribution of locus identifier

A Locus-ID will be assigned to all genetic objects having a unique position on the genome. This Locus ID provides a unique identifier initially provided after automatic annotation to a specific object along the genome. Locus-IDs under no circumstances can be re-used, but objects, like genes, can be changed when corrected. Initially, the numbering will be incremental along the chromosomes. With updates of the assembly, and the moving of unanchored contigs from chromosome "00" to their real location, new Locus-IDs will be created in series, as detailed in the "numeric code" section, replacing the chromosome "00"-related Locus-IDs. Merging (concatenating) gene models will follow the same rules, with the difference that one of the Locus-ID's will be discarded. In the case of splitting gene models, a new Locus-ID will be created and attributed to the new gene model. As such, Locus-IDs should not be seen as positional and derived products; however, transcripts and proteins will remain linked to these Locus-IDs. These rules can be virtually applied to any objects that are absent from the reference genome, such as genes that are only identified in other cultivars or *Vitis* species. Non-reference genes can then be

Table 1 Brief definition and example of the main elements of the gene nomenclature

Elements	Locus ID	Full name	Symbol	Synonyms
Example	Vitvi18g12230	(*Vitis vinifera*) Alcohol dehydrogenase 1	(Vvi)ADH1	GV-ADH1 aldehyde reductase, ethanol dehydrogenase
Description	Genome localization	Relatively descriptive function, include the level of curation (see Figure 2)	Concise (3–10 characters), should be descriptive of function when possible	Any known synonyms

referenced with their chromosome number (or "00" if unknown) and a numeric code can be stored in the ORCAE platform [25] that will be used for community annotation.

Taking into account previous experiences acquired through the previous grapevine locus ID schemes [26] and structures defined in other species, an ID containing the following elements was retained: Taxonomy ID/Chromosome number/Object type/Numeric code/Sequence variant/Version.

Each element separated by a slash has a specific function as described below.

Taxonomy ID For the reference genome of the *V. vinifera* var. PN40024, it was decided to follow the species abbreviation list that exists at UniProt [27], and the Supernomenclature Committee considered using this five-digit code for *V. vinifera* 'VITVI' (three letters for the genus and two for the species). This abbreviation is widely used in UniProt for gene abbreviation, but more rarely for locus name, but it was considered the best long-term solution. Other important plant species have their own strategies. In tomato (*Solanum lycopersicum)*, a five-letter code is used with two letters for the genus and three for the species; SOLYC instead of SOLLC as recommended at UniProt. Note that the *Brassica* community also uses a three-letter code [28], while most of the other species use two letter codes. For other *Vitis* species, the most widely occurring *Vitis* species already appear in the UniProt species list and this abbreviation should be used. Prefixes for other species must include the three letters 'VIT' and the code defined by the *Vitis* International Variety (VIV) Catalogue [29], for example the code for *Vitis berlandieri* should be VITVBR, with six letters. This code must be utilized when registering new genome sequencing of a *Vitis* species. No reference should be made to the cultivar in the taxonomy ID, which should be done in the sequence variant section.

Chromosome number The second item refers to the number of the chromosome to which the gene is predicted to be localized. The chromosome number is attributed as defined by the IGGP and ranges from 00 to 19. The chromosome "00" corresponds to an assembly, in a random order, of scaffolds that could not be positioned yet on the chromosomes.

Object type The third item represents the type of object corresponding to the molecular entity: **g** for gene; **t** for protein coding transcript; **p** for protein; **nc** for non-coding; **tr** for transfer RNA; **te** for transposable element; **rr** for ribosomal RNA; **mi** for microRNA; **ps** for pseudogene; **si** for small interfering RNA; **sn** for small nuclear RNA. Initially and before curation, the "Object types" referring to the DNA structure are labeled with the "**g**" code when referring to the locus, the "**t**" code when referring to the nucleic acid coding sequence of the transcript and the "**p**" code when referring to the amino acid sequence of the protein.

Numeric code The numeric code includes five digits that are initially defined in sequential order of the genes along a chromosome in ascending order from the telomere of the short arm (north side) to the telomere of the long arm (south side). In other species, it was decided to leave a gap between genes to allow the addition of further genes if new information was discovered. In *Arabidopsis* for instance, with a similar five-digit code, the gene IDs were numbered with an increment of 10 to allow room in-between currently annotated genes. In *Arabidopsis*, known gaps in the DNA sequence were assigned 200 'spare' identifiers per 100 Kb of gap [20]. In rice [21], a seven-digit code was used and genes were assigned in increments of 100. In tomato, a six-digit code was used and genes were assigned in increments of 10. In the *Vitis* Locus ID, because further improvements of the assembly are expected, we decided that no gaps would be left between the numeric codes of the genes (increments +1). If new objects have to be defined in the future, the next available number will be allocated as Locus-ID. Indeed, this means that after future rounds of improvement of this annotation the ID number will not reliably reflect the gene order along the chromosome. However, we think that this method presents several advantages. Given that the grapevine genome is still a work in progress with many unanchored scaffolds and whole regions with unsecure orientations, we can anticipate that scaffolds will be inserted or re-oriented and that the chosen numbering method will not lead to the risk of running out of numbers in the case that the gaps between two genes are larger than foreseen. Such an event will not impact the nomenclature; even if it involves chromosome changes, the old Locus ID will be stored as a synonym and a new Locus ID will be allocated, while in the case of a change of scaffold orientation, nothing would change. With a length of 5 digits for all the objects per chromosome (up to 99,999), the risk of running out of numbers is very low. The ORCAE platform [25] being used by the grapevine community can automatically handle any changes to ID numbers, decreasing the risk of errors.

Sequence variant This segment, which shall be preceded by a hyphen, will be used to discriminate molecular variants (allele, splice variant) that map to the same locus. The code can be numeric or alphabetic (e.g. for cultivar-specific polymorphism). If no allelic variant is present, one should refer to the primary sequence from the reference genome. Note that there would not be any cultivar-specific terms in the reference genome, these terms would

be addressed in the species' genomes. The splice variant is used only for object types "t" or "p".

The choice of numeric or alphabetic naming of the section (allele, splice variant, cultivar etc.) is left to the authors' discretion but it should be as concise as possible. As an example, it was identified that in the cultivar Tempranillo (abbreviated by the authors tp) that allele A produces mRNAs of splice form 1, 2, and 3; allele B produces mRNAs of splice form 1, 2, and 4; and Allele C produces mRNAs of splice form 1, 2, and 3. The sequence/splice variants as described above should be the following: –a1, –a2, –a3, –b1, –b2, –b4, –c1, –c2, –c3, or -tpa1, –tpa2, –tpa3, –tpb1, –tpb2, –tpb4, –tpc1, –tpc2, –tpc3, if the cultivar is mentioned. Authors must make sure that the code for the splice variant that they are defining is unique.

Version Any modification (addition, deletion) of any number of nucleotides, of the structural annotation of a gene will result in incrementing (+1) the version number. Version numbers are appended at the end of the locus ID, separated by a dot. If omitted, the most recent version of the gene model is implied. Versions are used when the modifications do not require Locus-ID change.

Definition of the nomenclature for assessing the level of confidence of the function as assigned to the full name

A guideline for defining the level of confidence of the annotation is presented in Figure 2. It is largely inspired by the guidelines proposed for the annotation of the rice [21] and tomato [23] genomes. Given that information obtained from experimental evidence is scarce in *Vitis*, it seems sensible to divide all loci into (i) those with defined, confirmed function (confirmed through biochemical characterization of the corresponding protein or the characterization of a mutant), (ii) those defined only by sequence similarity ('putative names") and (iii) genes of unknown function (including those with no match). Given the relative paucity of functional data available for grape it might be dangerous to suggest a "definitive" full name for a gene whose function has not been experimentally proven. On the other hand, not considering *in silico* inferred function would hide highly valuable information for hypothesis-driven experiments. We propose a set of guidelines that satisfy these considerations and

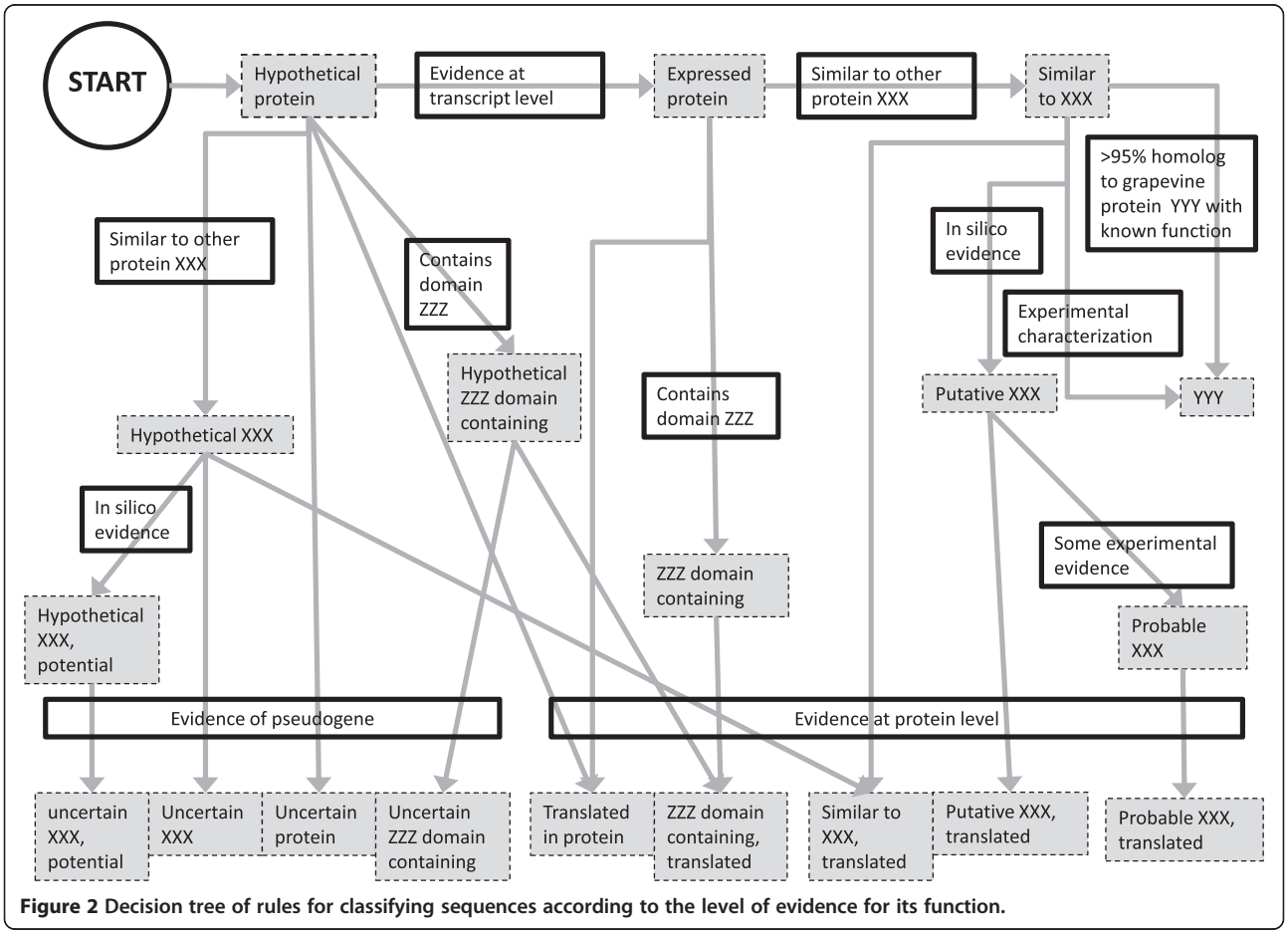

Figure 2 Decision tree of rules for classifying sequences according to the level of evidence for its function.

the recommendations of UniProt in terms of the degree of proof that defines the different levels of quality of the functional annotation [30]. Definition of the terms from Figure 2 is presented in Table 2. *In silico* evidence, *experimental characterization* and *some experimental evidence* should lead to the assignment of the GO annotation and the GO field in the ORCAE database should be edited complying with the Evidence Codes for the Gene Ontology (GO) [31].

Table 2 Definition of the level of curation terms

Value	Definition
Hypothetical protein	Allocated to each locus at the beginning of the process, meaning that the gene codes for a protein, for which no information regarding its function or actual existence is known. It should be removed only when existence of transcript is proven.
Expressed	Replaces "hypothetical" if existence of transcripts has been proven through expression data (proof of existence of RNA(s): RT-PCR, EST, RNA-seq, Northern blots, microarrays, etc.). The next step is to determine if similarity with sequences in other species can be observed.
ZZZ domain containing	Allocated if by comparison with other sequences or by performing a domain analysis, the highest level of information on the coding protein is the presence of a given domain ZZZ.
Similar to	Indicates that the existence of a protein is probable because a minimal level of similarity with a protein from a plant species was met. An e-value of e-20 is considered to be a reasonable cut-off or to have at least 30% identity for at least 80 contiguous amino acids, which places it into the "safe zone" as defined by [32]. The gene is labelled here as "similar to XXX", with "XXX" being the homologous protein from another species.
YYY	If the gene has been experimentally characterized and named YYY or if there is *>95% identical amino acids on the whole sequence to a grapevine protein YYY with a known function*, then the label should be the value "YYY" that corresponds to a gene whose function has been discovered and characterized in the *Vitis* Genus.
Putative	Derived from *in silico evidence* on function, indicates that there is some logical or conclusive evidence that the given annotation could apply. This non-experimental qualifier is often used to present results from protein sequence analysis software, which are only annotated if the result makes sense in the biological context of a given protein. A typical example is the annotation of N-glycosylation sites in secreted proteins.
Probable	Indicates stronger evidence than the qualifier "putative" on function. This qualifier implies that there must be at least *some experimental evidence*, which indicates that the information is expected to be found in the natural environment of a protein.
Uncertain	Indicates that the existence of the protein is unsure and that there is evidence that the sequence corresponds to a *pseudogene*.
Translated	Is acquired when experimental *evidence at the protein level* indicates that there is clear proof of the existence of the protein. The criteria include partial or complete Edman sequencing, clear identification by mass spectrometry, X-ray or NMR structure, good quality protein-protein interaction or detection of the protein by antibodies.

Definition of a convention for functional names and symbols

The adoption of a common nomenclature across diverse organisms facilitates structural, functional, and evolutionary comparisons of genes and genetic variation. From the onset of genetic research, genes were often named referring to the mutant the genes could be linked to. This is not only true for plants, but this gene-naming scheme can hardly be maintained across many species or is sometimes confusing or even misleading when looking deeper at the evidence compiled using cutting edge technologies. Indeed, most of the early gene names and symbols describing visible phenotypes provided by the earliest evidence for the existence of a gene might not have the same effect or worse more genes that lead to a certain phenotype would end up with related name while being completely different. In grapevine, there is much less mutational data than in *Arabidopsis*, and only a few genes were named after a phenotype. However, the naming system should be developed to be flexible enough to cope with the expansion of data that will be produced in the future, including from yet to be invented technology. Therefore the goal should be a system where both the full name and the symbol are composed by a descriptive (full name) and/or a short (symbol) name referring to the function of the coding protein and a number to discriminate the isoform. In rice, this later number is known as the locus designator and indicates the chronological order in which a particular gene or gene family member was identified [21]. In grapevine, the function of most genes is in the large majority inferred by sequence similarity. The 'guilt-by-association' approach, however, presents problems when a single-copy, well-characterized gene from one plant corresponds to multiple grapevine paralogs. In this case, a consistent individual numbering system in grapevine needs to be put in place. Another issue raises when, through independent studies carried out by different authors, multiple names and symbols were given to genes that converge to a single locus in grapevine. It is also very common for enzymes to be represented by different synonyms for the same function. The aim of the nomenclature system is to state on rules where only one full name and one symbol, consistent with each other, will be attributed and where all the other known names will be considered as synonyms. Rules for the attribution of both the main name and the numbering of the members of gene families are described below. When naming enzymes, the use of the Enzyme Commission nomenclature (EC) for the primary name should be preferred and when possible, a bibliographic reference for the synonym should be stored in ORCAE (doi, Pubmed ID…). Names corresponding to mutant phenotype should

be used when a mutant is available with the name describing some aspect of the corresponding phenotype. Names corresponding to gene product should be used regardless of the availability of a mutant when the symbol describes some aspect of gene structure or function.

The gene symbol should consist of two to five letters if possible and the corresponding locus designator consisting of one to three digits. In *Brassicaceae*, the gene symbol can have up to six digits. In *Arabidopsis* and rice the use of species-specific prefixes (At, Os) for the symbol and the full name in the official name is discouraged because of redundancy with species information already known elsewhere (in the Locus ID, for example), the same shall apply for *Vitis*. However, it could be added when specifically referring to the *Vitis* gene in publications, with the *vinifera* prefix being Vvi and the other prefixes as shown in the VIV catalogue [29]. Although *Vitis vinifera* genes were named with the vv (or Vv) prefix, this creates confusion with the bacteria *Vibrio vulnificus*, whose genome was published before the grapevine and "locked" the vv prefix into major databases. A two-letter code is also too short for discrimination between *Vitis* species. The intention of this paper is to strive to a consistent naming scheme that would avoid redundancy and confusion within and across gene families. When a mutant phenotype exists in *Vitis,* the root of the full name and the symbol will refer to it, else it is recommended to use when possible the same symbol as the corresponding gene family in the model plant Arabidopsis to facilitate cross-species comparisons since it is the best annotated plant to date. Bearing these crucial rules in mind, several strategies can be followed for the numbering of the members of a gene family. It is recommended to use numbers based on phylogenetic or 'guilt-by-association', homology based approaches although we recognize that phylogenetic trees may evolve as more species are sequenced in the future and that the functional information of such numbering may therefore be less relevant after several years, specifically when the gene belongs to a large family, alternative can be used: keep historical names when they do exist, numbering in a chronological order of discovery and random numbering. Use of the position on the chromosome is not recommended because it will be misleading when new genes in the family are found or segments of the genome are rearranged.

If an author plans to change or to update a name, we provide a summarizing decision tree in Figure 3, which we hope will allow one to evaluate what necessary steps to take that will lead to a appropriate naming. The next paragraphs give some case studies and recommendations for gene naming based on a phylogenetic approach.

Gene naming based on phylogenetic trees

In order to provide a reproducible phylogenetic tree, it is recommended to follow the instructions on homology determination provided by Gramene [33] (the method was published in [34]). Only orthologs one2one should be considered when allocating the *Arabidopsis*-like name to the *Vitis* gene. When the relationship is one-2-many or many-2-many, a new gene product symbol should be attributed. The new symbol will consist of a root with common protein group term (enzyme, transcription factor, transporter, elicitor family…) paired with a number higher than the highest number used already for both *Vitis* and *Arabidopsis*. Alternatively, as Gramene provides precomputed alignments and phylogenetic trees, we would recommend to use these and include the new *Vitis* genes, for the sake of uniformity. If a tree has to be generated *de novo*, curators can find useful resources at [35]. It is recommended to use branch support or bootstrapping to validate tree structure. Poorly supported branches, like bootstrap values below 70% should be collapsed, because values below this level imply a potentially misleading hierarchy. The phylogenetic trees are based on alignments that should be calculated from codons (at the nucleotide level) rather than with the amino acid sequences, to increase the discriminative power between closely related *Vitis* genes. Grapevine genes (two or more) at the same phylogenetic distance from a single homolog in *Arabidopsis* should be differentiated by a number. If the Arabidopsis gene name ends with a number, the characters used to differentiate the *Vitis* genes should be letters.

Examples of gene name confusion and the recommended nomenclature procedure

To highlight different gene name problems and the recommended resolution, four examples are described in the following section:

Example 1. Uncharacterized members in Arabidopsis and members with diverse names: the EIL family (Figure 4).

The four *Vitis* genes that have been identified as *EIN3*-like transcription factors (*EIL*) [7] were compared to the *EIL* genes of *Arabidopsis* found in the plantTFDB [40] and a phylogenetic tree was reconstructed. Plant transcription factor family symbols are available in plantTFDB or plnTFDB [41] and can be used for comparison with *Vitis*.

The gene *VIT06s0009g01380* is orthologous to *Arabidopsis EIN3*. Even though *EIN3* is the gene that gives its name to the whole family, it does not conform to the family name symbol and refers to a phenotype. In addition, there is no evidence that the grapevine gene induces the *EIN3* phenotype. Under these circumstances it is recommended to name the *Vitis* ortholog *EIL3*, because the number 3 is the next available numbers used for *Arabidopsis.* The symbol *VviEIN3* would then be used as a synonym. The choice of the lead symbol and the synonym should be left to the curator's discretion since it will depend on the history of the gene and additional evidences

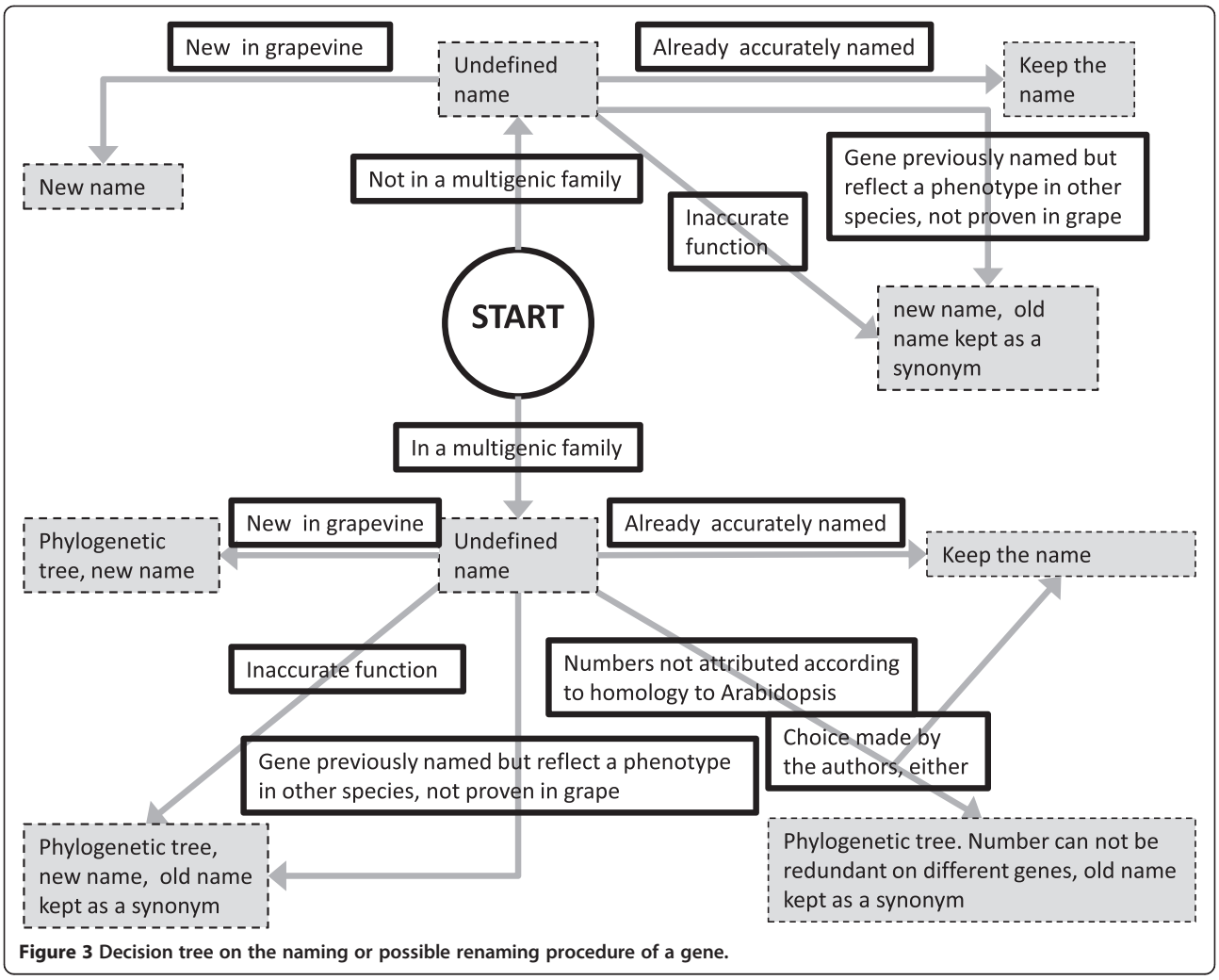

Figure 3 Decision tree on the naming or possible renaming procedure of a gene.

on the function (or phenotype). Only in the case that a similar function or phenotype, described for an *Arabidopsis* gene, could be experimentally demonstrated in *Vitis*, then only the name *EIN3* would be justified. In any other case *EIL3* should be favored.

Two genes are equally distant from *EIL2*. Since there are two genes, an additional letter should follow the symbol to differentiate them.

The last *Vitis* gene *VIT00s0357g00120* is equidistant from two unnamed and unclassified *EIL*s, and from *SLIM1* and *EIL1*. Therefore, the root will be 'EIL' and the index, the next available independent number. To avoid any confusion, the recommended symbol under these conditions should be *VviEIL4*.

There is no order in which *VIT06s0009g01380* and *VIT00s0357g00120* should be named; either one can be *VviEIL3* or *VviEIL4*.

Example 2. Genes already named in grapevine, but names inconsistent with Arabidopsis and Arabidopsis genes without symbols: sugar transporters.

The grapevine sugar transporter genes were classified by Afoufa-Bastien et al. [3]; when available, their classification was based on the literature. Three of the sugar transporter families provide examples for different scenarios.

The sucrose transporter family was classified by Davies et al. [42] as *SUCXX* and by Ageorges et al. [43] as *SUTXX* with the *SUC11/SUT1* gene being identified and named differently in the two papers. The phylogenetic tree drawn by [3] (adapted in Figure 5A) shows the genetic distance with the *Arabidopsis* genes and the proposed names of the symbols are shown in the middle column where the *SUCXX* format is prioritized as in *Arabidopsis*; as shown here *SUT1* should be used as a synonym for *SUC11*. *SUT2* should be kept as a synonym and a new name fitting the "SUC" format needs to be created. Since there is no closest ortholog, the number should be incremented after the highest number in both *Vitis* and *Arabidopsis*, which is *VviSUC28*. The names that would have been used if the genes were not named in earlier publications and only theoretically

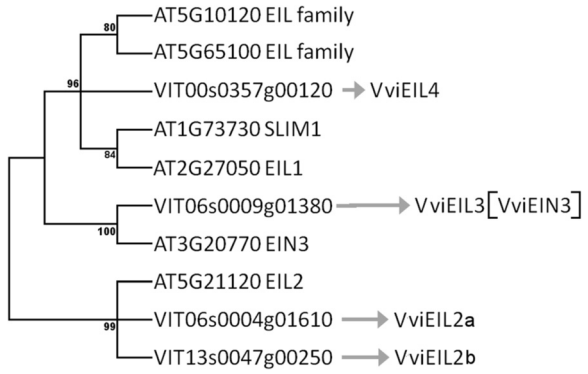

Figure 4 Molecular phylogenetic analysis of *Vitis vinifera* and *Arabidopsis thaliana* EIL gene models by the maximum likelihood method. Multiple sequence alignment for full-length transcription factors was inferred using MUSCLE [36]. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [37]. The bootstrap consensus tree inferred from 100 replicates [38] is taken to represent the evolutionary history of the taxa analyzed [38]. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [38]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 10 amino acid sequences. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 273 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [39]. Arrows point toward recommended *Vitis* symbols.

inferred by homology are indicated in the right section of Figure 5A.

The grapevine hexose transporters were symbolized as *HTXX* and functionally characterized [44] for *HT1*, [45] for *HT3*, *HT4*, *HT5*. Other sequences were identified and classified up to *HT24* [3]. However, in *Arabidopsis* this family is named sugar transporter proteins (*STP*). As a consequence, it is recommended that the symbols under the *VviHTXX* format should be kept as synonyms and the main symbol should be under the *VviSTPXX* format; the numbering of the genes should be in accordance with the phylogenetic tree performed in [3] as adapted in Figure 5B.

The grapevine sugar transporter *ERD6*-like family was also compared to *Arabidopsis* [3]; the phylogenetic tree was adapted in Figure 5C. In this work, no symbols were assigned to the *Arabidopsis* genes, probably because they were never published, even though a nomenclature existed and they appeared as full names in the UniProt and NCBI databases. As a consequence no symbols were transferred to the *Vitis* genes in that publication. In addition, since the symbol *ERD6* ends with a number it is recommended to add the letter L, for -like, between the family root of the symbol and the number as presented in Figure 5C. This family in *Vitis* contains also a branch that is not related to *Arabidopsis;* the numbers of the genes in this branch shall be incremented after the last known number for the *Arabidopsis* genes.

Example 3. When gene name and function change with new discoveries: the CCD family and the NCED subfamilies.

The *Vitis* genes for the *CCD/NCED* family were characterized and named according to homology with genes from *Arabidopsis* [41,42], although some were characterized in previous studies. The phylogenetic tree was independently rebuilt in Figure 6 and differs from the one presented in [46] since genes from non-*Arabidopsis* species were used. The tree is similar to [47] except for the genes not present in that study. Three previously undetected genes were added (*VviCCD8b* in [47] and *VviCCD4b VviCCD1b* in [46]), but the gene's nomenclature would have been relatively similar. The *NCED* genes are a subset of the *CCD* family and they share similar features, including sequence similarity and carotenoid double-bond-cleaving dioxygenase activity. *CCD*s are distinguished by the specificity of double bond cleavage and *NCED's* are plastid-localized [48]. Hereby, the genes belonging to the *NCED* family should only bear the *NCED* symbol, likewise for the *CCD* genes, to avoid confusion. However, two historical members were named *CCD1/NCED1*, and *CCD4/NCED4*. In this case both symbols should be kept with *CCD1* (or 4) as the main symbol and *NCED1* (or 4) as the synonym, since this gene presents a more *CCD*-like function as demonstrated in [47]. A note should be linked to the *NCED* synonym to indicate its obsolescence.

Since a second gene from grapevine appears to belong to the *CCD1* subgroup, the genes should be renamed with an extra character to differentiate them (*CCD1_1 and CCD1b*); however the symbols "*CCD1*" and "*NCED1*" were attributed to *CCD1a* and should be kept as synonyms for it. Since *VviCCD4b* was not identified in [47], authors named *VviCCD4c* with the letter b and [46] also named *VviCCD4b* with the letter b. To avoid any kind of confusion, new names can also be allocated to these genes and all the previous names should be reported as synonyms with a note indicating that a given synonym has been used for multiple genes.

Similarly, *VviNCED3* was incorrectly identified as *NCED1* in [49]. Therefore, *VviNCED1* should appear as a *VviNCED3* synonym but with a note indicating that this synonym is incorrect.

The gene *VIT04s0008g03510*, coding for a member of the well described *CCD8b* group of orthologous genes in the grapevine was named with this symbol even though no *Arabidopsis* gene belongs to this family, because it is a well described group of orthologous genes [46].

Example 4. Genes not present in Arabidopsis: the STS family.

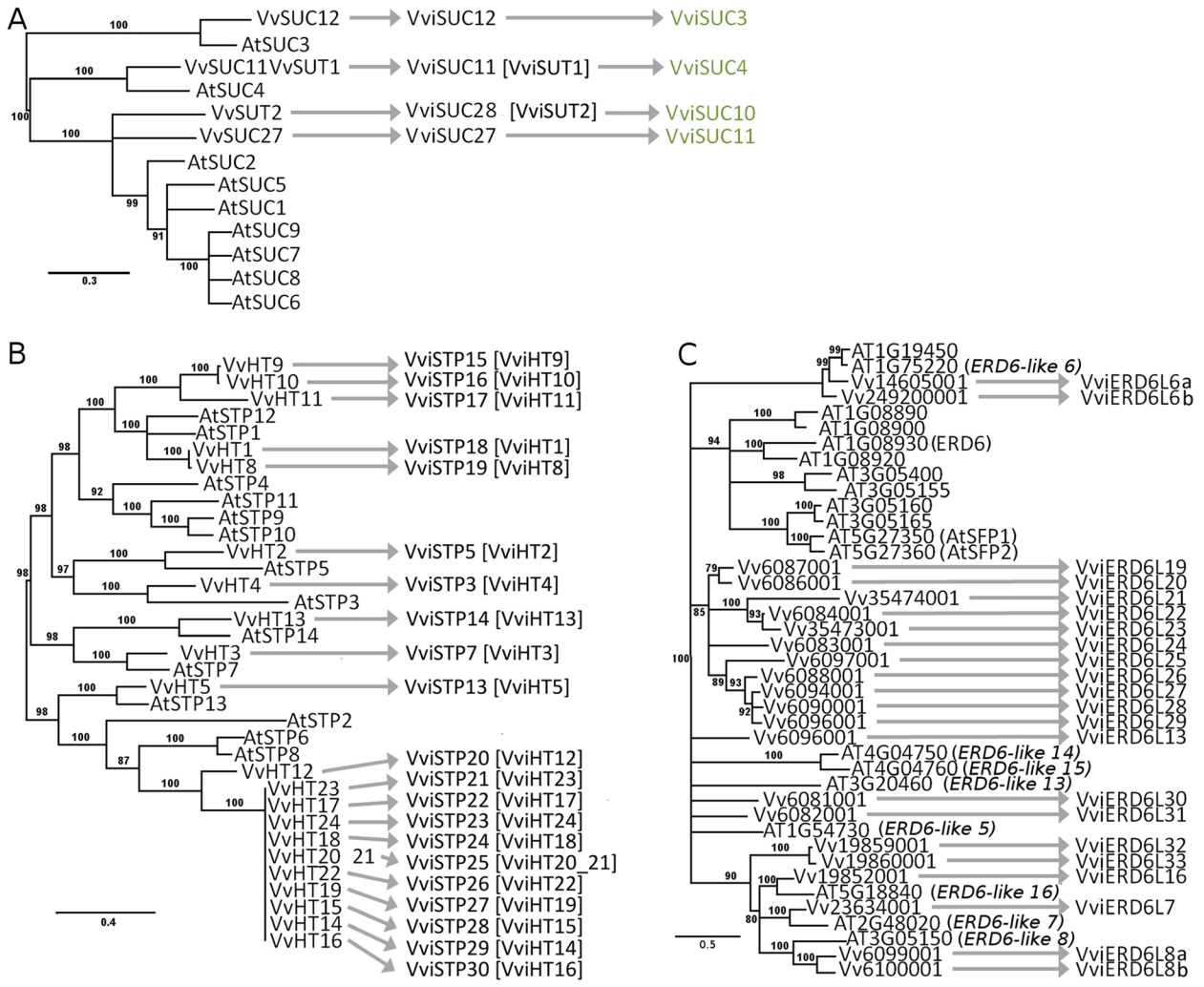

Figure 5 Molecular phylogenetic analysis of *Vitis vinifera and Arabidopsis thaliana* sugar transporter gene models by the Maximum Likelihood method. The trees are adapted from [3] and produced using MUSCLE [36] and PhyML with the JTT amino acid substitution model. Bootstrapping was performed with 100 replicates. In addition to the original picture, branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [38]. **A)** sucrose transporters **B)** hexose transporters **C)** ERD6-like proteins. Arrows point toward recommended *Vitis* symbols, the green symbols are the putative symbols that would be used had not the *Vitis* gene been previously annotated in the literature. Recommended synonyms are in brackets.

The grapevine trihydroxystilbene synthase (STS) gene family was characterized in two concomitant articles [4,5]. As this family is not present in *Arabidopsis*, it is not possible to rely on sequence similarity with the *Arabidopsis* genes to address the nomenclature. While describing the genes, both authors used the same strategy to name the genes according to the syntenic positions, which is logical since the genes are grouped in two clusters on chromosomes 10 and 16. The names in both studies are identical. However, some of the genes were already described in previous studies [50,51], and this was not taken into account for the naming of the members of the STS family. The genes were stored in public databases such as UniProt and Refseq under their original deno minations. The symbols are written differently, STS vs StSy, while the full names are both trihydroxystilbene synthase. This causes problems: for example, trihydroxystilbene synthase 5 may refer to two different genes (*Stsy5/VvSTS10* and *VvSTS5*); thus, the symbols are distinct but the full names are identical. There was one gene, however, (*VvSTS47*), that was previously named with an *STS*-like symbol (*STS2*) in addition to the synonyms (*VINST1*, *PSV25*, *VST1*). There is no problem in keeping *VvSTS47* as a synonym, but the symbol *STS2* refers to two different genes (*VvSTS2* and *VvSTS47*) which causes confusion. The strategy of ordering according to the chromosome position should be avoided. It presents the disadvantage of being invalidated each time changes occur at the level of

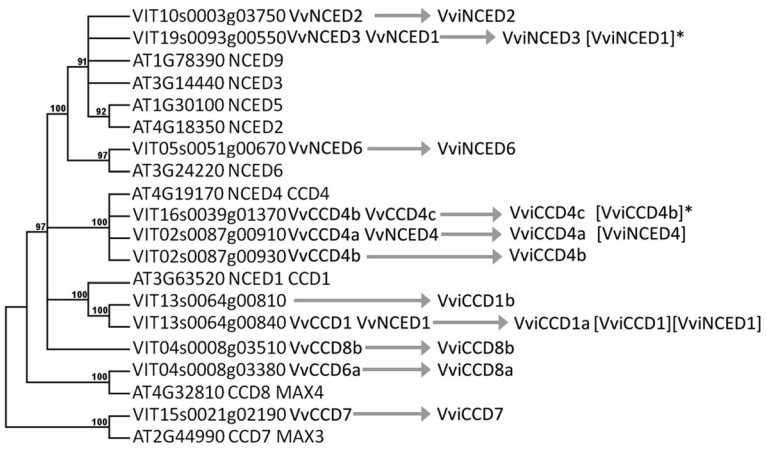

Figure 6 Molecular phylogenetic analysis of *Vitis vinifera and Arabidopsis CCD and NCED* gene models by the Maximum Likelihood method. Multiple sequence alignment for full-length carotenoid cleavage dioxygenases was inferred using MUSCLE [36]. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [37]. The bootstrap consensus tree inferred from 100 replicates [38] is taken to represent the evolutionary history of the taxa analyzed [38]. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [38]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 20 amino acid sequences. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated. There were a total of 225 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [39]. Arrows point toward recommended *Vitis* symbols. Asterisks indicate redundant synonyms.

the genome assembly or when new members of the family are discovered. It is therefore recommended to conserve the phylogenetic tree strategy for gene naming (Figure 7).

Annotation platform and informatics tools

There is a need for a centralized online platform that allows manual curation of gene-models and their functional annotation by experts. Besides the central repository, several other (offline) resources are available that can be used to improve the annotation.

Platform for community curation of grapevine gene annotation

The annotation platform for the grapevine genome is centralized and maintained in the ORCAE database with online interface from the VIB [25] and was chosen to perform community annotation for *Vitis*. ORCAE was developed with a gene-centric vision, meaning that the gene information pages are the central access points instead of a genome browser. The basic setup of ORCAE can be compared to a wiki system with information pages for each gene like a 'topic' page of a traditional wiki text. ORCAE was designed to suit the needs of genome sequencing projects from small consortia, like the grapevine. Like wikis, the data stored in ORCAE is never removed and a complete history of the changes applied by curators is kept. Also a number of analyses are run and updated in the background after changes affecting the gene structures have been supplied. Updates to central repositories, like NCBI, will be organized on a six months basis, if the number of modifications can be considered as worthwhile. Users, willing to manually curate data will have to register with the ORCAE system, mostly to allow communication between curators worldwide. Also accounts are a way to remediate when erroneous modifications occur or to track errors in the input data, and discuss with the authors that mistakenly entered incorrect data. The whole systems history of modifications allows the retrieval of previous versions of gene models. Furthermore, to limit simple errors, tests have been implemented for checking the editing process, via the GenomeView application. These checks result in the ability of the system to reject genes models that contain obvious errors after user's modifications. Genes that would be missing from the current genome assembly, but are proven to be in Vitis, will be added to ORCAE as standalone genes, although, only after thorough checking to ensure that they are actually real. As for the genes represented in the reference genome, they will follow the same process for submitting annotation to NCBI and their nomenclature will follow the same rules as for other genes.

Guidelines for community gene functional and structural annotation

The sNCGGa can be contacted from the IGGP website at http://www.vitaceae.org/index.php/Annotation. Official announcement from the committee can be found at that address. A preliminary functional annotation tutorial is

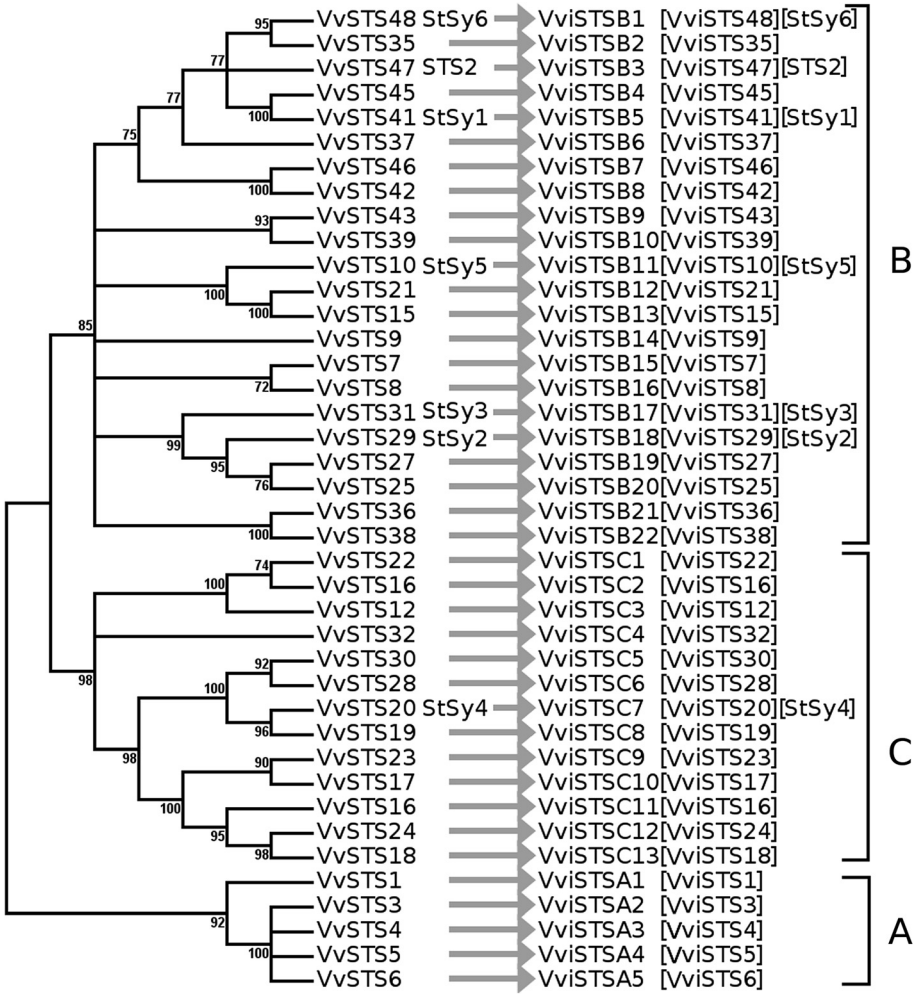

Figure 7 Molecular phylogenetic analysis of *Vitis vinifera* trihydroxystilbene synthase gene models by the Maximum Likelihood method. Multiple sequence alignment for full-length trihydroxystilbene synthases was inferred using MUSCLE [36] from the nucleotide sequence. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [37]. The bootstrap consensus tree inferred from 100 replicates [38] is taken to represent the evolutionary history of the taxa analyzed [38]. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [38]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 40 amino acid sequences. The coding data was translated assuming a standard genetic code table. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 292 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [39]. Arrows point toward recommended *Vitis* symbols. **A,B,C** refer to the groups in [4].

also available [52] and will be updated with the present paper. Topics described in this tutorial are open to debate and can be amended during the process of community annotation. The sNCGGa can be contacted for enquiries at the Google group.

One of the major goals is to bring together experts for each gene family to allow them to perform their annotation through the ORCAE annotation website, which in due time will synchronized with major public databases such as NCBI or Uniprot. The annotation should fit the IGGP Committee guidelines in terms of nomenclature and rules for addressing the level of confidence. In any case where possible, it is advised to annotate complete gene families or all the enzymes involved in a metabolic pathway, rather than a single isolated member of a larger group of genes.

Conclusions

The intent of the grapevine nomenclature standardization is, taking into account the accumulated experience from other species and in grapevine, to clear up gene name confusion and redundancy. In particular we want to

anticipate on the ever-growing amount of new sequencing data. It is important to consider that the collection of experimental evidence for grapevine genes will most likely be limited and that the community is forced to opt for a strategy that can consider annotation inferred from similarity to other species. This is a problem endemic to small and medium-sized research communities. With the current paper, it was chosen to propose a set of guidelines aiming at a harmonized nomenclature for the full names and symbols of *Vitis* genes that allow easy correspondence with other species, without being restrictive or too rigid. On the other hand the attribution of the locus ID is done automatically and will be systematically attributed to each new gene according to internal rules. This rule-based nomenclature system is intended to reduce confusion, improve gene and protein comparisons, and facilitate the comparison of functions across species. The success of a nomenclature system requires the participation of the grape community, who by contributing will share the knowledge through discussions and through implementation of the system to improve grape gene nomenclature and annotation.

Methods

Phylogenetic analysis

Multiple sequence alignment was inferred using MUSCLE [36]. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [37]. The bootstrap consensus tree inferred from 100 replicates [38] is taken to represent the evolutionary history of the taxa analyzed [38]. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The coding data was translated assuming a Standard genetic code table. All positions containing gaps and missing data were eliminated.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JG coordinated the committee and drafted the manuscript. GC initiated the project. JG, AFAB, PFB, OB, DC, CD, SD, MP, SR and GC participated in the meetings and took part in the elaboration of the nomenclature. All authors read, contributed and approved the final manuscript.

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